Impact of Ozone on the Reproductive Biology of *Brassica campestris* L. and *Plantago major* L.

by

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ABSTRACT

Although sexual reproductive development is a critical phase in the life cycle of plants, the effects of air pollutants on the processes involved have not been investigated extensively. This thesis describes experiments undertaken to determine the direct effects of short-term exposures to ozone on the reproductive biology of species with contrasting reproductive growth habits, *Brassica campestris* L., an indeterminate species, and *Plantago major* L., a more determinate species. Two purpose-designed exposure chambers were constructed, each of which permitted the reproductive structures of up to twelve plants to be isolated from the vegetative parts and exposed simultaneously to ozone-enriched or charcoal-filtered air. The design of these chambers also permitted whole plant exposures.

The occurrence of significant decreases in stomatal conductance and the net rate of photosynthesis following exposure to 70 ppb ozone established that the vegetative structures of both *B. campestris* and *P. major* were sensitive to ozone. Visible leaf injury also developed in *B. campestris*, the extent of which varied with the stomatal conductance of plants prior to fumigation and therefore ozone uptake. The growth of *B. campestris* and *P. major* was also reduced following 10 and 14 days of exposure respectively to 70 ppb ozone for 7 h d^{-1}. The effects of ozone on growth differed between the four populations of *P. major* examined in this study.

While a single 6 h exposure of the terminal inflorescence of *B. campestris* to 100 ppb ozone had no significant effect on reproductive development, repeated exposures over four consecutive days increased the abortion of seeds in apical pods and the precocious germination of seeds in older pods. However, the impact on final seed yield was dependent upon the timing of exposure; thus seed yield was significantly reduced following exposure during the early flowering phase, but was unaffected following exposure during the later stages of reproductive development. The compensation for seed losses in more mature plants resulted from their ability to retain naturally aborting seeds in older pods. Nonetheless, single and multiple exposures to ozone both reduced the rate of germination of harvested seeds and multiple exposures affected seed colour. In *P. major*, exposure of the first spike to 120 ppb ozone for 7 h d^{-1} during flowering primarily affected seed number per capsule. Both significant increases and decreases in seed number were observed in the four populations examined, and seed number was also affected in spikes which had not been exposed to ozone, indicating the existence of possible compensation mechanisms. Pollen from both species was also shown to be sensitive to a 6 h *in vivo* exposure to ozone.

The complex responses of *B. campestris* and *P. major* following direct exposure of the reproductive structures to ozone suggest that factors other than the type of reproductive growth habit are important in determining the extent of ozone damage and the ability of plants to compensate for reproductive site losses.

ACKNOWLEDGEMENTS

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF PLATES

LIST OF UNITS

d	day
h	hour
min	minute
s	second
$h\ d^{-1}$	hours per day
m	metre
cm	centimetre
mm	millimetre
μm	micrometre
nm	nanometre
mm^2	square millimetre
$mm^2\ mg^{-1}$	millimetre square per milligram
cm^2	square centimetre
cm^3	cubic centimetre
kg	kilogram
g	gram
l	litre
ml	millilitre
μl	microlitre
$l\ min^{-1}$	litres per minute
ppb	parts per billion
ppm	parts per million
°C	degree centigrade
%	percent
M	molar
$mmol\ m^{-2}\ s^{-1}$	millimoles per square metre per second
$\mu mol\ m^{-2}\ s^{-1}$	micromoles per square metre per second
$\mu mol\ mol^{-1}$	micromoles per mole
$\mu mol\ m^{-2}\ s^{-1}$	micromoles per square metre per second
V	volt
$W\ m^{-2}$	watts per square metre

LIST OF ABBREVIATIONS

A	net rate of photosynthesis
ANOVA	analysis of variance
AOT40	accumulated exposure over a threshold of 40 ppb ozone
CEGC	controlled environment growth cabinet
C_i	intercellular CO_2 concentration
CIRAS	combined infra-red gas analysis system
CO_2	carbon dioxide
DAS	days after sowing
g_s	stomatal conductance
H_2O	water
K	allometric root:shoot coefficient
NO	nitrogen oxide
NO_2	nitrogen dioxide
NO_x	nitrogen oxides
O_3	ozone
PAR	photosynthetically active radiation
$\overline{R}$	mean relative growth rate
SO_2	sulphur dioxide
UV	ultra-violet radiation
VOCs	volatile organic compounds

CHAPTER ONE - INTRODUCTION

Sexual reproductive development is a crucial stage in the life cycle of plants because any impairment of the processes involved may have significant implications for the yield of crop plants and the survival of native species. Although reproductive processes are known to be sensitive to a number of environmental stresses such as temperature and drought (Stirling and Black, 1991; Rao et al., 1992; Ney et al., 1994; Lardon and Triboiblondel, 1995) there has, with the exception of pollen (Feder, 1968; Cox, 1983; O'Conner et al., 1987), been relatively little investigation of the effects of air pollutants on reproductive development. Although air pollution has been an issue of global environmental significance since the start of the industrial revolution c.150 years ago, it has only been demonstrated in the last two to three decades that pollutants such as sulphur dioxide (SO_2), nitrogen oxides (NO_x), and ozone (O_3) may adversely affect plant growth and development. Of these major pollutants, ozone is considered to be the most important, not only because of its phytotoxicity but because its concentration in the troposphere has risen considerably over the last c.60 years (Anfossi et al., 1991) and is likely to continue to increase for the foreseeable future. Because of the increasing threat of pollution stress to plants and the dearth of existing information, it is essential to ascertain whether elevated concentrations of ozone can adversely affect the reproductive biology of both crop and native species. It is of value to establish not only the mechanisms of ozone damage but also whether plants have the ability to resist or compensate for damage to their reproductive structures so that these attributes may be exploited, for example, in the development of new crop varieties capable of maintaining their yield in polluted environments. In addition, these studies are important because more information regarding the ozone sensitivity of plants, particularly native species, is required so that effective pollution control strategies can be developed to protect natural and semi-natural communities.

In the light of these concerns, this thesis describes a series of laboratory experiments which investigated the effects of short-term exposures to elevated concentrations of ozone on the physiology, growth and reproduction of two plant species, *Brassica campestris* L. and *Plantago major* L. In particular, this study aimed to identify whether reproductive processes such floral initiation, pollen germination, seed yield and seed germination may be damaged directly by exposure to ozone, and whether the nature of the responses induced differ between species.

In order to place this study in context, this chapter reviews the formation and distribution of ozone in the atmosphere and outlines the known effects of ozone on the physiology, growth and reproduction of plants.

1

1.1. OZONE IN THE ATMOSPHERE

1.1.1. Ozone formation

Ozone is a natural constituent of the atmosphere formed by photochemical reactions involving oxides of nitrogen (NO_x) and volatile organic compounds (VOCs; Wayne, 1987; Chameides and Lodge, 1992). 90 % of the ozone in the atmosphere is formed and located in the stratosphere at altitudes between 12 and 40 km, where it is important in preventing damaging short wavelength UV radiation from reaching the earth's surface (Crutzen and Golitzyn, 1992). In the troposphere, ozone originates from the occasional downward transport of stratospheric ozone and from photochemical reactions *in situ* (Chameides and Lodge, 1992). The formation of ozone involves many complex chemical reactions but basically depends on the following reactions:

$$hv + NO_2 \rightarrow NO + O \quad (1)$$
$$O + O_2 + M \rightarrow O_3 + M \quad (2)$$
$$O_3 + NO \rightarrow NO_2 + O_2 \quad (3)$$
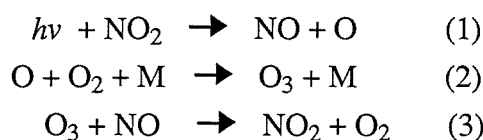
Nitrogen dioxide (NO_2) absorbs solar radiation with wavelengths between 280 and 430 nm and is split into nitric oxide (NO) and atomic oxygen (O; eq. 1). O then reacts with molecular oxygen (O_2) via a molecule or atom (M; e.g. nitrogen or oxygen) which can absorb the energy of the reaction as O_3 is formed (eq. 2). Ozone can then react rapidly with NO to form NO_2 and O_2 (eq. 3). The equilibrium reached by these reactions is determined by the concentrations of NO and NO_2 in the atmosphere, although this balance can be altered by the presence of biogenic hydrocarbons (isoprene, terpenes), which are evolved in appreciable amounts by woody plants (Moldau, 1993; Arey *et al.*, 1995). Photochemical degradation of these hydrocarbons produces peroxy radicals (RO_2; where R is a hydrogen atom or an organic radical) which react with NO, converting it to NO_2; this, in turn, releases atomic oxygen (eq. 1), shifting the balance of photochemical reactions towards ozone formation (Mansfield and Pearson, 1993). The amount of ozone which is formed during daylight hours depends upon the concentrations of the precursor chemicals in the air, although the concentration of ozone in the troposphere at any one time is a balance between input from downward transport from the stratosphere and photochemical production *in situ* and losses by chemical reaction (e.g. with NO) and deposition onto vegetation and soil. Removal of ozone by deposition to terrestrial surfaces (dry deposition) is the major sink for tropospheric ozone (UKPORG, 1993). The above is a simplified account of ozone chemistry; more detailed accounts of the complex reactions involved in ozone formation

and degradation can be found in Wayne (1987), Chameides and Lodge (1992) and UKPORG (1997).

1.1.2. Ozone as an air pollutant

Anthropogenic emissions of nitrogen oxides and VOCs (primarily hydrocarbons) into the atmosphere from the combustion processes of industry and vehicle exhausts have caused ozone concentrations over Europe to increase significantly since about 1940 (Anfossi *et al.*, 1991). Because ozone is formed from the reaction of primary air pollutants and is not emitted directly into the atmosphere, it is termed a secondary pollutant. Using early measurements of ozone concentrations in Paris between 1876 and 1910, as well as recent ozone measurements at remote locations in Europe and the USA, it appears that the average daily concentration of ozone at mid to high latitudes has approximately doubled during the past 100 years to between 20-40 ppb (Hough and Derwent, 1990). Models using estimates for future emissions of nitrogen oxides up to the year 2020 suggest that ozone concentrations will continue to increase at a rate faster than during the past 100 years (Hough and Derwent, 1990). The potential for further increases in tropospheric ozone must therefore be taken into consideration when assessing the environmental impact of this pollutant.

1.1.3. Spatial and temporal distribution of ozone

The concentration of ozone in the atmosphere is not uniform but varies both spatially and temporally. Importantly, ozone concentrations vary depending on the timing of release and quantity of precursor chemicals in the atmosphere, although a number of other factors influence the pattern of ozone concentrations. Since ozone production is driven by solar radiation, concentrations rise during the day to a peak just after midday, at night, in the absence of ozone formation, the concentration of ozone tends to fall because of reactions with NO and deposition to the earth's surface (UKPORG, 1987). Ozone concentrations also vary with the seasons and tend to be greater during the warmer summer months because of the increased quantity and intensity of solar radiation (Logan, 1985). Year to year variations also occur because of differences in meteorological conditions. In polluted atmospheres, short periods of high ozone concentrations or 'peak episodes' may occur during which concentrations exceed 100 ppb (Guicherit and Van Dop, 1977; UKPORG, 1993). These episodes may last for several days and are generally associated with warm anticyclonic weather conditions, when irradiances are high and the wind speeds are low (Guicherit and Van Dop, 1977). Such conditions prevent the dispersion of precursor chemicals and

provide the solar energy and high temperatures essential for the photochemical reactions involved in ozone formation.

The diurnal cycle of ozone concentrations has been shown to vary with altitude (UKPORG, 1993). At high altitude (above 800 m) there is often little diurnal variation because the atmosphere is well mixed and the supply of ozone to the surface exceeds its rate of deposition. In contrast, at low level sites, there is commonly a marked diurnal cycle because the development of a nocturnal inversion restricts the downward transport of ozone such that the concentration near the surface declines as ozone is deposited or removed by reactions with NO. Since ozone and its precursors can readily travel downwind of pollution sources, elevated concentrations of ozone are not confined to urban areas. Indeed, ozone concentrations are generally lower in urban than in rural areas because ozone reacts with NO at the source of pollution to form NO_2 (Altshuller, 1987; UKPORG, 1997). In 1977, Ashmore et al. (1978) used the ozone sensitive cultivar of Nicotiana tabacum, Bel W-3, to survey ozone levels at 52 locations over the whole of the British Isles. Using visible leaf injury as an indication of ozone exposure, a correlation was found between injury and the quantity of solar radiation received at each site, rather than the location of the site relative to pollution sources. Natural vegetation in rural areas is therefore particularly at risk of being exposed to high concentrations of ozone during the spring and summer growing season.

The presence of elevated concentrations of ozone in the British Isles was first recorded in 1971 (Atkins et al., 1972) and since then ozone concentrations have been well documented through a national network of ozone monitoring stations which was established to characterise the distribution of ozone across the country (UKPORG, 1987, 1993, 1997). Present concentrations of ozone in the UK frequently exceed 60 ppb, but regional variation exists, with the frequency of peak episodes in excess of 60 and 90 ppb being greatest over the south coast of England (UKPORG, 1993) due to the more favourable meteorological conditions for ozone formation, greater release of primary pollutants and the transport of pollution from the Continent by easterly winds. Ozone concentrations also vary from year to year; for example, during the exceptionally warm summer of 1976, concentrations as high as 250 ppb were recorded downwind of London (UKPORG, 1987).

Ozone concentrations are greatest in the most densely populated and industrialised regions on the northern hemisphere (Logan, 1985). However, with increasing industrialisation and traffic in the third world, it is likely that ozone concentrations will also increase in future in these areas.

1.1.4. Importance of ozone in the atmosphere

Increases in ozone concentrations are of concern because ozone has an important modifying influence on the climate of the troposphere and because the gas can have deleterious effects on non-biological materials, human health and vegetation.

Ozone is an important constituent of the atmosphere because it absorbs radiation at wavelengths below 300 nm and thus limits the quantity of harmful UV-B radiation reaching the earth's surface (UKPORG, 1987). The increased concentrations of tropospheric ozone may be important in offsetting the effects of the reduction in stratospheric ozone (Logan, 1985) which has occurred as a result of emissions of chlorofluorocarbon aerosol propellants and oxides of nitrogen from supersonic aircraft (Colbeck and Harrison, 1984). However, ozone also absorbs longer wave radiation emitted from the earth's surface and is therefore considered a 'greenhouse gas'. It has been calculated that a doubling of current tropospheric ozone concentrations may increase global surface temperatures by 0.9 °C (Hov, 1984).

Ozone is extremely reactive with many organic polymers and is known to degrade materials such as rubber, fabrics, dyes, plastics and paints, leading to cracking, brittleness and fading (Lanting, 1984). In humans, ozone has been linked with mucosal irritation, headaches, reduced physical performance, alterations of lung function, reduced resistance to infections and an increase in the prevalence and severity of asthma (UKPORG, 1987). In 1987, the World Health Organisation (WHO) suggested that in order to protect human health ozone should not exceed 76-100 ppb averaged over 1 h or 50-60 ppb averaged over 8 h. However, these values were only set as guidelines, since there is still a degree of uncertainty about the toxicity of ozone to human health. To warn people about the possibility of poor air quality, information concerning the concentrations of ozone and other pollutants (SO_2 and NO_x) in particular areas of the country is provided to the public during weather forecasts and in daily newspapers. Up to date details of ozone concentrations are also available via a freephone telephone number, through TELETEXT/CEEFAX and on the internet (UKPORG, 1997). During 1997, the health standard of 50 ppb averaged over 8 h was exceeded at a number of sites in the UK. Lullington Heath in East Sussex was the most polluted site, exceeding this standard 55 times during the year (Foster, 1997).

1.2. EFFECTS OF OZONE ON PLANTS

Damage to vegetation as a result of exposure to high levels of photochemical smog was first reported in the Los Angeles Basin in 1944 (Bell, 1978). Subsequently, the phytotoxic components of the smog were identified as two oxidants, ozone and peroxyacetylnitrate (PAN). As a consequence of the increased emissions of ozone precursors, ozone is now considered to be the most important air pollutant in North America. In 1988, Adams, Glyer and McCarl estimated that, through crop loss, existing levels of ozone cost the US economy $3 x 10^9 per annum. The first observations of ozone injury to vegetation in Europe were made in West Germany in 1963 (Saxe, 1990), but it was not until 1978 that experimental evidence showed that ambient ozone caused visible foliar injury to commercial crop species in the UK (Ashmore *et al.*, 1980). Since then ozone has been shown to have significant effects on the growth of many crop species (Jäger *et al.*, 1993) and also in native British herbaceous and tree species, although at present the amount of information available concerning native vegetation is considerably less than for crops.

Many methods have been employed to study the effects of ozone on plants, including closed chambers, open-top field chambers and open-air fumigation systems (Ashenden and McLeod, 1993). In general, these systems compare the responses of plants grown in filtered air with those of plants exposed to ambient or elevated concentrations of ozone, and each has its own advantages and disadvantages. For the study of plant responses in the field, large, cylindrical, plastic, glass or polycarbonate-covered open-top chambers (OTCs) have been developed. The airflow into these chambers can be modified, allowing the removal or addition of particular pollutants without greatly altering other environmental factors. However, these environmental variables have to be monitored and, because of the sheltering effect of the chamber, the environment and therefore the responses of plants grown in open-top chambers may differ from those of plants grown in the open air. Field exposures in ambient air with no interfering chambers or other apparatus represent the most natural systems for investigating plant responses to ozone. In this approach, ozone or charcoal-filtered air is introduced to the experimental plots through pipes placed either on the ground or above ground level, although other pollutants cannot be excluded entirely, making it difficult to attribute responses specifically to ozone. In addition, there can be problems in creating control plots under similar environmental conditions.

Considerable research has been conducted in closed chambers. In these systems, plants are subjected to modified environmental conditions which can be carefully controlled and readily manipulated. An advantage of this approach is that it allows the

6

precise control required for the replication of experiments; however, the exclusion of other abiotic and biotic variables means that extrapolation of the data to field conditions may not be possible (Krupa and Manning, 1988). For a number of reasons, closed chambers were employed for the investigations reported here. Firstly, in order to determine the direct effects of ozone on reproductive development, the reproductive structures of plants had to be isolated from the vegetative parts for fumigation. The plants also had to be exposed to known concentrations of ozone under controlled conditions of light, temperature and humidity, so that the observed responses could be attributed to ozone. These requirements could only be achieved using the closed chamber method. Details of the system used in this study are presented in Chapter 2.

1.2.1. Ozone uptake

Although it has been shown that ozone may react with the epicuticular waxes of the leaf surface (Barnes et al., 1988), the quantity of ozone taken up through the cuticle is negligible (Moldau et al., 1990). Primarily, ozone enters leaves through the stomata during the normal process of gas exchange (Rich et al., 1970). The quantity of ozone absorbed is determined by the external concentration and the resistances to gaseous diffusion (Heath, 1994). Boundary layer and stomatal resistances are almost entirely responsible for controlling ozone uptake (Reich, 1987), with stomatal resistance accounting for 90-100 % of the total pathway resistance at ozone concentrations below 250 ppb (Leuning et al., 1979). Genetic variability in the number and size of stomata therefore affects the quantity of ozone taken up by leaves (Iqbal et al., 1996), although uptake may be modified by environmental factors which influence stomatal aperture such as light (Dugger et al., 1963), relative humidity (McLaughlin and Taylor, 1981) and soil moisture (Olszyk and Tibbitts, 1981b). The rate of ozone uptake may also be enhanced by higher wind speeds which reduce boundary layer resistance, thereby allowing increased pollutant entry into the leaves (Iqbal et al., 1996). The structure of the canopy and the density of the foliage may influence the concentration of ozone to which individual leaves are exposed and therefore the quantity of ozone absorbed (Runeckles, 1992). It has been clearly shown that the concentration of ozone decreases within the canopy (Bennett and Hill, 1973), with the result that leaves deep within a dense canopy experience lower concentrations than those at the surface or at its edges. The spacing of the plants in crops results in a more open canopy structure, increasing the transport of ozone to all leaves (Runeckles, 1992). It has only been recently accepted that the flux of ozone to leaves is more important in determining plant responses than the dose (concentration x time) of ozone to which the plants are exposed (Musselman et al., 1994). An increasing number of studies are now measuring ozone fluxes in order to relate ozone uptake with observed plant responses.

7

1.2.2. Cellular damage

Once inside the leaf, ozone is very reactive and can dissolve in water in the apoplast to produce active decomposition products that include superoxide ($O_2^-\cdot$), hydroxyl ($OH\cdot$) and peroxy ($HO_2\cdot$) radicals (Heath, 1987). It is also thought that ozone may react with hydrocarbons such as ethylene which are emitted by plants, leading to free radical formation (Melhorn and Wellburn, 1987; Hewitt *et al.*, 1990; Salter and Hewitt, 1992). Since ozone is a natural component of the atmosphere, plants have evolved a range of mechanisms which enable them to tolerate the presence of low concentrations of ozone (Mansfield and Pearson, 1993; Iqbal *et al.*, 1996). Biochemical resistance to oxidative stress is brought about by antioxidants (e.g. enzymes such as peroxidases, catalase and superoxide dismutase) which react readily with oxidising agents, preventing harmful effects to the plant. Although evidence suggests that ozone exposure may induce biochemical defence systems (Kangasjarvi *et al.*, 1994), the operational capacity of these defences may be exceeded at elevated concentrations such that, disturbances to the cells may occur above a certain threshold level of ozone uptake (Moldau, 1993).

The cell membranes are thought to be the primary sites of ozone attack. In particular changes in the permeability of the plasmalemma have often been observed, causing cells to leak essential sugars, ions (especially potassium) and amino acids (Evans and Ting, 1974; Heath, 1975; Sutton and Ting, 1977; Dominy and Heath, 1985). At present, there is some uncertainty as to whether ozone *per se* or its reaction products are directly responsible for the various phytotoxic effects attributed to ozone, and also whether these compounds are capable of penetrating beyond the plasmalemma (Runeckles, 1992). The observation of Laisk *et al.* (1989) that the ozone concentration in the intercellular air spaces of sunflower (*Helianthus annuus*) was close to zero, even when plants were exposed to concentrations as high as 1000 ppb, suggests that ozone is highly reactive and that the apoplastic space and plasmalemma are major sinks for ozone. It has also been suggested that the oxidising compounds resulting from ozone breakdown cannot travel far before reacting (Heath, 1987), indicating that ozone and its reaction products may not be able to penetrate deeper into the cell (Heath, 1994). Nevertheless, exposure to ozone has been shown to cause ultrastructural changes within plant cells, including swelling of the thylakoids (Guderian *et al.*, 1985), rupturing of the chloroplast envelope (Sanders *et al.*, 1992) and injury to the mitochondria (Pell and Weissberger, 1976).

1.2.3. Effects on physiological processes

1.2.3.1. Stomatal conductance

The cells most exposed to attack by ozone are the stomatal guard cells and the mesophyll cells surrounding the substomatal cavity (Mansfield and Pearson, 1993). Numerous studies have shown that ozone can affect stomatal conductance, and many of these are summarised by Darrall (1989). In general, the responses of stomatal conductance are variable following exposure to ozone concentrations below 100 ppb (Darrall, 1989), as stomatal conductance may either increase (Evans and Ting, 1974; Olszyk and Tibbitts, 1981a; Reich and Lassoie, 1984; Eamus *et al.*, 1990; Hassan *et al.*, 1994) or decrease (Reich and Lassoie, 1984; Mulchi *et al.*, 1988; Fernandez-Bayon *et al.*, 1993; Weber *et al.*, 1993) depending upon species, cultivar and exposure conditions. At concentrations above 200 ppb, stomatal closure has been found in the majority of species examined (Darrall, 1989). As a result of ozone-induced reductions in stomatal conductance, injury to cells may be reduced or prevented due to the decreased uptake of ozone by the leaves; however, such a reduction in conductance may also decrease photosynthesis as a result of CO_2 limitation. Because of the interaction between photosynthesis and stomatal conductance, it is difficult to differentiate between direct effects of ozone on the guard or subsidiary cells and indirect responses resulting from effects on photosynthesis and intercellular CO_2 concentrations. Aben *et al.*, (1990) showed that the stomatal conductance of faba bean (*Vicia faba*) can be reduced by exposure to ozone without a concomitant decrease in the net rate of photosynthesis, suggesting a direct effect upon the stomata. Such an effect on the stomata could be brought about by changes in the elastic properties of the cell wall and the permeability of the plasmalemma (Heath, 1987). There is also evidence, however, that stomatal conductance may be affected indirectly through effects on photosynthesis (Weber *et al.*, 1993). For example, Temple (1986) reported a decrease in stomatal conductance in ozone-exposed cotton (*Gossypium hirsutum*) resulting from the increased intercellular CO_2 concentration which occurred following inhibition of photosynthesis.

The response of stomatal conductance to ozone exposure differs between species (Hassan *et al.*, 1994), populations (Reiling and Davison, 1995), cultivars (Guzy and Heath, 1993) and individuals (Grulke *et al.*, 1996), and these responses may be important in determining sensitivity to ozone. For example, Knudson Butler *et al.* (1979) noted that resistant cultivars of pinto bean (*Phaseolus vulgaris*) closed their stomata more completely than susceptible cultivars in the presence of ozone, while Hassan *et al.* (1994) showed that exposure to 80 ppb ozone for 8 h d^{-1} for 7 d

9

decreased conductance in turnip (*Brassica rapa* cv. Sultani) but increased conductance in radish (*Raphanus sativus* cv. Baladey), a more ozone-sensitive species. In contrast, Dijak and Ormrod (1982) found a greater decrease in stomatal conductance in response to ozone in a sensitive than in a resistant cultivar of pea (*Pisum sativum*), suggesting that resistance may also depend upon internal tolerance and repair mechanisms.

1.2.3.2. Photosynthesis

Although there have been reports that ozone may increase the net rate of photosynthesis (Eamus *et al.*, 1990), the majority of studies have shown that both short-term (hours) exposures to high concentrations of ozone (>100 ppb; Hill and Littlefield, 1969; Bennett and Hill, 1973; Farage *et al.*, 1991) and long-term (weeks) exposures to low concentrations (<100 ppb; Barnes, 1972; Reich and Amundson, 1985; Gagnon and Karnosky 1992; Pearson, 1995) can result in rapid (minutes or hours) reductions in net photosynthesis. Such effects may be transient (Dann and Pell, 1989) or permanent. Black *et al.* (1982), for example, observed that net photosynthesis was decreased by a 4 h exposure of faba bean (*Vicia faba*) to ozone concentrations between 50-300 ppb, although the rate returned to prefumigation values in plants which had been exposed to less than 90-100 ppb, while above this concentration the effect on photosynthesis was irreversible. The response of photosynthesis to ozone exposure has also been shown to vary with leaf age, with the youngest and oldest leaves being least sensitive (Taylor *et al.*, 1982; Myhre *et al.*, 1988).

The effect of ozone on photosynthesis was initially ascribed to a decrease in stomatal conductance and a consequent reduction in the availability of CO_2 for photosynthesis (Hill and Littlefield, 1969). Subsequently, evidence was produced to suggest a direct effect on photosynthesis; for example, Myhre *et al.* (1988) reported a 10-25 % reduction in photosynthesis in the flag leaves of oats following a 2 h exposure to 150 ppb ozone, without any concomitant change in stomatal conductance. Changes in the net rate of photosynthesis may be brought about by effects on photosynthesis and/or respiration, although the precise mechanism(s) involved remain unclear. Because ozone appears to be highly reactive within the cell walls and plasmalemma (Laisk *et al.*, 1989) it is unlikely that significant quantities of ozone actually reach the chloroplast. It is therefore thought that alterations within the chloroplast may arise indirectly through the generation of oxidising radicals or alterations to the plasmalemma (Guderian *et al.*, 1985; Heath, 1994). Studies have shown that exposure to ozone may affect the photosystems (Chang and Heggestad, 1974), the activity and quantity of RuBisCO (Pell and Pearson, 1983; Lehnherr *et al.*, 1987; Farage *et al.*, 1991; Pell *et*

al., 1992; Mulholland et al., 1997b), the chlorophyll pigment system (Reich, 1983), electron transport (Schrieber et al., 1978) and the chloroplast membranes (Mudd, 1984).

1.2.3.3. Respiration

Increases in dark respiration are often observed following exposure to ozone (Todd, 1956; Botkin et al., 1971; Barnes, 1972; Pell and Brennan, 1973; Reich, 1983; Skärby et al., 1987), but decreases have also been reported (Yang et al., 1983; Lehnherr et al., 1987). Increases in respiration are thought to occur because of the extra energy required for repair, maintenance and detoxification processes (Sutton and Ting, 1977). Thus, Skärby et al., (1987) attributed an accumulated 60 % increase in dark respiration during and after a one month exposure of Scots pine (Pinus sylvestris) to an increasing concentration of ozone (60-200 ppb), to direct effects on cell metabolism and an after-effect associated with the repair of damaged membranes. Following longer exposures, the respiration rates of various organs, particularly the roots, declined, possibly as a consequence of a reduced assimilate availability caused by decreases in photosynthesis and increased consumption of assimilates to support maintenance and repair processes (Miller, 1987).

1.2.4. Visible injury

Ozone has been reported to cause a range of visible foliar symptoms which vary depending upon the species and variety of plant and the concentration and duration of ozone exposure (Krupa and Manning, 1988). Characteristic symptoms of acute ozone injury appear as white necrotic lesions, often in the form of stipples or flecks on the upper surface of the foliage, as a result of localised cell death adjacent to the stomata. Acute exposure to ozone may also induce other symptoms of damage, including tissues which appear waterlogged, brown necroses, bronzing of leaves, interveinal necrotic streaks, reddish pigmentation of leaves and tip burn on conifer needles (Hill et al., 1970). At lower ozone concentrations, chronic symptoms of injury include chlorosis (Bell, 1978) and premature senescence of the leaves (Reich and Lassoie, 1985; Held et al., 1991). Visible foliar damage can reduce crop quality, particularly when the marketable parts of the plant are the leaves. A good example is the tobacco (Nicotiana tabacum) cultivar, Bel W-3, which can be injured by hourly mean concentrations of ozone above 40 ppb (Hewitt et al., 1990). This cultivar is regarded as being highly sensitive to ozone and has been used as a biomonitor of ozone in many studies (Bell and Cox, 1975; Ashmore et al., 1978; Koppel and Sild, 1995; Gimeno et al., 1995).

In experiments where plants have been exposed to ozone under similar environmental conditions, the extent of visible injury has been shown to vary between species and cultivars (Dijak and Ormrod, 1982; Berrang *et al.*, 1989) as well as between leaves of different age (Heath, 1994). It has been suggested that this may be due to variability in ozone uptake resulting from differences in stomatal conductance (Nebel and Fuhrer, 1994), stomatal density (Knudson Butler and Tibbitts, 1979b; Evans *et al.*, 1996) or stomatal responses to ozone exposure (Knudson Butler *et al.*, 1979). Other studies, however, have shown little or no correlation between stomatal conductance and visible leaf damage (Olszyk and Tibbitts, 1981a). More recently, it has been recognised that the interaction of ozone with stress ethylene may be important in determining visible injury (Mehlhorn and Wellburn, 1987; Hewitt *et al.*, 1990; Mehlhorn *et al.*, 1991). In a study by Mehlhorn and Wellburn (1987), seedlings of pea (*Pisum sativum*) were exposed to ozone concentrations ranging from 50-150 ppb for 7 h d^{-1} for the first three weeks of growth, or to charcoal-filtered air for three weeks followed by the same concentrations of ozone for a single 7 h period. In seedlings fumigated with ozone for three weeks no visible leaf damage occurred, whereas severe leaf necrosis developed in those fumigated for only one day. Further studies suggested that these differences could be attributed to the quantity of ethylene being produced since plants exposed for three weeks to 150 ppb ozone emitted ethylene at about one tenth of the rate of the clean air control plants, while ethylene production was almost doubled in plants grown for three weeks in clean air prior to exposure to 150 ppb for 7 h. It has been proposed that ozone may react with ethylene to produce a series of free radicals which then elicit injury symptoms (Hewitt *et al.*, 1990).

The extent of visible leaf damage has been correlated with decreased growth in some (Pääkkönen *et al.*, 1993) but not all species (Kress and Skelly, 1982; Fuhrer *et al.*, 1993). Indeed, effects on physiology and reductions in growth may occur in the absence of visible leaf injury (Adams, Kelly and Edwards, 1988; Gagnon and Karnosky, 1992), indicating that visible injury may not be a useful indication of plant sensitivity to ozone.

1.2.5. Effects of ozone on growth and biomass allocation

It is well documented that exposure to ozone can have deleterious effects on growth (e.g. Reich and Amundson, 1985; Kobayashi *et al.*, 1995), but there are also reports that ozone may promote growth (Rajput and Ormrod, 1986), especially following exposure to low concentrations (Deveau *et al.*, 1987). Reductions in growth have been related to a decline in photosynthesis (Reich and Amundson, 1985), although this relationship is not unequivocal since decreases in growth have been observed in the

absence of any decrease in net photosynthesis (Chevone and Yang, 1985; Takemoto *et al.*, 1989). Changes in assimilate partitioning between the roots and shoots also occur in response to ozone stress (Cooley and Manning, 1987). In general, the allocation of carbohydrates to the roots is decreased, resulting in a decrease in the root:shoot ratio (Tingey *et al.*, 1971; Oshima *et al.*, 1978, 1979; Bennett *et al.*, 1979; Walmsley *et al.*, 1980; Deveau *et al.*, 1987; Held *et al.*, 1991), and potentially increasing sensitivity to frost, heat and water stress. An increase in leaf initiation has also been observed in response to ozone, and may represent a mechanism which enables plants to replace injured leaf tissue, thereby maintaining assimilation and relative growth rates (Walmsley *et al.*, 1980; Held *et al.*, 1991). In contrast, one of the most noticeable effects of exposure to ozone at the plant level is accelerated leaf senescence (Reich and Lassoie, 1985; Held *et al.*, 1991). The precise mechanism by which ozone alters resource partitioning has not been elucidated, although there is evidence to suggest that ozone may inhibit translocation as a result of effects on phloem loading (Spence *et al.*, 1990; Pausch *et al.*, 1996).

1.2.6. General effects of ozone on crops

Because of the economic significance of crop loss, a considerable amount of research has examined the effects of both ambient and elevated concentrations of ozone on crop species (Jacobson, 1982; Heagle, 1989). Although the effects of ozone on crops can be assessed in closed chambers (e.g. Endress and Grunwald, 1985), more realistic responses are observed when plants are exposed in the field in open-top chambers (Jacobson, 1982). The first large scale research programme into the effects of ozone on crop species was conducted between 1980 and 1985 by the US National Crop Loss Assessment Network (NCLAN) established by the US Department of Agriculture and the US Environmental Protection Agency. The primary purpose of the NCLAN programme was to assess the effects of ozone on the yield of 14 economically important crop species and to use this information to develop predictive models of yield losses resulting from ozone exposures for use in establishing national air quality standards (Heck *et al.*, 1988). Comparisons were made in open-top chambers of crops grown in charcoal-filtered air with those grown in unfiltered air. Responses to increased concentrations of ozone were also investigated. The results showed that ambient concentrations of ozone in the US caused substantial crop losses and that an increase in the mean ozone concentration from 25 to 60 ppb reduced yields by up to 20 % in 13 of the 14 species examined (Lesser *et al.*, 1990). It was estimated that a reduction in ozone concentrations of 40 % would benefit the US economy by $2.0 billion a year (Heck *et al.*, 1988). The results of the NCLAN study could not be used to predict crop responses in Europe because of differences between the two continents

in climatic conditions, ozone exposure regimes and crop cultivars. In Europe, the European Open-Top Chamber (EOTC) network, sponsored by the Commission of the European Communities, was conducted between 1985 and 1991 (Unsworth and Geissler, 1993). Using a similar approach to that of NCLAN, the EOTC programme aimed to quantify the effects of ozone on crop species and establish the mechanisms of ozone action and the influence of other environmental factors which may modify plant responses to ozone (Jäger et al., 1993). Controlled filtration studies showed that the ambient air pollution climate over Europe was sufficient to reduce crop yields, particularly in spring wheat (Triticum aestivum), although there was no consistent effect of ambient air pollution on yield in other species including green beans (Phaseolus vulgaris; Unsworth and Geissler, 1993). Studies of yield-concentration relationships using multiple ozone treatments indicated that a 10 ppb increase in the seasonal mean ozone concentration might decrease the yield of green beans and spring wheat by about 25 and 10 % respectively (Unsworth and Geissler, 1993). More recent work has suggested that the increasing concentrations of other atmospheric gases such as CO_2 may be important in influencing the impact of ozone on the growth and yield of crop plants (Mulholland et al., 1997a, b, 1998a, b).

The increased awareness of and concern over the effects of acidifying gaseous emissions have led to the development of the critical levels concept as a scientific means of assessing and planning air pollution control strategies both nationally and internationally (Bull, 1991). In order to protect the most sensitive species, critical levels for ozone have been established based on the cumulative exposure over a threshold concentration of 40 ppb ozone or AOT40 (Accumulated exposure Over a Threshold of 40 ppb), calculated as the sum of the differences between the hourly ozone concentration and the threshold value of 40 ppb for each hour that the concentration exceeds 40 ppb. Using data from exposure-response relationships of wheat, an AOT40 value for the protection of crops against a mean annual yield loss of 5 % was set at 3000 ppb.h accumulated during daylight hours for the three months when the crop is most sensitive to ozone (May, June and July for Northern Europe). Over substantial areas of the UK, it is apparent that this critical level is being exceeded (CLAG, 1996). However, there is still some uncertainty about the influence that other factors, such as environmental conditions and the presence of other pollutants, may have on plant responses to ozone. Further research is required to incorporate these factors into the critical levels approach and to validate critical levels for other crop species and cultivars for which limited data are available regarding ozone sensitivity (CLAG, 1996).

1.2.7. General effects of ozone on forests and wild species

Reports of forest decline in the USA (Miller, 1983) and Europe (Ashmore et al., 1985) were among the first to suggest that ozone may affect native plant species. In Europe, this premature defoliation and dieback, or 'Neuartige Waldschäden' (new kind of forest damage), first appeared in Germany in the early 1970s but has since spread to other countries including Austria, Switzerland, France, the Netherlands and southern Sweden (Ashmore et al., 1985). Despite more than 10 years of research into forest decline, it is still not clear whether ozone causes damage by acting directly or indirectly through interactions with other environmental stresses, although increasing evidence suggests that the interaction of ozone with acid mist may result in mineral nutrient deficiency (Schmieden and Wild, 1995) and/or an increased sensitivity of trees to drought, winter desiccation and freezing (Barnes and Davison, 1988; Barnes et al., 1990; Brown et al., 1987).

In contrast to crops and economically important tree species, relatively little is known about the impact of ozone on native species and semi-natural ecosystems. Controlled fumigations of individual species have shown that there is a wide range in ozone sensitivity in both tree (Pye, 1988; Kickert and Krupa, 1990) and herbaceous species (Ashmore et al., 1988; Reiling and Davison 1992a; Mortensen, 1992; Mortensen and Nilsen, 1992; Gagnon and Karnosky, 1992; Nebel and Fuhrer, 1994; Bergmann et al., 1995; Warwick and Taylor, 1995; Potter et al., 1996; Bergmann et al., 1996). Ozone sensitivity, assessed in terms of visible leaf injury, of c. 150 native British herbaceous species exposed to 250 ppb for 4 h, has shown that certain families, such as the Papilionaceae, contain a high proportion of sensitive species, while others, such as the Compositae, contain very few (Ashmore et al., 1988). In addition, those species characteristic of calcareous habitats tend to be more sensitive than those from acid habitats, suggesting a link between ozone sensitivity and calcium nutrition (Ashmore et al., 1988). A study by Reiling and Davison (1992a) has shown a wide range in ozone sensitivity among a number of native British species when exposed to 70 ppb ozone for 7 h d^{-1} over a two week period. In particular, it appeared that *Plantago major* was as sensitive to ozone as the tobacco cultivar Bel W-3 in terms of growth reduction, although, unlike Bel W-3, *P. major* showed no visible foliar symptoms. Results from this study also suggested that the response of individual species in terms of the relative growth rate may be related to their intrinsic growth strategy as defined by Grime et al. (1989), with ozone having a greater effect on species with a highly competitive or ruderal growth habit than on species categorised as stress-tolerators.

15

While controlled fumigation studies may prove useful in defining the responses of individual species, the data obtained cannot necessarily be extrapolated to predict the response of natural communities since the extent to which plants respond to ozone depends not only on the characteristics of the dose expressed in terms of concentration, duration and frequency, but also on other factors such as the age of the plant (Evans and Ting, 1974; Hanson *et al.*, 1975; Blum and Heck, 1980; Richards *et al.*, 1980; Pääkkönen *et al.*, 1995), environmental conditions such as light, temperature and humidity (Guderian *et al.*, 1985; Mortensen, 1989; Mortensen and Nilsen, 1992), water stress (Amundson *et al.*, 1986; Temple, 1986; Vozzo *et al.*, 1988), nutrient availability (Guderian *et al.*, 1985), and the presence of other pollutants (Darrall, 1989). Exposure to ozone may also predispose plants to other stresses such as frost (Foot *et al.*, 1997), insects (Stark *et al.*, 1968; Holopainen *et al.*, 1997) or fungal attack (Tiedemann and Fehrmann,1988). In both natural and agricultural communities, the competitive ability of individual species may be altered by ozone treatment (Bennett and Runeckles, 1977). Several studies of grassland communities have examined the influence of ozone on competition by exposing artificially composed communities in open-top or closed chambers (Ashmore and Ainsworth, 1995; Ashmore *et al.*, 1995; Mortensen, 1997). These studies have shown that the effect of ozone on sensitive species is greater when plants are grown in an inter-specific competitive environment than when grown in monocultures because decreases in the growth of ozone-sensitive species allow ozone-tolerant species to compete more effectively for available resources. This suggests that, in the long-term, sensitive species could disappear from natural vegetation communities due to a decrease in their competitive ability.

Predictions of future changes in ecosystem structure are complicated further because natural variability between plants may lead to selection for ozone resistance (Berrang *et al.*, 1989; Heagle *et al.*, 1991; Reiling and Davison, 1992b). Reiling and Davison (1992b), for example, have shown that populations of *Plantago major* from areas of high ozone pollution are more ozone-tolerant than populations from areas where ozone concentrations are lower, suggesting that there may be natural selection for resistance to ozone. However, it is difficult to prove that differences in resistance between populations result from the local pollution climate rather than other environmental factors which show similar spatial or temporal variability, for example, frequency of high radiation levels. Further work by Davison and Reiling (1995) suggested that ozone tolerance in *P. major* may evolve rapidly in response to year-to-year changes in ozone concentrations, although it is still unclear whether differences in ozone resistance between populations are the result of adaptation of individuals to ozone stress, natural selection within populations for resistance, or the spread of more tolerant genotypes into existing populations.

Because of the longevity and size of plants, their complex interactions and their differing responses, very few studies have attempted to examine the integrated response of ecosystems to ozone stress. The limited information available and uncertainty concerning the responses of semi-natural plant communities have therefore made it difficult to set critical levels of ozone for natural vegetation (Ashmore and Davison, 1996). Future research is required to determine the response of other native plant species to ozone stress, not only in terms of effects on vegetative growth but also in terms of reproductive output, since this is a major determinant of the long-term success of many species, particularly annuals.

1.3. EFFECTS OF OZONE UPON REPRODUCTION

Since sexual reproduction is critical to the economic value of many crops and the survival of many wild plant species, any adverse effects of air pollution on reproductive biology and seed yield may have important consequences for both agricultural and natural ecosystems. Although reproductive development is known to be sensitive to environmental stresses such as temperature extremes (Rao *et al.*, 1992) and drought (Ney *et al.*, 1994), and the vegetative structures of plants have been shown to be sensitive to air pollutants (cf. Section 1.2.3), there has been relatively little investigation of the impact of air pollution on reproductive biology. Several studies have shown that exposure to pollutants such as SO_2 (Roberts, 1984) and ozone (Jäger *et al.*, 1993) may decrease crop yields, but there is little information pertaining to the effects of air pollutants on native trees or herbaceous species. Moreover, those studies which have examined the impact of SO_2 and ozone on crop yields have generally exposed both the vegetative and reproductive structures to the pollutant, making it impossible to separate direct effects on the reproductive structures from indirect effects mediated via damage to the vegetative parts of the plant. Several potential points of interaction between reproductive elements and air pollutants have been identified (Fig. 1.1.; Wolters and Martens, 1987), all of which may be affected either directly and/or indirectly by air pollutants. The present investigation attempted to investigate the direct effects of ozone on reproductive development in *Brassica campestris* and *Plantago major*. The following sections review the known impacts of ozone on the reproductive biology of both crop and native plant species and consider the direct effects of ozone on reproductive processes.

1.3.1. Timing of reproduction

Exposure to ozone has been shown to delay flowering in a number of species, including soybean (*Glycine max*; Amundson *et al.*, 1986), cotton (*Gossypium*

Developmental stage   **Air pollution influence**

flowers

pollen production   ⟵———— reduced pollen production

pollination   ⟵———— reduced pollen distribution
                                 and germination

fertilisation   ⟵———— reduced pollen growth

fruit and seed set   ⟵———— reduced fruit or seed production

seeds

seed germination   ⟵———— reduced seed germination

seedlings

seedling development   ⟵———— restricted seedling growth

juvenile development

mature plants

flower initiation   ⟵———— reduced flower initiation

senescent plants

Figure 1.1. Potential points of interaction between air pollutants and sexual reproductive processes in higher plants (after Wolters and Martens, 1987).

hirsutum; Oshima *et al.*, 1979), duckweed (*Lemna perpusilla*; Feder and Sullivan, 1969b), geranium (Feder, 1970) and carnation (*Dianthus caryophyllus*; Feder and Campbell, 1968). For example, in soybean plants exposed over an eight week period for 6.8 h d^{-1} to 10, 50, 90 or 130 ppb ozone, flowering started at 37, 40, 42 and 44 days after seedling emergence respectively (Amundson *et al.*, 1986). In contrast, Bergmann *et al.* (1996) showed that the time of flowering and seed set in 17 herbaceous species was not significantly affected by exposure throughout the growing season to 1.5 times the ambient ozone concentration when compared to plants which had experienced ambient concentrations. Potentially, any alteration in the time to first flowering may have consequences for seed set, particularly for those species which require flowering to be synchronous with the presence of pollinator species.

1.3.2. Fruit and seed yield

1.3.2.1. *Crop species*

Although there is evidence that ambient (e.g. Vandermeiren *et al.*, 1995) and elevated (e.g. Mulchi *et al.*, 1988) concentrations of ozone may reduce the yield of seed crops, there has been relatively little investigation of the effects of ozone on specific yield components or seed development (Ormrod, 1996).

In seed crops, ozone has been shown to reduce yield by decreasing the number of pods (Vandermeiren *et al.*, 1995), seeds (Shannon and Mulchi, 1974), individual seed weight (Sanders *et al.*, 1992), and both seed number and seed weight (Fuhrer *et al.*, 1989; Finnan *et al.*, 1996). The relative effects of ozone on the various components of seed yield have been shown to vary depending on factors such as the ozone concentration and exposure regime and the timing of exposure. Finnan *et al.* (1996), for example, reported that exposure to ozone during the growing season decreased yield in spring wheat (*Triticum aestivum* cv. Promessa) by reducing both grain weight and the number of grains per ear; however, for a given ozone dose, short-term, high-concentration (ambient + 50 ppb ozone) exposures had a greater effect on grain yield than long-term, low-concentration (ambient + 25 ppb ozone) exposures, suggesting that the response of the plant is more dependent upon the concentration of ozone than on the duration of exposure. In contrast to the effects observed by Finnan *et al.* (1996), Amundson *et al.* (1987) reported that exposure of wheat from anthesis reduced yield by decreasing only the grain weight, suggesting that the timing of exposure may be important in determining the response of individual yield components.

Shannon and Mulchi (1974) also showed that the effects of ozone on the components of seed yield varied between cultivars. In their study, two cultivars of wheat were exposed from anthesis to 200 ppb ozone for 4 h d^{-1} for one week; it was noted that, although yield was reduced in both cultivars, grain weight in cv. Arthur '71 was more sensitive to ozone exposure than grain number, while in cv. Blueboy, grain number appeared to be the most sensitive yield parameter. Evidence from other studies suggests that the effect of ozone on grain weight in wheat results from a decrease in kernal growth rate rather than the duration of kernal fill (Slaughter et al., 1993; Mulholland et al., 1998b).

The adverse effects of ozone on crop yield have been shown to increase with increasing concentrations of ozone; for example, a season-long exposure of spring wheat cv. Minaret to a seasonal mean of 60 ppb ozone (7 h d^{-1}) was shown by Mulholland et al. (1997a) to have no significant effect on grain yield. However, when the seasonal concentration was increased to 84 ppb ozone, there was a significant 30 % reduction in grain yield relative to control plants resulting from a combination of decreases in the number of ears, the numbers of grains per ear and per spikelet, and individual grain weight (Mulholland et al., 1998a). Retzlaff et al. (1997) have also shown that the number of fruits per tree, and therefore the yield of plum (Prunus salicina, cv. Casselman), decreased as ozone concentrations increased, while Kress and Miller (1983) reported a decrease in the number of filled pods per plant and seeds per filled pod in soybean (Glycine max) in response to increased ozone stress. In snap bean (Phaseolus vulgaris), pod dry weight was decreased more by two 1.5 h exposures to 60 ppb than by two exposures to 30 ppb irrespective of the stage of plant development (Blum and Heck, 1980).

There is also evidence to suggest that ozone sensitivity may differ between the vegetative and reproductive structures. For example, when Oshima et al. (1975) exposed tomato (Lycopersicon esculentum) plants to 200 or 350 ppb ozone for 2.5 h d^{-1}, 3 d wk^{-1} for 15 weeks, the weight of the stems and leaves was reduced by both exposures; however, the weight of fruit was only reduced following exposure to 350 ppb ozone, indicating that the reproductive structures were less sensitive than the vegetative components. This decrease in the yield of tomato resulted from the production of fewer fruits rather than a decrease in fruit weight (Oshima et al., 1975). Similarly, Oshima et al. (1979) reported that exposure to ozone decreased boll production in cotton (Gossypium hirsutum), although the size of the bolls and the proportions of boll components were unaffected by treatment. Ozone has also been reported to decrease floral initiation in muskmelon (Cucumis melo) and watermelon (Citrullus lanatus; Fernandez-Bayon et al., 1993). In general, because both the

vegetative and reproductive structures of plants were exposed to ozone in the above studies, it was impossible to determine the extent to which effects on reproductive output resulted from the direct impact of ozone on the reproductive structures rather than indirect effects mediated via damage to the vegetative structures.

In contrast to the reductions in yield which have generally been reported, long-term exposures to low concentrations of ozone have been shown to increase seed yield in wheat (*Triticum aestivum*, Finnan *et al.*, 1996) and soybean (*Glycine max*, Endress and Grunwald, 1985), and to increase the number of pods per plant and hence seed yield in *Phaseolus vulgaris* (Sanders *et al.*, 1992).

1.3.2.2. *Native species*

Relatively few studies have examined the effects of ozone on the reproductive output of native species. In a study of white pine and red pine, Houston and Dochinger (1977) showed that polluted air decreased seed number, seed weight and percentage pollen germination. Cela Renzoni *et al.* (1990) showed that the germination of *Pinus pinea* pollen was adversely affected to a greater extent in plants from sites which experienced high levels of pollution than in plants grown at cleaner locations. However, in both studies the effects could not be attributed specifically to ozone.

In a recent study, exposure of 17 herbaceous species to 27 ppb (24 h mean) ozone throughout the growing season was shown to decrease the number of reproductive organs in the majority of the species examined, and this decrease appeared to be related to the extent of visible leaf injury (Bergmann *et al.*, 1996). However, despite a decrease in leaf biomass in response to ozone-treatment, *Trifolium arvense* and *Papaver dubium* respectively produced increased numbers of inflorescences and seeds. *Malva sylvestris* was shown to be the most ozone-sensitive species in terms of effects on reproduction since there were significant decreases in both seed number and 1000 seed weight. Ozone has also been shown to cause significant reductions in the number and weight of reproductive structures of certain grass species (Price and Treshow, 1972). Two studies have shown that exposure to ozone may reduce seed yield in *Plantago major*, but that the responses vary depending upon both the population and ozone exposure regime (Pearson *et al.*, 1996) and the stage of development during exposure (Reiling and Davison, 1992b).

1.3.3. Seed quality

Because seeds are an important source of nutrition, several studies have investigated the effect of ozone on the quality of seeds from crop plants. Investigations in the field have shown that ambient air pollution can have significant effects on both seed (Scotti *et al.*, 1994) and fruit (Crisosto *et al.*, 1993) quality; however, in these studies it was impossible to identify the pollutant(s) responsible for inducing the observed effects. In controlled fumigations of soybean (*Glycine max*, Kress and Miller, 1983; Mulchi *et al.*, 1988; Pleijel *et al.*, 1989) and wheat (*Triticum aestivum*, Kress and Miller, 1983; Mulchi *et al.*, 1986; Fuhrer *et al.*, 1990; Finnan *et al.*, 1996; Rudorff *et al.*, 1996; Pleijel *et al.*, 1997) seed protein content was observed to increase with increasing ozone concentration. In contrast, Grunwald and Endress (1988) noted a significant increase in the oil content of soybeans following exposure to ozone but not in protein content. In Lima bean (*Phaseolus limensis*), ozone exposure increased the concentration of nitrogen and several essential amino acids in the seeds (Meredith *et al.*, 1986), while in spring wheat, Fuhrer *et al.* (1990) showed that ozone increased the mineral content (Ca, Mg, K, P) of the grain. Such effects on seed quality may potentially affect the viability, dormancy, germination or longevity of seeds, and in wild plant species may have potential consequences for the development of the progeny. To date, however, there has been no examination of the impact of ozone on the seed quality of wild species.

1.3.4. Direct impact of ozone upon reproduction

The adverse effects of ozone on reproductive development have generally been attributed to injury to the vegetative structures because it was thought that this would decrease the quantity of photosynthate available to support reproduction and/or alter the partitioning of assimilates to the reproductive structures (Cooley and Manning, 1987). However, while damage to reproductive structures may occur in this way, flowers and fruit structures may also come in contact with ozone and be damaged directly. Since most previous studies have exposed the vegetative and reproductive structures simultaneously to ozone, it has been impossible to separate any direct effects on the reproductive structures from indirect effects mediated via injury to the vegetative component. To date, the majority of evidence for a direct effect of ozone on reproduction has come from studies of pollen germination and pollen tube growth. An early study of petunia (*Petunia hybrida*) showed that ozone may alter the topography of the stigmatal surface (Harrison and Feder, unpublished data, cited in Feder and Shrier, 1990), suggesting a direct effect on the maternal structures. A more recent study of *Brassica napus* by Bosac (1992) provided evidence that ozone may affect the

reproductive structures directly, resulting in significant effects on floral development, seed yield and seed quality. The present study follows a similar approach to that of Bosac (1992) by investigating the direct effects of ozone on the reproductive development of *Brassica campestris* and *Plantago major*. The following sections review current knowledge concerning the direct effects of ozone on pollen and on other aspects of reproductive development in *Brassica napus*.

1.3.4.1. Pollen

The first observations that ozone may directly affect pollen were made by Feder in 1968. In their study, exposure of pollen from the ozone-sensitive tobacco (*Nicotiana tabacum*) variety Bel W-3 to 100 ppb of ozone for 5.5 h, either *in vitro* on agar plates or *in vivo* on the anthers, caused a significant c.50 % reduction in both pollen germination and pollen tube growth. Subsequent studies showed that ozone may affect pollen in a number of species, although there are also reports which suggest that pollen is insensitive to ozone (Bosac *et al.*, 1993), even at high concentrations (Krause *et al.*, 1975; Masaru *et al.*, 1976). As well as varying between species, the sensitivity of pollen has been shown to vary within species. Feder and Sullivan (1969b), for example, exposed pollen from two cultivars of tobacco (Bel W-3 and Bel B) and petunia (*Petunia hybrida,* White Cascade and Blue Lagoon), one of which was ozone-tolerant and the other ozone-sensitive with regards to visible injury, to 100 ppb ozone for 5.5 h and showed that the reductions in pollen germination and pollen tube growth were positively correlated with the ozone sensitivity of the parent leaf. For a number of species, the reductions in germination and pollen tube growth have been shown to be greater at higher ozone concentrations (Feder, 1981; Hormaza *et al.*, 1996), and for this reason Feder (1981) suggested that pollen from ozone-sensitive species could be used as a bioassay for air quality.

Although pollen germination and/or pollen tube growth may be affected by exposure to ozone either *in vivo* on the anthers (Feder, 1968) or *in vitro* (Feder, 1968; Masaru *et al.*, 1976; Hormaza *et al.*, 1996), there is evidence to suggest that pollen may be protected from ozone damage when exposed on the stigma. Krause *et al.* (1975) observed that the germination of pollen from petunia (cv. White Bountiful) and tomato (*Lycopersicon esculentum* cv. Tiny Tim) was reduced significantly by *in vitro* exposure to 10 ppb ozone for 3 h, whereas germination was unaffected when pollen was exposed to 80 ppb of ozone for 6 hours after direct transfer to the stigma. Therefore, although *in vitro* exposure and germination of pollen is a useful tool in the study of pollen physiology, it may not provide a realistic indication of the susceptibility of pollen exposed and germinated *in vivo*.

The precise mechanism(s) by which ozone damages pollen have not been established. Feder (1970) showed that the inhibition of pollen germination observed following exposure to low concentrations of ozone could be reversed by returning the pollen to a normal environment, and suggested that a chemical regulator of pollen tube growth was temporarily inactivated by the oxidising effect of ozone. Subsequently, Harrison and Feder (1974) showed that exposure of petunia pollen to 500 ppb ozone for 3 h reduced germination by 80 % in an ozone-sensitive cultivar (White Bountiful) and 15 % in an ozone-tolerant cultivar (Blue Lagoon). Detailed examination following ozone exposure showed that there was a peripheral band of cytoplasm which was free of all organelles except ribosomes in over 50 % of the pollen from the ozone-sensitive cultivar, while in the ozone-tolerant cultivar fewer pollen grains showed this cytoplasmic change. The authors suggested that this change in the internal organisation of the pollen grain may have contributed to the observed effects on pollen germination.

Although significant effects of ozone on pollen germination and tube growth have been demonstrated, the consequent effects on fertilisation and seed yield have not been determined. In addition, there is no information concerning the influence of ozone on the quantity of pollen produced by individual flowers and whether this may have consequences for successful pollination. In wind pollinated species, air pollution is unlikely to affect the movement of pollen between plants (Wolters and Martens, 1987), whereas in insect pollinated species, ozone may affect pollen distribution by influencing insect behaviour and hence pollinator visitation, although there have been no investigations of this aspect to date.

1.3.4.2. *Other reproductive processes*

A previous study of two oilseed rape (*Brassica napus*) cultivars, Tapidor and Libravo, demonstrated that a single 6 h exposure of the reproductive organs to 100 ppb ozone or 100 ppb ozone plus 30 ppb sulphur dioxide can have significant effects on reproductive development (Bosac, 1992). Following exposure to 100 ppb ozone, significant increases in bud abortion and abscission were observed 2 and 5 d after exposure in cv. Tapidor and 5 d after exposure in cv. Libravo (Bosac *et al.*, 1994). Different racemes exhibited varying sensitivity to ozone, with reproductive site losses being greatest in the second and third laterals (Bosac, 1992). Ozone-treated plants of both cultivars continued to exhibit greater fertile site losses up to 25 d after exposure, although the number of fertile sites present was not significantly different from the control plants, indicating that the former were able to compensate for the loss of fertile sites (Bosac *et al.*, 1994). Despite the apparent compensation for losses of fertile sites in cv. Libravo, seed yield at final harvest was significantly reduced, whereas in cv. Tapidor, which had

24

initially sustained greater fertile site losses, seed yield was maintained (Bosac *et al.*, 1999). Exposure to ozone also produced significant effects on seed quality in cv. Libravo, in which significant reductions in the oil, protein and soluble carbohydrate content of the seeds were observed (Bosac *et al.*, 1998). Although the germination of seeds was unaffected by treatment, the root and shoot growth of seedlings from ozone-treated plants of cv. Libravo was significantly reduced, indicating that the effects of ozone on the parent plant may be carried through to the next generation (Black *et al.*, 1993).

Because the seeds of oilseed rape are the harvestable component, the direct effects of ozone on final seed yield and seed quality may be of agricultural significance. It is therefore important to establish whether single or multiple short-term exposures to ozone may bring about similar direct effects, not only in other species and cultivars of seed and fruit crops, but also in native species which rely on sexual reproduction for their ecological success. Detailed examinations of the effects of ozone on reproductive biology would also be valuable to ascertain whether plants have mechanisms which allow them to compensate for ozone-induced damage to the reproductive structures and thereby maintain seed yield and quality under stress conditions.

1.3.5. Reproductive growth habit

Although the observed ozone induced increases in bud abortion in *Brassica napus* cv. Tapidor, might have been expected to reduce seed yield, no long-term effect on seed yield was detected because of the intrinsic ability of oilseed rape plants to compensate for the loss of reproductive sites (Bosac 1992). This was possible because the indeterminate reproductive growth habit of *B. napus* provided the flexibility to produce additional reproductive sites following exposure to ozone. In *B. napus*, although the number of flower initials which can develop on individual inflorescences is ultimately limited, under normal field conditions as little as 2-10 % of the flowering potential of rapeseed is realised because many floral initials fail to develop to the point of flower opening (McGregor, 1981). Thus, because flowering occurs over an extended period, the plants have the potential to compensate for the loss of floral sites during early reproductive development caused by herbivory or adverse environmental conditions by retaining or developing more sites to maturity.

In contrast to *B. napus*, some species have developed a reproductive strategy which provides less flexibility to compensate for reproductive site losses. For example, in species with a determinate or semi-determinate reproductive growth habit, a limited number of flowers are produced over a relatively short time period; thus a single ozone

episode may have a dramatic effect on reproductive output if it coincides with a particularly sensitive stage of reproductive development. In other species, for example *P. major*, individual inflorescences are determinate because all of the floral initials are formed before flowering begins; since the apical meristem eventually forms a terminal floral initial, the plants cannot produce additional flowers on individual spikes. It could be hypothesised therefore that plants with a determinate reproductive growth habit may be affected more severely by exposure to ozone because of their limited capacity to compensate for damage.

The study reported here examines the direct effects of ozone on the reproductive development of two species with contrasting reproductive habits; the rapid-cycling *Brassica campestris*, a species with an indeterminate reproductive growth habit, and *Plantago major,* a wild species with a more determinate reproductive structure.

1.4. AIMS OF THESIS

The primary aim was to establish whether the reproductive structures of *Brassica campestris* and *Plantago major* were affected by exposure to elevated concentrations of ozone. For this purpose, exposure chambers which allowed the inflorescences to be isolated for fumigation from the vegetative components were designed and constructed (Chapter 2). The specific aims were:

i) to determine the vegetative sensitivity of *B. campestris* and *P. major* by investigating the effects of ozone on physiology (photosynthesis and stomatal conductance) and growth (Chapter 3), to allow a comparison between the vegetative and reproductive sensitivities of these species;

ii) to investigate the direct effects of ozone on the reproductive development of *B. campestris* and to compare the responses with those observed for *B. napus* (Bosac, 1992) (Chapter 4);

iii) to establish the direct effects of ozone on the reproductive development of *P. major* and to determine whether responses varied between populations reported to differ in ozone sensitivity (Chapter 5);

iv) to determine whether the differing reproductive growth habits adopted by *B. campestris* and *P. major* influence the sensitivity and responses of the reproductive structures to ozone.

CHAPTER TWO - EXPOSURE SYSTEM AND PLANT MATERIAL

2.1. INTRODUCTION

This chapter details the construction and operation of the exposure chambers and exposure system used for the fumigation of *Brassica campestris* and *Plantago major* to ozone. The methods used to prepare both of these species for fumigation are also documented. Separate experimental methodologies are detailed in the relevant chapters.

Two exposure chambers, one treatment and one control, were designed and constructed for the main purpose of exposing the flowering structures of both *Brassica campestris* and *Plantago major* to ozone. An advantage of the design was that it also allowed whole plant exposures to be undertaken. The chambers were incorporated into an existing exposure system, used previously by Bosac (1992), with the addition of minor modifications.

The exposure chambers were assembled from perspex and chipboard to permit the reproductive structures of up to twelve plants to be exposed simultaneously in each chamber. A single light source was held above each chamber and the humidity, temperature and ozone concentration of the airflow through each chamber was controlled and monitored continuously throughout the experimental period.

The whole system was housed in a darkened room to limit radiative exchanges. The temperature of the room was kept constant with the aid of a cooling fan which was set at 22 °C, to keep the vegetative parts of the plants outside the exposure chambers under similar growth temperatures to the reproductive structures within. A schematic representation of the whole system is shown in Figure 2.1.

2.2. EXPOSURE CHAMBERS: CONSTRUCTION

Two exposure chambers with interior dimensions 50 x 50 x 30 cm were constructed from 6 mm thick ICI Perspex Acrylic Sheet and white melamine covered chipboard. The top, base, front and back walls were made from perspex allowing light to enter the chamber and pass through to the vegetative parts below. The perspex front also facilitated the visual inspection of plants during exposures. The side walls were assembled from chipboard to strengthen the structure and to allow attachment of the chamber onto a Dexion framework support. The white melamine walls also reflected light back into the chamber to maintain a high internal irradiance.

AIR INLET

Light Source
↓ ↓ ↓
Heat Filter

Mixing
Tank

Controlled Temperature Room

EXHAUST

Humidity
Control

Thermocouple CONTROL CHAMBER

Pollution Scrubbers

Dewpoint
Hygrometer

Environmental
Logger

Ozone
Analyser

Relay Box

Dewpoint
Hygrometer

Pumps

Thermocouple

Light Source
↓ ↓ ↓
Heat Filter

Temperature
Control

Ozone Generator

Ozone
Introduction

EXHAUST

ROOM
AIR

Pollution Scrubbers   Pump

TREATMENT CHAMBER

Figure 2.1. Schematic representation of the exposure system.

The top, sides and back of each chamber were permanently adjoined. To allow for the introduction of flowering structures, the base was removable. This was achieved by affixing 6 mm wide plastic runners along the edge of both sides and the back wall for the base to slide into position. The base was cut into four 11.5 x 50 cm sections and one 4 x 50 cm section which was attached to the back wall so that a space was provided between the plants and the air circulation equipment mounted on the back wall. Two of the 11.5 x 50 cm perspex sections had four 1 cm diameter semi-circular holes cut out on one long edge, 6.25 cm and 18.75 cm from each end to give a spacing of 12.5 cm between each hole. The other two sections had these holes cut out along both long edges. By sliding the four sections in turn along the runners on the side walls, a base with twelve 1 cm diameter holes was formed as shown in Figure 2.2. The inflorescences of plants could easily be placed between the perspex strips, which were then brought together to form a seal around the plant stem. The holes were lined with high density foam so that the plants were held in position without damage. This also blocked off any space between the enclosed reproductive shoots and the base, thus preventing ozone leakage from the chamber to the vegetative structures below. A complete seal of the base was achieved when the plants were in position by taping the joins between the perspex strips with transparent Sellotape 'All Weather Sealing Tape'.

Flush around the front edge of the chamber a 5 cm wide perspex lip was attached perpendicular to the base, top and side walls. Eighteen 6 mm diameter holes were spaced out evenly around the lip, 2 cm from the edge. The lip was permanently adjoined to the top and two side edges, but along the base of the chamber the lip was removable so that the sliding base could be withdrawn.

The removable front piece of perspex (60 x 40 cm) was lined 1 cm from the edge with a 5 cm wide strip of 6 mm thick high density foam. Eighteen 6 mm diameter holes mirroring those on the perspex lip permitted the front to be bolted onto the main chamber. When in place, the foam on the inner side gave a good seal between the lip and the front. To prevent leakage from the exposure chambers, all permanent joints were sealed on the inside of the chamber with the ozone resistant sealant, Silcoset.

The whole chamber was joined at each corner to four 54.5 cm long pieces of Dexion which were attached to the corners of a 50 x 50 x 2 cm wooden base. This base made the whole structure rigid, while the Dexion legs allowed the attachment of a movable shelf on which to position the plants. This was constructed from a 47 x 50 x 2 cm piece of wood and by affixing strips of Dexion to the sides of this base, it could be raised up and down the Dexion legs by bolt attachments to achieve the most suitable position for the introduction of the flowering parts into the chamber. Because of the

Figure 2.2 Plan view of exposure chamber base.

Back

Front

├─────────┤
10 cm
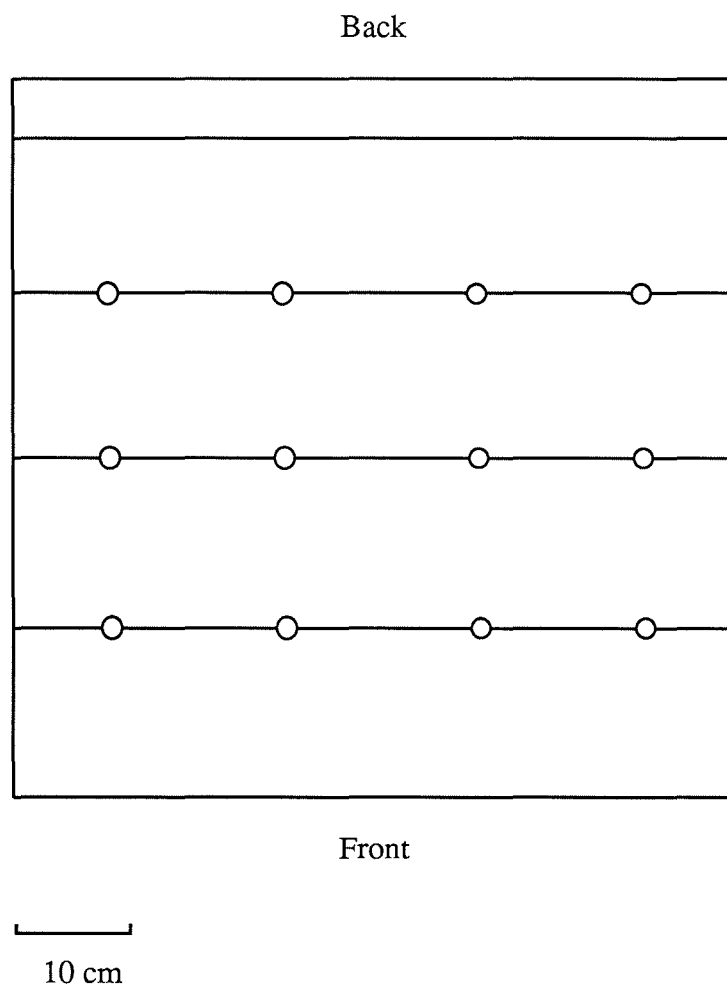
variability within plant populations, the exact positioning of each plant beneath the chamber was achieved using a variable number of thin wooden squares, each 5 mm thick, placed beneath the plant pot. Plate 2.1 shows the inflorescences of *Brassica campestris* introduced into the exposure chambers.

The chambers were positioned about 1.5 m from the floor by resting them on a purpose built metal framework, painted white to increase reflectiveness. This framework also held the lights in position above each chamber.

2.3. EXPOSURE CHAMBERS: ENVIRONMENTAL CONDITIONS

2.3.1. Light source

A single Osram Powerstar HQI-E 400W/D lamp with a 48 cm diameter circular reflector was held 41.5 cm above each chamber. To reduce the heat load from the lamps, a perspex waterbath, 70 x 60 x 15 cm and lined with polythene sheeting to prevent leakage, was positioned 19.5 cm above each chamber. The waterbath was filled to a 7 cm standing depth of water and this helped to maintain the temperature within each chamber between 21 °C and 25 °C during exposures.

The quantity of Photosynthetically Active Radiation (PAR) within each chamber was recorded using a Li-Cor LI-185B Quantum/Radiometer/Photometer. PAR was measured within the chambers at regular intervals to check for any deterioration in the emission of the light bulbs. Typical PAR measured at the floor of each chamber was 200-250 μmol m^{-2} s^{-1} and below the base, 150-200 μmol m^{-2} s^{-1}.

The light quality, or spectral composition of radiation emanating from the lamps was recorded using a Li-Cor Portable Research Spectroradiometer. This was used to produce spectra for light quality above and below the base of each chamber where plant material would be placed (Figures 2.3-2.6). Figures 2.3-2.6 show that there was no detectable difference in the spectral distribution within or below the chambers, although the light quantity is significantly less below the base of each chamber. For comparison, the spectral distribution of daylight is shown in Figure 2.7.

2.3.2. Air flow

The air used for exposures was drawn from outside the building through 6.35 mm diameter internal bore tubing held several metres above ground level using two diaphragm pumps (Charles Austin Pumps Ltd, Weybridge). Before entering the
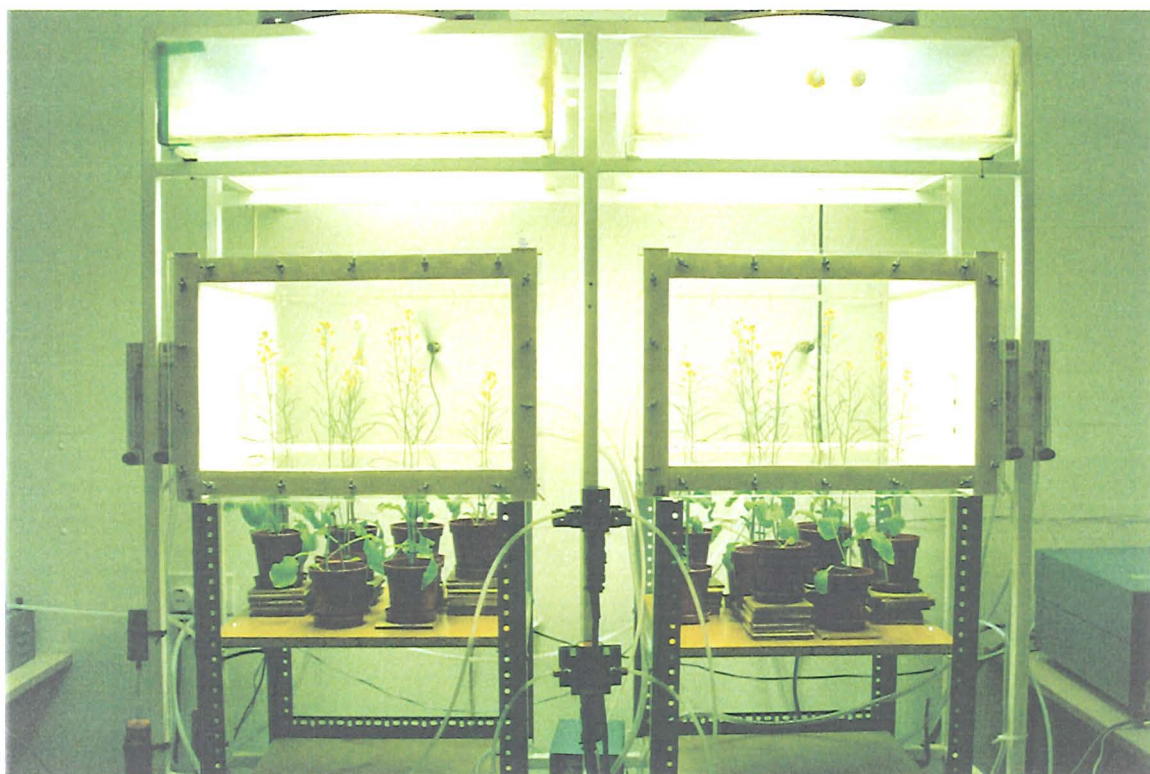
Plate 2.1. Plants of *Brassica campestris* being introduced into the two exposure chambers.

Figure 2.3 Spectral distribution of radiation within the control chamber
(Chamber 1).
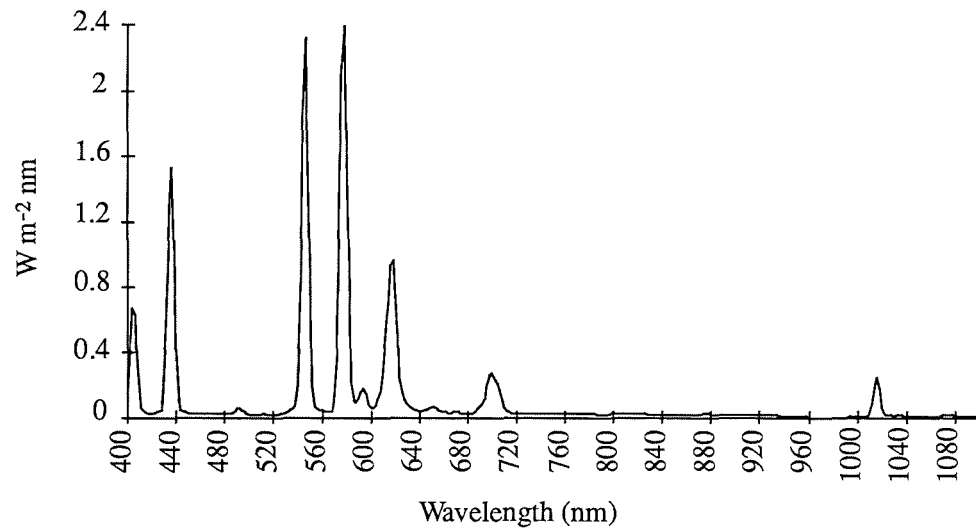
Figure 2.4 Spectral distribution of radiation beneath the control chamber
(Chamber 1).
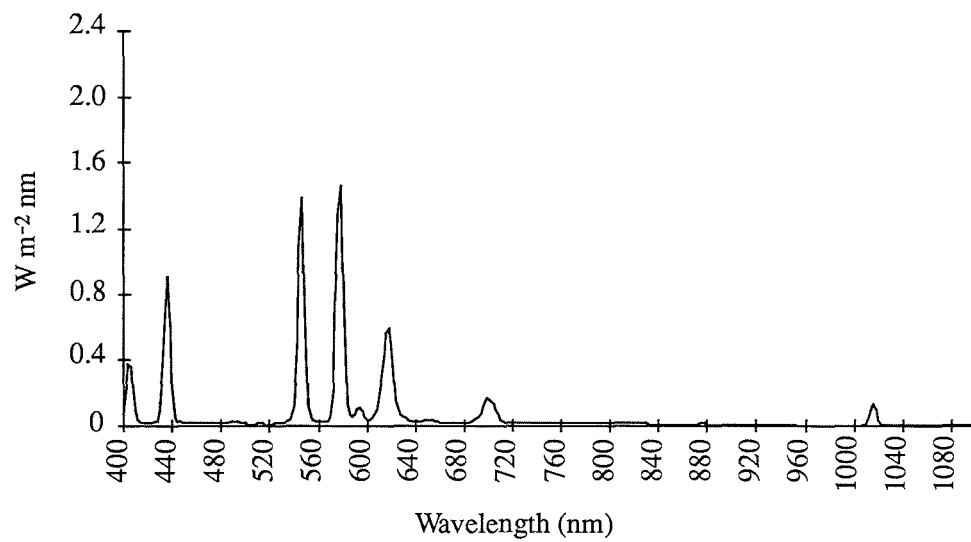
Figure 2.5 Spectral distribution of radiation within the treatment chamber
(Chamber 2).
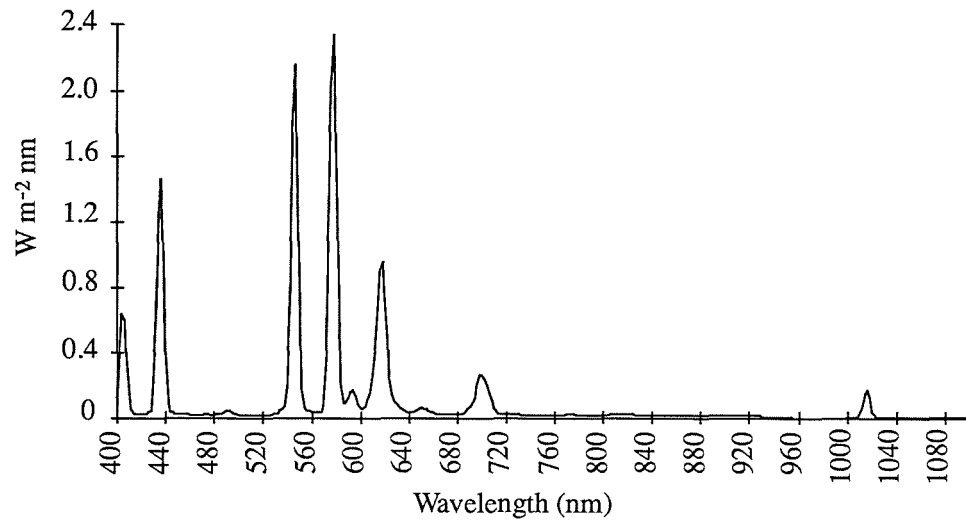
Figure 2.6 Spectral distribution of radiation beneath the treatment chamber
(Chamber 2).
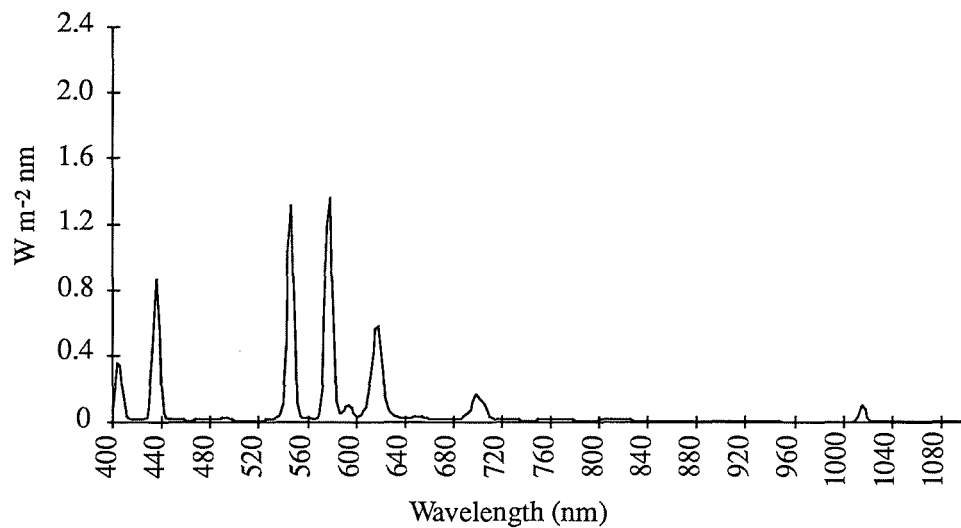
Figure 2.7 Spectral distribution of natural direct sunlight from an open cloudy sky.
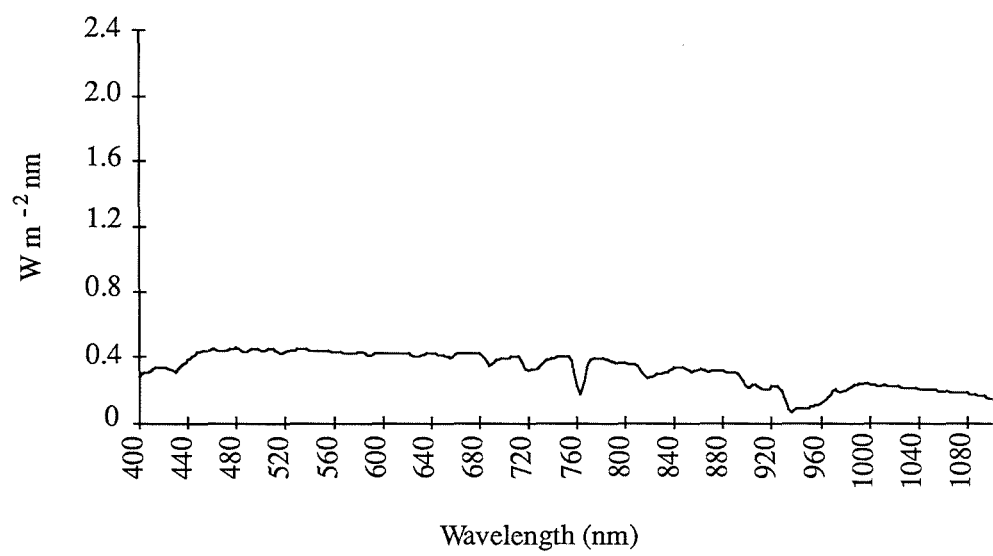
building, the air passed through a 46 litre plastic tank which served to mix the air and to deposit any rainwater or solid contaminants which may have entered the airline. The air was then drawn through the humidification system and after passing through the pumps was conditioned for temperature and pollutants before entering the exposure chambers.

2.3.2.1. *Humidity*

Air humidity is known to interact with plant responses to ozone because of its influence on stomatal aperture (Mortensen, 1989). Therefore air entering the exposure chambers needed to be at a constant relative humidity so that the plants were not subjected to fluctuating humidity or the additional stress of dry air.

The air was conditioned to achieve a relative humidity within the chambers of over 50 % by passing air in through the base of a sealed 5 l glass aspirator bottle standing in a controlled temperature water bath (model Y28, Grant Instruments, Cambridge). Water within the aspirator was at the same level as in the water bath so that air bubbled through it. The resulting percentage relative humidity of the air stream was dependent upon the temperature of the water bath. By using a cooling coil (Grant Instruments, Cambridge), the temperature of the water bath was held at 10 °C which was sufficient to give a relative humidity for the air entering each chamber of between 50 % and 60 %. Air then passed out of the top of the aspirator bottle to enter the diaphragm pumps which served to pull the air through the humidification system.

2.3.2.2. *Air pumps*

After passing through the humidification aspirator, the airline was split and diverted to two separate diaphragm pumps. This provided an airflow of 13 l min^{-1} to each 75 l chamber, resulting in an air change every 5 min 45 s. A frequent air change and a turbulent airflow were required so that ozone uptake by the plant was not restricted by high boundary layer resistances and CO_2 and pollutant concentrations could be maintained (Unsworth and Ormrod, 1982). Once through the pumps, each airline passed through a particle filter (Charles Austin, Weybridge) to remove any particulate material generated by the pumps and then the airlines were rejoined by entering at one end of a 50 cm long, 4 cm internal diameter, PVC tube. This served to mix the air from each pump, before the airflow was conditioned for temperature.

2.3.2.3. *Temperature control*

To counteract the heating effect of the diaphragm pumps, the airline was passed on through a 1.6 m long 9 mm diameter copper coil standing in a 7 l water bath at room temperature ($\sim$ 22 °C). Because copper is a good heat conductor, air passing through the coil was quickly cooled to the temperature of the surrounding water. However the air temperature was subsequently raised 2-3 °C to 24-25 °C by the light source on entry to the chambers.

2.3.2.4. *Pollution control*

Air leaving the temperature controlling waterbath was split into two streams, one to supply each chamber, before being scrubbed of ambient air pollutants. This ensured that ozone, subsequently introduced into the treatment chamber airline, would not contaminate the control chamber by a backflow of air. Each airline passed through three separate pollution scrubbers, two containing charcoal and one containing Purafil. These pollution scrubbers each consisted of a 11.5 cm long 4 cm internal diameter piece of perspex tubing with a rubber bung fitted in each end. Each bung was penetrated by a piece of glass tubing which extended 4 cm from the bung to allow for the attachment of the airline. Each tube was filled with 80 cm^3 of charcoal or Purafil as required. Several layers of 50 μm fine nylon mesh over the internal end of each bung prevented charcoal and Purafil dust contaminating adjoining scrubbers or the airline. Initially, each airline passed through a charcoal scrubber to remove any sulphur dioxide and nitrogen dioxide and then through a Purafil scrubber to remove ozone and convert any nitric oxide present to nitrogen dioxide. This nitrogen dioxide was subsequently removed by the second charcoal scrubber. Each airline then passed through a particle filter (Charles Austin, Weybridge) to remove any charcoal or Purafil dust.

2.3.2.5. *Air circulation within the chambers*

After exiting the pollution scrubbers, the airline was split to pass through two 12 l min^{-1} flowmeters which were used to control the flow of air into the chamber. The line was then rejoined before it was split (16.5 cm) before entering through two 14 mm diameter holes in the back of the chamber. These holes were positioned 5 cm and 15 cm from the top of the chamber and 17.5 cm from the left hand side wall. The division of the airline served to improve the distribution of air within the chamber. This was further enhanced by an internal fan which comprised a model aeroplane propeller with a 15.2 cm span attached to a 5 cm long hollow metal spindle. The spindle was attached to a small motor (MFA/ Como Drills, 3-7.2V) which was connected outside

the chamber, 10 cm from the top and 17.5 cm from the right hand side wall. This allowed the propeller to cut across and distribute air equally from both inlet ports. Location of the motor outside the chamber prevented contamination by soot and heat build up produced by its operation. The fan was driven by a power pack supplying 3 V.

Air exited the chamber through two 12 mm diameter holes positioned on the right hand wall 5 cm and 10 cm from the base and 10 cm from the front wall. The positioning of these holes encouraged the air to pass through the entire volume of air contained within the chamber before exiting. The two separate exit lines were joined 14 cm from the chamber and the air was then expelled outside the building.

2.3.3. Ozone generation

Air from the room containing the exposure system was used to generate ozone for introduction into the treatment chamber. Air was pumped through a purpose built ozone generator by a small bench top pump (Charles Austin, Weybridge) via a 4 l min^{-1} flowmeter and a set of pollution scrubbers, as described previously for the main airline (Section 2.3.2.4.).

The ozone generator comprised a 52 x 29 x 29 cm chipboard box with two 9 " long Pen-Ray UV light bulbs positioned horizontally 12 cm from the base. Air entered the generator through two 3 cm diameter UV penetrable quartz glass tubes, each located 1 cm above one of the UV bulbs. O_2 was converted to ozone by photochemical reactions as air passed through the glass tubes. Aluminium foil was used to line the box to increase reflectance and therefore ozone generation. The two exit lines from the generator were combined and the ozonated air was carried to the treatment chamber airline through ozone-resistant Teflon tubing. This line was introduced to the main airline 12 cm before it divided to enter the treatment chamber, thus diluting the concentration of ozone prior to entry into the chamber. By altering the flow of the air into the generator and the proportion of air passing over each UV bulb by using Hoffman clips, it was possible to alter the final concentration of ozone produced within the treatment chamber. To compensate for the flow of air into the treatment chamber from the ozone generator, the main airline flow was reduced by an equivalent amount using the flowmeters.

2.3.4. Monitoring

2.3.4.1. *Temperature*

Air temperature within each chamber was measured using a copper/constantan thermocouple (Gauge, 28 SWG) inserted through the left hand side wall, 15 cm from the base and 25 cm from the front of each chamber, and sealed in position. The thermocouple projected 2 cm into the chamber and was shielded above by aluminium foil so that there was no radiative heating of the thermocouple. The thermocouple was directly attached to a micrologger (Model 21XL, Campbell Scientific, Shepshed, UK) which transformed the differential voltage produced by the thermocouple into a temperature and this was logged at 1 min intervals. Means were calculated every ten minutes and these were stored for downloading to a Personal Computer at the end of the exposure period.

2.3.4.2. *Humidity*

To calculate and check the humidity of air entering the chambers, a sample line was inserted in the airline between the pollution scrubbers and the chambers which diverted air through a dewpoint hygrometer, see Figure 2.1. The dewpoint of the air flowing to each chamber was recorded prior to ozone introduction because of the damaging effect ozone may have had on the measuring equipment. Two dewpoint hygrometers were used; a General Eastern System 1100DP for one chamber and a General Eastern System 1100AD for the other. Although the models differed, there was no difference in the precision of measurement of dewpoint as both models were calibrated before use. The airflow to each dewpoint hygrometer was controlled by Hoffman clips so that a flow rate of $0.5 \ l \ min^{-1}$ was supplied to the respective dewpoint sensors. The dewpoint values were recorded by the micrologger at 1 min intervals, converted to 10 min means and stored for downloading. Using the dewpoint and the internal chamber temperature, it was possible to calculate the relative humidity of the air entering the chambers as follows:

$$\% \text{ Relative Humidity} = 100 \ Exp \ 17.2694 \left(\frac{T°C_{dewpoint}}{T°C_{dewpoint} + 238.3} - \frac{T°C_{ambient}}{T°C_{ambient} + 238.3} \right)$$

2.3.4.3. Ozone

Ozone was sampled from each chamber through a 4 mm diameter Teflon tube positioned 5 cm above the top air exit on the right hand side wall. The sample line from each chamber was passed through a particle filter (Type FH, 0.5 µm, Millipore) and then through a chemically inert three-way solenoid valve (Model LFYA1226032H, The Lee Company, Essex, UK). On exiting the valve, the selected line passed on to the ozone analyser (Model 8810, Environmental Technology, Stroud, UK). Prior to use, the ozone analyser was calibrated with the ozone generation and monitoring system at the University of Nottingham field station, Sutton Bonnington, UK. A relay controller connected to the solenoid valve was used to select between the two sample lines; this was controlled externally by the micrologger. During exposures, samples from each chamber were drawn alternately every ten minutes. To allow the ozone analyser time to equilibrate after each switch over, and unless otherwise stated, the concentration of ozone was recorded every minute from 5-10 minutes of the sampling interval, and the mean of these six values was stored for downloading. Thus three mean values for each chamber were recorded every hour. An example of the typical output from the ozone analyser over a two day period of ozone introduction at 100 ppb for 6 h is shown in Figure 2.8. The concentration of ozone within the control chamber was invariably below the detection level of the ozone analyser. Tests also showed that the concentration of ozone was uniform throughout each chamber and that outside the treatment chamber, particularly below the base, it rarely exceeded 5 ppb.

2.4. PLANT MATERIAL

2.4.1. Rapid-cycling *Brassica campestris* L.

Brassica campestris (syn. *Brassica rapa*) occurs both as a weed and as one of the six cultivated species of the genus *Brassica* which are economically important as crop plants all over the world, producing vegetables, oil and animal fodder (Tomkins and Williams, 1990). *B. campestris* is a native of Europe, where it has been cultivated since the 13th century. Prior to this, oil from the seeds of *B. campestris* growing wild in grain fields was used for illumination and soap making (Appelvist and Olsen, cited by Vaughan, 1977). Today there are a number of forms of this species which represent divergent selection during domestication. These include the varieties of Chinese cabbage, turnip and pak choi which are consumed in various Oriental cultures and the oilseed varieties of Sarson and Toria grown in the northern parts of the Indian subcontinent (Williams and Hill, 1986).

Figure 2.8 Typical time courses of ozone concentration over two days of exposure to 100 ppb of ozone for six hours (1015 to 1615h).
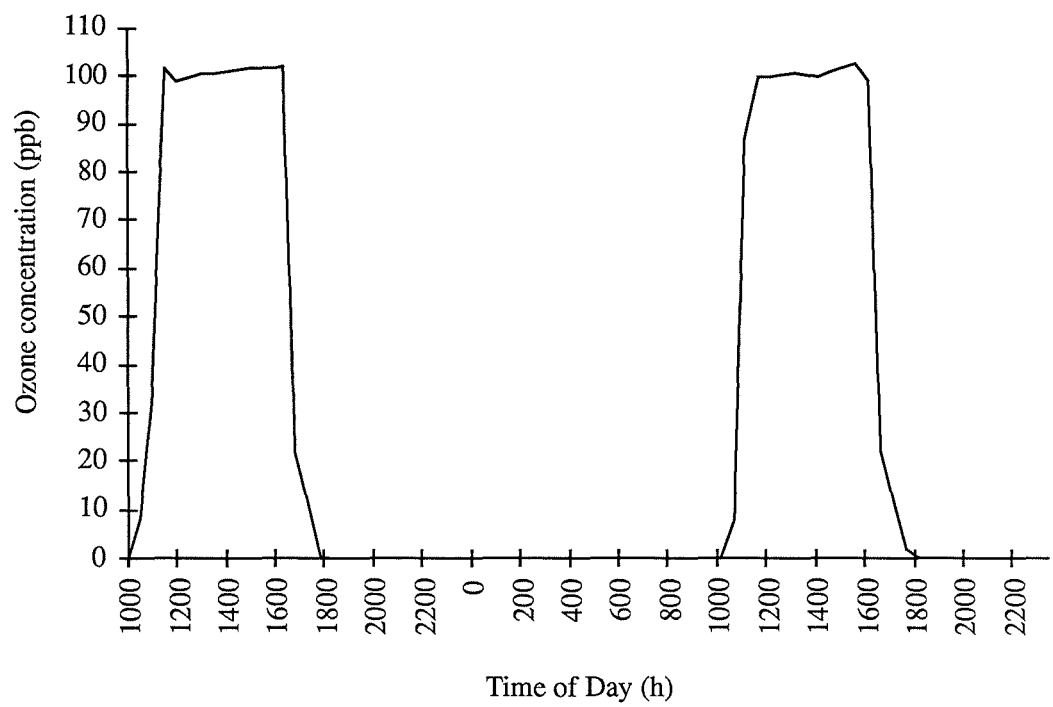
Rapid-cycling *B. campestris* was developed by scientists at the University of Wisconsin- Madison, U.S.A. who were searching for disease resistant genes for transfer to other agricultural species of *Brassica*. Among the six cultivated species of *Brassica* being screened, it was noticed that some individuals flowered significantly earlier than others. Because most species of *Brassica* are biennial or annuals, their normal growth cycle is too long to be convenient to plant breeders who are interested in rapid gene transfer. Therefore, it was decided to develop populations from these individuals which would reduce the time between generations and provide plants suitable for use under controlled laboratory conditions. This was achieved by recurrent mass selection whereby plants within each species, which showed the desired characteristics, were cross-pollinated. Over a 15 year period selection was made for;

1. Minimum time from sowing to flowering.
2. Rapid seed maturation.
3. Absence of seed dormancy.
4. Small plant size.
5. High female fertility.

Selection ceased for each species when 50% of the plants first flowered within a 2-3 day period of each other (Williams and Hill, 1986). The resulting growth characteristics of the six species are summarised in Table. 2.1.

These six rapid-cycling species are now widely used in research and as instructional materials in schools, where they are used to teach students about plant growth and development, physiology, reproduction, ecology (Hafner, 1990) and genetics (Hawk and Crowder, 1978). The use of rapid-cycling *Brassicas* within schools has been developed both in the U. S. A, where they originated, and also within England and Wales where the use of rapid-cycling *B. campestris* has been promoted by Science and Plants for Schools, Cambridge (SAPS, 1990a).

Rapid-cycling *B. campestris* was chosen for use in the present study for several reasons. Firstly *B. campestris* is a progenitor of *Brassica napus*, the flowering structures of which have already been shown to be sensitive to ozone (Bosac, 1992). *B. campestris* also has a similar although less flexible indeterminate reproductive growth habit to that of *B. napus*, a feature important to achieving the aims of this study. The growth characteristics of *B. campestris* were also more amenable to replicated exposure experiments compared to *B. napus*; the small size allowed many plants to be introduced simultaneously into an exposure system and relatively large self-incompatible flowers (about 1 cm diameter) permitted manipulation of pollination and easier observation. As this species was also developed for growth under continuous fluorescent light, this removed any complication of diurnal rhythms associated with

Table 2.1. Phenotypic characterisation of rapid-cycling *Brassica* populations grown at 24 °C under continuous fluorescent light in minipots (10 cm^3). After Williams and Hill (1986).

Species	Days to flower	Length (cm) to first flower	Seeds per plant	Days for cycle	Cycles per year
B.campestris	16	11.9	78	36	10
B. nigra	20	27.1	69	40	9
B. oleracea	30	22.6	18	60	6
B. juncea	19	29.6	107	39	9
B. napus	25	35.3	76	55	6
B. carinata	26	41.7	67	56	6

flowering, for example, pollen production and flower opening. Another advantageous feature is that the seeds mature quickly and have no dormancy period, with the result that germination experiments could be conducted directly after harvesting. Finally, the short life cycle of *B. campestris*, at around 40 days, permitted many replications of an experiment over a short space of time. Various stages of development in the life-cycle of *B. campestris* are shown in Plate 2.2.

2.4.1.1. *Seed source and growth conditions*

Seeds of rapid-cycling *Brassica campestris* were supplied by MacIntyre Mottingham Garden Centre, London and growing instructions by Science and Plants for Schools (SAPS 1990b) and Tompkins and Williams (1990). When not being used, the seeds were stored in the refrigerator at 4 °C in paper envelopes contained within a plastic screw top bottle with a quantity of silica gel to remove moisture. This procedure was recommended to prolong the viability of the seeds.

For experimentation, seeds were germinated in a 10 cm pot of Levingtons Multipurpose Compost (Fisons, Loughborough) in a G3600THTL Fisons Fitotron growth cabinet with continuous fluorescent and tungsten lighting giving a mean PAR flux of 230 μmol m^{-2} s^{-1} at a distance of 60 cm beneath the light source. The spectral distribution of radiation within the cabinet was determined using a Li-cor Spectroradiometer (Figure 2.9). Temperature and relative humidity within the growth cabinet were maintained at 24 °C and 70 % respectively.

Although it is recommended that *B. campestris* is grown in a minipot (10 cm^3) to limit plant growth in classroom studies, it was decided that in the present study, three day old germinated seedlings would be potted on into 10 cm diameter pots of Levingtons Multipurpose compost. Williams and Hill (1986) have suggested that rapid-cycling *Brassicas* will respond to this larger rooting volume by developing larger plants with more seeds. This was favourable since it would provide a slightly larger reproductive structure for ease of handling and introduction into the exposure chambers.

The plants were supplied with adequate water and placed as close to the light source as possible during their growth, being lowered down within the growth cabinet as required. The plants were frequently rotated around the growth cabinet to prevent any variation in growth due to small variations in environmental conditions within the growth cabinets.
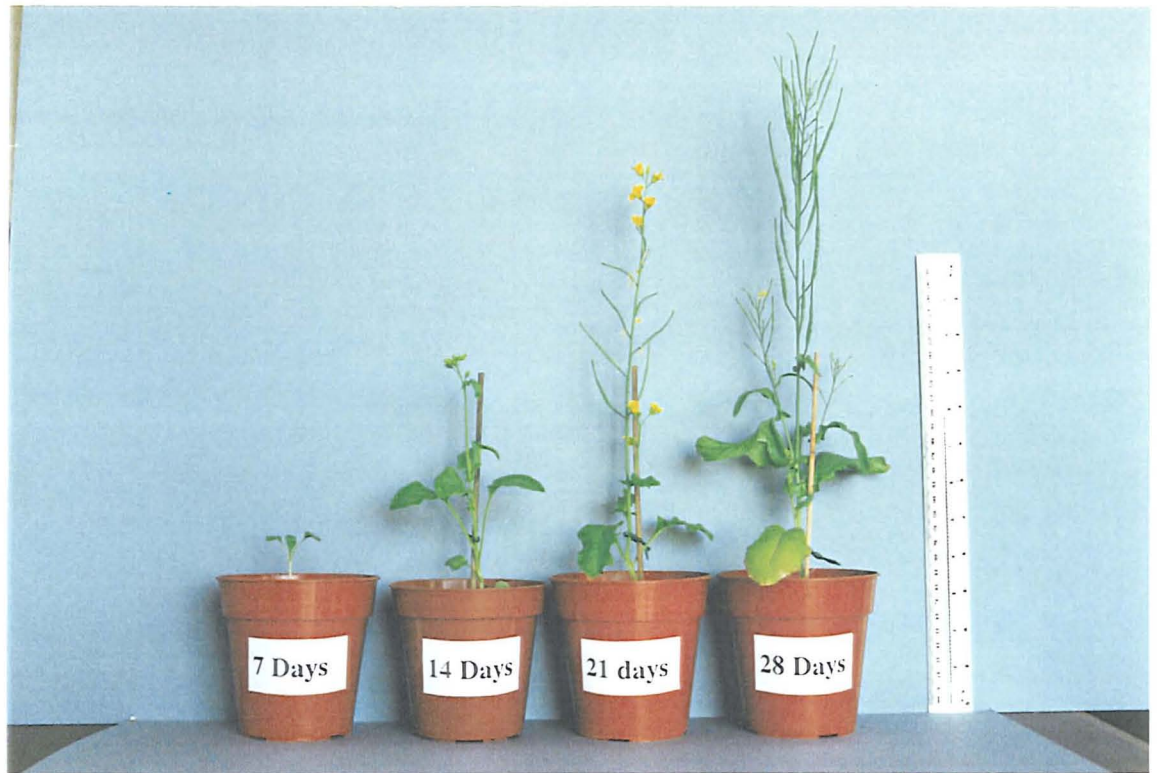
Plate 2.2. Stages of plant development in *Brassica campestris* at various days after sowing.

Figure 2.9   Spectral distribution of radiation within the 600G3 THTL growth
cabinet 30 cm beneath the light source.
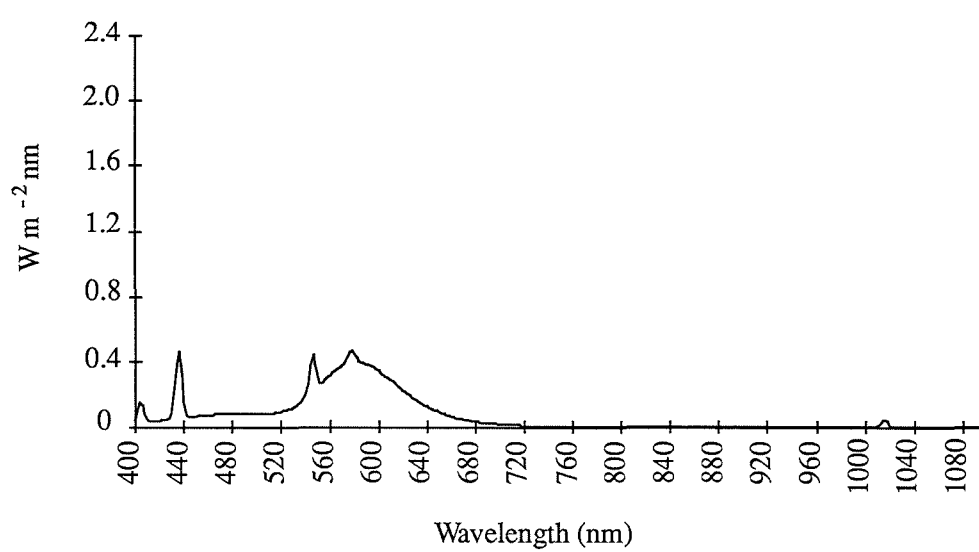
As the plants grew taller they were tied to small stakes to encourage straight stems to facilitate the introduction of inflorescences into the exposure chambers. The axillary racemes of each plant were removed as close to the main stem as possible, when and if they developed leaving only the terminal raceme for exposure. This not only simplified the structure of the plants for exposure but also directed resources into one raceme and confined the period of flowering to under 30 days.

The plants were kept within the growth cabinet until transfer to the exposure system and then returned to the growth cabinet to complete their life cycle post exposure. Watering was stopped 35 days after sowing (DAS) so that the plants could dry out and the seeds be harvested. Drying out, which normally took around 10-15 days from the last watering, was required because the seed would germinate within the siliqua if the plants were provided with water after 40 days (Tompkins and Williams, 1990). Due to the larger plant size under these conditions, the life cycle of *Brassica campestris* was slightly longer than suggested by Williams and Hill (1986), (Table 2.1.) at around 45 days. First flowering remained unchanged at around 16 DAS, but the extended period of flowering resulted in a larger number of seeds per plant (300-450).

2.4.1.2. *Cross-pollination*

Being a self-incompatible species, *B. campestris* required cross-pollination. Flower buds borne on the terminal raceme began to open about 16 DAS in a regular progression, with one bud opening about every 8 hours. In nature, *B. campestris* is pollinated by honeybees (*Apis mellifera*). A 'beestick' as described in SAPS (1990b), was used as a natural alternative for controlled cross-pollination by hand. The anthers dehisced by the second day of opening and pollen was easily collected and transferred to receptive stigmas which were receptive for the first 2-3 days after anthesis. Pollination was performed daily until all of the flowers had opened on the terminal raceme, around 30 DAS, in order to ensure maximum seed set.

2.4.2. *Plantago major* **L.**

Plantago major is a perennial herb species, native to Europe, which has been distributed around the world anthropogenically. Indeed the name *Plantago* is derived from; planta: sole of the foot and agere: to stride (Kuiper and Bos, 1992). Being found on all continents except Antarctica, the introduction and spread of *P. major* has probably been due to its occurrence as an impurity in hay or crop seeds which have been carried long distances over land or sea (Cox and Moore, 1995).

In Great Britain, *P. major* is very widely distributed since it is tolerant of regional variations in climate (temperature and water availability). It occurs in a whole range of soil types being absent only from extremely acid peat and mountain grassland (Sagar and Harper, 1964). *P. major* grows best in open ground since it requires full sunlight for germination and efficient photosynthesis (Kuiper and Bos, 1992) and commonly establishes in grassland habitats where disturbance by humans and animals keeps the vegetation short e.g. cultivated soil, lawns, and pathways. Because *P. major* consists of a short subterranean stem, or caudex, from which arise a rosette of oval leaves and adventitious roots, its flattened form can withstand the pressures of grazing and trampling. Since *P. major* has no economic value it is usually regarded as a noxious weed.

From early Spring, the seeds of *P. major* germinate sporadically throughout the growing season and in undisturbed habitats shoot development continues until August. Since this species does not reproduce freely by vegetative means, reproduction is primarily sexual. In the field, flowering occurs for 3 months from early June, as soon as 6 weeks after germination (Sagar and Harper, 1964). The plants produce one or more reproductive spikes comprising many small (2-4 mm diameter) wind pollinated flowers held above the basal rosette of leaves on long scapes (10-15 cm). The flowers are protogynous and capable of full self-fertilisation. As the flowers on each spike open in acropetal succession, only the first opened flowers are certain to be cross pollinated. Each flower develops into a capsule with 3-28 seeds, which are often not dispersed until the following spring or summer (Sagar and Harper, 1964). *P. major* overwinters with an above ground rosette of leaves, however in sever winters or exposed areas it will overwinter either at or below ground level.

This species was chosen for study for several reasons. Firstly, the vegetative structures of many populations of *P. major* have been shown to differ in sensitivity to ozone (Reiling and Davison, 1992d) and seed yield has been reduced in the most sensitive of these populations after exposure of the whole flowering plant (Reiling and Davison, 1992b). Using several different populations ranging in sensitivity to ozone, this study could examine the direct effects on reproduction and compare the responses with those of the vegetative structures. Secondly, *P. major* has a more determinate reproductive growth habit than that of *B. campestris* and this would allow an examination of the extent to which the reproductive growth habit modifies the response to ozone. In addition, the flowering spikes of *P. major* were very suitable for exposure as they are held well above the basal rosette of leaves by long scapes, as an adaptation to wind pollination. This facilitated the separation of the reproductive and vegetative structures within the exposure system.

2.4.2.1. *Seed source and growth conditions*

Four different populations of *P. major* were selected for study. Seeds of one population were obtained from the NERC Unit of Comparative Plant Ecology, University of Sheffield, UK. This population from High Low, Derbyshire, National Grid reference SK 157684, was collected in 1985 and was found to be the most sensitive of 28 populations screened for vegetative sensitivity by Reiling and Davison (1992d). Seeds from the other three populations, two resistant and one sensitive to ozone, were obtained from Prof. A. Davison, Newcastle University, UK. These three populations were chosen to cover the range of vegetative sensitivities found within this species (Reiling and Davison, 1992d) and were as follows:

Population	Location	Year of Harvest	Sensitivity (A.Davison, pers. comm.)
Penicuik	NT 24 63	1991	Sensitive
Sibton	TM 37 69	1991	Resistant
Lullington	TQ 53 01	1991	Resistant

(Location obtained from Reiling and Davison (1992d) and Pearson *et al.* (1996)).

Seeds were germinated in pots of John Innes No. 2 potting compost within a SGC1700/FM/HQI Fisons Fitotron growth cabinet with a 15 h photoperiod (0600-2100 h). As well as using this type of compost in their studies, Reiling and Davison (1992a,b,c,d) also utilised this photoperiod since germination is promoted in *P. major* by light (Kuiper and Bos, 1992) and flowering has been shown to require a photoperiod greater than 13 h (Hawthorn, 1974). PAR at leaf height averaged 260 μmol m^{-2} s^{-1} and the spectral distribution of radiation within the growth cabinet was recorded using a Li-cor Spectroradiometer Photometer (Figure 2.10). Temperature was set at 22 °C day and 15 °C night and relative humidity at a constant 50 % to try and reduce infection by powdery mildew (*Erysiphe pisi*), to which *P. major* is susceptible.

Seedlings were transplanted after 10-15 days into 9 cm diameter pots containing John Innes No. 2 potting compost and returned to the growth cabinet in trays and watered when required. During growth, powdery mildew was treated with Safers Natural Organic Garden Fungicide, a formulation which has been shown to have no effect on the sensitivity of *P. major* to ozone (personal communication, A.W. Davison). All leaves on each plant were sprayed at the first signs of any infection regardless of whether or not they were individually affected. Spraying was repeated as necessary but at not less than the recommended 7 d interval.

Figure 2.10 Spectral distribution of radiation within the SGC1700/FM/HQI growth
cabinet, 100 cm beneath the light source.
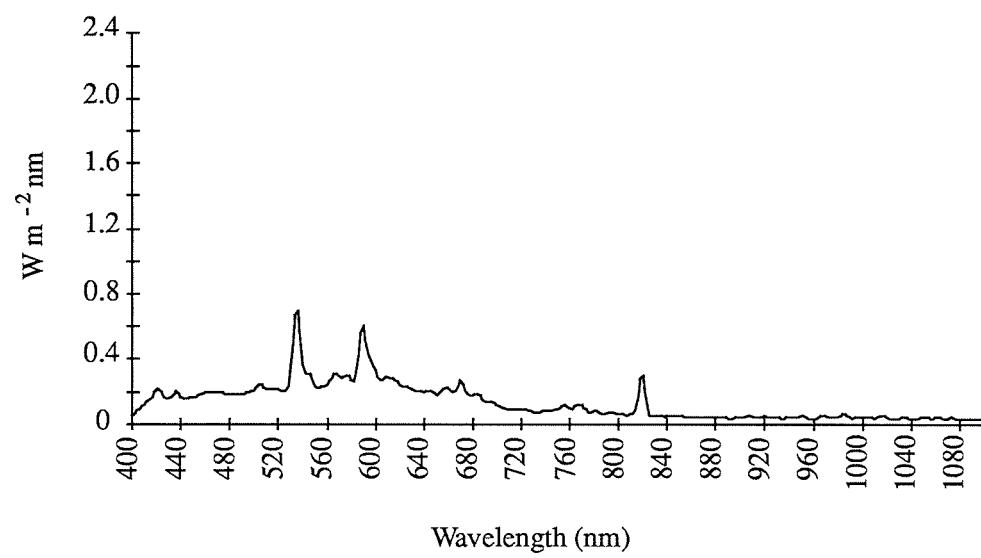
Plants were grown within the growth cabinets prior to exposure and then returned to complete their life cycle after exposure. Because *P. major* is a self-compatible and wind pollinated species, no cross-pollination was required.

2.5. PREPARATION OF PLANT MATERIAL FOR EXPOSURE

Plants for exposure were transferred in polystyrene lined boxes from the growth cabinets to the exposure system in an adjacent building, a distance of around 200 m.

The plants were placed within the exposure chambers before the system was started up. In the majority of experiments, the plants were introduced on the day prior to the first day of exposure to ozone to allow them to acclimatise to their new environmental conditions (cf. Sections 2.3.1.-2.3.2.). The light environment was set specifically for each species, with *B. campestris* receiving continuous illumination and *P. major* receiving a 15 h photoperiod (0600-2100 h) controlled by a timer switch. With the plants in position, the timed fumigation period started when the required concentration of ozone was achieved within the exposure chamber. This concentration of ozone was usually achieved 15-30 min after the commencement of ozone generation. The plants were maintained within the exposure chambers between fumigations and were only removed for measurements to be taken. Water was supplied as required to a saucer placed beneath each plant pot. For whole plant exposures, where the entire plant and pot were to be placed within the exposure chamber, a layer of sharp sand was placed on top of the soil to prevent excessive water loss and a corresponding increased humidity within the chambers. At the end of the exposure period, the plants were returned to the appropriate growth cabinet.

2.6. TEST FOR EXPOSURE CHAMBER UNIFORMITY

To check that there was no significant effect on the growth of plants due to differing environmental variables within the exposure chambers, 14 plants of *Plantago major*, (population High Low), were grown for nine consecutive days within each chamber without the introduction of ozone. Seeds were germinated in John Innes Compost No. 2 and seedlings transplanted into individual 9 cm pots after 12 days. These seedlings were allowed to establish for three days within the growth cabinet before being transferred to the exposure chambers. Plants were allocated randomly to each chamber, where they were spaced out regularly on the base and remained until the end of the exposure period. The photoperiod was 15 h (0600-2100 h), and the average day and night temperatures and relative humidities were calculated and compared by means of a paired, two-tailed t-test to test for significance ($P<0.05$) using the statistical package

Excel 4 (Table 2.2). The ozone concentration within both chambers was invariably below 1 ppb. The plants were harvested after nine days of exposure and divided into the root and shoot before being dried in an oven at 60°C for 48 h and weighed. The dry weights of the root and shoot, total dry weight and root: shoot ratio were compared by means of a t-test (Table 2.3). The results showed that there was no significant difference in the environmental conditions between the two exposure chambers during the exposure period and no significant effect on the growth of *P. major* (population High Low).

Table 2.2. Average temperature and percentage relative humidity within the two exposure chambers during exposure of *Plantago major* (population High Low) for 9 days (8 nights) to test for chamber uniformity. Standard errors are shown, n=9 (day), n=8 (night).

	Day	Night
Temperature (°C)		
Chamber 1	24.1 ± 0.39	22.4 ± 0.12
Chamber 2	23.6 ± 0.09	22.0 ± 0.07
Relative Humidity (%)		
Chamber 1	57.4 ± 1.63	62.1 ± 0.98
Chamber 2	56.9 ± 0.86	62.2 ± 1.00

Table 2.3. Growth of *Plantago major* (population High Low) within the two exposure chambers after a 9 day exposure to test for chamber uniformity. Standard errors are shown, n=14.

	Chamber 1	Chamber 2
Mean root dry weight (mg)	6.4 ± 0.45	6.2 ± 0.73
Mean shoot dry weight (mg)	34.9 ± 2.87	33.2 ± 4.04
Mean total dry weight (mg)	41.3 ± 3.21	39.3 ± 4.80
Mean root:shoot ratio	0.19 ± 0.009	0.19 ± 0.012

CHAPTER THREE - THE EFFECTS OF OZONE ON PHYSIOLOGY, GROWTH AND REPRODUCTION.

3.1. INTRODUCTION

It has been widely reported that exposure to elevated concentrations of ozone can affect the physiology (Darrall, 1989), growth (Cooley and Manning, 1987) and yield of plants (Jager *et al.*, 1993) but that the type of response varies between species and genotypes. This chapter presents data from a series of experiments undertaken to assess the effects of ozone on the physiology and growth of *Brassica campestris* and four populations of *Plantago major*. In addition, data are presented from an experiment to examine the effect of ozone on the reproductive development of *B. campestris*.

Many studies have shown that the stomatal conductance and net rate of photosynthesis of plants can be reduced and sometimes increased following exposure to ozone, although the mechanisms behind such responses are not clearly understood (Darrall, 1989). In this study, stomatal conductance, net rate of photosynthesis and intercellular CO_2 concentration were measured simultaneously in the leaves of *B. campestris* and each population of *P. major* prior to, during and following fumigation with 70 ppb ozone for 7 h day^{-1} on two consecutive days, in order to determine their physiological responses. This daily ozone dose was chosen because it has been used previously in the Integrated Screening Programme (Reiling and Davison, 1992c) and in studies with *Plantago major* (Reiling and Davison, 1992b,d) where it was chosen to be within the range of episodes that occur in England during warm summers.

Many assessments of plant sensitivity to ozone have been based on visible symptoms (Reinert *et al.*, 1972; Knudson Butler and Tibbits, 1979a). However, Reiling and Davison (1992a) have shown that a reduction in growth rate is more useful for determining ozone sensitivity than an assessment of visible injury, since the growth of many species, including that of *P. major*, can be reduced by exposure to ozone without the development of visible symptoms. Effects of ozone on the growth of *P. major* are also known to differ between populations (Reiling and Davison, 1992d). In this study, four populations of *P. major* were exposed for two weeks to 70 ppb ozone for 7 h day^{-1} and measurements were made of growth, in order to determine the relative sensitivity of each population when exposed in the present system and to compare this with the data from a similar study by Reiling and Davison (1992d). In addition, a separate experiment examined the effects of ozone on biomass allocation in *B. campestris*.

The effect of ozone on the reproductive development of *B. campestris* was also examined in this study when entire plants were exposed to 70 ppb ozone for 7 h day^{-1} for two or 10 days prior to first flowering. Measurements were made of floral development, pollen germinability, seed yield and seed germination.

3.2. MATERIALS AND METHODS

For each experiment, seeds of *B. campestris* or *P. major* were germinated within the controlled environment growth cabinet (CEGC) as detailed in Sections 2.4.1.1 and 2.4.2.1, transplanted into 7.5 cm diameter pots and prepared for exposure (Section 2.5). In each of the experiments, whole plants were exposed to filtered air (control treatment) or to a target concentration of 70 ppb of ozone for 7 h d^{-1} (0930-1630 h), for a variable number of days particular to specific experiments.

For those experiments in which the net rate of photosynthesis (A), stomatal conductance (g$_s$) and intercellular CO_2 concentration (C$_i$) were recorded, these parameters were measured using a PP Systems (Hitchin, UK) Combined Infrared Gas Analysis System (CIRAS-1) and a Parkinson Leaf Cuvette (window size: broad, leaf area: 2.5 cm^2) supplying 355 ppm CO_2, a concentration chosen to be close to the current long term ambient average (IPCC, 1995). Measurements were taken daily at 0900 h, 1300 h and 1700 h to provide a record of A, g$_s$ and C$_i$ prior to, during and after ozone introduction. To gain access to the plants, the front of each exposure chamber was removed for the duration of the measurement period, resulting in temporary reductions in temperature of ~1 °C, ozone concentration to < 5 ppb and an increase in relative humidity of 3 % within the chambers. The leaf cuvette was clamped onto the middle of the oldest true leaf (*Brassica campestris*) or the largest expanding leaf (*Plantago major*) and held as close to the light source as possible, providing a PAR flux of 180-250 µmol m^{-2} s^{-1}. When the readings for A, g$_s$ and C$_i$ became steady, the values were recorded. The stored data were downloaded from the CIRAS-1 to a PC at the end of the experimental period.

3.2.1. Experiment 1: The physiological and yield responses of *Brassica campestris* to a two day fumigation with ozone

Three separate experiments using *B. campestris* were undertaken, each involving 20 plants, 10 treatment and 10 control. In two of these experiments, the plants were grown within the CEGC (Experiments 1a & 1b) and transferred into the exposure chambers 12 days after sowing (DAS) to allow the plants to acclimatise to their new environment prior to fumigation. In the third experiment, the plants were transferred to

the exposure chambers at 4 DAS until the end of the fumigation period. In all three experiments, the plants were exposed to filtered air (control treatment) or 70 ppb ozone for 7 h d^{-1} (0930-1630 h) at 14 and 15 DAS, when the first true leaf of each plant was large enough to allow physiological measurements to be made. The physiological parameters A, g_s and C_i were recorded in each experiment (Section 3.2.1.1) and in one experiment (Experiment 1b) seed yield was also recorded (Section 3.2.1.2). A summary of the three experiments is shown in Table 3.1.

3.2.1.1. Measurement of physiological parameters

A, g_s and C_i were recorded each day between 13-16 DAS, to include the day prior to and the day after two days of fumigation with ozone. Any visual foliar injury developing during the experimental period was recorded photographically.

3.2.1.2. Seed yield

By the last day of physiological measurements (16 DAS), *B. campestris* had begun to flower. To determine whether there was any subsequent effect of two days of exposure to ozone on seed yield, the plants of Experiment 1b, which had been grown initially in the CEGC, were returned to the CEGC to complete their life cycle. Axillary racemes were removed from each plant to leave only the terminal raceme, and all of the flowers opening on this were cross-pollinated using pollen from control plants. At the end of the life cycle, the seeds were harvested. In *B. campestris*, as for many other species (e.g. pea, Ney *et al.*, 1994), it is natural for some of the seeds in each pod to abort before maturity. In *B. campestris* some seeds were identified as being small (< 1 mm), dark brown and flat, giving the appearance that only the testa remained and that seed filling was incomplete. Germination tests showed that > 90% of the seeds within this category did not germinate within 7 days, substantiating the view that these were aborted seeds. Invariably, a small proportion (< 1 %) of the seeds per plant had also germinated within the pods by the time of harvest, these being easily identified by the emergence of the radicle. Thus, for each plant, the number of mature, germinated and aborted seeds contained within each pod were recorded.

3.2.1.3. Germination of seeds

Mature seeds were germinated in petri dishes containing 1% agar in order to provide a constant moisture supply, since preliminary tests of germination on filter paper showed that it was difficult to prevent drying out of the incubation medium. 20 randomly

Table 3.1. Summary of three separate fumigations undertaken in Experiment 1.
✓ indicates measurements made.

Experiment	Growth outwith exposure period	Measurements		n
		A, Ci, gs	Yield	
1 a	CEGC	✓		10
1 b	CEGC	✓	✓	10
1 c	Exposure chamber	✓		10

chosen seeds from each plant, giving a total of 200 seeds per treatment, were tested for germination. The 20 seeds from each plant were put onto an agar plate, placed within the CEGC under the conditions set for *B. campestris*, and the number of germinated seeds was counted at 24 h intervals over the following seven days. After this time, the majority of seeds had germinated and fungal contamination of the agar and seeds precluded longer term observation. A seed was identified as having germinated if the testa had ruptured to reveal the cotyledons and radicle, or if the radicle alone had emerged through the testa.

3.2.2. Experiment 2: Effects of a 10 day exposure to ozone on growth and reproduction of *Brassica campestris*

Three days after sowing, 40 seedlings of *B. campestris* were placed in the exposure chambers, 20 as treatment and 20 as control. After 24 h of acclimation, the plants were exposed to filtered air (control treatment) or 70 ppb ozone for 7 h d^{-1} from 4-13 DAS. Exposure lasted for 10 days during the main vegetative phase of development prior to first flowering, during which time all of the true leaves and initial floral racemes developed.

3.2.2.1. *Measurement of physiological and growth parameters*

A, g_s and C_i were measured on the last day of exposure (13 DAS) prior to, during and after ozone introduction at 0900 h, 1300 h and 1700 h respectively, using 10 plants per chamber. The following measurements were then made at 14 DAS.

1. Number of true leaves.
2. Number of leaflets.
3. Number of axillary racemes.
4. Total number of reproductive sites on the terminal and axillary racemes, including open flowers and buds >1 mm in diameter.
5. Length of terminal raceme, measured as the distance between the points where the first and last reproductive sites were initiated.
6. Total height, including the stem and the terminal raceme.
7. Leaf area.
   The leaves of each plant were removed at the junction with the stem and an outline of each leaf (± petiole) was traced onto paper and labelled cotyledon, true leaf (plus number in order of development) or leaflet. The area of each leaf was then determined using a Digital-Planimeter (A.OTT, Kempten, Germany, model Ottplan 700/710).

8. Dry weights.

The plant was divided into root, leaves, stem (= stem plus terminal raceme minus reproductive sites) and reproductive sites (= flowers plus buds) and oven-dried at 60 °C for 72 h before being weighed. From the dry weights obtained, the Root:Shoot Ratio and Specific Leaf Area (SLA) were calculated.

3.2.2.2. Measurements of reproductive biology

The remaining 10 plants per chamber were returned to the CEGC where the axillary racemes were removed for the reasons discussed in Section 2.4.1.1. All flowers opening on the terminal raceme were cross-pollinated using pollen from unexposed donor plants, and then allowed to set seed. As well as recording final seed yield, various aspects of reproduction were monitored throughout the period of flowering extending from 14 to 26 DAS. The period of flowering was advanced by two to three days as a result of 10 days of growth within the exposure chambers.

Reproductive sites and terminal raceme development

Each day throughout the period of flowering, the length of the terminal raceme was measured as the distance between the points where the first and last floral sites were initiated. The total number of floral sites present on the terminal raceme was also recorded daily throughout the period of flowering. In addition, the stage of floral development was recorded for each site at 24 h intervals. The following 5 stages of development were identified for *Brassica campestris*:

1. **Bud** - >1 mm in diameter.
2. **Open flower** - either opening or fully opened.
3. **Developing pod** - flowers with petals wilting and reflexing forward or petals, sepals and anthers abscinding, ovary enlarging.
4. **Aborted post flowering** - flowers with petals wilting and reflexing forward or petals, sepals and anthers abscinding, ovary not enlarging.
5. **Aborted buds** - non-abscinding yellow buds which failed to open.

The number of reproductive sites on the terminal raceme at each stage of floral development was calculated daily to determine whether treatment with ozone affected the number of sites aborting or progressing through to each stage of floral development.

Pollen

To examine the effect of ozone upon the germination and tube growth of pollen produced following exposure, pollen was collected from the most recently dehiscent flower of each plant, when all of the plants were flowering (15 DAS). Using an acrylic paintbrush, pollen from each flower was lightly dusted from the anthers into separate petri dishes. As suggested by Science and Plants for Schools (SAPS, 1990c), a pollen germination medium consisting of two separate solutions was utilised for *B. campestris*. These solutions comprised:

Mineral salt solution

0.417 g	$Ca(NO_3)_2$
0.200 g	H_3BO_3
0.101 g	KNO_3
0.217 g	$MgSO_4.7H_2O$
3.5 cm³ of 1.0 M	NH_4OH

Made up to 1 litre with deionised water.

Sugar Solution

1.2 M Sucrose solution

Equal volumes of each solution were mixed to provide a medium at pH 8.8 which was ideal for the growth of *B. campestris* pollen grains. The pollen from each flower was dusted onto a 50 μl drop of this medium on a microscope slide and placed within a petri dish on a moist filter paper to maintain a high relative humidity. Although pollen grains of *Brassica campestris* should begin to germinate within an hour (SAPS, 1990c), the samples were placed within the CEGC, under conditions set for *B. campestris*, for 24 hours to allow completion of germination. For observation under the microscope, a permanent preparation of the pollen grains was made by placing 2-3 drops of melted glycerol jelly onto the germination medium and positioning a coverslip on top. Pollen grains were observed at 100 x magnification. For germination measurements, a total of 200 pollen grains per slide were counted. A transect through the centre of the sample was used and if this did not provide sufficient pollen grains, transects adjacent to the centre were also counted. Germination was deemed to have occurred if the length of the pollen tube was equal to or greater than the diameter of the pollen grain. In addition, the length of 20 randomly chosen pollen tubes per slide was measured using a graticule.

Seed yield

The total number of developing pods per plant was counted and the length of each fully elongated ovary was measured at 30 DAS. The seeds from each pod were harvested at 45 DAS and the number of mature, germinated and aborted seeds counted. The mature seeds were pooled for each plant and weighed to calculate the total mature seed weight per plant and the average individual mature seed weight per plant. The remainder of each pod was oven-dried at 60 °C for 72 h before being weighed collectively for each plant.

Seed colour

During harvest it was noted that a range of seed colours was produced both within individual plants and between treatments. The mature seeds from each treatment were therefore pooled and subsequently divided into 3-4 colour categories. The following categories were used for each treatment.

Control - Brown, Purple/Brown and Light Brown
Ozone - Brown, Green, Yellow/Green and Yellow

The seeds within each colour category were counted and weighed before carrying out germination and imbibition tests.

Germination of seeds

Before the seeds from each treatment were pooled and separated into colour categories (above), 20 randomly chosen seeds from each plant were tested for germination as detailed in Section 3.2.1.2. After the seeds from each treatment had been pooled and separated into colours, four replicates containing 20 seeds per colour category were germinated in the same manner and germination was scored at 24 h intervals over a seven day period.

Imbibition of Seeds

Imbibition is the adsorption of water by seeds and is the first stage in germination. The rate of imbibition by differently coloured seeds was calculated to determine whether there was any relationship with the rate or extent of germination. For each treatment, 10 seeds per colour category were weighed collectively and then placed in a beaker containing 20 ml of deionised water. Since imbibition is a passive process, this

method allowed more rapid water uptake than if the seeds had been placed on moist filter paper. The seeds were removed from the water at one hour intervals and towel-dried before being weighed and replaced in fresh deionised water. The measurements continued until there was no more weight gain, usually after 6 h. Water uptake per seed was calculated as a percentage of the initial weight. This process was repeated to provide three replicates per colour category.

Light microscopy study of seeds

Several seeds from each colour category were fixed overnight in 3 % glutaraldehyde in 0.02 M phosphate buffer (pH 7) and then washed overnight in 0.02 phosphate buffer (pH 7). The seeds were dehydrated in series of ethanol solutions, allowing > 2 h each in 70, 80, 90 and 100 % ethanol. The seeds were then embedded in LR white (London Resin Company; medium grade resin) for two weeks, changing the resin frequently, to allow complete infiltration of the tissue. At the end of this period, the seeds were transferred to fresh resin and placed in gelatine capsules, covered to exclude air, and left overnight in an oven at 60 °C to harden the resin. Using a Reichert OM U2 Ultramicrotome, 5 μm thick sections were cut from each seed with a glass knife and placed onto glass microscope slides. Sections were stained with a 0.1 % aqueous solution of Toluidine Blue before being mounted in Euparal Mountant (Fisons, UK). Sections were observed and photographed using a Leica DMRB photomicroscope.

3.2.3. Experiment 3: Effects of ozone on the physiology and growth of *Plantago major*

Three experiments were undertaken involving 2, 6 or 14 days of exposure to ozone and measurements of A, g_s and C_i and/or biomass partitioning and mean relative growth rate were made using the *P. major* populations shown in Table 3.2.

3.2.3.1. Experiment 3a. Physiological responses of Plantago major *to a two day fumigation with ozone.*

In a similar way to *Brassica campestris* (Section 3.2.1.), this experiment set out to examine the response of A, g_s and C_i in four populations of *P. major* when exposed over two days to filtered air (control treatment) or air containing 70 ppb ozone for 7 h d^{-1}. Due to limited space within the exposure chambers, each population was exposed separately to ozone. Twenty plants, 10 treatment and 10 control, from each of the four populations were used for exposure. The plants were grown within the

Table 3.2. Summary of the three exposure period applied in Experiment 3. ✓ indicates measurement made.

Experiment	Days of fumigation with O_3	Population	Days after sowing when fumigated	Measurements		n
				A, Ci, gs	Biomass	
3 a	2	High Low	28	✓		10
		Lullington	26			
		Penicuik	28			
		Sibton	30			
3 b	6	High Low	25	✓	✓	8
		Lullington	25			
		Penicuik	25			
3 c	14	High Low	28		✓	9
		Lullington	28			
		Sibton	28			

CEGC and then transferred to the exposure chambers 48 h prior to fumigation, which started from 26-30 DAS depending on the population involved (Table 3.2). A, g_s and C_i were first measured three days prior to ozone exposure while the plants were still growing within the CEGC and the first true leaves were large enough to allow physiological measurements to be taken. This approach enabled A, g_s and C_i to be compared in the CEGC and the exposure chambers. Within the CEGC, the PAR flux reaching the leaf cuvette was c.220 μmol m^{-2} s^{-1}. On the morning following these measurements, the plants were transferred into the exposure chambers where, after 24 h, A, g_s and C_i were recorded for four consecutive days, including the day prior to and the day after two days of fumigation with ozone.

3.2.3.2. Experiment 3b. Physiological and growth responses of Plantago major *to a six day fumigation with ozone*

The mean relative growth rate, $\overline{R}$, and the allometric root/shoot coefficient, K, (Hunt, 1990) have been utilised in previous studies (Reiling, 1990; Reiling and Davison, 1992d) as a measure of the effect of ozone on growth and the balance of growth between the root and shoot. To determine the effect of six days of exposure to ozone on $\overline{R}$ and K, this experiment utilised 24 plants from each of three *P. major* populations: High Low, Lullington and Penicuik. Due to a lack of germinated seedlings, the Sibton population had to be omitted from this experiment.

From each population, eight plants were harvested at 23 DAS, divided into root and shoot, oven-dried at 60 °C for 72 h and weighed; the remaining 16 plants, 8 treatment and 8 control, were randomly placed within the exposure chambers and exposed to filtered air (control treatment) or 70 ppb ozone for 7 h d^{-1} from 25-30 DAS and then harvested, divided into root and shoot, oven-dried at 60 °C for 72 h and weighed. The following growth parameters were then calculated for each population.

Mean relative growth rate was calculated as:

$$\overline{R} = (\log_e W_2 - \log_e W_1)/(t_2 - t_1) \tag{1}$$

where W_1 and W_2 represent total dry weights at times 1 and 2, and t_1 and t_2 represent times 1 and 2.

The allometric root:shoot coefficient was calculated as:

$$K = \overline{R}_R/\overline{R}_S \tag{2}$$

where $\overline{R}_R$ = root relative growth rate and $\overline{R}_S$ = shoot relative growth rate.

In addition, ozone resistance (O_{res}), defined by Reiling and Davison (1992d) as the relative growth rate of ozone-treated plants expressed as a percentage of that in charcoal-filtered air, was calculated in order to allow a comparison with published data for various *P. major* populations. O_{res} was calculated as:

$$O_{res} = \overline{R}_{ozone}/\overline{R}_{control} \times 100 \qquad (3)$$

A, g_s and C_i were also measured prior to exposure on the final day of growth within the CEGC (23 DAS). These parameters were measured again 24 h after the plants had been transferred to the exposure chambers at 24 DAS, and then repeated on the first, second, third and sixth days of exposure to ozone.

3.2.3.3. Experiment 3c. The growth responses of Plantago major *to a 14 day exposure to ozone*

As a comparison with the data reported by Reiling and Davison (1992d), this experiment set out to determine the effect of 14 days of exposure to ozone on the growth rate and biomass partitioning of *P. major* populations under the experimental conditions employed in the present study. Only three populations: High Low, Lullington and Sibton were used since insufficient seedlings of the Penicuik population had established.

Using a total of 27 plants from each population, $\overline{R}$, K and O_{res} were calculated as described in Section 3.2.3.2., with 9 plants being harvested at 27 days after sowing, divided into root and shoot, oven-dried at 60 °C for 72 h and weighed. 18 plants, 9 treatment and 9 control, were then placed within the exposure chambers and exposed for 14 days to filtered air (control treatment) or 70 ppb ozone for 7 h d^{-1} from 28-42 DAS and then harvested and analysed as described above.

3.2.4. Data Analysis

3.2.4.1. *Experiment 1*

Physiological parameters

For each experiment, the mean values for stomatal conductance, net rate of photosynthesis and intercellular CO_2 concentration for ozone-treated and control plants were analysed separately for each time interval using paired, two tailed t-tests, to establish significance ($P<0.05$).

Floral development and seed yield

For each plant of *Brassica campestris*, the following variables were recorded:

1. Total number of floral sites on the terminal raceme.
2. Total number of floral sites which aborted, either as buds or post pollination.
3. Number of axillary racemes which developed and were subsequently removed.
4. Number of mature, germinated and aborted seeds in each pod.
5. Total mature seed weight per plant.

For each plant, the total number of mature, germinated and aborted seeds, the average number of mature, germinated and aborted seeds per pod and average individual mature seed weight were calculated. The treatment means for these variables were tested for significance ($P<0.05$) using a paired, two tailed t-test ($n=10$).

Germination

The data for percentage germination were transformed using an arc sine transformation prior to analysis. Means were compared for ozone-treated and control plants at each time interval using a paired, two tailed t-test, to establish significance ($P<0.05$).

3.2.4.2. *Experiment 2*

Physiological parameters

A, g_s and C_i were analysed as for Experiment 1, above.

Growth parameters

Means parameters were compared for ozone-treated and control plants using a paired, two tailed *t*-test (n=10), to establish significance (P<0.05): the data for plant height, length of the terminal raceme, the numbers of axillary racemes, number of reproductive sites on the terminal and axillary racemes, true leaves and leaflets, root, shoot, stem, leaf, reproductive and total dry weights, root:shoot ratio, specific leaf area, total leaf area and the area of each leaf type; cotyledons, true leaves and sessile leaves, were all analysed separately.

Reproductive biology

a) Daily measurements

The following measurements were recorded daily throughout the period of flowering:

1. Terminal raceme length.
2. Total number of reproductive sites on the terminal raceme.
3. The number of reproductive sites present as buds, open flowers, developing pods, aborted post flowering and aborted buds.

The means for each of these parameters were compared for ozone-treated and control plants at each time interval using paired, two tailed *t*-tests.

b) Pollen

The number of germinated pollen grains in each sample was converted to a percentage and then transformed using an arc sine transformation prior to analysis (Gomez and Gomez, 1984). The sample means were then compared using a paired, two tailed t-test to identify significant differences (P<0.05). The 20 measurements of pollen tube length from each sample were used collectively for analysis to give n=200. Means for pollen tube length were analysed by calculating *d*, the 'standardised normal deviate' (Parker, 1979):

$$d = \frac{\overline{X}_1 - \overline{X}_2}{\sqrt{\left(\frac{s_1^2}{N_1} + \frac{s_2^2}{N_2}\right)}}$$

where $\overline{X}_1$ and $\overline{X}_2$ represent the means of samples 1 and 2, s_1^2 and s_2^2 denote the variance of samples 1 and 2, and N_1 and N_2 indicates the number of measurements in samples 1 and 2.

Significance (P<0.05) was determined by comparing the calculated value for d with the tabulated values for d (equivalent to tabulated values for t with an infinite number of degrees of freedom).

c) Seed yield parameters

The following variables were recorded for each plant:

1. Total number of pods.
2. Length and weight (minus seeds) of each pod.
3. Number of mature, germinated and aborted seeds per pod.
4. Total weight of mature seeds.

For each plant, the total number of mature, germinated and aborted seeds, average individual pod weight and average individual mature seed weight were calculated. Means for all of the above parameters except the number of mature, germinated and aborted seeds per pod were compared for ozone-treated and control plants using a paired, two tailed t-test, to establish significance (P<0.05). The numbers of mature, germinated and aborted seeds in each pod were analysed collectively for all plants in each treatment and the means for ozone-treated and control plants were analysed for significant differences by calculating the standardised normal deviate (above).

d) Seed germination and imbibition

The data for percentage germination were transformed using an arc sine transformation prior to analysis. Means for the germination of seeds prior to separation into different colour categories were compared for ozone-treated and control plants at each time interval using a paired, two tailed t-test, to establish significance (P<0.05). Since the range of seed colours differed between treatments, the mean values for germination of different coloured seeds were analysed separately for ozone-treated and control plants at each time interval using a single factor Analysis of Variance (ANOVA) in combination with Duncan's Multiple Range Test to identify significant differences (P<0.05; Gomez and Gomez, 1984). The means for percentage water uptake during

imbibition for the different seed colour categories were also analysed in this way using the untransformed percentage data.

3.2.4.3. *Experiment 3*

Experiment 3a

Since each population of *Plantago major* was exposed independently to ozone, direct statistical comparison of the responses of stomatal conductance, net rate of photosynthesis and intercellular CO_2 concentration was not possible; a separate analysis was therefore made between the treated and control plants of each population. A, g_s and C_i were analysed for each population as for Experiment 1 (Section 3.2.4.1).

Experiment 3b

a) Physiological parameters

Since the plants from each of the three populations of *Plantago major* had been exposed simultaneously in this experiment, the data for A, g_s and C_i were analysed at each time interval using a two factor ANOVA to test for significant treatment effects (P<0.05). For each population, the means for ozone-treated and control plants were compared at each time interval using a paired, two tailed *t*-test, to identify significant differences (P<0.05).

b) Growth parameters

The means for relative growth rate and the allometric root:shoot coefficient were analysed using a two factor ANOVA in combination with Duncan's Multiple Range Test. This type of analysis was also used in Experiment 3c.

3.3.1. Experiment 1: The physiological and yield responses of *Brassica campestris* to a two day fumigation with ozone

Environmental conditions

Environmental conditions within the control and treatment chambers during the four day period of measurement in Experiments 1a, 1b and 1c are shown in Table 3.3. Mean air temperature varied between 24.4 °C and 25.1 °C over the three experiments with no significant difference between the chambers. Relative humidity varied between 42.0-45.0 % in the control chamber and between 46.0-49.4 % in the treatment chamber. The higher relative humidity recorded within the treatment chamber may have been due to problems encountered with the dewpoint hygrometer used to monitor the airstream passing through this chamber. The mean concentration of ozone within the treatment chamber was close to the target of 70 ppb in all three experiments, while the concentration recorded within the control chamber was invariably below 1 ppb.

3.3.1.1. *Physiology and visible damage*

Experiment 1a

Figure 3.1 shows that there were significant effects of ozone on stomatal conductance (g_s), the net rate of photosynthesis (A) and intercellular CO_2 concentration (C_i). Stomatal conductance (Fig. 3.1a) varied between 311 and 433 mmol m^{-2} s^{-1} during the four days of growth under constant environmental conditions. Ozone caused a progressive reduction in stomatal conductance throughout the first day of exposure to a minimum value of 279 mmol m^{-2} s^{-1} at 1700 h. This reduction of 87 mmol m^{-2} s^{-1} had been almost entirely restored by 0900 h on day 3, before a similar pattern of response was exhibited during the second exposure. There was little recovery of stomatal conductance up to 24 h after the end of this exposure, suggesting a persistent effect had developed after only two days of exposure to 70 ppb ozone. The net rate of photosynthesis (Fig. 3.1b) varied during the four day experimental period in control plants exposed to filtered air, but this did not obscure the significant reduction induced by ozone at 1700 h on day two. This reduction was still apparent at 0900 h on day three and, during the second day of exposure, the net rate of photosynthesis fell further so that by 1700 h it was 2.4 μmol m^{-2} s^{-1} lower than in the control plants, at 4.5 μmol m^{-2} s^{-1}. On day four, the values for A followed a similar pattern to control plants, with the values recorded at 0900 h and 1700 h being lower than at 1300 h;

70

Table 3.3. Mean air temperature and relative humidity over the four day experimental period within the control and treatment chambers during the three separate fumigations undertaken in Experiment 1. Double standard errors are shown. ** and *** indicate significance at P<0.01 and P<0.001. NS, non significant.

Experiment 1a	Control	Ozone	Significance
Temperature (°C)			
Outwith exposure [1]	25.0 ± 0.09	25.1 ± 0.03	NS
During exposure [2]	24.9 ± 0.01	24.7 ± 0.03	NS
Relative humidity (%)			
Outwith exposure [1]	42.9 ± 0.26	47.7 ± 0.08	***
During exposure [2]	43.3 ± 0.04	48.7 ± 0.09	***
Ozone concentration (ppb)	71.9 ± 1.81	< 1.0	***

Experiment 1b	Control	Ozone	Significance
Temperature (°C)			
Outwith exposure [1]	24.6 ± 0.08	24.6 ± 0.07	NS
During exposure [2]	24.4 ± 0.05	24.5 ± 0.04	NS
Relative humidity (%)			
Outwith exposure [1]	42.0 ± 0.25	46.0 ± 0.08	***
During exposure [2]	42.3 ± 0.16	46.5 ± 0.13	***
Ozone concentration (ppb)	71.8 ± 2.30	< 1.0	***

Experiment 1c	Control	Ozone	Significance
Temperature (°C)			
Outwith exposure [1]	24.7 ± 0.03	24.9 ± 0.08	NS
During exposure [2]	24.4 ± 0.05	24.7 ± 0.05	**
Relative humidity (%)			
Outwith exposure [1]	44.4 ± 0.12	49.0 ± 0.30	***
During exposure [2]	45.0 ± 0.15	49.4 ± 0.18	***
Ozone concentration (ppb)	69.0 ± 2.64	< 1.0	***

[1] 0000-0930 h and 1630-0000 h; [2] 0930-1630 h.

Figure 3.1. Experiment 1a. Responses of a) stomatal conductance, b) net rate of photosynthesis and c) intercellular CO_2 concentration over a four day period in *Brassica campestris*. Plants were exposed to filtered air (control) or 70 ppb ozone for 7 h d^{-1} (0930-1630 h) on days two and three. Solid squares, controls; open squares, ozone-treated. Double standard errors are shown, except when these are smaller than the symbols. * and ** indicate significance at $P<0.05$ and $P<0.01$; n=10.
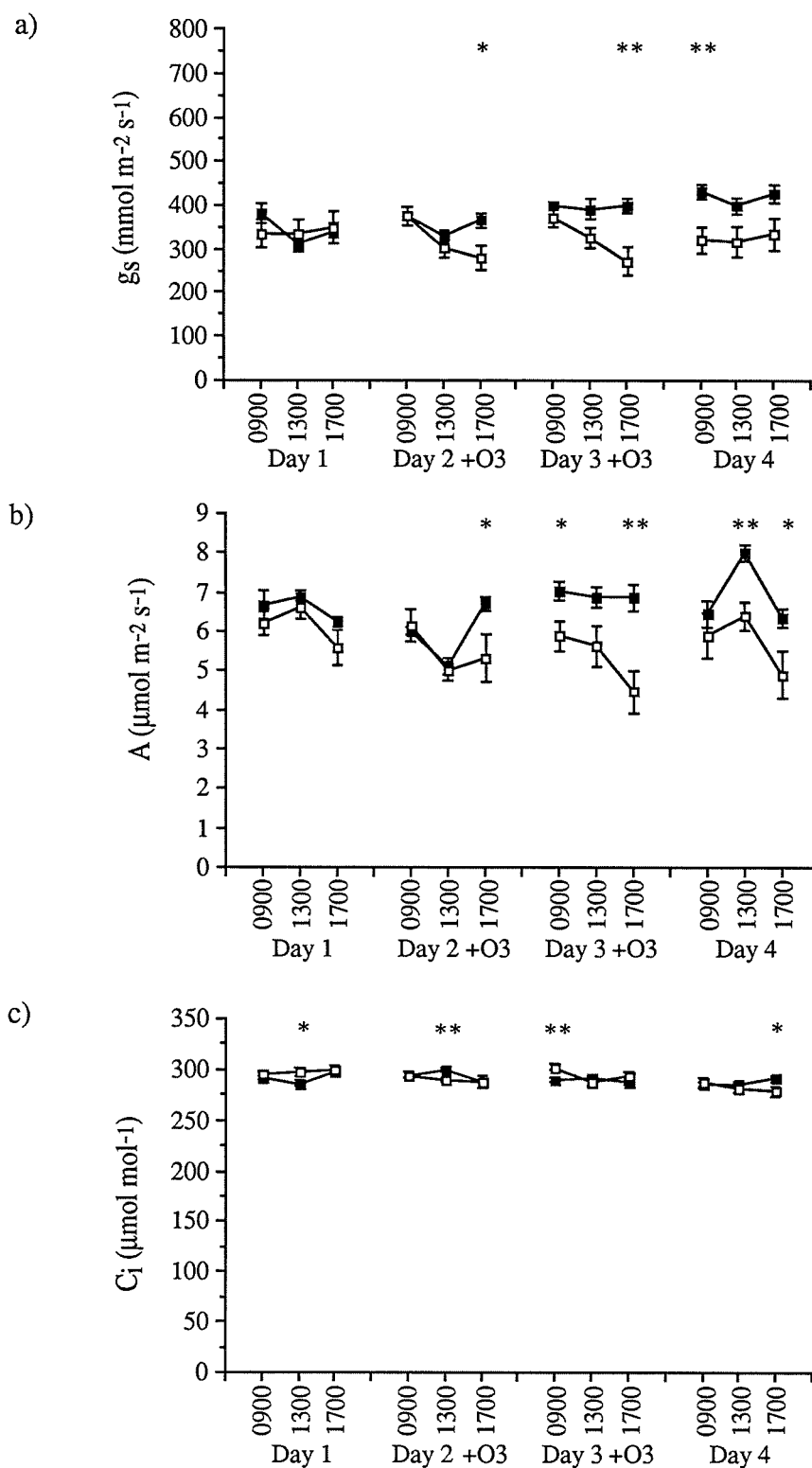
however the net rate of photosynthesis was lower than in the controls at all times, with no recovery evident. The intercellular CO_2 concentrations (Fig. 3.1c) in control and treated plants varied between 278 and 302 μmol mol^{-1} throughout the four day period of measurement. There was no consistent effect of ozone on C_i, although significant differences between control and treated plants were apparent throughout the four day period, including day one at 1300 h even though no ozone had been applied. No visible foliar injury was observed by the end of the four day period.

Experiment 1b

Prior to fumigation with ozone, stomatal conductance (Fig. 3.2a) ranged between 490-564 mmol m^{-2} s^{-1}. After commencing ozone fumigation on day two, stomatal conductance showed a significant reduction at 1700 h. There was little recovery by 0900 h on day three and this effect persisted throughout the following 24 h, with no evidence of recovery to control levels. The net rate of photosynthesis (Fig. 3.2b) followed a similar pattern to stomatal conductance, but the significant reduction on day two was evident earlier at 1300 h, 3.5 h after the start of ozone exposure. This reduction in photosynthesis persisted throughout the second day of exposure and the subsequent 24 h. Significant differences were seen in intercellular CO_2 concentration, which tended to be higher in the ozone-treated plants following the start of exposure on day two (Fig. 3.2c).

Foliar injury was observed on the second day of exposure to ozone (day 3). This took the form of both interveinal chlorosis of the true leaves (Plate 3.1) and small necrotic flecks on both the true leaves and sessile leaves. Injury was not universal, with some cotyledons and leaves exhibiting no damage.

Experiment 1c

The stomatal conductance of control plants ranged between 512 and 586 mmol m^{-2} s^{-1} throughout the four day period (Fig. 3.3a). The plants in the ozone treatment exhibited significantly higher stomatal conductances than the control plants prior to fumigation, averaging 653.7 mmol m^{-2} s^{-1}, suggesting the possible existence of a chamber effect since these plants had been grown for 9 days within the exposure chambers prior to the first measurements. After applying ozone, stomatal conductance decreased to values similar to the control plants by the end of the first exposure. By the following morning, there had been no recovery of stomatal conductance to prefumigation levels; indeed stomatal conductance was less than the control plants throughout the second day of exposure, although not significantly. Stomatal conductance recovered to control

Figure 3.2. Experiment 1b. Responses of a) stomatal conductance, b) net rate of photosynthesis and c) intercellular CO_2 concentration over a four day period in *Brassica campestris*. Plants were exposed to filtered air (control) or 70 ppb ozone for 7 h d^{-1} (0930-1630 h) on days two and three. Solid squares, controls; open squares, ozone-treated. Double standard errors are shown, except when these are smaller than the symbols. *, ** and *** indicate significance at P<0.05, P<0.01 and P<0.001; n=10.
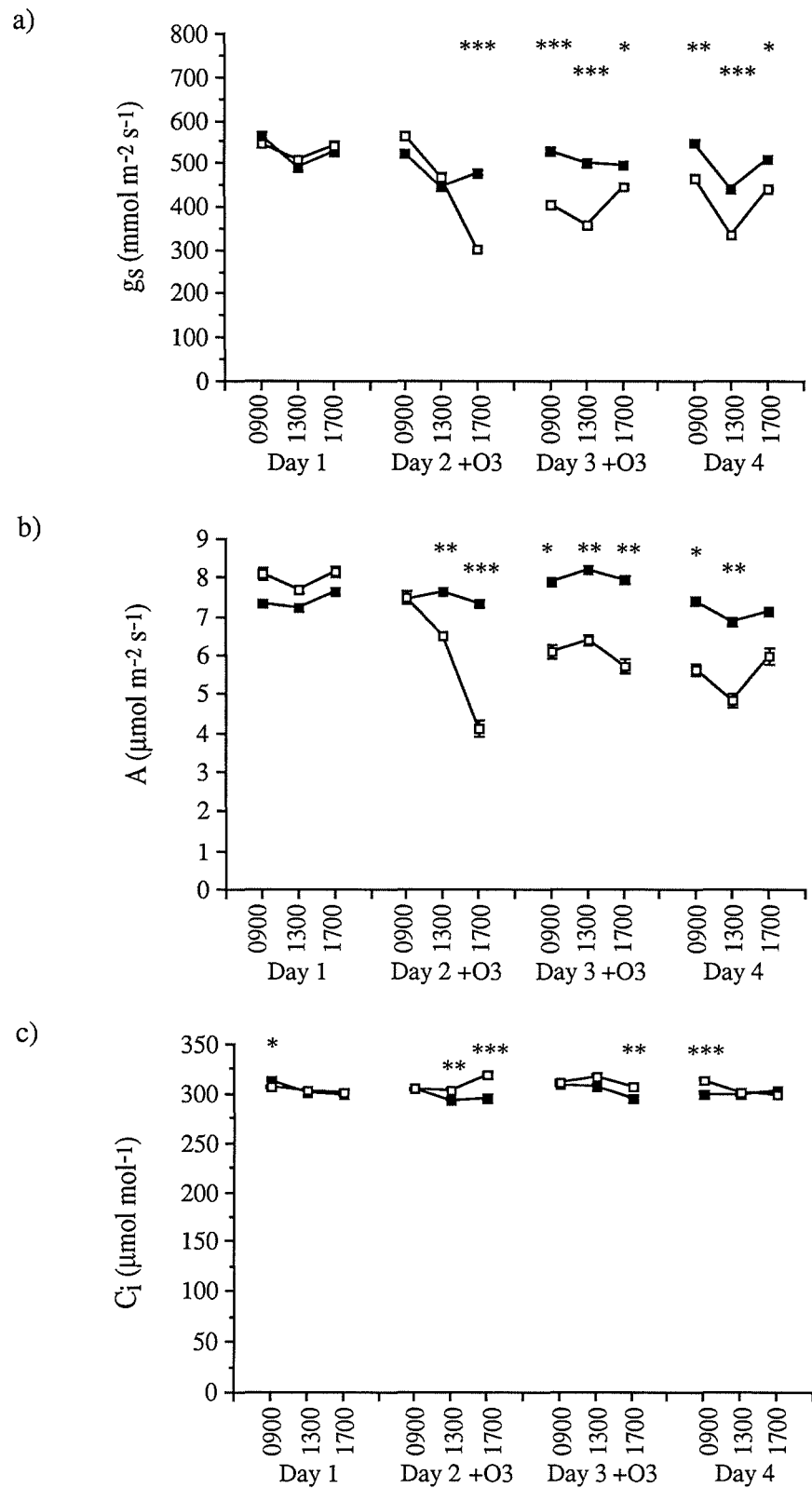
Plate 3.1.   Experiment 1b: leaf of *Brassica campestris* showing interveinal chlorosis 24 h after two days of exposure to 70 ppb ozone for 7 h d^{-1} at 14-15 DAS.

Figure 3.3. Experiment 1c. Responses of a) stomatal conductance, b) net rate of photosynthesis and c) intercellular CO_2 concentration over a four day period in *Brassica campestris*. Plants were exposed to filtered air (control) or 70 ppb ozone for 7 h d^{-1} (0930-1630 h) on days two and three. Solid squares, controls; open squares, ozone-treated. Double standard errors are shown, except when these are smaller than the symbols. *, ** and *** indicate significance at $P<0.05$, $P<0.01$ and $P<0.001$; n=10.
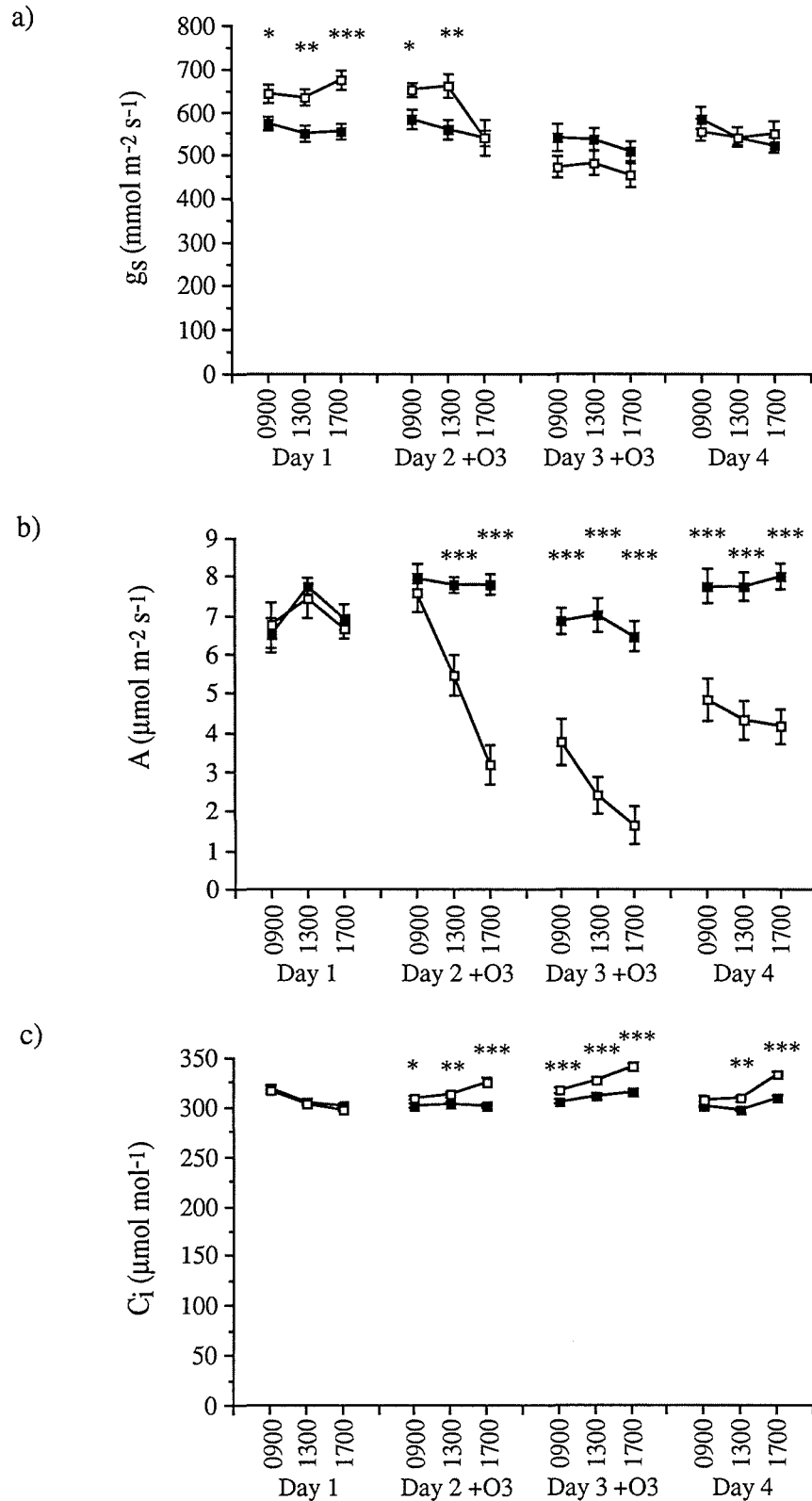
levels in the subsequent 24 h however, although the values obtained were lower than the prefumigation conductances of these plants on day one. In contrast to stomatal conductance, the net rate of photosynthesis (Fig. 3.3b) did not differ between treatments prior to ozone introduction, although there was a rapid and extensive decline in A from 7.6 $\mu mol \ m^{-2} \ s^{-1}$ to 3.2 $\mu mol \ m^{-2} \ s^{-1}$ during the 7 h exposure. There was very little recovery by 0900 h on the following day, when photosynthesis declined further throughout the second exposure to a minimum of 1.7 $\mu mol \ m^{-2} \ s^{-1}$. By day four there was again little recovery of photosynthesis, which remained significantly lower than in the control plants for up to 24 h after the last exposure. Intercellular CO_2 concentration (Fig. 3.3c) increased significantly throughout each of the two days of fumigation, recovering partially by the following morning at 0900 h. Although there was no further introduction of ozone on day four, C_i continued to rise in a similar way to that seen on the previous two days of exposure.

By the end of the first 7 h exposure, extensive foliar damage was evident. This injury appeared as areas where turgor had been lost (Plate 3.2a), and was found on most leaves. With this type of damage, there was also a general 'stickiness' of the leaves, suggesting a release of cell contents. Seventeen hours later, prior to the second exposure, these areas had developed into necrotic patches extending over the leaf and leaf margins, where the leaf had started to curl (Plate 3.2b). By the end of the second exposure, the necrotic patches were clearly evident (Plate 3.2c).

3.3.1.2. *Seed yield*

In Experiment 1b, two days of exposure to ozone had no significant effect on the number of reproductive sites produced or aborted on the terminal raceme, or on the number of axillary racemes which developed (Table 3.4). There were also no significant differences in the numbers of mature, germinated or aborted seeds per pod or plant and no effect on either the individual or total seed weight per plant (Table 3.5).

3.3.1.3. *Germination of seeds*

Germination began within 24 h of sowing for seeds from ozone-treated and control plants and by 96 h the majority of seeds (80-85 %) had germinated (Fig. 3.4). Ozone had no significant effect on the timecourse of germination, although the percentage germination was less than for control seed at each time interval.

Plate 3.2a. Experiment 1c: Typical leaf of *Brassica campestris* immediately after the first of two 7 h exposures to 70 ppb ozone at 14 and 15 DAS showing loss of turgor particularly in the interveinal areas.

Plate 3.2b. Experiment 1c: Typical leaf of *Brassica campestris* 17 h after the first of
two 7 h exposures to 70 ppb ozone at 14 and 15 DAS showing marginal
leaf curl, the development of necrotic patches and general leaf chlorosis.
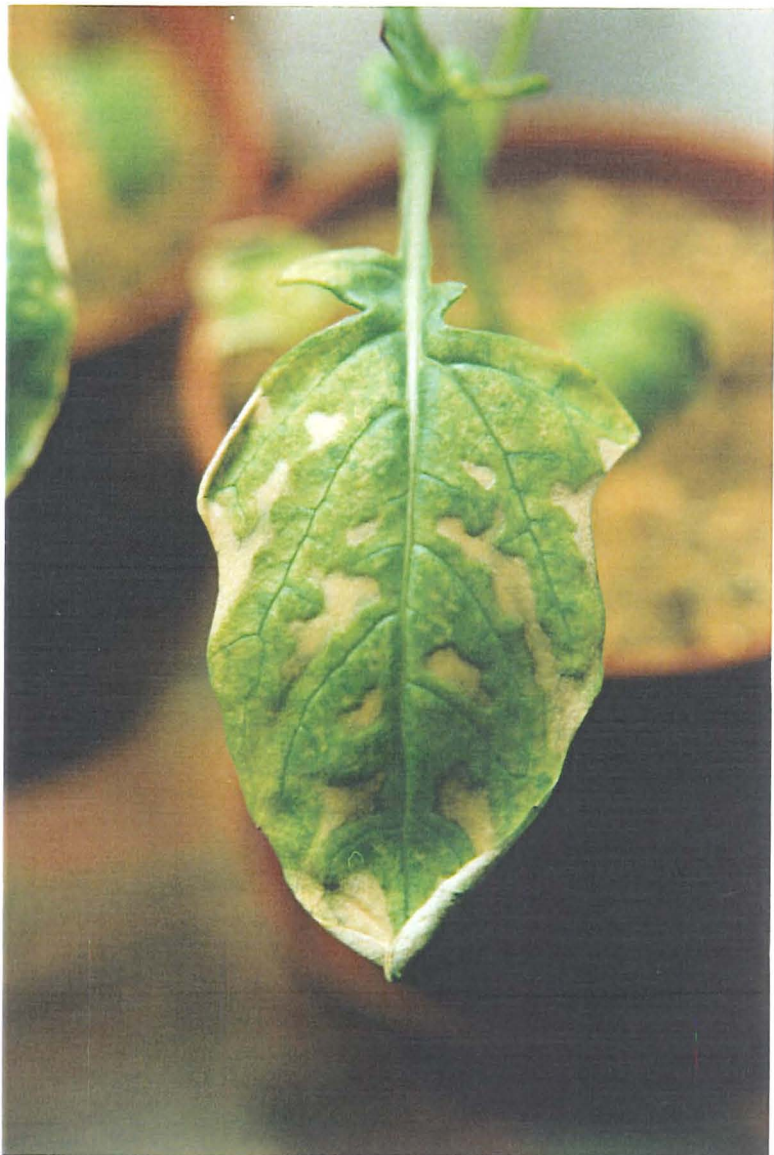
Plate 3.2c. Experiment 1c: Typical leaf of *Brassica campestris* immediately after the second of two 7 h exposures to 70 ppb ozone at 14 and 15 DAS showing marginal leaf curl, necrotic patches and general leaf chlorosis.

Table 3.4. Experiment 1b: Summary of flower production in *Brassica campestris* after two days of exposure to filtered air (control) or 70 ppb ozone for 7 h d^{-1}. Means and standard errors are shown. NS, non significant; n=10.

	Control	Ozone	Significance
Total number of reproductive sites on terminal raceme	23.3 ± 0.24	24.8 ± 0.61	NS
Number of aborted reproductive sites on terminal raceme	1.9 ± 0.16	2.6 ± 0.23	NS
Number of axillary racemes removed	8.1 ± 0.27	7.8 ± 0.36	NS

Table 3.5. Experiment 1b: Summary of seed yield at final harvest in *Brassica campestris* following two days of exposure to filtered air (control) or 70 ppb ozone for 7 h d^{-1}. Means and standard errors are shown. NS, non significant; n=10.

	Control	Ozone	Significance
Number of seeds per pod:			
Mature	15.1 ± 1.35	15.0 ± 0.64	NS
Germinated	0.1 ± 0.05	0.4 ± 0.19	NS
Aborted	1.7 ± 0.29	1.3 ± 0.35	NS
Total number of seeds per plant:			
Mature	325.1 ± 34.03	330.6 ± 21.13	NS
Germinated	2.2 ± 1.12	8.7 ± 3.61	NS
Aborted	37.3 ± 8.04	29.5 ± 8.74	NS
Total seed weight per plant (g)	0.339 ± 0.0329	0.360 ± 0.0181	NS
Individual seed weight (mg)	1.054 ± 0.0329	1.126 ± 0.0793	NS

Figure 3.4. Timecourse of germination of seeds from plants exposed to filtered air (control) or 70 ppb ozone for 2 days (7 h d^{-1}). Solid circles, controls; open circles, ozone-treated. Double standard errors are shown. n=10. There were no significant differences between control and ozone-treated plants at any time during the germination period.
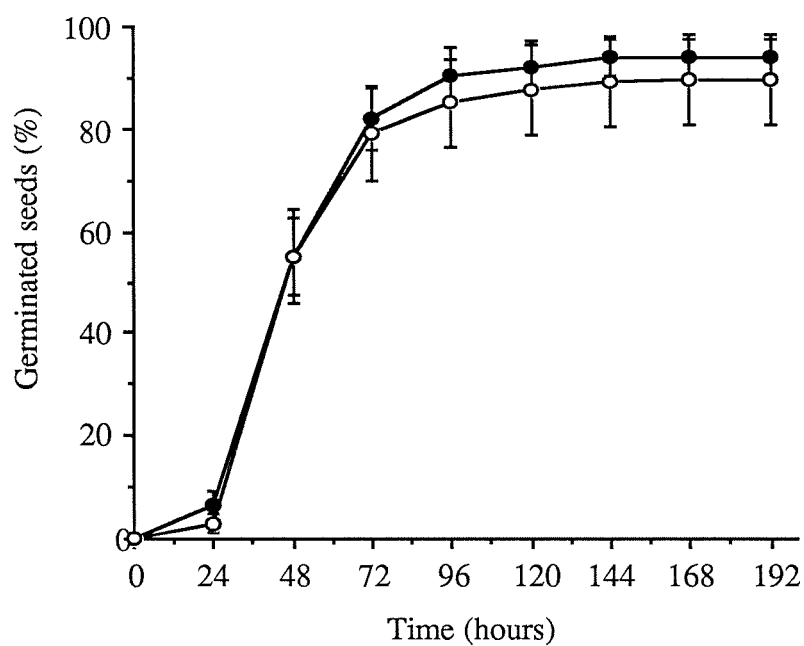
3.3.1.4. Summary of Experiment 1

Exposure to 70 ppb ozone for 7 h d^{-1} on two consecutive days during vegetative growth significantly reduced stomatal conductance and the net rate of photosynthesis in *Brassica campestris*, to an extent which varied between replicate experiments. The observed reductions were greater in those plants in which the stomatal conductance and net rate of photosynthesis were larger prior to exposure. In experiments where the reduction in photosynthesis was greater, visual foliar damage was also increased. In addition to this decrease in photosynthesis, intercellular CO_2 concentration increased during the day in ozone-treated plants. For all experiments, there was little recovery in either stomatal conductance or photosynthesis between the first and second exposures and the effects persisted throughout the following 24 h. Where recorded (Experiment 1b), there was no effect on seed yield at maturity.

3.3.2. Experiment 2: Effects of a 10 day exposure to ozone on growth and reproduction in *Brassica campestris*

Environmental conditions

Environmental conditions during the 10 day period of ozone introduction are summarised in Table 3.6. Mean air temperature was 0.85 °C higher and relative humidity 2.6 % greater in the treatment chamber, with these differences being significant. The ozone concentration was slightly below the target concentration of 70 ppb, averaging 65.6 ppb. For this experiment, the target concentration will be referred to hereafter for the sake of clarity.

3.3.2.1. Physiological and growth parameters

Physiology

Ten days of exposure to 70 ppb ozone for 7 h d^{-1} induced a range of effects on the physiology, growth and reproduction of *Brassica campestris*. On the final day of exposure, stomatal conductance (Fig. 3.5a) was significantly lower than in the control plants at 0900 h, suggesting a persistent effect of the previous nine days of exposure to ozone. This reduction in g_s increased in significance by the end of ozone fumigation. The net rate of photosynthesis (Fig. 3.5b) did not differ significantly between control and treated plants, but began to fall once ozone fumigation commenced, to become significantly lower by the end of the exposure period. There was no significant effect

Table 3.6. Mean temperature and relative humidity over the 10 day exposure period within the control and treatment chambers in Experiment 2. Double standard errors are shown. *** indicates significance at P<0.001.

Experiment 2	Control	Ozone	Significance
Temperature (°C)			
Outwith exposure [1]	24.5 ± 0.17	25.4 ± 0.12	***
During exposure [2]	24.4 ± 0.17	25.2 ± 0.09	***
Relative humidity (%)			
Outwith exposure [1]	42.8 ± 0.49	45.3 ± 0.25	***
During exposure [2]	43.2 ± 0.51	45.9 ± 0.33	***
Ozone concentration (ppb)	65.6 ± 2.01	< 1.0	***

[1], 0000-0930 h and 1630-0000 h; [2], 0930-1630 h.

Figure 3.5. Responses of a) stomatal conductance, b) net rate of photosynthesis and c) intercellular CO_2 concentration in *Brassica campestris* during the tenth day of exposure to filtered air (control) or 70 ppb ozone for 7 h d^{-1} (0930-1630 h). Solid squares, controls; open squares, ozone-treated. Double standard errors are shown, except when these are smaller than the symbols. *, ** and *** indicate significance at $P<0.05$, $P<0.01$ and $P<0.001$; n=10.
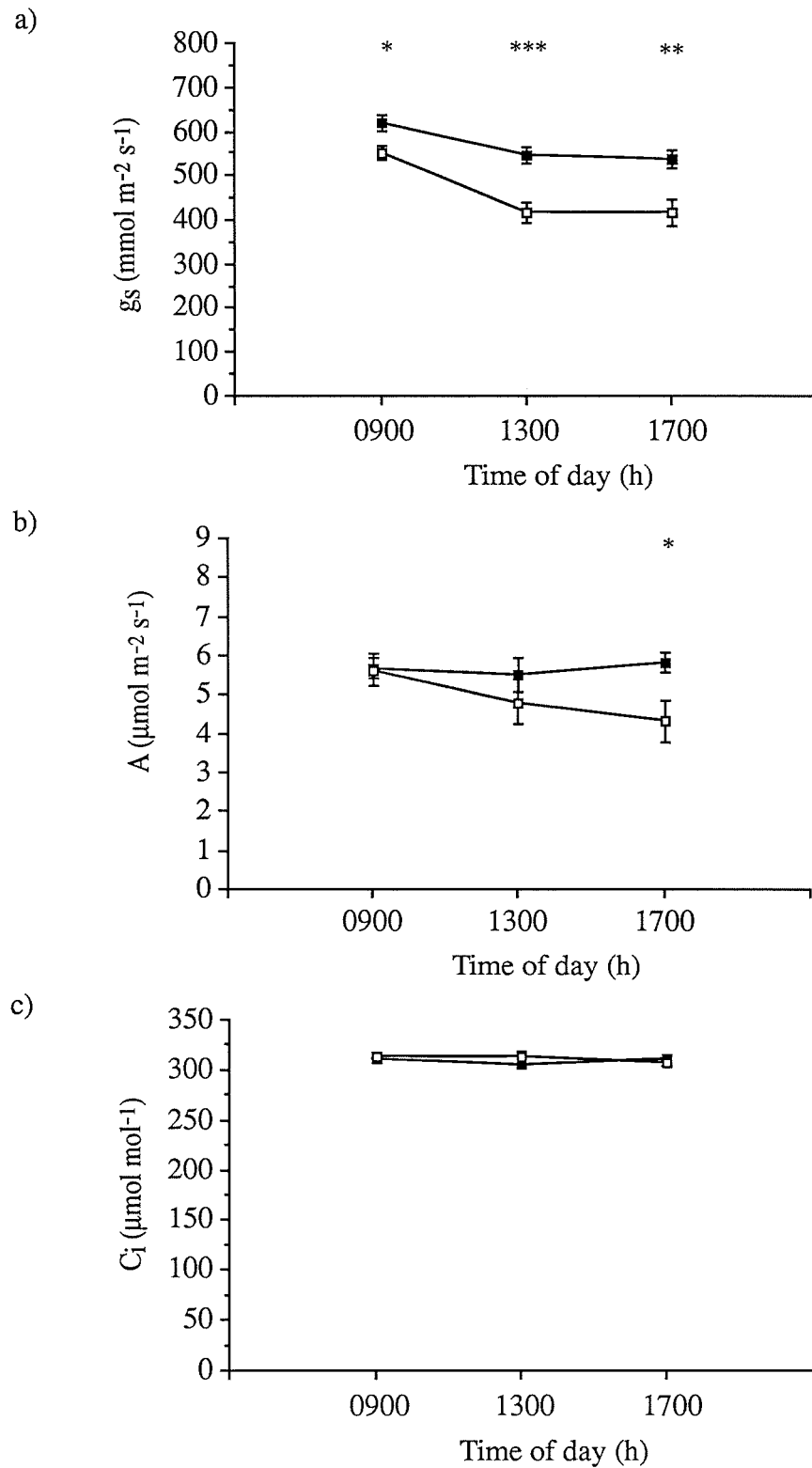
on intercellular CO_2 concentration at any time during the measuring period (Fig. 3.5c).

Organ initiation

Table 3.7 shows that the initiation of organs in *Brassica campestris* was unaffected by exposure to ozone since the numbers of leaves, leaflets and axillary racemes were similar in both treatments. By the end of the exposure period, there was also no detectable effect on plant height or the length of the terminal raceme, although the total number of reproductive sites was lower in ozone-treated plants than in the controls. This was due to the presence of fewer reproductive sites on both the terminal and axillary racemes; however, this reduction was not statistically significant.

Dry weight

Exposure to ozone induced reductions in both root dry weight (32 %, $P<0.05$) and shoot dry weight (14 %, $P<0.05$; Table 3.8). The reduction in shoot biomass resulted from decreases in stem, leaf and reproductive dry weight, with the reduction in leaf dry weight being significant ($P<0.05$); however, due to the high variability between plants, the reductions in shoot and total dry weight were not significant. As the decrease in root dry weight was greater than that in shoot dry weight in ozone-treated plants, the root:shoot ratio was reduced.

Foliar injury and leaf area

Visible foliar injury became apparent during the final days of exposure to ozone. This injury appeared either as a general chlorosis or necrotic flecks and occurred on all foliar organs, especially the cotyledons and the two oldest true leaves. In addition, many of the leaves showed marginal leaf curl, as can be seen in Plate 3.3. Figure 3.6 shows that the total leaf area per plant was decreased by ozone exposure, although the 16.5 % reduction proved insignificant because of variability between individual plants. However, when individual leaves are examined, significant reductions in the area of the cotyledons, first two true leaves and sessile leaves are apparent, with no effect being observed for the subsequent true leaves. Specific leaf area (Table 3.8), which is a measure of leaf area per unit dry weight, was unaffected by ozone; thus the observed significant reduction in leaf dry weight resulted primarily from a reduction in leaf area.

Table 3.7. Summary of growth parameters in *Brassica campestris* following 10 days of exposure to filtered air (control) or 70 ppb ozone for 7 h d^{-1}. Means and standard errors are shown. NS, non significant; n=10.

	Control	Ozone	Significance
Plant height (mm)	214.5 ± 10.31	220.8 ± 18.90	NS
Length of terminal raceme (mm)	18.2 ± 4.13	17.4 ± 2.58	NS
Number of axillary racemes	2.4 ± 0.16	2.4 ± 0.37	NS
Total number of reproductive sites:	37.1 ± 3.76	31.5 ± 3.58	NS
terminal raceme	17.8 ± 1.94	16.3 ± 1.51	NS
axillary racemes	19.3 ± 3.26	15.2 ± 3.70	NS
Number of true leaves	4.0 ± 0.26	4.5 ± 0.31	NS
Number of leaflets	1.1 ± 0.53	2.2 ± 0.63	NS

Table 3.8. Summary of biomass production and partitioning in *Brassica campestris* following ten days of exposure to filtered air (control) or 70 ppb ozone for 7 h d^{-1}. Means and standard errors are shown. * indicates significance at $P<0.05$. NS, non significant; n=10.

	Control	Ozone	Significance
Dry weight (mg):			
Root	31.5 ± 3.27	21.4 ± 1.96	*
Shoot;	241.8 ± 14.42	207.9 ± 17.64	NS
- stem	83.9 ± 6.87	75.9 ± 9.27	NS
- leaf	129.3 ± 9.04	102.2 ± 7.36	*
- reproductive	36.7 ± 3.23	29.8 ± 4.57	NS
Total	273.3 ± 17.06	229.3 ± 19.14	NS
Root:Shoot ratio	0.15 ± 0.012	0.12 ± 0.009	NS
Specfic leaf area (mm^2 mg^{-1})	32.5 ± 2.11	33.3 ± 0.61	NS
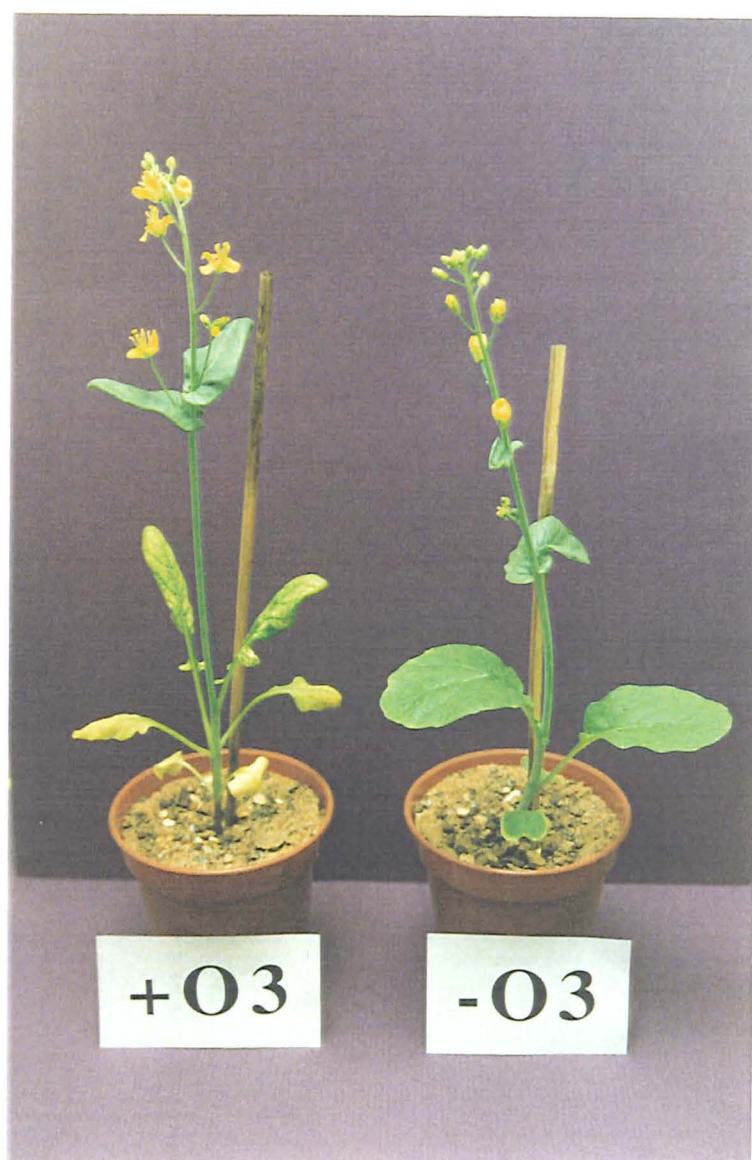
Plate 3.3. Plants of *Brassica campestris* after 10 days of exposure to filtered air or 70 ppb ozone for 7 h d^{-1}.

Figure 3.6. Mean area of leaves in *Brassica campestris* following ten days of exposure to filtered air (control) or 70 ppb ozone for 7 h d^{-1}. Leaves are numbered from the base of the stem. Open bars, control; shaded bars, ozone-treated. Single standard errors are shown. * and *** indicate significance at P<0.05 and P<0.001; n for each leaf type is shown in brackets.
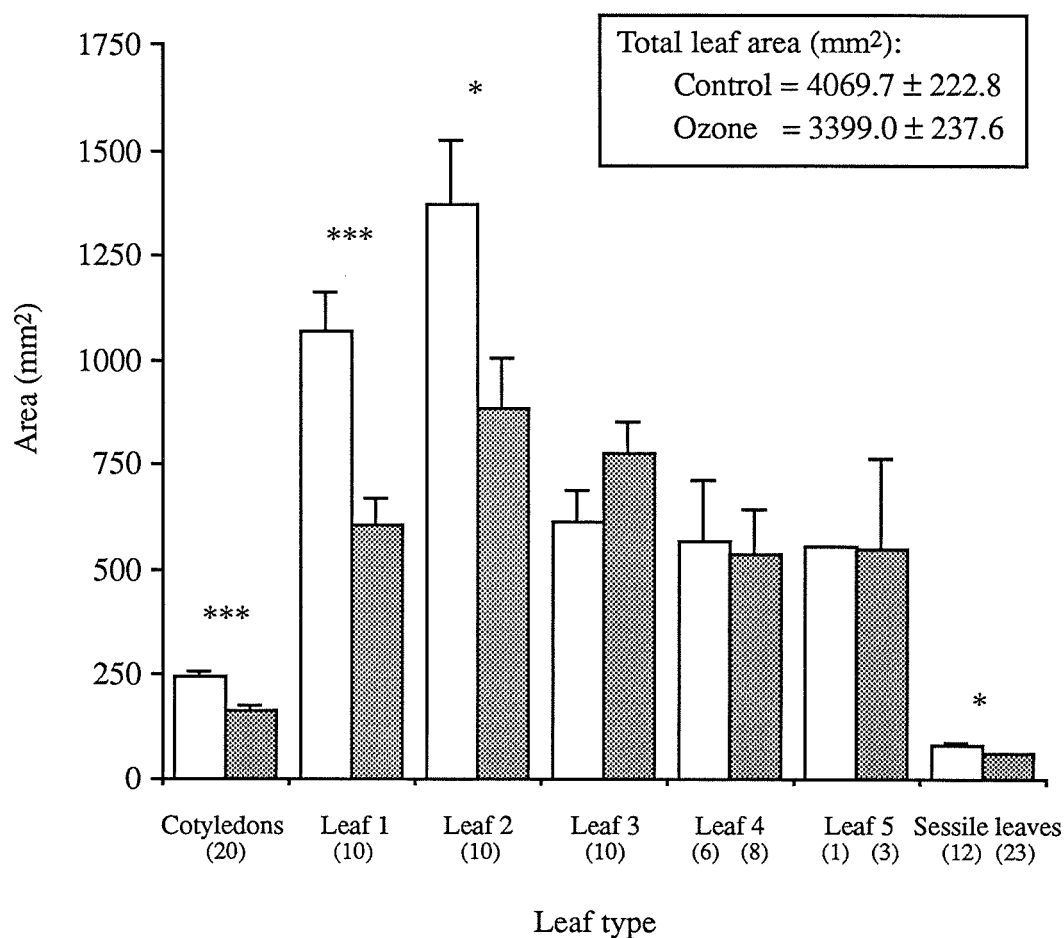
3.3.2.2. Reproductive biology

Terminal raceme and reproductive sites

Figure 3.7 shows that there was no effect of ozone on the length of the terminal raceme for the first two days after exposure, but as the flowering period proceeded the extension of the terminal raceme decreased to produce significantly shorter racemes from 18 DAS onwards (P<0.05). The majority of floral sites were present on the terminal raceme of both ozone-treated and control plants by the start of flowering at 14 DAS (Fig. 3.8). The number of sites produced on control plants continued to increase until day 24 (Fig 3.8a) whereas the maximum number was attained much earlier in ozone-treated plants, at 18 DAS (Fig. 3.8b). From the start of flowering, the total number of reproductive sites on the terminal raceme was significantly lower in ozone-treated than in control plants (P<0.05) and this difference became more significant as flowering proceeded. The shorter terminal raceme of the ozone-treated plants probably resulted from the production of fewer reproductive sites. More flowers opened each day in control than in ozone-treated plants, but this was only significant at 18-20 DAS (Fig. 3.8) since the progressive reduction in the number of flowers which opened each day occurred earlier in ozone-treated than in control plants. Although more sites flowered in control plants, significantly more of these sites were aborted post-flowering from 20 DAS, resulting in no significant difference between ozone-treated and control plants in the total number of pods which formed on the terminal raceme (Fig. 3.8). Fewer buds aborted in the ozone treatment as compared to the controls; however this difference between treatments was not significant.

Pollen

Using the techniques described in Section 3.2.2.2, the mean percentage germination of pollen from control plants of *Brassica campestris* was found to be only 34.4 %. Germination was significantly reduced to 20.9 % in pollen from ozone-treated plants, but no significant effect on pollen tube length was observed (Table 3.9).

Pod development

A summary of pod development is shown in Table 3.10. As well as having fewer reproductive sites on the terminal raceme (Fig. 3.8b), ozone-treated plants also produced fewer pods, although this effect was not significant. Final pod length was not reduced significantly in ozone-treated plants, suggesting that ozone did not affect ovary expansion. Since pod length is partly determined by the number of developing

Figure 3.7. Change in the length of the terminal raceme of *Brassica campestris* during the period of flowering, following 10 days of exposure to filtered air (control) or 70 ppb ozone for 7 h d^{-1}. Solid circles, control; open circles, ozone-treated. Double standard errors are shown. n=10. There was a significant treatment effect from 18 DAS (P<0.05).
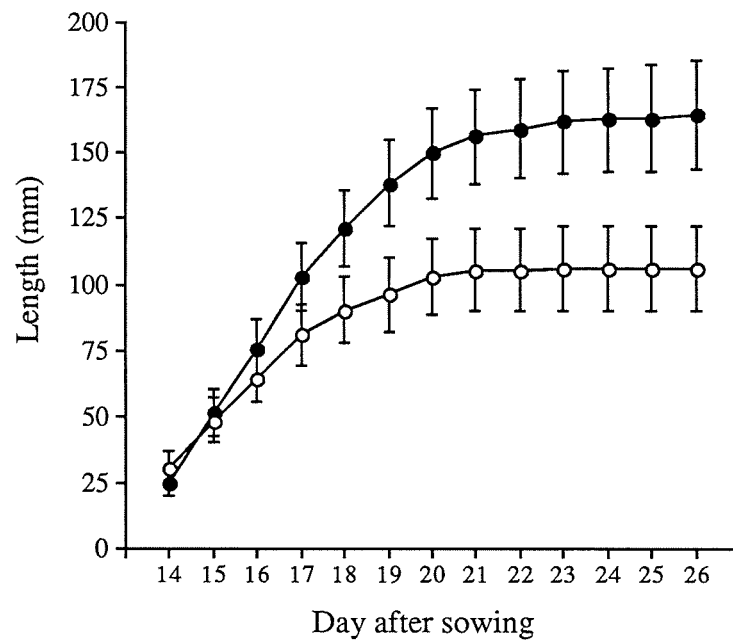
Figure 3.8. Accumulative mean number of floral sites on the terminal raceme of *Brassica campestris* at each stage of development throughout the flowering period after exposure to a) filtered air (control) or b) 70 ppb ozone for 7 h on 10 consecutive days, 4-13 DAS. The table shows significant differences between ozone-treated and control plants in the number of sites at each stage of floral development during the flowering period. *, ** and *** indicate significance at $P<0.05$, $P<0.01$ and $P<0.001$. NS, non significant; n=10.
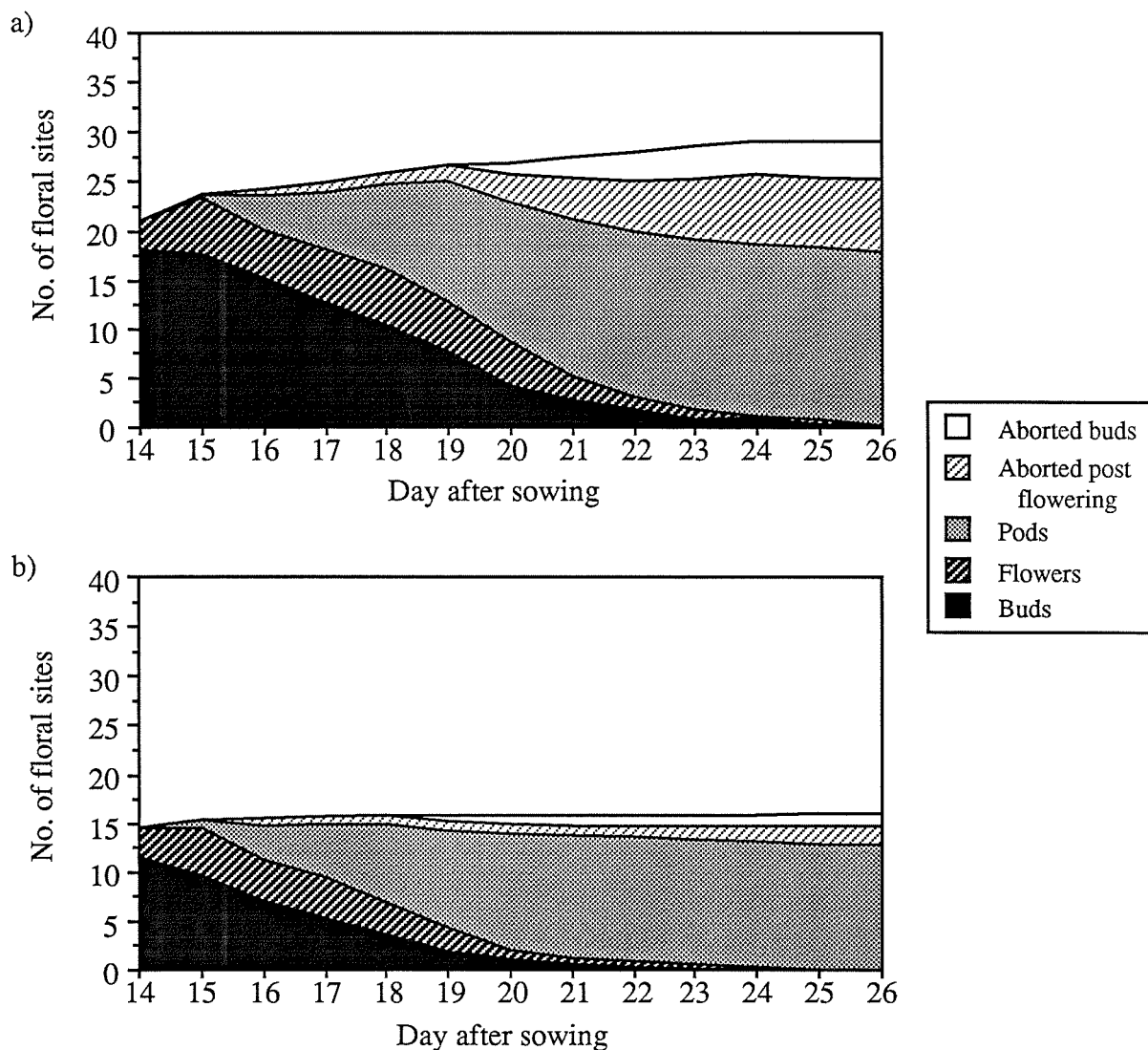
Stage of floral development	Days after sowing												
	14	15	16	17	18	19	20	21	22	23	24	25	26
Buds	*	*	*	**	**	**	NS	NS	NS	NS	NS	NS	NS
Flowers	NS	NS	NS	NS	*	*	***	NS	NS	NS	NS	NS	NS
Pods	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Aborted pods	NS	NS	NS	NS	NS	NS	*	*	**	**	**	**	**
Aborted buds	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Total	*	*	*	*	*	*	*	**	**	**	**	**	**

Table 3.9. Mean percentage germination and tube length for pollen collected from *Brassica campestris* after 10 days of exposure to filtered air (control) or 70 ppb ozone for 7 h d^{-1}. Standard errors are shown. * indicates significance at P<0.05. NS, non significant; n is shown in brackets.

	Control	Ozone	Significance
Percentage germination	34.3 ± 4.11 (10)	20.9 ± 4.25 (10)	*
Pollen tube length (μm)	103.1 ± 4.35 (200)	95.6 ± 3.92 (200)	NS

Table 3.10. Summary of pod development in *Brassica campestris* after 10 days of exposure to filtered air (control) or 70 ppb ozone for 7 h d^{-1}. Means and standard errors are shown. * indicates significance at P<0.05. NS, non significant; n is shown in brackets.

	Control	Ozone	Significance
Number of pods per plant	17.2 ± 1.37 (9)	14.0 ± 1.19 (9)	NS
Pod length (mm)	37.5 ± 0.51 (155)	36.55 ± 0.75 (125)	NS
Dry pod weight per plant (minus seeds) (mg)	357.8 ± 13.40 (9)	292.0 ± 26.78 (9)	*
Individual dry pod weight (minus seeds) (mg)	21.6 ± 1.43 (9)	21.9 ± 2.65 (9)	NS

seeds within the pod, the observed decrease in pod length in ozone-treated plants may have been related to the smaller number of seeds present (Table 3.11). Pod dry weight, excluding seeds, was unaffected, but since slightly fewer pods developed in ozone-treated plants, there was a significant reduction in total pod dry weight per plant.

Seed yield

Table 3.11 shows that the total number of seed sites per pod, determined as the total number of mature, germinated and aborted seeds, was significantly reduced following exposure to ozone. Of this total, the number developing into mature seeds was reduced by ozone in the majority of pods along the terminal raceme (Fig. 3.9). However, because ozone significantly increased the number of seeds germinating within the pod prior to harvest, the total number of mature seeds was not significantly affected by treatment, with 14.5 ± 0.5 and 13.7 ± 0.6 seeds being produced in the control and ozone environments respectively. The number of mature seeds per pod was maintained under ozone by a significant 35 % reduction (P<0.05) in the total number of seed sites aborting. Similar patterns were seen for the numbers of mature, germinating and aborting seeds per plant, since ozone had little effect on the total number of pods. However, due to the variability between plants, the only significant effect of ozone was on the total number of seed sites per plant (Table 3.11). The individual and total weight of mature seeds per plant was not significantly affected by treatment, although total seed weight per plant was lower under ozone due to the production of fewer mature seeds.

The mature seeds from each treatment were separated into colour categories, as shown in Plate 3.4. The colour of seeds obtained differed between treatments; whilst both treatments produced large numbers of brown seeds, the control plants also produced light brown and brown/purple seeds and the ozone-treated plants produced green, yellow/green and yellow seeds. Table 3.12 shows that the majority of seeds in both treatments were brown, comprising 81 % of the total in control and 72 % in ozone-treated plants. Each of the other colour categories comprised 8 to 10 % of the total number of seeds in each treatment. The individual weight of each seed type varied, with the green seeds produced by ozone-treated plants being heaviest and the purple/brown seeds produced by the controls being the lightest. The brown seeds were similar in weight in both treatments.

Table 3.11. Summary of seed yield in *Brassica campestris* after 10 days of exposure to filtered air (control) or 70 ppb ozone for 7 h d^{-1}. Means and standard errors are shown. * and *** indicate significance at P<0.05 and P<0.001. NS, non significant.

	Control	Ozone	Significance
Number of seeds per pod;			
Mature	14.2 ± 0.53	12.9 ± 0.57	NS
Germinated	0.3 ± 0.09	0.8 ± 0.21	*
Aborted	4.3 ± 0.35	2.8 ± 0.29	***
Total	18.8 ± 0.46	16.5 ± 0.64	***
n	155	125	
Total number of seeds per plant;			
Mature	242.7 ± 23.52	178.8 ± 27.36	NS
Germinated	5.1 ± 3.16	10.9 ± 7.15	NS
Aborted	61.9 ± 14.38	38.2 ± 12.05	NS
Total	319.7 ± 20.16	227.9 ± 33.62	*
n	9	9	
Total seed weight per plant (mg)	165.0 ± 15.09	130.7 ± 18.34	NS
n	9	9	
Individual seed weight per plant (mg)	0.69 ± 0.032	0.74 ± 0.056	NS
n	9	9	

Figure 3.9. Profiles of the mean number of mature seeds per pod at each position along the terminal raceme of *Brassica campestris* after exposure of plants on 10 consecutive days to filtered air (control) or 70 ppb ozone for 7 h d^{-1} between 4-13 DAS. Solid circles, control; open circles, ozone-treated. Single standard errors are shown.
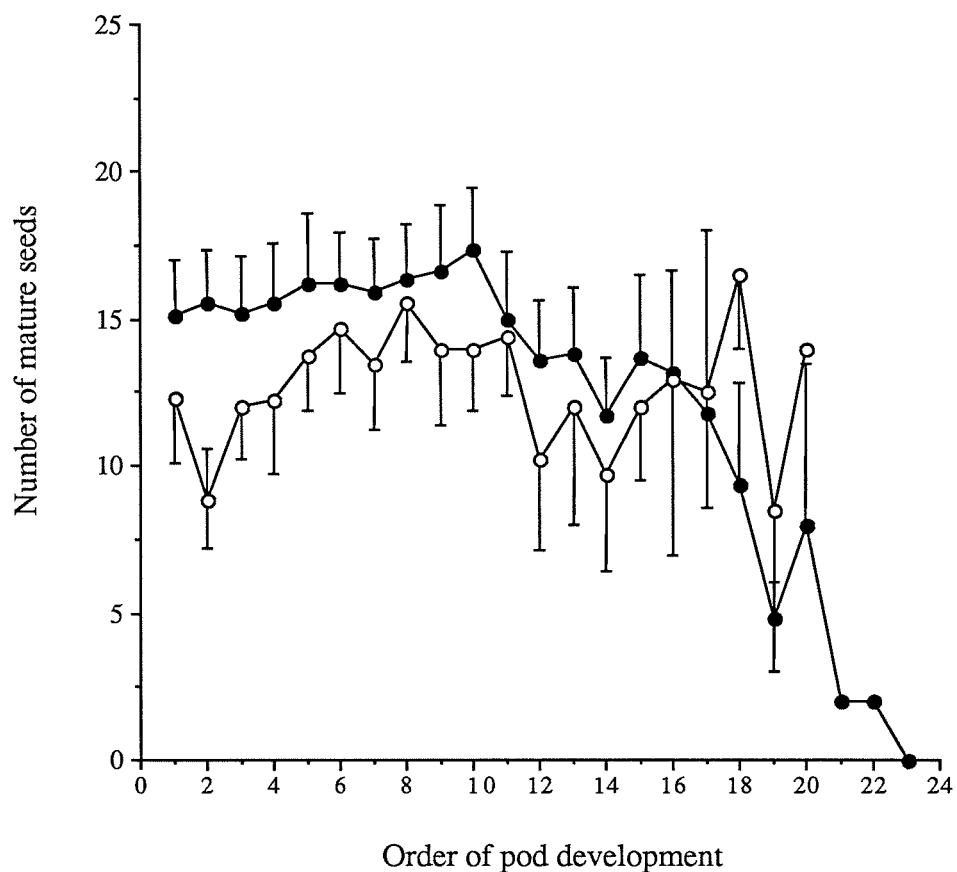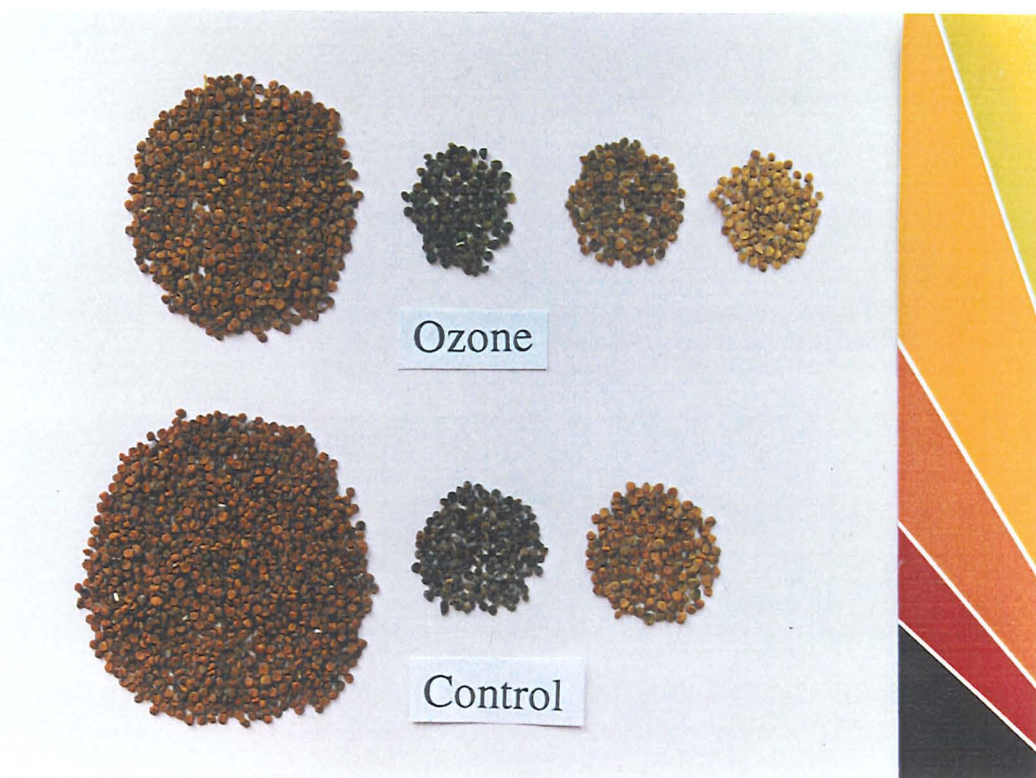
Plate 3.4. Seed yield within various colour categories in *Brassica campestris* following exposure on 10 consecutive days to filtered air (control) or 70 ppb ozone for 7 h d^{-1} between 4-13 DAS. Seed colours from left to right: ozone; brown, green, yellow/green, yellow; control, brown, brown/purple, light brown.

Table 3.12. Summary of mature seed yield for various colour categories in *Brassica campestris* after 10 days of exposure to filtered air (control) or 70 ppb ozone for 7 h d^{-1}.

Treatment	Colour Category				
Control	Brown	Brown/ Purple	Light Brown		Total, All Colours
Number of seeds	1560	184	185		1929
Percentage of total	81	9.5	9.5		
Total weight of seeds (g)	1.050	0.104	0.151		1.305
Mean individual seed weight (mg)	0.674	0.565	0.814		
Ozone	Brown	Green	Yellow/ Green	Yellow	Total, All Colours
Number of seeds	974	112	149	125	1360
Percentage of total	72	8	11	9	
Total weight of seeds (g)	0.670	0.103	0.113	0.090	0.976
Mean individual seed weight (mg)	0.687	0.919	0.758	0.717	

Seed germination

Using seeds sampled randomly from each treatment, it was found that the majority of seeds germinated within 72 h (Fig. 3.10). Ozone significantly reduced the rate of germination up to 48 hours, but had no effect on the final percentage germination. When the different seed colour categories were examined separately (Fig. 3.11), the majority were again found to germinate within 72 h, but with some variation between categories. The lightest coloured seeds from both treatments exhibited the highest percentage germination values, with 98 % of the light brown control and 100 % of the yellow, ozone-treated seeds germinating within seven days. The germination of brown seeds from the control and ozone treatments was similar, with 93 and 98 % germination respectively. The rate and extent of germination were lowest in the brown/purple seeds from control plants, which achieved only 30 % germination within seven days. The rate of germination was also slower in the green seeds from ozone-treated plants, although 67 % of these eventually germinated.

Seed imbibition

Water uptake was rapid, with all colour categories at least doubling their weight within one hour (Fig. 3.12). The rate and percentage water uptake were similar for the brown and lighter coloured seeds from both treatments. The brown/purple seeds of the control plants imbibed significantly more water ($P<0.05$) than any other seed colour, absorbing over 2.5 times their own weight of water within two hours. The green and yellow/green seeds from the ozone-treated plants also imbibed faster and took up significantly more water ($P<0.05$) than either the brown or yellow seeds in the first two hours of imbibition (Fig. 3.12).

Light microscopy study of seed structure

Whilst embryonic structures were identified in seeds from each colour category of both control (Plate 3.5) and ozone-treated (Plate 3.6) plants, there were visible differences in seed coat structure between categories. In all seed categories, the testa comprised the three single-celled layers typically found in *Brassica campestris* (Rathore and Singh, 1968); an outer epidermal layer, a dense layer of thickened supporting cells and an inner layer. Generally, the number of cells comprising each layer increased around the micropylar end of the seed. The structure of the seed coat was similar for the brown seeds from control plants (Plates 3.5a, 3.7a) and for the brown (Plates 3.6a, 3.8a) and yellow (Plates 3.6d, 3.8b) seeds from ozone-treated plants, and appeared to have developed normally. Although three cell layers were present in the testa of

Figure 3.10. Timecourse of germination of seeds from plants exposed to filtered air (control) or 70 ppb ozone for 10 days (7 h d^{-1}). Solid circles, controls; open circles, ozone-treated. Standard errors are shown. * and ** indicate significance at P<0.05 and P<0.01; n=10.
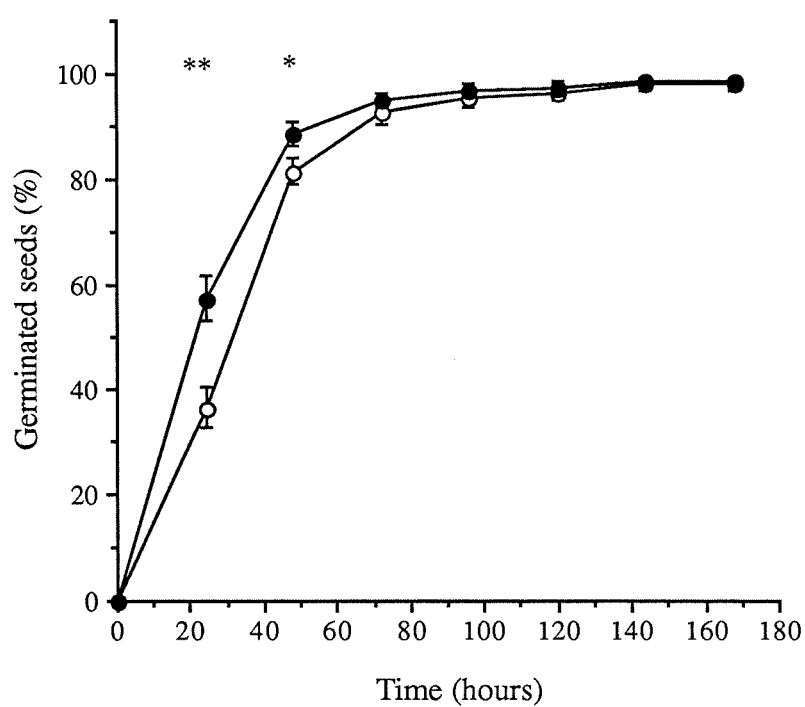
Figure 3.11. Timecourse of germination for different seed colour categories from plants of *Brassica campestris* exposed on 10 consecutive days to a) filtered air (control) and b) 70 ppb ozone for 7 h d^{-1}. Keys indicate seed colour. Double standard errors are shown; n=4. Significant differences between ozone-treated and control seeds are shown in the tables; for each time interval, seed colours followed by a common letter were not significantly different at the 5 % level.
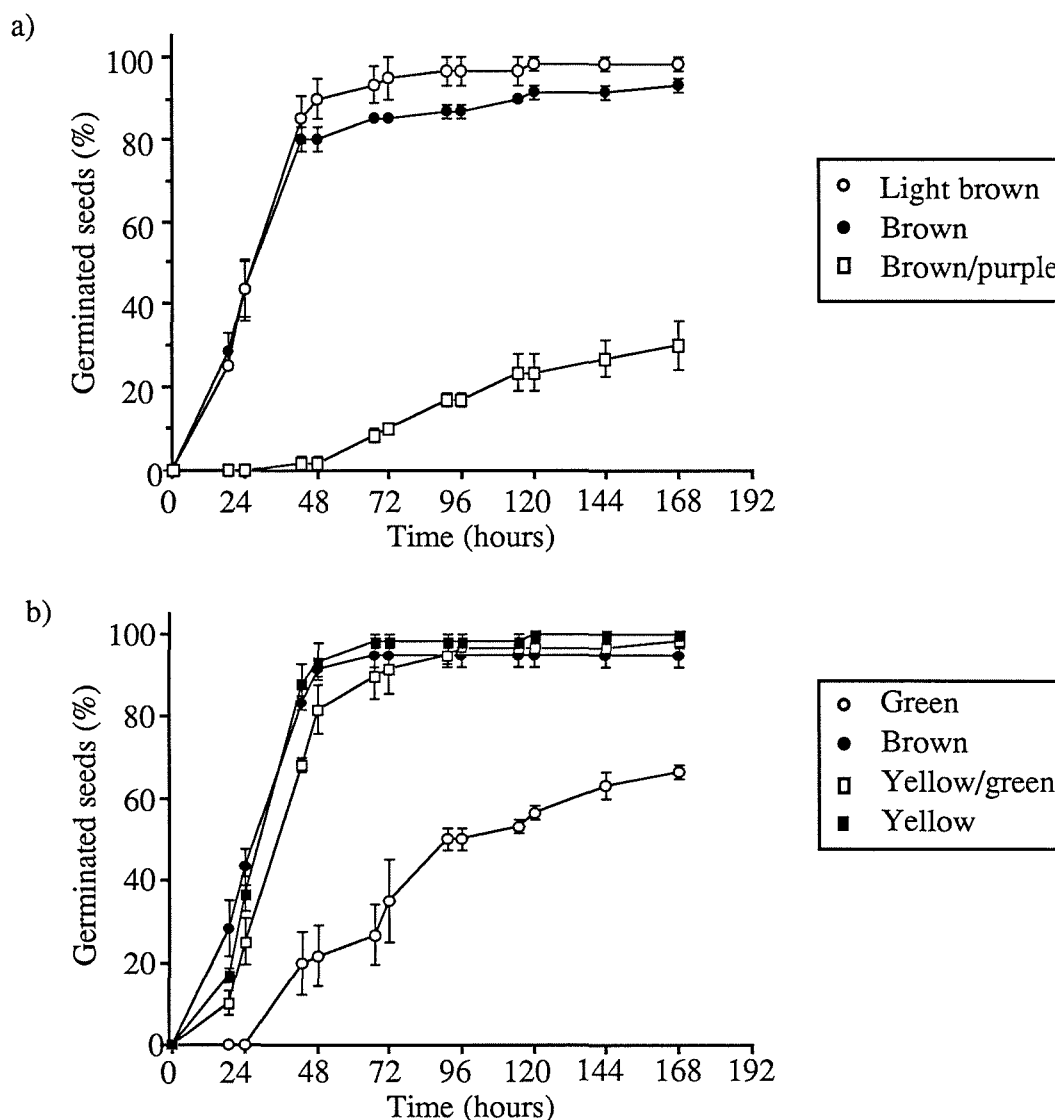
Control	Time (h)											
Seed colour	19	24	43	48	67	72	91	96	115	120	144	168
Light brown	a	a	a	a	a	a	a	a	a	a	a	a
Brown	a	a	a	a	a	b	b	b	a	b	b	a
Brown/purple	b	b	b	b	b	c	c	c	b	c	c	b

Ozone	Time (h)											
Seed colour	19	24	43	48	67	72	91	96	115	120	144	168
Green	c	c	c	b	b	b	b	b	b	b	b	b
Brown	a	a	a	a	a	a	a	a	a	a	a	a
Yellow/green	b	b	b	a	a	a	a	a	a	a	a	a
Yellow	b	ab	a	a	a	a	a	a	a	a	a	a

Figure 3.12. Imbibition by different seed colour categories of *Brassica campestris* from plants exposed on 10 consecutive days to a) filtered air (control) and b) 70 ppb ozone for 7 h d⁻¹. Keys indicate seed colour. Single standard errors are shown; n=3. Significant differences between ozone-treated and control seeds are shown in the tables; for each time interval, seed colours followed by a common letter were not significantly different at the 5 % level.

a)

b)
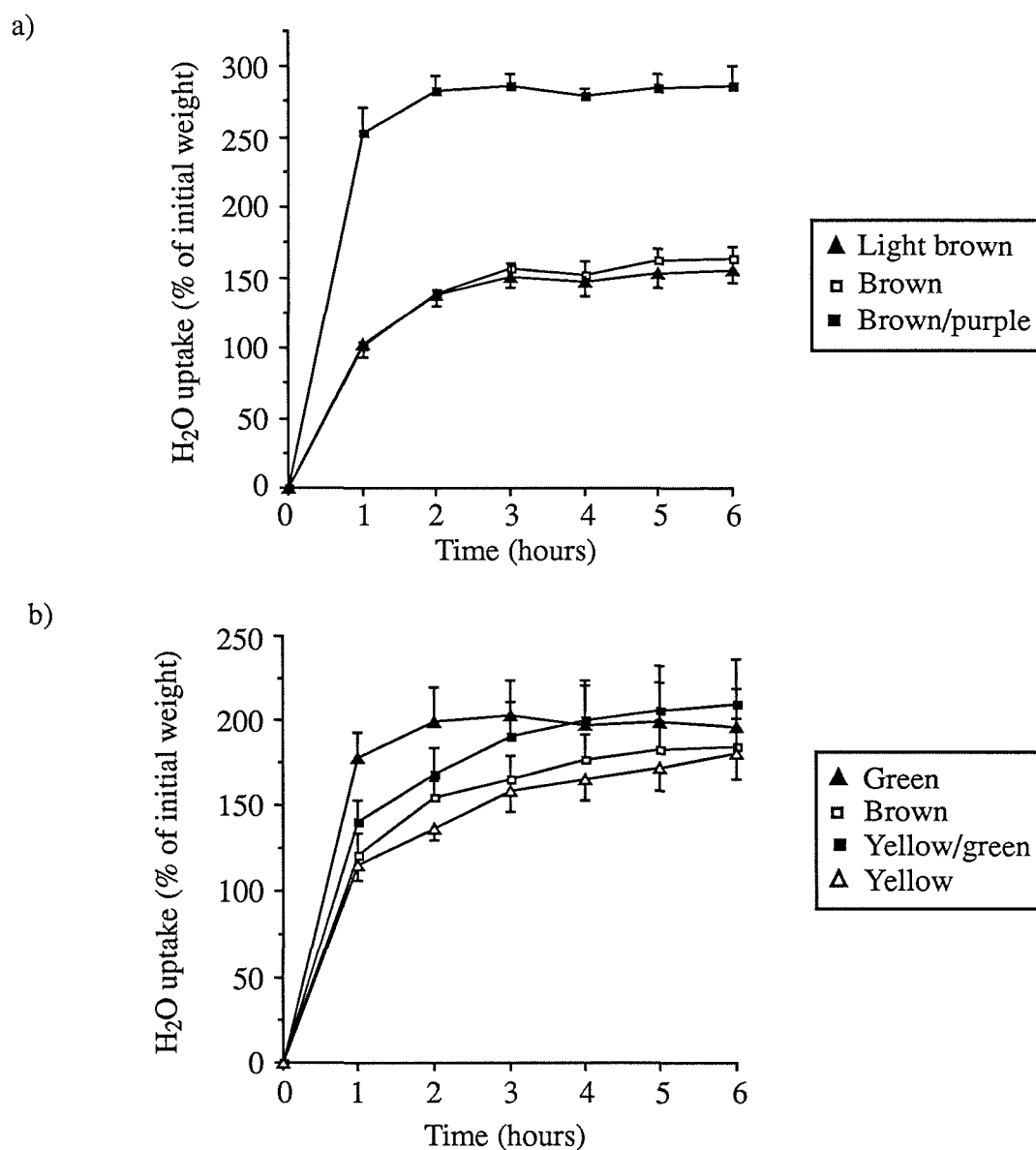
Control	Time (h)					
Seed colour	1	2	3	4	5	6
Light brown	b	b	b	b	b	b
Brown	b	b	b	b	b	b
Brown/purple	a	a	a	a	a	a

Ozone	Time (h)					
Seed colour	1	2	3	4	5	6
Green	a	a	a	a	a	a
Brown	b	ab	a	a	a	a
Yellow/green	ab	ab	a	a	a	a
Yellow	b	b	a	a	a	a

102
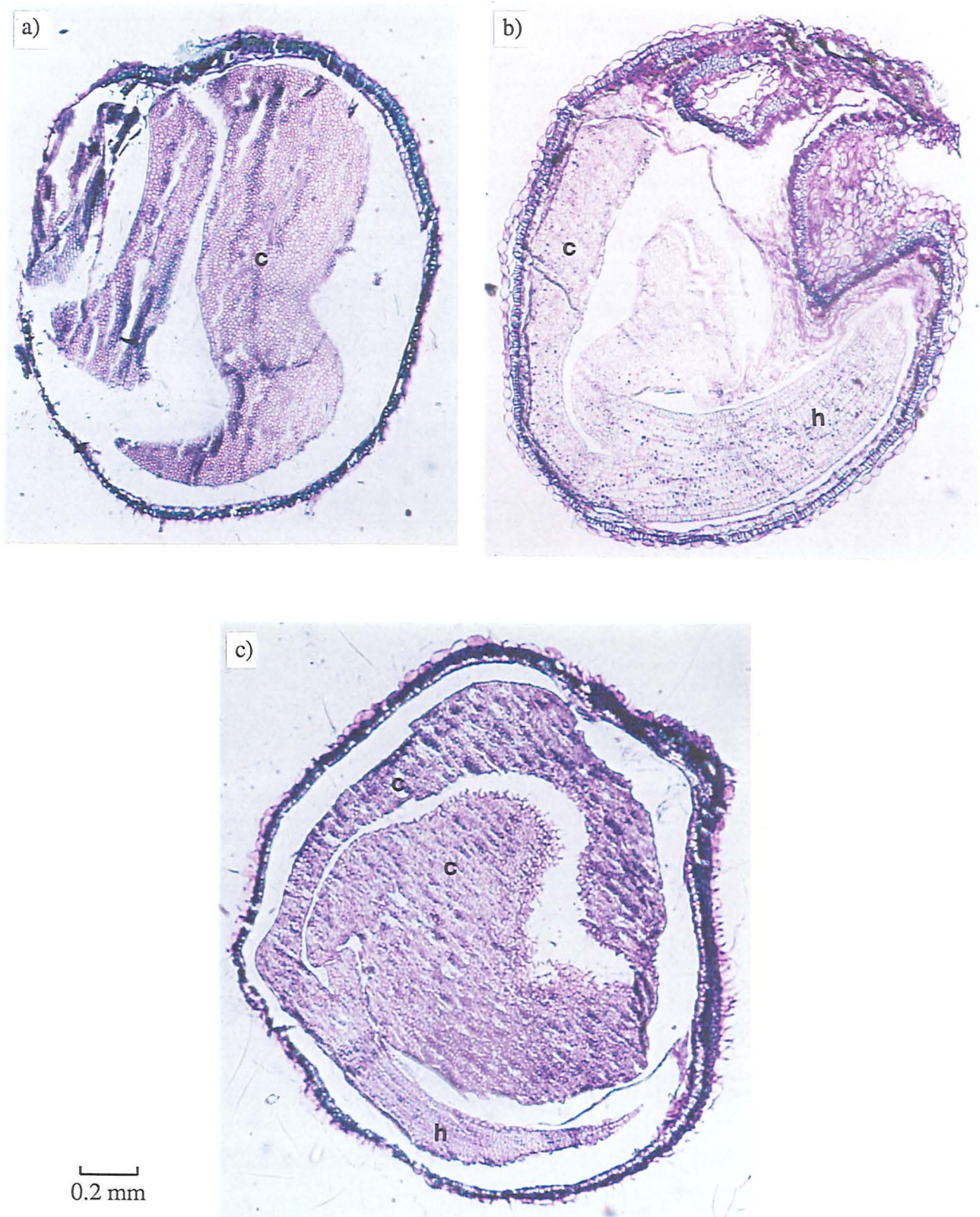
Plate 3.5.   Sections through a) brown, b) purple and c) light brown seeds of *B. campestris* harvested from plants exposed on 10 consecutive days to filtered air (control treatment) between 4-13 DAS showing embryonic structures: (h) hypocotyl, (c) cotyledon
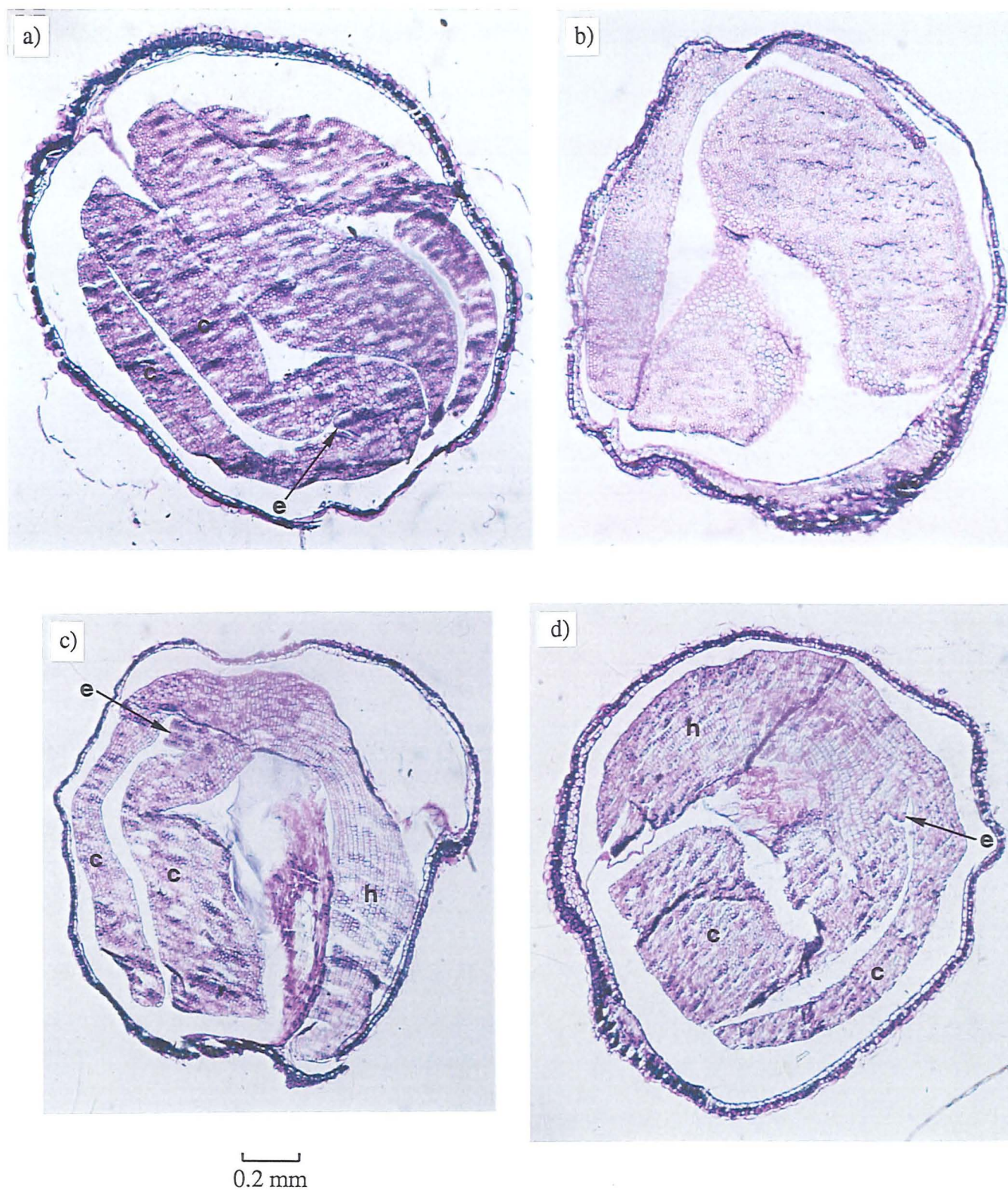
Plate 3.6. Sections through a) brown, b) green c) yellow/green and d) yellow seeds of *B. campestris* harvested from plants exposed on 10 consecutive days to 70 ppb ozone for 7 h d^{-1} between 4-13 DAS, showing embryonic structures: (h) hypocotyl, (c) cotyledon, (e) epicotyl.
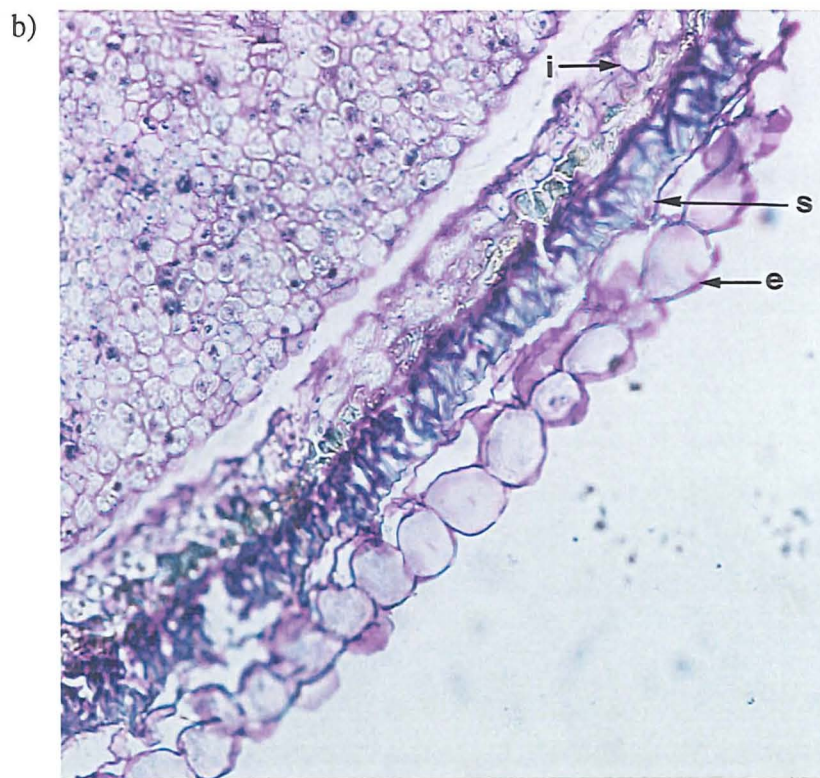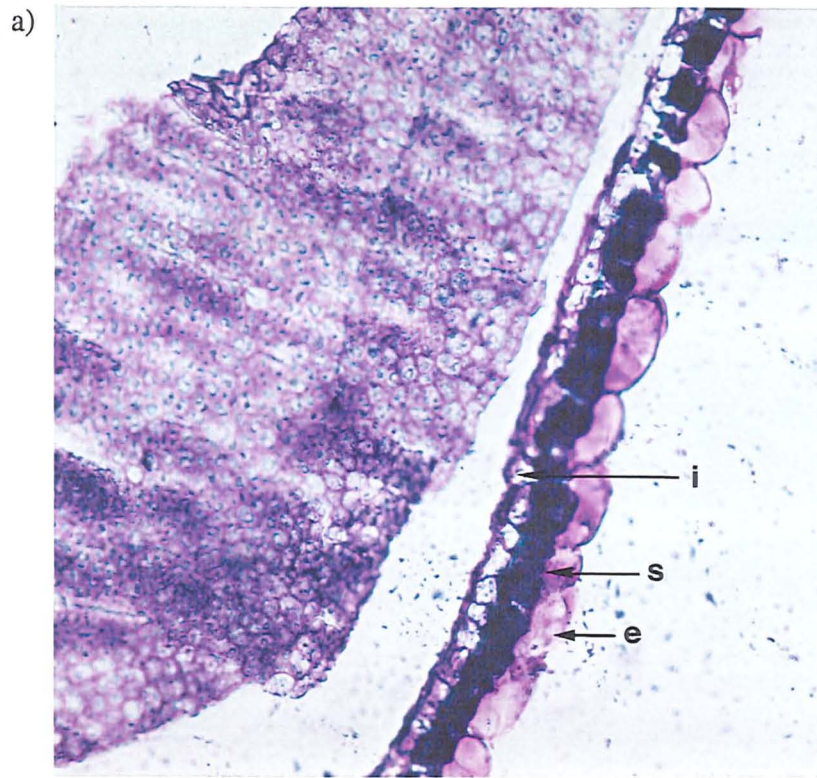
33 µm

Plate 3.7.   Sections through the seed coat of a) brown and b) purple seeds of
             B. campestris harvested from plants exposed on 10 consecutive days to
             filtered air (control treatment) between 4-13 DAS: (e) epidermal layer,
             (s) supporting layer and (i) inner layer of testa.

a)

b)
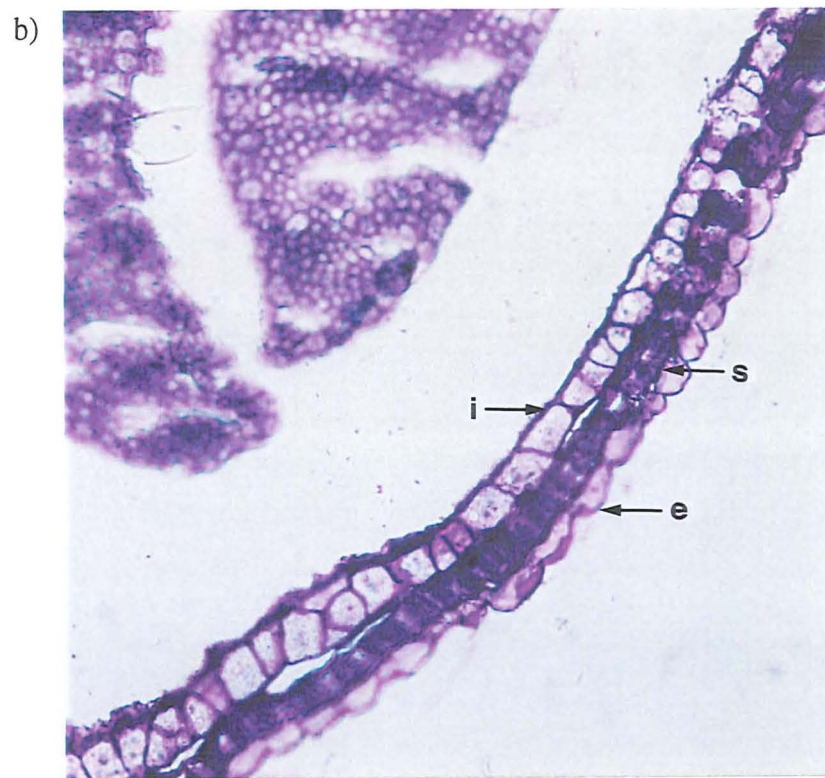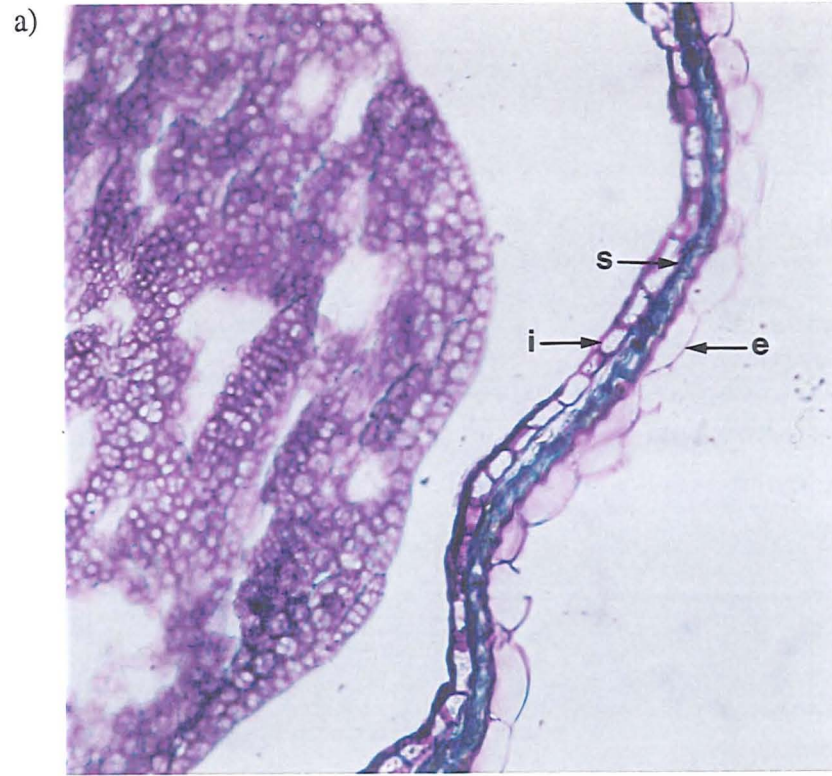
33 µm

Plate 3.8.   Sections through the seed coat of a) brown and b) yellow seeds of
            B. campestris harvested from plants exposed on 10 consecutive days to
            70 ppb ozone for 7 h d^{-1} between 4-13 DAS: (e) epidermal layer,
            (s) supporting layer and (i) inner layer of testa.

brown/purple seeds (Plate 3.7b) from control plants, the structure was non-uniform, with an abnormal indentation and proliferation of cells in one particular area of the seed coat (Plate 3.5b). Of the seeds from ozone-treated plants, the structure of the seed coat was similar in green (Plates 3.6b, 3.9b) and yellow/green seeds (Plates 3.6c, 3.9a) but contrasted to that of brown and yellow seeds (Plate 3.8), in that the outer epidermal layer was not as apparent and the cells of the inner layer were not as well defined.

3.3.3. Experiment 3: Effects of ozone on the physiology and growth of *Plantago major*

Environmental conditions

The environmental conditions experienced in the three separate experiments are summarised in Tables 3.13, 3.14 and 3.15. Temperature varied little between the chambers and decreased by about 2.5 °C during the dark period. Problems arose with the dewpoint hygrometers which may have accounted for the significant differences in relative humidity between the chambers recorded in all three experiments. In two of the three fumigations the concentration of ozone achieved was close to the target of 70 ppb; however problems were experienced with the ozone generation system during Experiment 3b in which the concentration of ozone averaged 26.4 ppb. In the following text, the target concentration will be referred to for Experiments 3a and 3b.

3.3.3.1. Experiment 3a. Physiological responses of Plantago major *populations to a two day fumigation with 70 ppb ozone for 7 h d^{-1}.*

The plants from each population of *P. major* designated for particular treatments showed little difference in stomatal conductance, net rate of photosynthesis or intercellular CO_2 concentration within the controlled environment growth cabinet prior to being transferred to the exposure chambers for fumigation (Figs. 3.13-3.16).

For each population the maximum values for stomatal conductance recorded within the charcoal filtered exposure chambers varied between 478-581 mmol m^{-2} s^{-1} (Figs. 3.17a-3.20a) and were similar to the values recorded for each population within the CEGC (Figs. 3.13a-3.16a). Within the exposure chambers however, each population exhibited a pronounced daily pattern for conductance, which was highest in the morning and decreased throughout the day. The extent of this decline varied between populations, with High Low and Sibton typically showing a decrease of 145-165 mmol m^{-2} s^{-1} and Lullington and Penicuik a decrease of 40-60 mmol m^{-2} s^{-1} from 0900 h to 1700 h each day. Figures 3.17b-3.20b show that for each population the net rate

a)

b)

33 μm
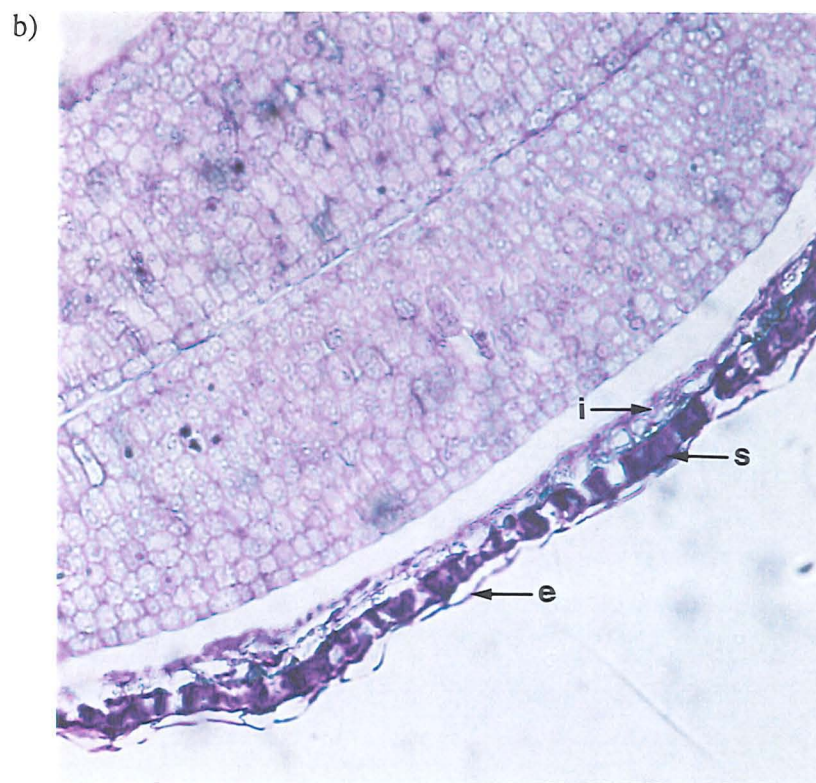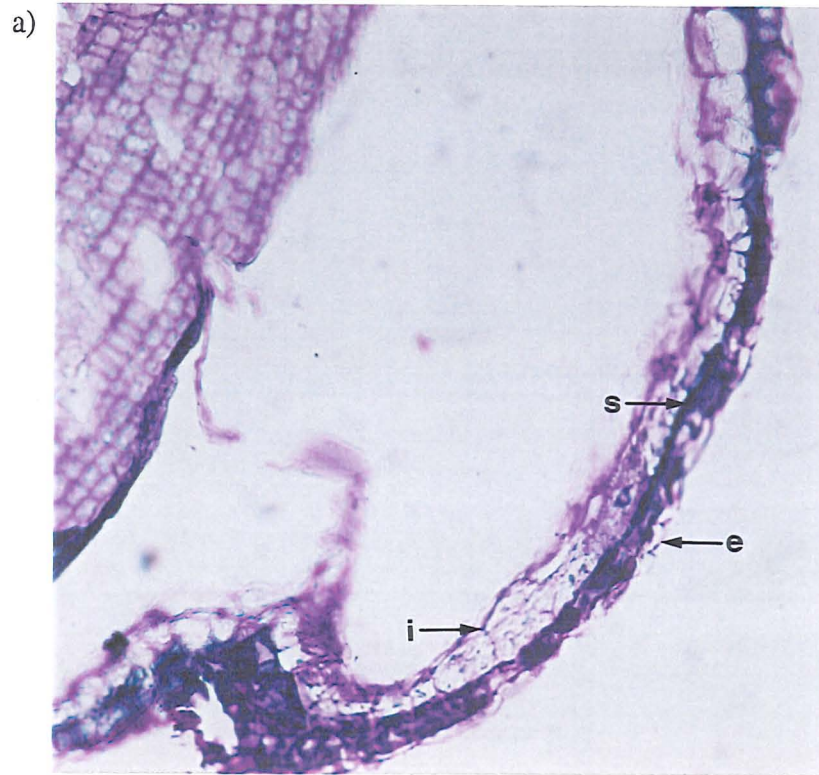
Plate 3.9. Sections through the seed coat of a) yellow/green and b) green seeds of
B. campestris harvested from plants exposed on 10 consecutive days to
70 ppb ozone for 7 h d^{-1} between 4-13 DAS: (e) epidermal layer,
(s) supporting layer and (i) inner layer of testa. Note the incomplete
development of the epidermal layer in both seeds.

Table 3.13. Mean temperature and relative humidity within the control and treatment chambers for each of four *Plantago major* populations in Experiment 3a. Standard errors are shown. *, ** and *** indicate significance at P<0.05, P<0.01 and P<0.001. NS, non significant.

a) Population High Low

	Control	Ozone	Significance
Temperature (°C)			
Day;			
Outwith exposure [1]	23.4 ± 0.09	24.0 ± 0.12	*
During exposure [2]	23.6 ± 0.06	24.2 ± 0.08	*
Night	21.5 ± 0.02	21.4 ± 0.02	NS
Relative humidity (%)			
Day;			
Outwith exposure [1]	45.6 ± 0.29	48.4 ± 0.34	***
During exposure [2]	45.1 ± 0.07	47.6 ± 0.08	**
Night	50.5 ± 0.05	55.8 ± 0.03	***
Ozone concentration (ppb)	71.9 ± 4.50	< 1.0	***

b) Population Lullington

	Control	Ozone	Significance
Temperature (°C)			
Day;			
Outwith exposure [1]	23.6 ± 0.31	24.3 ± 0.38	NS
During exposure [2]	23.4 ± 0.03	24.1 ± 0.01	**
Night	21.9 ± 0.46	22.1 ± 0.61	NS
Relative humidity (%)			
Day;			
Outwith exposure [1]	45.3 ± 0.64	47.9 ± 0.93	NS
During exposure [2]	45.5 ± 0.23	47.9 ± 0.21	*
Night	50.5 ± 1.34	55.7 ± 1.37	*
Ozone concentration (ppb)	70.8 ± 0.96	< 1.0	***

[1] Ozone introduction between 0930-1630 h during the 15 h photoperiod.

[2] Period during the 15 h photoperiod outwith ozone introduction; 0600-0930 and 1630-2100 h.

c) Population Penicuik

	Control	Ozone	Significance
Temperature (°C) Day;			
Outwith exposure [1]	23.8 ± 0.04	24.1 ± 0.03	**
During exposure [2]	23.9 ± 0.07	24.2 ± 0.04	NS
Night	21.5 ± 0.01	21.5 ± 0.01	NS
Relative humidity (%) Day;			
Outwith exposure [1]	45.0 ± 0.14	49.7 ± 0.14	***
During exposure [2]	45.1 ± 0.25	49.5 ± 0.23	**
Night	51.3 ± 0.23	57.6 ± 0.19	***
Ozone concentration (ppb)	67.6 ± 3.67	< 1.0	***

d) Population Sibton

	Control	Ozone	Significance
Temperature (°C) Day;			
Outwith exposure [1]	23.8 ± 0.16	24.6 ± 0.22	*
During exposure [2]	23.6 ± 0.19	24.4 ± 0.25	NS
Night	23.4 ± 0.95	24.0 ± 1.27	NS
Relative humidity (%) Day;			
Outwith exposure [1]	45.3 ± 0.13	49.8 ± 2.09	NS
During exposure [2]	45.9 ± 0.11	48.0 ± 0.01	**
Night	46.8 ± 2.10	49.7 ± 3.31	NS
Ozone concentration (ppb)	71.1 ± 3.4	< 1.0	***

[1] Ozone introduction between 0930-1630 h during the 15 h photoperiod.

[2] Period during the 15 h photoperiod outwith ozone introduction; 0600-0930 and 1630-2100 h.

Table 3.14. Mean temperature and relative humidity within the control and treatment chambers in Experiment 3b. Standard errors are shown. ** and *** indicate significance at P<0.01 and P<0.001. NS, non significant.

Experiment 3b	Control	Ozone	Significance
Temperature (°C) Day;			
Outwith exposure [1]	24.7 ± 0.16	24.8 ± 0.21	NS
During exposure [2]	24.7 ± 0.28	25.1 ± 0.18	NS
Night	21.5 ± 0.08	21.5 ± 0.05	NS
Relative humidity (%) Day;			
Outwith exposure [1]	42.2 ± 0.80	46.7 ± 0.93	**
During exposure [2]	43.1 ± 1.27	46.5 ± 1.14	NS
Night	51.4 ± 0.99	56.7 ± 0.91	**
Ozone concentration (ppb)	26.4 ± 1.15	< 1.0	***

[1] Ozone introduction between 0930-1630 h during the 15 h photoperiod.

[2] Period during the 15 h photoperiod outwith ozone introduction; 0600-0930 and 1630-2100 h.

Table 3.15. Mean temperature and relative humidity within the control and treatment chambers in Experiment 3c. Standard errors are shown. * and *** indicate significance at P<0.05 and P<0.001. NS, non significant.

Experiment 3c	Control	Ozone	Significance
Temperature (°C) Day;			
Outwith exposure [1]	24.8 ± 0.07	24.9 ± 0.07	NS
During exposure [2]	25.3 ± 0.03	25.4 ± 0.03	*
Night	22.6 ± 0.06	22.5 ± 0.07	NS
Relative humidity (%) Day;			
Outwith exposure [1]	54.2 ± 0.22	63.8 ± 0.27	***
During exposure [2]	52.4 ± 0.07	61.6 ± 0.09	***
Night	61.9 ± 0.20	73.8 ± 0.27	***
Ozone concentration (ppb)	67.0 ± 1.20	< 1.0	***

[1] Ozone introduction between 0930-1630 h during the 15 h photoperiod.

[2] Period during the 15 h photoperiod outwith ozone introduction; 0600-0930 and 1630-2100 h.

Figure 3.13. Changes in a) stomatal conductance, b) net rate of photosynthesis and c) intercellular CO_2 concentration of *Plantago major* population High Low 25 days after sowing within the controlled environment growth cabinet. Solid squares, control; open squares, ozone-treated. Double standard errors are shown, except when these are smaller than the symbols. n=10.
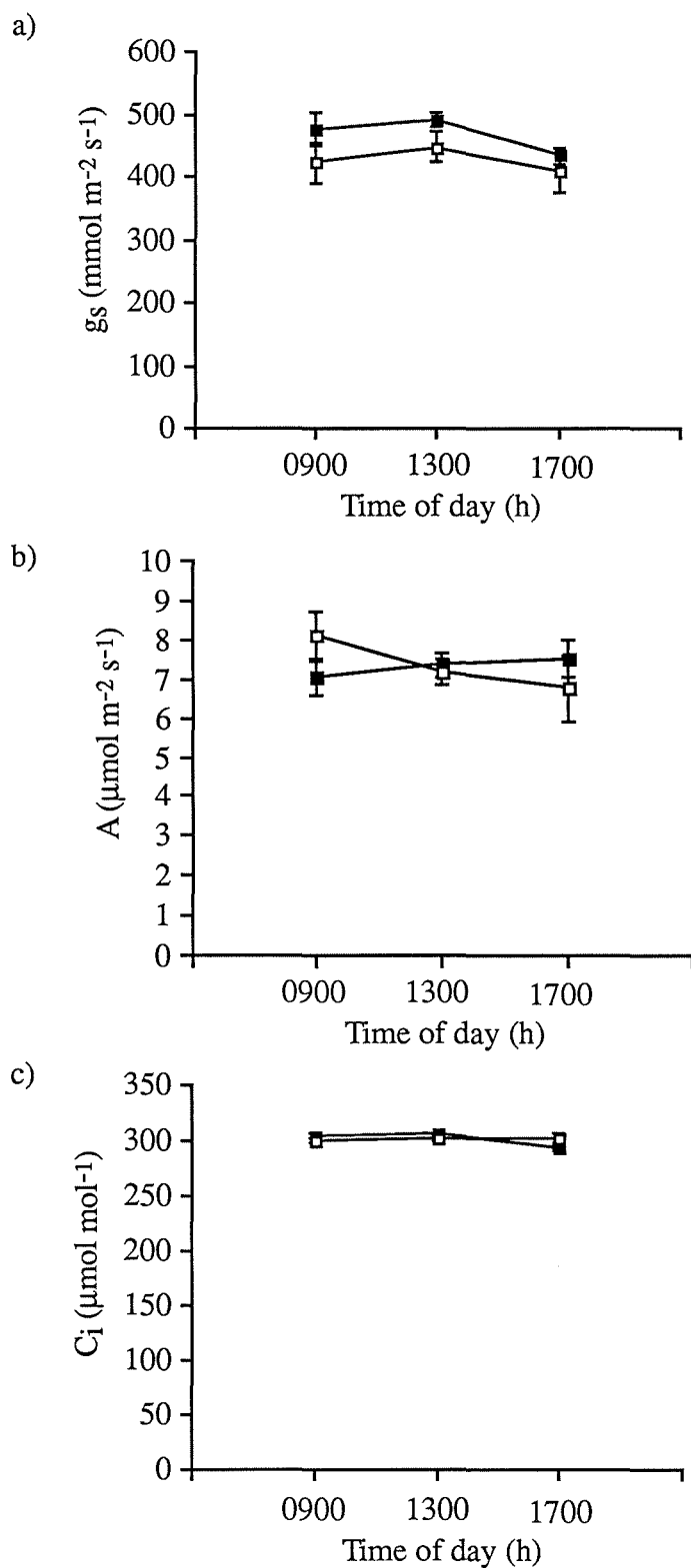
Figure 3.14. Changes in a) stomatal conductance, b) net rate of photosynthesis and c) intercellular CO_2 concentration of *Plantago major* population Lullington 23 days after sowing within the controlled environment growth cabinet. Solid squares, control; open squares, ozone-treated. Double standard errors are shown, except when these are smaller than the symbols. n=10.
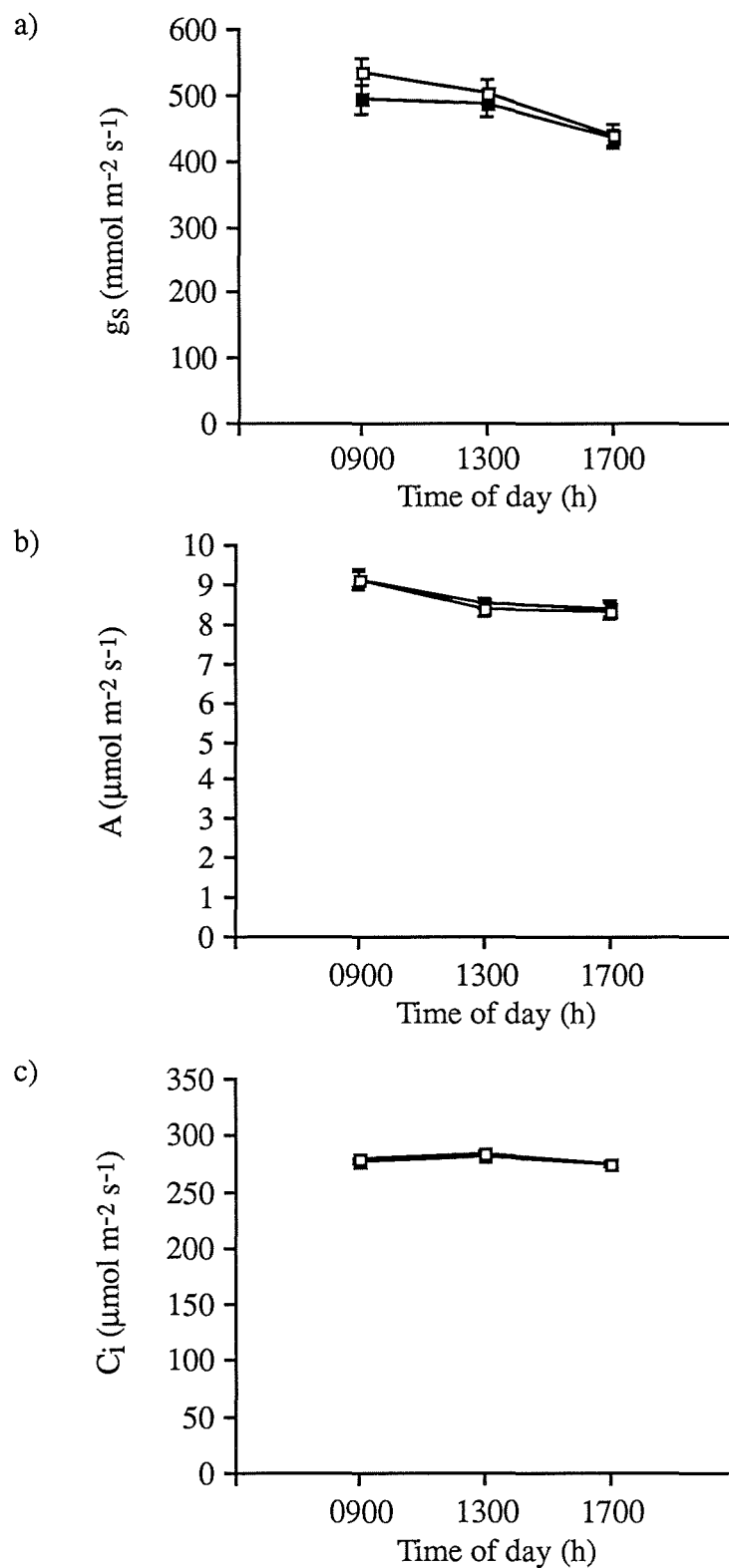
Figure 3.15. Changes in a) stomatal conductance, b) net rate of photosynthesis and c) intercellular CO_2 concentration of *Plantago major* population Penicuik 25 days after sowing within the controlled environment growth cabinet. Solid squares, control; open squares, ozone-treated. Double standard errors are shown, except when these are smaller than the symbols. ** indicates significance at $P<0.01$; n=10.
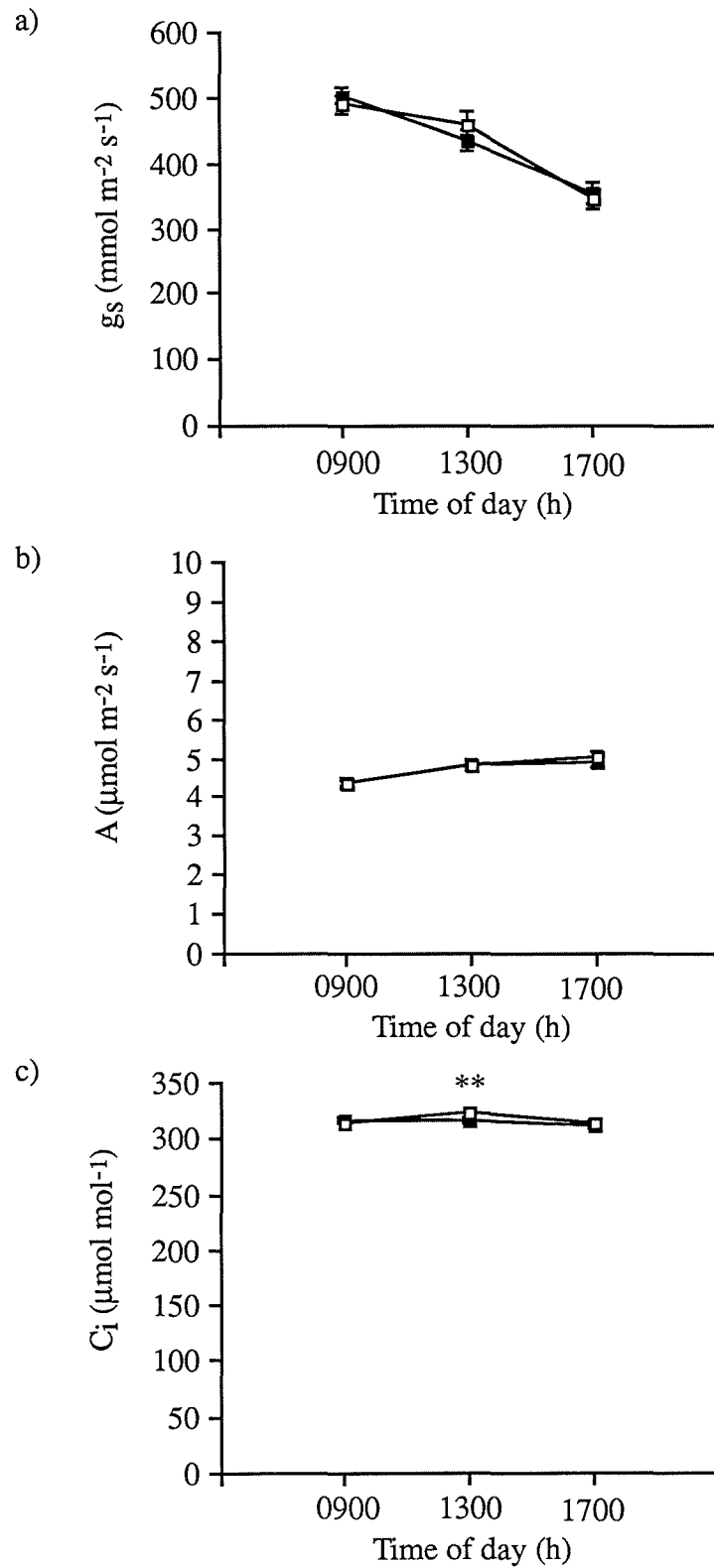
Figure 3.16. Changes in a) stomatal conductance, b) net rate of photosynthesis and c) intercellular CO_2 concentration of *Plantago major* population Sibton 27 days after sowing within the controlled environment growth cabinet. Solid squares, control; open squares, ozone-treated. Double standard errors are shown, except when these are smaller than the symbols. *** indicates significance at P<0.001; n=10.
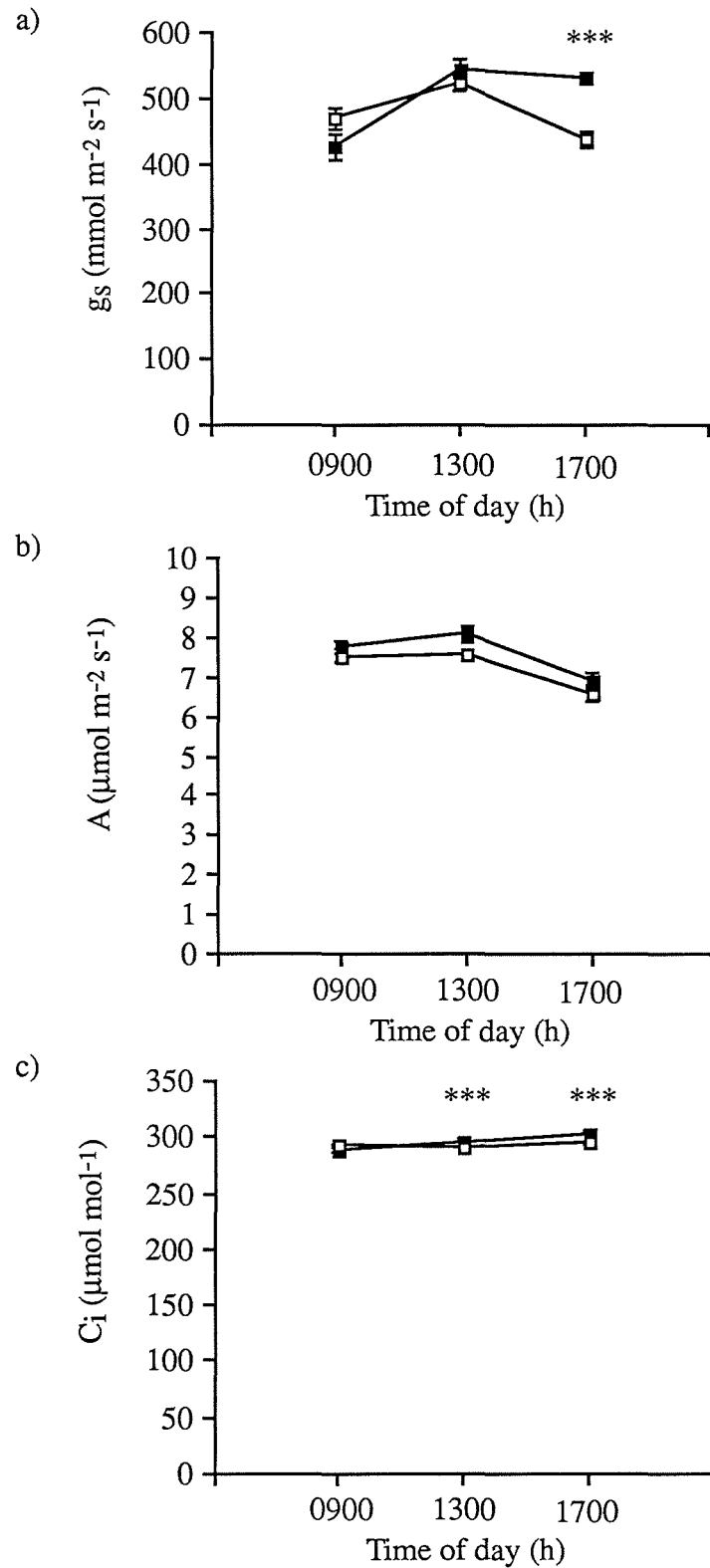
Figure 3.17. Responses of a) stomatal conductance, b) net rate of photosynthesis and c) intercellular CO_2 concentration over a four day period in *Plantago major* population High Low. Plants were exposed to filtered air (control) or 70 ppb ozone for 7 h d^{-1} (0930-1630 h) on days two and three. Solid squares, controls; open squares, ozone-treated. Double standard errors are shown, except when these are smaller than the symbols. *, ** and *** indicate significance at $P<0.05$, $P<0.01$ and $P<0.001$; n=10.
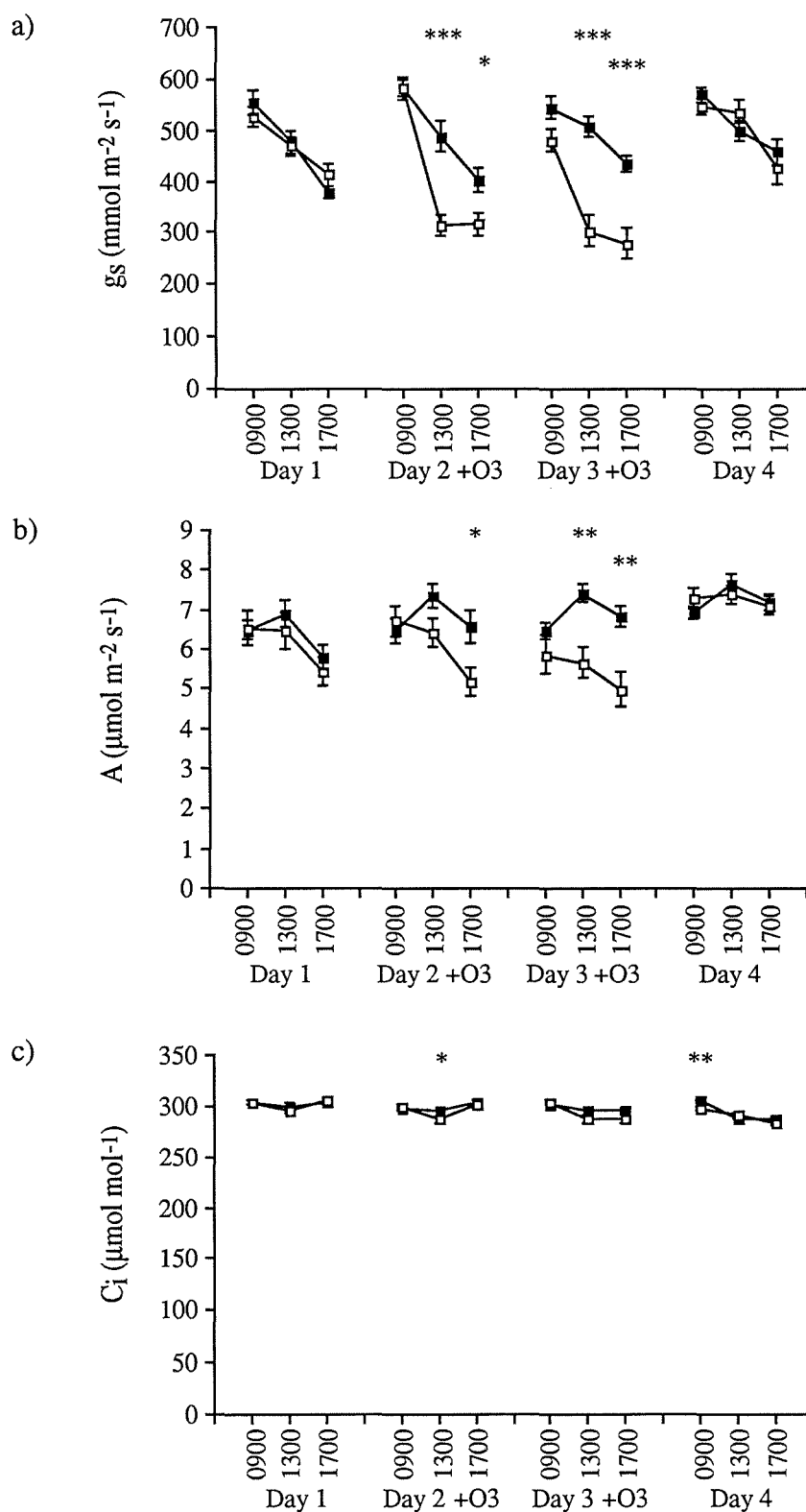
Figure 3.18. Responses of a) stomatal conductance, b) net rate of photosynthesis and c) intercellular CO_2 concentration over a four day period in *Plantago major* population Lullington. Plants were exposed to filtered air (control) or 70 ppb ozone for 7 h d^{-1} (0930-1630 h) on days two and three. Solid squares, controls; open squares, ozone-treated. Double standard errors are shown, except when these are smaller than the symbols. *, ** and *** indicate significance at P<0.05, P<0.01 and P<0.001; n=10.

a)

b)

c)
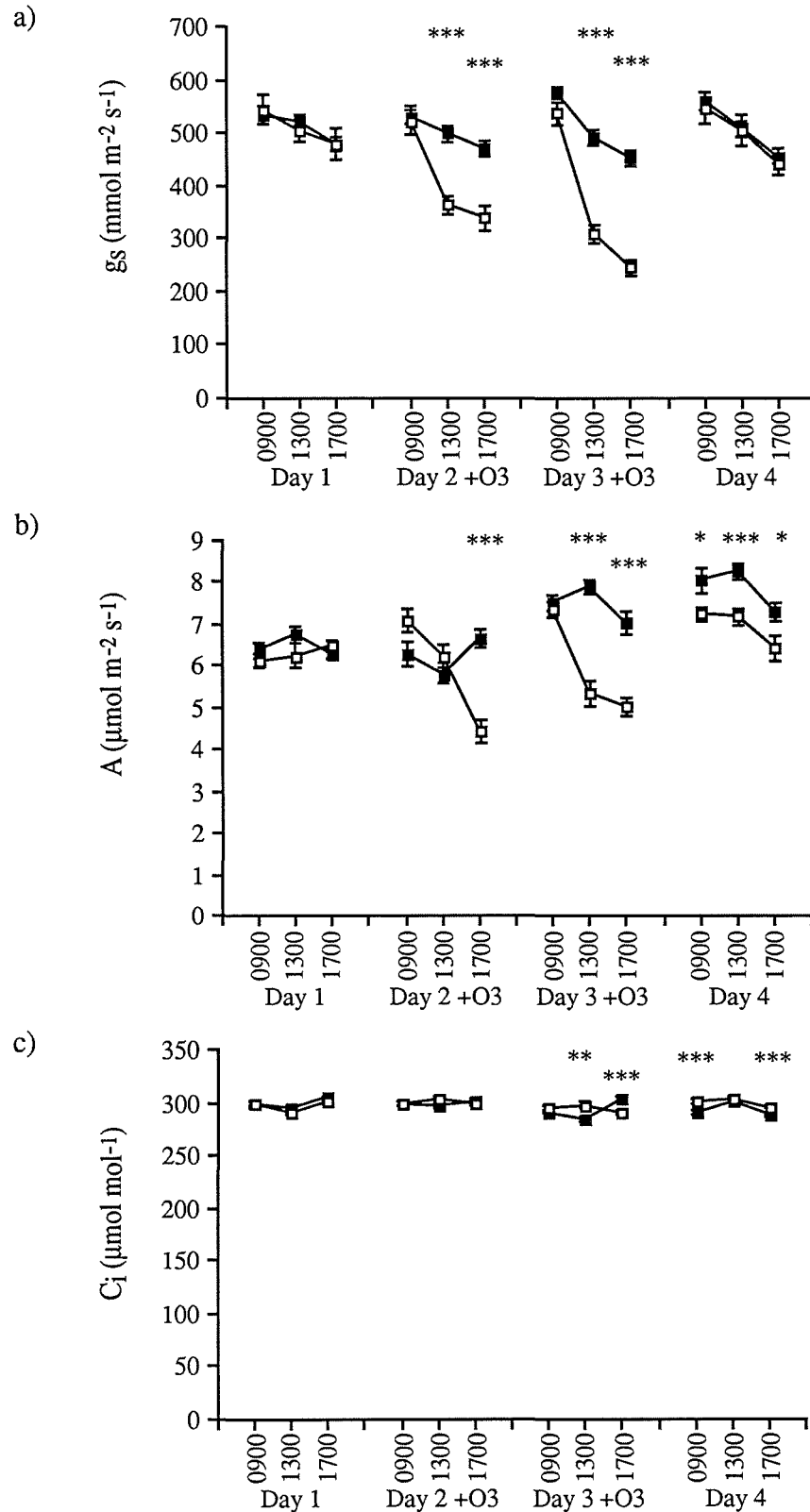
Figure 3.19. Responses of a) stomatal conductance, b) net rate of photosynthesis and c) intercellular CO_2 concentration over a four day period in *Plantago major* population Penicuik. Plants were exposed to filtered air (control) or 70 ppb ozone for 7 h d^{-1} (0930-1630 h) on days two and three. Solid squares, controls; open squares, ozone-treated. Double standard errors are shown, except when these are smaller than the symbols. *, ** and *** indicate significance at $P<0.05$, $P<0.01$ and $P<0.001$; n=10.
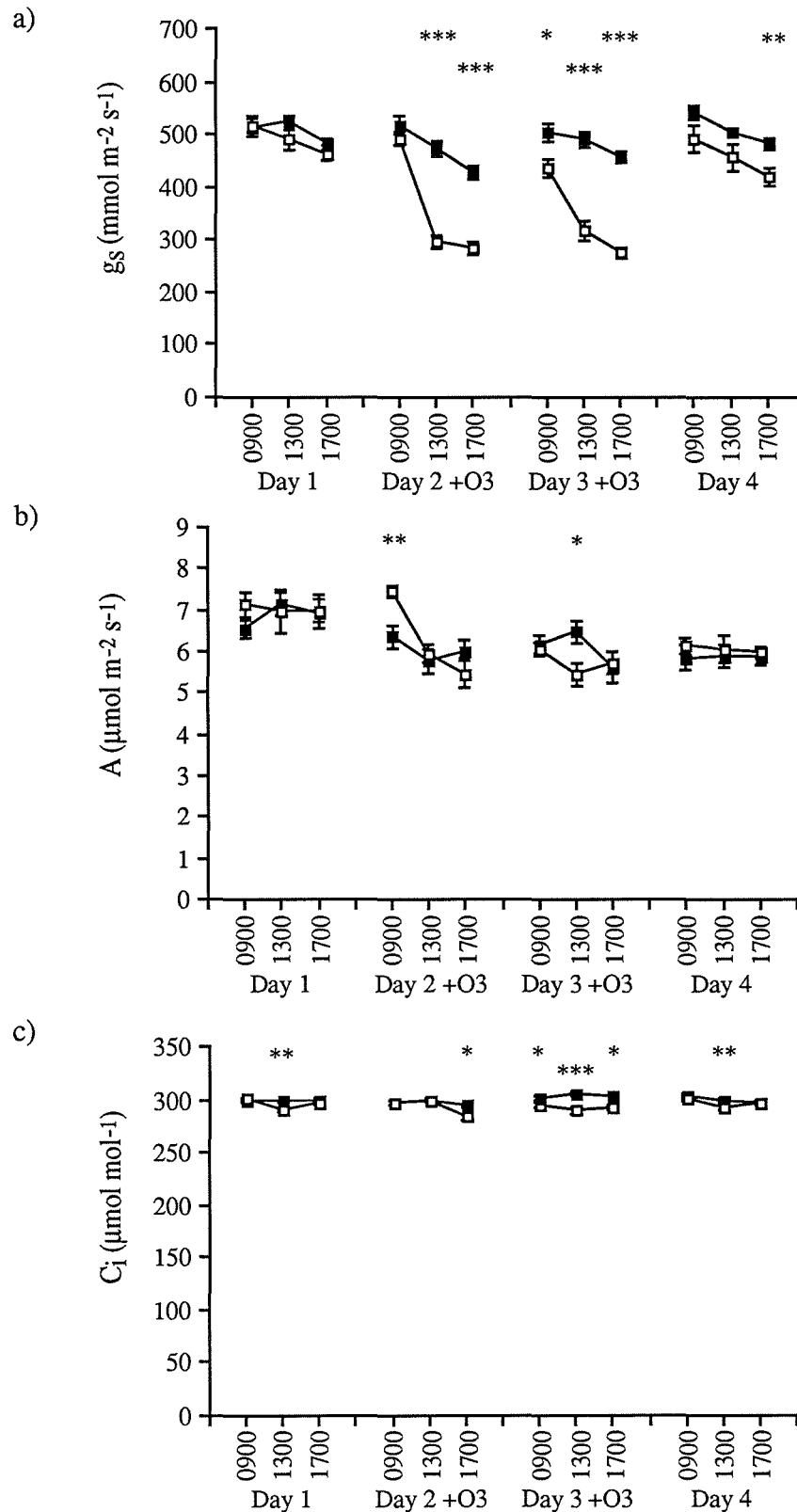
Figure 3.20. Responses of a) stomatal conductance, b) net rate of photosynthesis and c) intercellular CO_2 concentration over a four day period in *Plantago major* population Sibton. Plants were exposed to filtered air (control) or 70 ppb ozone for 7 h d^{-1} (0930-1630 h) on days two and three. Solid squares, controls; open squares, ozone-treated. Double standard errors are shown, except when these are smaller than the symbols. * and *** indicate significance at $P<0.05$ and $P<0.001$; n=10.
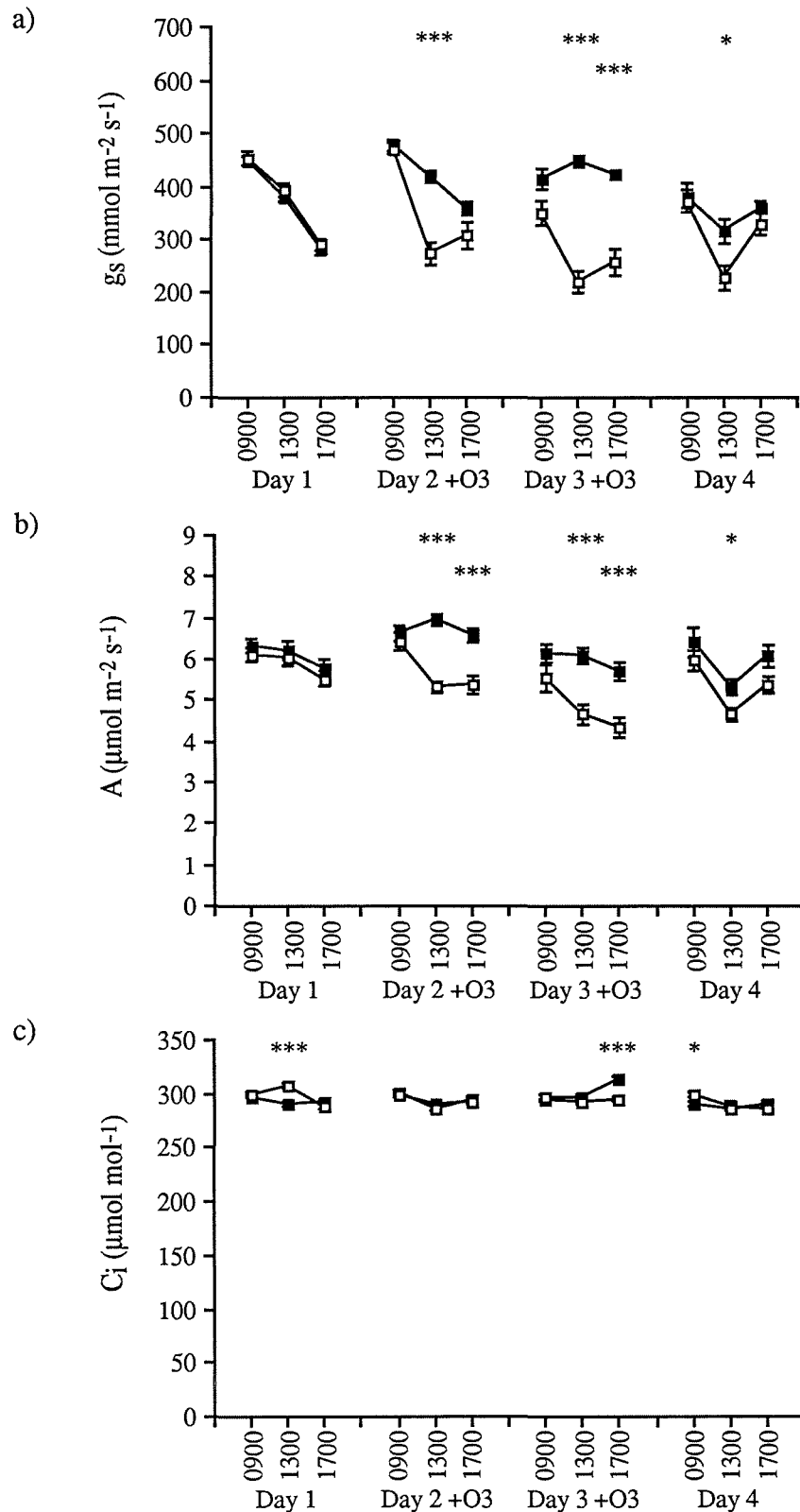
of photosynthesis changed when the plants were moved from the CEGC to the exposure chambers. In general, A increased in Penicuik by about 2.2 $\mu mol\ m^{-2}\ s^{-1}$ to around 7.0 $\mu mol\ m^{-2}\ s^{-1}$ whereas the other three populations A decreased by about 1-2 $\mu mol\ m^{-2}\ s^{-1}$. Unlike stomatal conductance, no consistent daily pattern of A developed within the exposure chambers, although in High Low photosynthesis rose to a maximum at 1300 h on each day. For each population, C_i remained at around 300 $\mu mol\ mol^{-1}$ throughout the day (Figs 3.17c-3.20c), similar to values recorded within the CEGC. Prior to ozone introduction (day 1), there were no significant differences in A, g_s and C_i between chambers except in the Penicuik and Sibton populations where there was a significant difference in C_i at 1300 h.

Figures 3.17a-3.20a show that ozone significantly reduced the stomatal conductance of all four populations of *P. major* within 4 h of the start of each fumigation. The reduction in g_s was greatest within this first 4 h, decreasing by 159-197 $mmol\ m^{-2}\ s^{-1}$ in the Lullington, Penicuik and Sibton populations and 271 $mmol\ m^{-2}\ s^{-1}$ in the High Low population on the first day of exposure. Conductance declined further during fumigation from 1300 h to 1700 h but this reduction was not as extensive as during the first four hours of fumigation. An exception was the Sibton population, where conductance was greater at 1700 h than at 1300 h (Fig. 3.20a). Stomatal conductance recovered almost fully between exposures except in Penicuik where, prior to the second exposure (0900 h, day 3), g_s was still significantly lower than in the control plants (Figure 3.19a). Stomatal conductance was reduced to a similar extent on the second day of exposure to ozone except in Lullington where g_s was decreased by a further 108 $mmol\ m^{-2}\ s^{-1}$ (Fig. 3.18a). In the High Low and Lullington populations, a complete recovery of stomatal conductance was achieved on day four, following the two days of exposure. In the Penicuik and Sibton populations, stomatal conductance did not recover fully up to 24 h after the end of ozone exposure; indeed the conductance of ozone-treated plants remained less than controls, with the reduction in g_s being significant at 1300 h for Sibton and at 1700 h for Penicuik.

Ozone significantly reduced the net rate of photosynthesis in all populations except Penicuik, where only one significant reduction in A was recorded at 1300 h on the second day of exposure (Figs. 3.17b-3.20b). As for stomatal conductance, the reduction in A was detectable within 4 h of the start of exposure; however, compared to g_s, the decline of A was more gradual over the 8 h measuring period. In a similar way to g_s, the net rate of photosynthesis of the High Low, Lullington and Sibton populations recovered between exposures but declined again to a similar extent with subsequent exposure to ozone. The recovery of A after two days of exposure to ozone differed for each population. In High Low, A recovered fully, showing a similar

response to g_s. In Lullington and Sibton, the net rate of photosynthesis remained lower than controls up to 24 h after exposure; in Lullington this decrease was significant throughout the day whereas for Sibton, A was significantly lower at 0900 h.

C_i differed significantly between the treatment and control plants of all four populations at various times during the four day measurement period (Figs. 3.17c-3.20c). A consistent trend in the response of C_i to ozone was not apparent partly because there were both significant increases and decreases in C_i in the Lullington and Sibton populations as well as significant effects appearing on day one, prior to ozone introduction, in the Penicuik and Sibton populations. For High Low and Penicuik, the significant effects of ozone recorded were all decreases in C_i.

3.3.3.2. Experiment 3b. Physiological and growth responses of Plantago *major populations to a six day fumigation with 26 ppb ozone for 7 h d^{-1}*

Physiology

Figures 3.21-3.23 show that there were few significant differences in stomatal conductance, the net rate of photosynthesis or intercellular CO_2 concentration in plants from each of the three populations of *P. major*; High Low, Lullington and Penicuik, prior to being placed within the exposure chambers. However, small but statistically significant differences in intercellular CO_2 concentration were detected between the plants from High Low allocated to each chamber (Fig. 3.21c), while the stomatal conductance of plants from Penicuik designated for ozone treatment was consistently greater at all times during the measurement period (Fig. 3.23a).

The stomatal conductance of plants from each of the three populations increased after transfer to the exposure chambers, where g_s exhibited a daily pattern starting highest in the morning and decreasing throughout the day (Figs. 3.24a-3.26a). The increase in g_s following transfer from the CEGC was greater for each population within the ozone treatment chamber, resulting in significant differences between chambers prior to ozone exposure. The net rate of photosynthesis also increased in each population by about 3 μmol m^{-2} s^{-1} after transfer to the exposure chambers (Figs. 3.24b-3.26b). For each population, the net rate of photosynthesis fluctuated in both treatments during the day and at 0900 h was significantly lower for each population within the ozone treatment chamber even though no ozone had been applied. The intercellular CO_2 concentration was not significantly different between chambers for Lullington but for High Low and Penicuik, C_i was significantly greater within the ozone treatment chamber at 0900 h and 1300 h (Figs. 3.24c-3.26c).

Figure 3.21. Responses of a) stomatal conductance, b) net rate of photosynthesis and c) intercellular CO_2 concentration in *Plantago major* population High Low 22 days after sowing within the controlled environment growth cabinet. Solid squares, controls; open squares, ozone-treated. Double standard errors are shown, except when these are smaller than the symbols. * and *** indicate significance at $P<0.05$ and $P<0.001$; n=8.

a)

g_s (mmol m⁻² s⁻¹) vs Time of day (h): 0900, 1300, 1700

b)

A (μmol m⁻² s⁻¹) vs Time of day (h): 0900, 1300, 1700

c)

C_i (μmol mol⁻¹) vs Time of Day (h): 0900, 1300, 1700
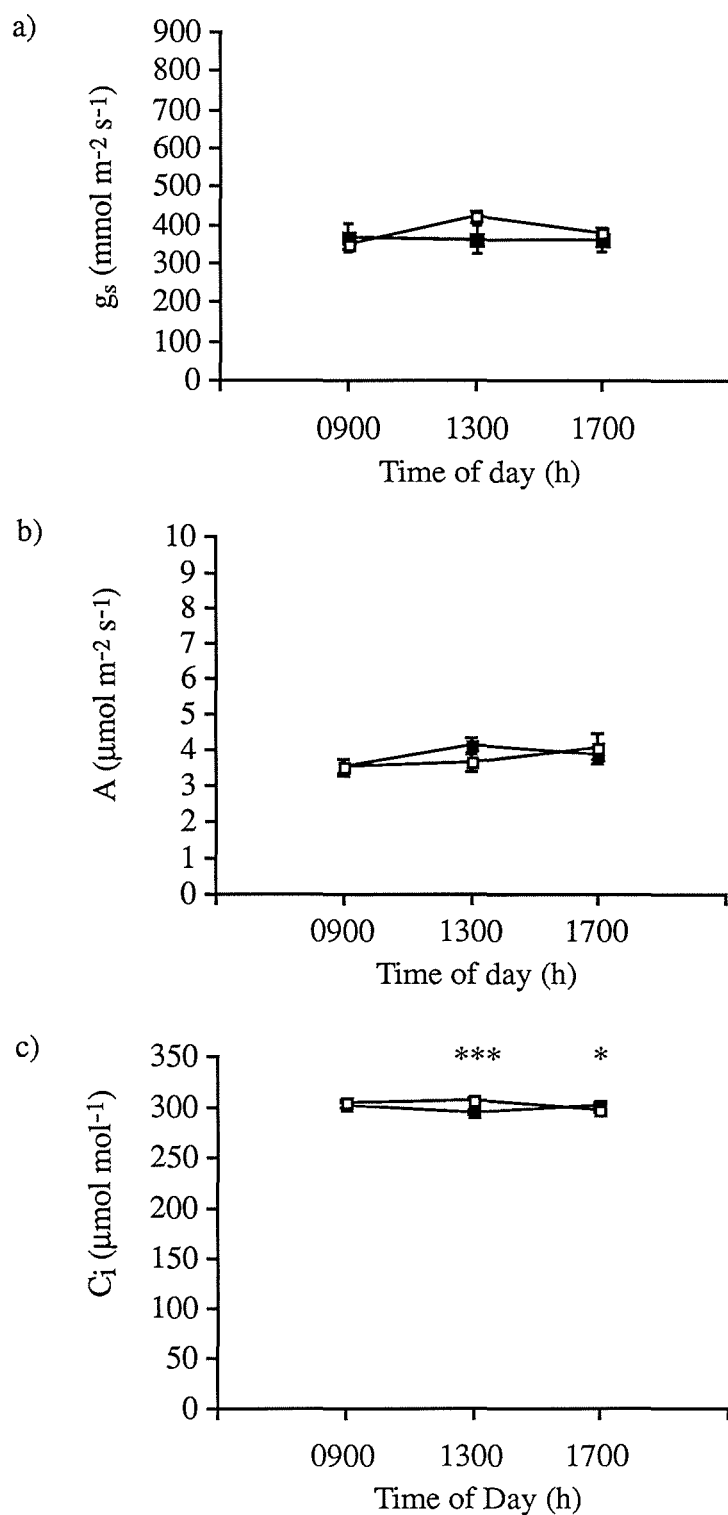
Figure 3.22. Responses of a) stomatal conductance, b) net rate of photosynthesis and c) intercellular CO_2 concentration in *Plantago major* population Lullington 22 days after sowing within the controlled environment growth cabinet. Solid squares, controls; open squares, ozone-treated. Double standard errors are shown, except when these are smaller than the symbols; n=8.

Figure 3.23. Responses of a) stomatal conductance, b) net rate of photosynthesis and c) intercellular CO_2 concentration in *Plantago major* population Penicuik 22 days after sowing within the controlled environment growth cabinet. Solid squares, controls; open squares, ozone-treated. Double standard errors are shown, except when these are smaller than the symbols. ** indicates significance at $P<0.01$; n=8.

a)

b)

c)

Figure 3.24. Responses of a) stomatal conductance, b) net rate of photosynthesis and c) intercellular CO_2 concentration in *Plantago major* population High Low, on day 24 after transfer to exposure chambers, prior to the introduction of ozone. Solid squares, controls; open squares, ozone-treated. Double standard errors are shown, except when these are smaller than the symbols. * and *** indicate significance at $P<0.05$ and $P<0.001$; n=8.
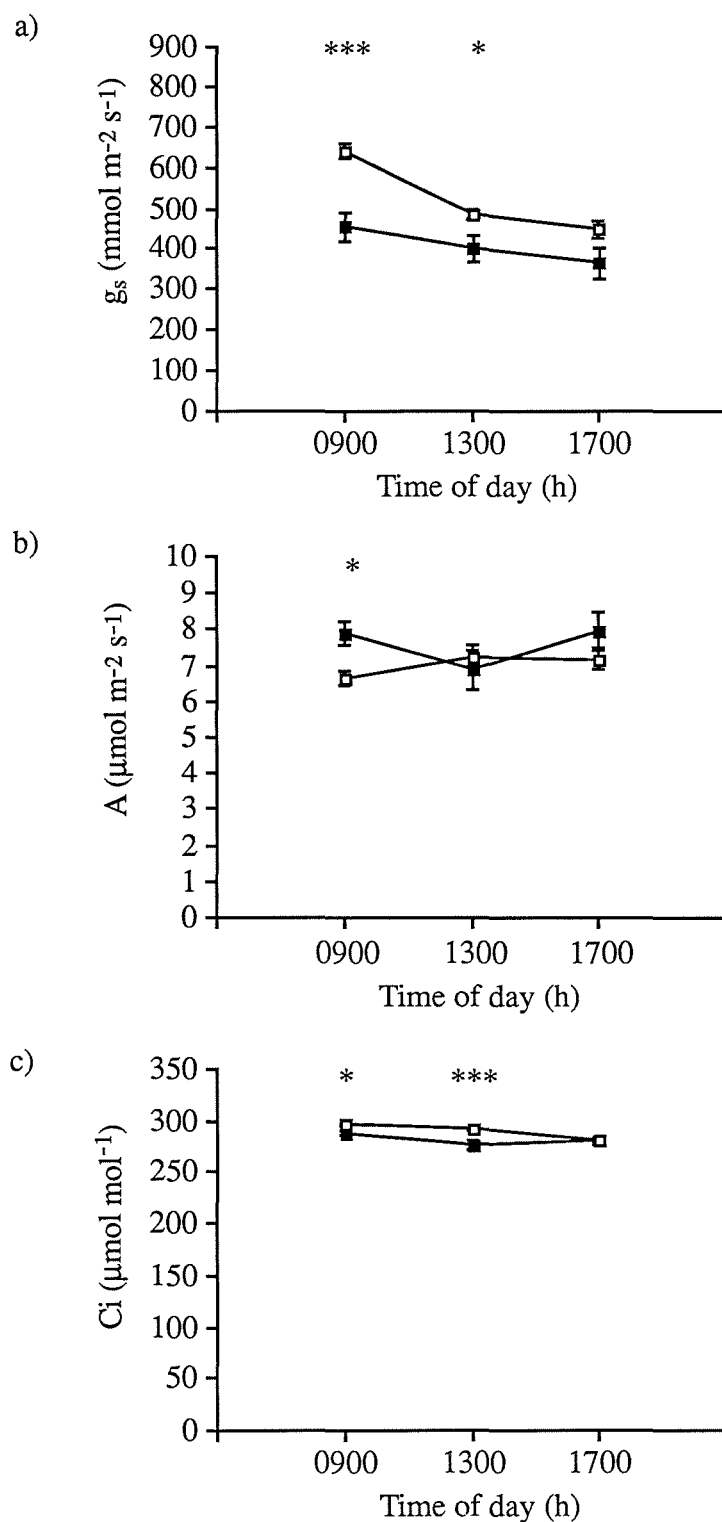
Figure 3.25. Responses of a) stomatal conductance, b) net rate of photosynthesis and c) intercellular CO_2 concentration in *Plantago major* population Lullington, on day 24 after transfer to exposure chambers, prior to the introduction of ozone. Solid squares, controls; open squares, ozone-treated. Double standard errors are shown, except when these are smaller than the symbols. *, ** and *** indicate significance at $P<0.05$, $P<0.01$ and $P<0.001$; n=8.
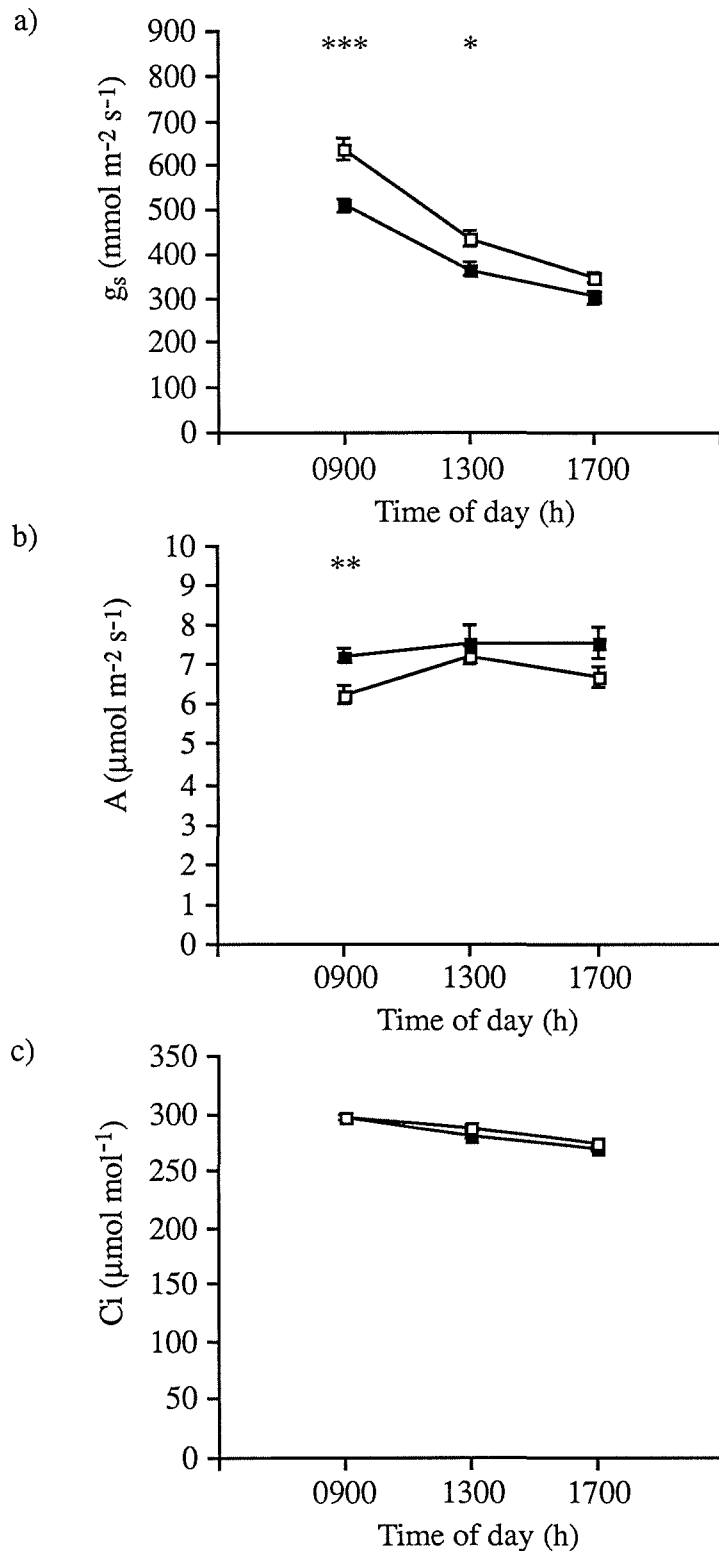
Figure 3.26. Responses of a) stomatal conductance, b) net rate of photosynthesis and c) intercellular CO_2 concentration in *Plantago major* population Penicuik on day 24 after transfer to exposure chambers, prior to the introduction of ozone. Solid squares, controls; open squares, ozone-treated. Double standard errors are shown, except when these are smaller than the symbols. *, ** and *** indicate significance at $P<0.05$, $P<0.01$ and $P<0.001$; n=8.

a)

g_s (mmol m^{-2} s^{-1})

900
800
700
600
500
400
300
200
100
0

*** ** **

0900 1300 1700

Time of day (h)

b)

A (μmol m^{-2} s^{-1})

10
9
8
7
6
5
4
3
2
1
0

** *** *

0900 1300 1700

Time of day (h)

c)

Ci (μmol mol^{-1})

350
300
250
200
150
100
50
0

*** ***

0900 1300 1700

Time of day (h)
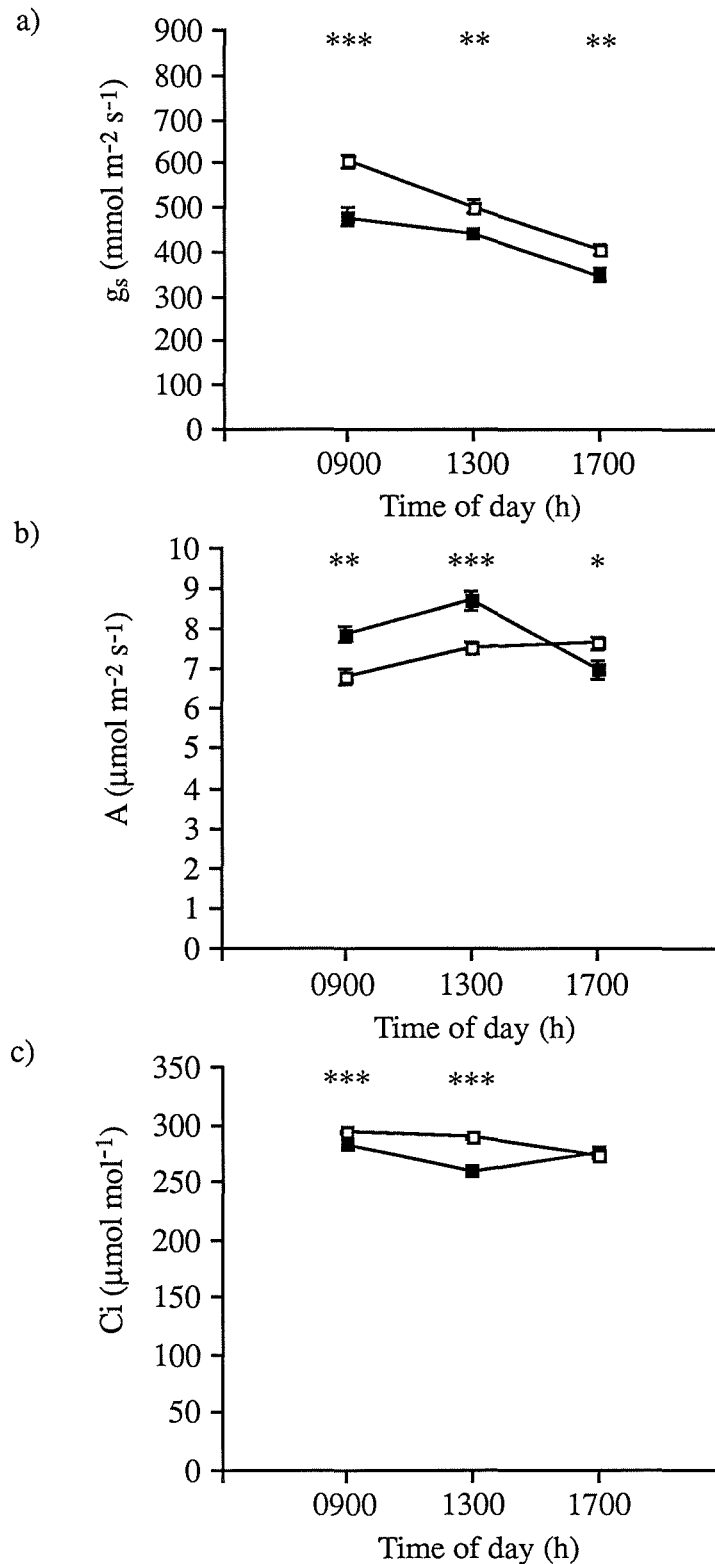
For each population, ozone significantly reduced stomatal conductance on each day of measurement during the six day exposure period (Figs. 3.27a-3.29a). Stomatal conductance recovered between exposures and this recovery showed a progressive increase following each exposure to ozone, so that by 0900 h on the sixth day of exposure g_s was significantly greater than in control plants. The results show that, despite this initial daily increase in g_s prior to the exposure period, ozone continued to reduce conductance below that of the control plants; indeed for Lullington and Penicuik, g_s was reduced to an increasing extent with each day of exposure.

The net rate of photosynthesis varied considerably in all populations throughout the six days of exposure. Ozone had little effect on the net rate of photosynthesis in High Low but caused significant reductions in A in Lullington and Penicuik during each day of exposure (Fig. 3.27b-3.29b). In Lullington and Penicuik, the net rate of photosynthesis recovered to control levels between consecutive exposure period, while in High Low there was the tendency for A to be significantly greater than in control plants at 0900 h on each day. With exposure to ozone, intercellular CO_2 concentration varied considerably in all three populations, showing both significant increases and decreases relative to the control plants over the four days of measurement.

Growth

Table 3.16 shows that there was no effect of ozone on the mean relative growth rate, $\overline{R}$, or the allometric root/shoot coefficient, K, of the High Low, Lullington or Penicuik populations. The mean relative growth rate in ozone-treated plants expressed as a percentage of that in charcoal-filtered air, O_{res}, suggested that the growth of Penicuik was stimulated by ozone while that of Lullington was reduced and High Low was unaffected.

3.3.3.3. *Experiment 3c. Growth responses of* Plantago major *to a 14 day exposure to 70 ppb ozone for 7 h d^{-1}*

Table 3.17 shows that fourteen days of exposure to 67 ppb ozone for 7 h d^{-1} had no significant effect on the allometric root/shoot coefficient or the mean relative growth rate of the High Low, Lullington or Sibton populations. However, the small reduction in mean relative growth rate under ozone resulted in an O_{res} value for each population of less than 100%, suggesting that the growth of these three populations was sensitive to ozone, with Sibton being the most sensitive and High Low the least sensitive populations.

Figure 3.27. Responses of a) stomatal conductance, b) net rate of photosynthesis and c) intercellular CO_2 concentration in *Plantago major*, population High Low, on the first, second, third and sixth days of fumigation with filtered air (control) or 26 ppb ozone for 7 h d^{-1} (0930-1630 h). Solid squares, controls; open squares, ozone-treated. Double standard errors are shown, except when these are smaller than the symbols. *, ** and *** indicate significance at P<0.05, P<0.01 and P<0.001; n=8.
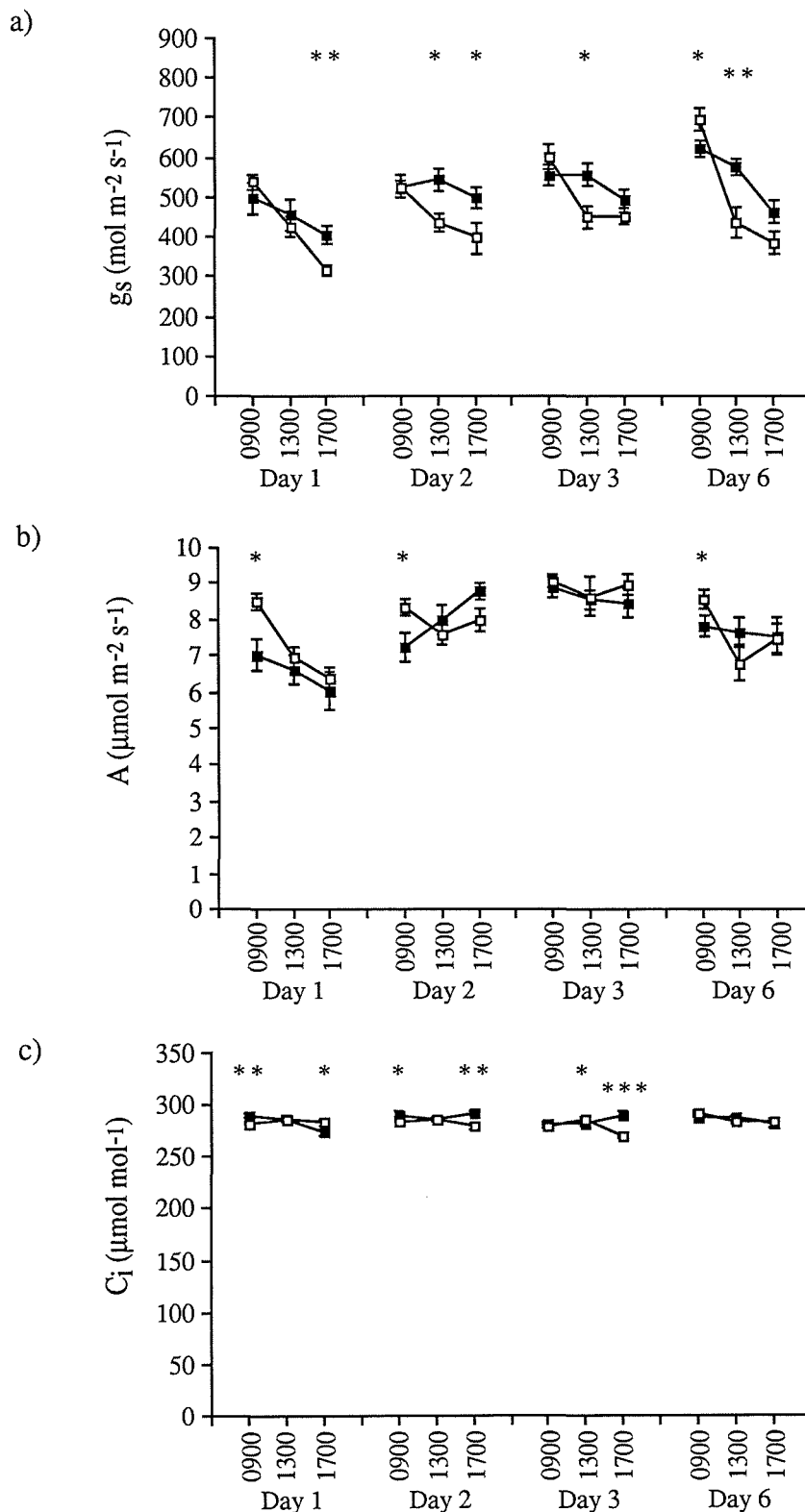
Figure 3.28. Responses of a) stomatal conductance, b) net rate of photosynthesis and c) intercellular CO_2 concentration in *Plantago major*, population Lullington, on the first, second, third and sixth days of fumigation with filtered air (control) or 26 ppb ozone for 7 h d^{-1} (0930-1630 h). Solid squares, controls; open squares, ozone-treated. Double standard errors are shown, except when these are smaller than the symbols. *, ** and *** indicate significance at P<0.05, P<0.01 and P<0.001; n=8.
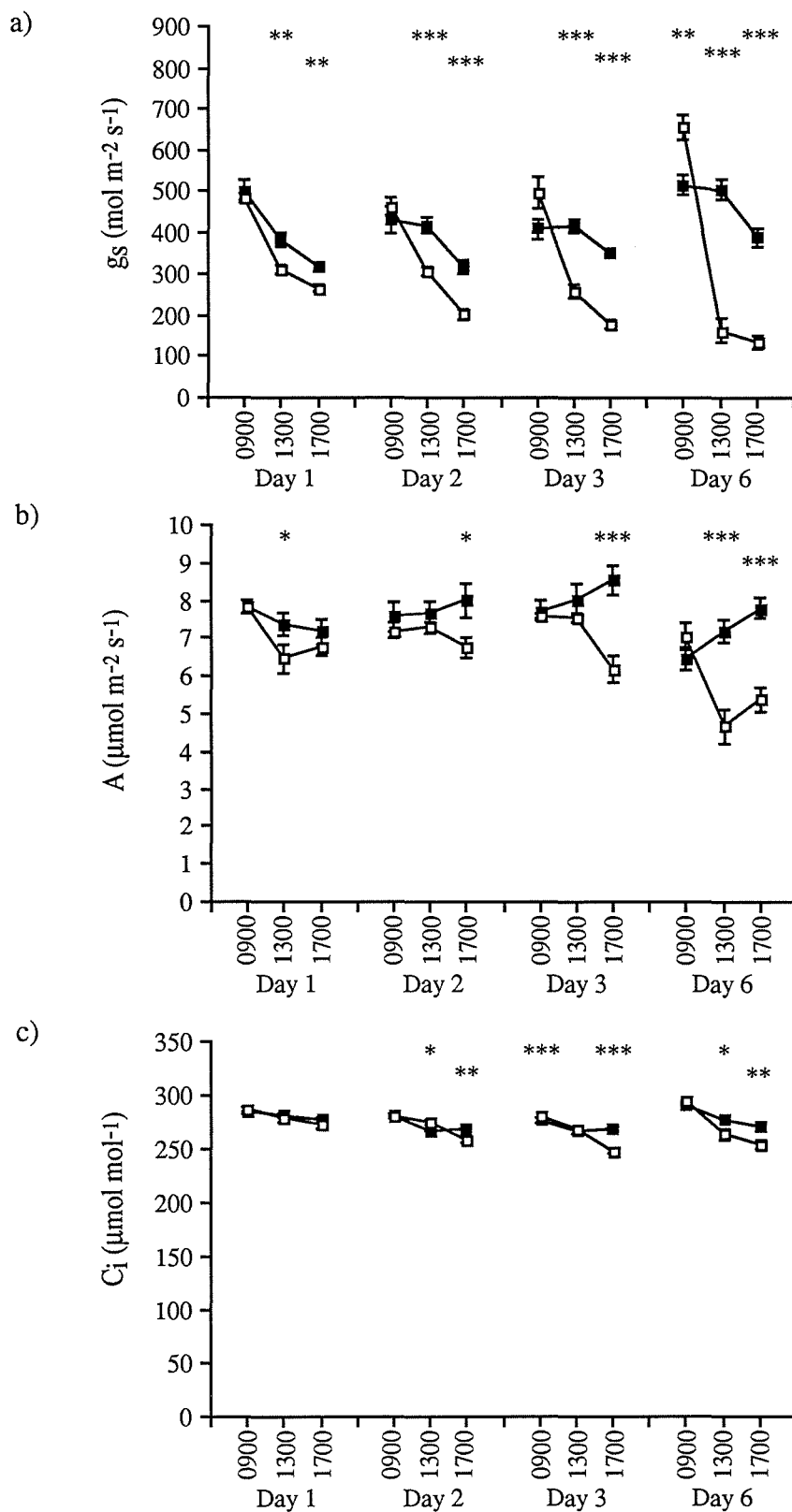
Figure 3.29. Responses of a) stomatal conductance, b) net rate of photosynthesis and c) intercellular CO_2 concentration in *Plantago major*, population Penicuik, on the first, second, third and sixth days of fumigation with filtered air (control) or 26 ppb ozone for 7 h d^{-1} (0930-1630 h). Solid squares, controls; open squares, ozone-treated. Double standard errors are shown, except when these are smaller than the symbols. *, ** and *** indicate significance at $P<0.05$, $P<0.01$ and $P<0.001$; n=8.
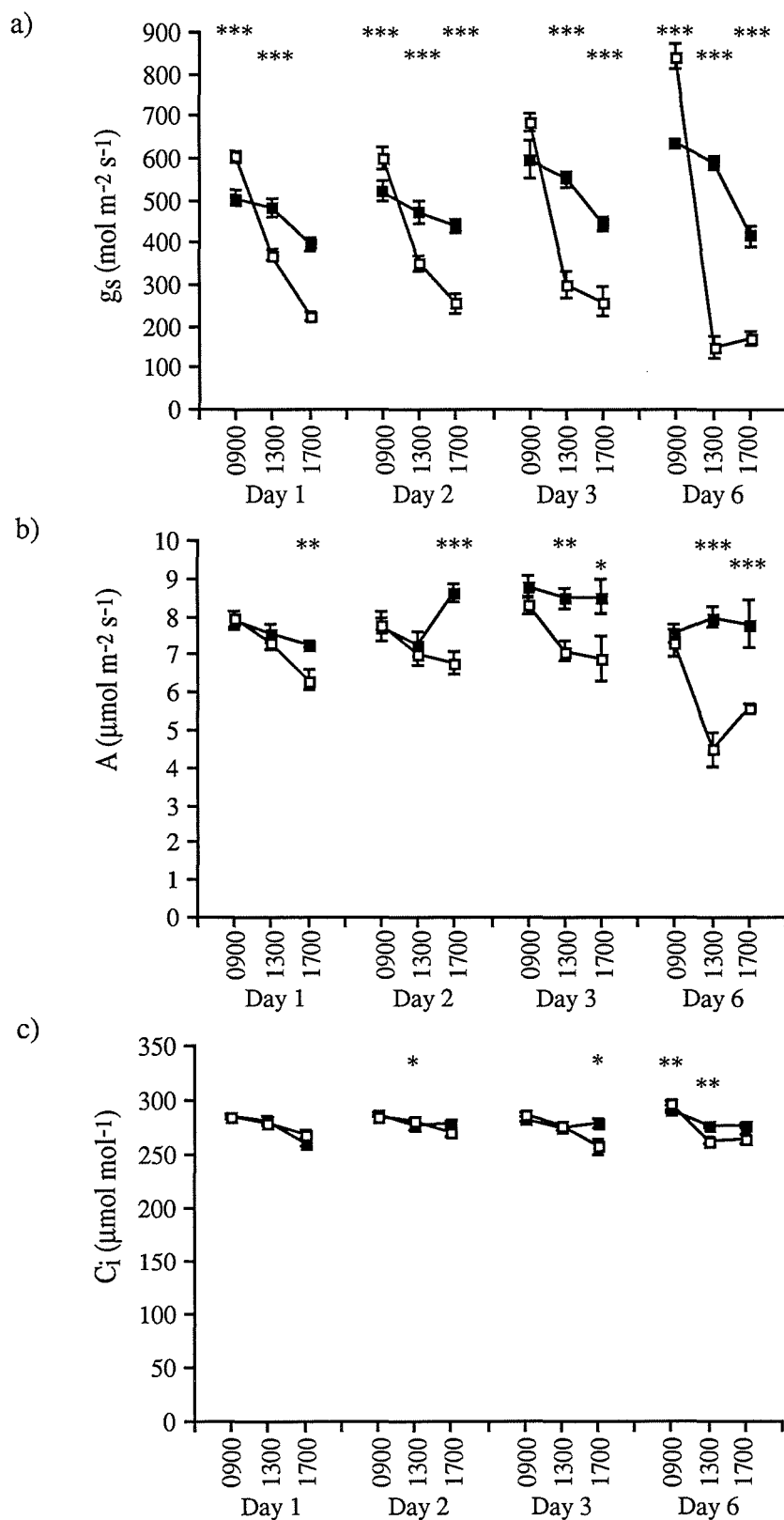
Table 3.16. Summary of growth for *Plantago major* populations after 6 days of exposure to filtered air (control) or 26 ppb ozone for 7 h d⁻¹. Means and standard errors are shown; n=8.

Population	Treatment	Total $\overline{R}$ (week⁻¹)	K	O₃ resistance (%)
High Low	Control	3.1 ± 0.21	0.97 ± 0.020	
	Ozone	3.1 ± 0.20	0.82 ± 0.026	101.6
Lullington	Control	2.5 ± 0.13	1.01 ±0.122	
	Ozone	2.2 ± 0.08	0.92 ± 0.017	88.7
Penicuik	Control	2.2 ± 0.13	0.79 ± 0.035	
	Ozone	2.5 ± 0.11	0.86 ± 0.018	113.0

Table 3.17. Summary of growth for *Plantago major* populations after 14 days of exposure to filtered air (control) or 67 ppb ozone for 7 h d⁻¹. Means and standard errors are shown; n=9.

Population	Treatment	Total $\overline{R}$ (week⁻¹)	K	O₃ resistance (%)
High Low	Control	3.9 ± 0.31	1.25 ± 0.097	
	Ozone	3.4 ± 0.26	1.30 ± 0.109	86.8
Lullington	Control	3.6 ± 0.40	1.04 ± 0.081	
	Ozone	3.0 ± 0.42	0.90 ± 0.108	81.5
Sibton	Control	3.6 ± 0.36	1.27 ± 0.100	
	Ozone	2.9 ± 0.33	1.32 ± 0.093	79.7

132

3.4. DISCUSSION

The data presented in this chapter clearly demonstrate that the physiology and vegetative growth of both *Plantago major* and *Brassica campestris* may be affected by exposure to ozone. In addition, various aspects of reproductive development were affected in *B. campestris* when plants were exposed to ozone for several days prior to first flowering.

3.4.1. Effects of ozone on physiology

The initial response of *B. campestris* and *P. major* to two days of fumigation with 70 ppb ozone was a rapid decline in stomatal conductance (g_s) and the net rate of photosynthesis (A) within 3.5 h of the start of fumigation (Figs. 3.1-3.3, 3.17-3.20). In both species, the decline in g_s and A was generally synchronous and increased progressively as ozone exposure continued. Reductions in stomatal conductance and the net rate of photosynthesis are consistent phenomena related to ozone stress, with responses occurring either immediately or within hours of the start of fumigation (Reich and Lassoie, 1984; Temple, 1986; Darrall, 1989; Moldau *et al.*, 1990; Sanders *et al.*, 1992; Hassan *et al.*, 1994; Reiling and Davison, 1995). It has been suggested that stomatal closure is the result of a direct effect on guard cell membranes which induces a loss of turgor (Faensen-Thiebes, 1983, cited by Saxe, 1990), and that this response may be a stress avoidance mechanism which limits further ozone uptake by the plant (Takemoto *et al.*, 1988). The decrease in A observed in both *B. campestris* and *P. major* has been recorded for many other species (Darrall, 1989) and has been attributed to a direct effect of ozone on stomatal conductance which reduces the availability of CO_2 for photosynthesis (Temple, 1986; Amundson *et al.*, 1987). However, ozone exposure may also decrease the net rate of photosynthesis by affecting the chloroplasts directly and/or enhancing dark respiration (Barnes, 1972; Reich and Amundson, 1985). If ozone reduces CO_2 fixation before affecting the stomata, this would lead to an increase in intercellular CO_2 concentration and a consequent reduction in stomatal conductance through feedback control (Runeckles and Chevone, 1992). Despite the complicated interaction of stomatal conductance and photosynthesis, there is evidence to suggest that there are separate effects of ozone on both of these physiological processes. For example, Faensen-Thiebes (1983, cited by Saxe, 1990) found that transpiration in *Nicotiana tabacum* and *Phaseolus vulgaris* was affected by low doses of ozone in the absence of any photosynthetic response, indicating an independent effect on the stomata. Aben *et al.* (1990) showed that ozone can have a direct effect on the stomata as well as on the photosynthetic system. They exposed *Vicia faba* plants to 60 ppb ozone for 8 h d^{-1} over a two week period and found

differences in the response at different times of year. Plants fumigated in open top chambers between May and June did not develop visible injury but stomatal conductance was decreased without an accompanying decline in photosynthesis. In contrast, plants exposed between August and October became chlorotic and the rate of photosynthesis was reduced. These results suggested that the stomata were more sensitive to ozone than the photosynthetic system. Investigations have shown that different parts of the photosynthetic system may be affected by ozone, including the chloroplast envelope (Sanders et al., 1992), chlorophyll pigments (Runeckles and Chevone, 1992), photosystems (Chang and Heggestad, 1974), and the CO_2 fixation enzyme RUBISCO (Pell and Pearson, 1983; Lehnherr et al., 1987; Aarnes, 1993), although it is still contentious whether ozone actually reaches the chloroplast or whether its effects arise indirectly from the reaction products of ozone (Runeckles and Chevone, 1992).

Ozone affected the net rate of photosynthesis in B. campestris to an extent related to the stomatal conductance of the plants prior to exposure. Initial stomatal conductance varied between the three, two day exposure experiments (Figs. 3.1-3.3), possibly because of differences in the growth conditions experienced by the plants prior to exposure; thus, the plants grown within the exposure chambers experienced different environmental conditions during early vegetative development compared to those grown within the CEGC. Exposure to 70 ppb ozone reduced stomatal conductance by a similar amount (c. 150-200 mmol m^{-2} s^{-1}) in each of the three exposure experiments, suggesting that the degree of change in stomatal conductance was dependent on the concentration of ozone rather than the flux of ozone into the leaf. Despite a similar effect on g_s in each experiment, the decrease in A was larger in plants with greater stomatal conductances (and hence greater ozone uptake), suggesting that ozone was damaging the chloroplasts directly. In two of the three experiments, this was confirmed by the development of visible damage to the leaves as well as a rise in intercellular CO_2 concentration (C_i). Stomata would normally respond to this increase in C_i by partially closing, causing C_i to return to its original level but, particularly in those plants exhibiting the greatest damage, this did not occur, suggesting that the stomata were dysfunctional.

In P. major, the rapid decline in g_s following exposure to 70 ppb ozone was similar in each of the four populations and g_s was reduced more during the first than during the second half of each daily exposure period (Figs 3.17-3.20). A reduction in g_s in response to 70 ppb ozone has been reported previously for P. major by Reiling and Davison (1995). However, in their study, the extent of the decline varied between populations, being greatest in the ISP population (equivalent to High Low in the

present study) and least in the Lullington and Sibton populations. In the present study, the decrease in g_s was generally accompanied by a similar decline in A, although it is not known whether stomatal closure was the result or the cause of the reduction in photosynthesis. A notable exception was the Penicuik population, in which the decline in net photosynthesis was smaller than in the other populations and the intercellular CO_2 concentration was also reduced. This suggests that ozone may have affected the stomata directly, with little or no effect on CO_2 fixation. Normally the stomata would open to increase C_i when the intercellular CO_2 concentration falls, but this response may have been overridden by direct effects of ozone on the stomata, such that a lower stomatal conductance was maintained. The reduction in A recorded for High Low is consistent with the results of Reiling and Davison (1995). However, their results for Lullington contrast to those obtained in the present study since they showed that photosynthesis was unaffected in this population despite a reduction in stomatal conductance. There is clear evidence presented here to show that ozone can reduce photosynthesis in the Lullington population. In the present study g_s and A also declined after exposure of *P. major* to 26 ppb ozone (Figs. 3.27-3.29), indicating that these physiological processes are sensitive to low concentrations of ozone. In each of the three populations exposed to this concentration, High Low, Lullington and Penicuik, stomatal conductance tended to increase from day to day between the first and the last day of exposure in both ozone-treated and control plants; this may have been a consequence of the plants acclimatising to the different environmental conditions experienced in the exposure chambers or a natural increase in g_s as the leaves aged. Although not consistent, there was a trend for C_i to decrease in all three populations during exposure to 26 ppb ozone, suggesting that the greater effect was on stomatal conductance.

The ability of g_s and A to recover from exposure to 70 ppb ozone differed between species and also between populations of *P. major*. In *B. campestris*, the reduction in g_s and A persisted between and after the two 7 h exposures to ozone (Figs 3.1-3.3). This effect would have been at least partly attributable to the loss of photosynthetic tissue resulting from the development of chlorosis and necrosis, although similar reductions were also evident in plants which exhibited no foliar damage, suggesting that they had little capacity to repair damage. A reduction in g_s was also evident in *B. campestris* during the final day of a 10 d exposure to 70 ppb ozone (Fig 3.5), although A was similar to that of control plants prior to the last exposure, suggesting that there may have been a long-term mechanism acting to restore net photosynthetic rates, although photosynthesis was again reduced by the following ozone exposure. Unlike *B. campestris*, there was generally a partial or total recovery of g_s and A in clean air between and after the two days of exposure to ozone in each of the four populations of

P. major, although there were differences between populations, particularly during the 24 h period following exposure (Figs. 3.17-3.20). In High Low, there was a full recovery of g_s and A, while in Sibton there was only a partial recovery in both processes. There was a complete recovery of stomatal conductance in Lullington but only a partial recovery in A. As there was also a rise in intercellular CO_2 concentration, this suggested that the reduction in A had resulted from damage to the chloroplasts. In Penicuik, the net rate of photosynthesis was not affected greatly during exposure and A remained similar to control plants up to 24 h after exposure; however, there was a persistent reduction in g_s, further demonstrating a direct effect of ozone on the stomata of this population. It is not known whether the effects on g_s and A in these populations were permanent or would eventually have been reversed. This study has not identified the mechanisms responsible for the differing responses observed in each population; further investigations are required to establish the precise nature of the injury induced and responses exhibited by each population.

3.4.2. Visible foliar injury

Visible foliar injury was observed in *B. campestris* but not in *P. major* following exposure to ozone. The lack of visible injury in *P. major* following 14 days of exposure to 70 ppb ozone for 7 h d^{-1} is consistent with other studies (Reiling, 1990; Reiling and Davison, 1992b). The leaves of *B. campestris* developed chlorosis and necrosis, both of which are characteristic symptoms of ozone damage (Hill *et al.*, 1970, Krupa and Manning, 1988).

In *B. campestris,* symptom expression differed during each of the three, two day exposure experiments, and these effects may be related to stomatal conductance and hence ozone uptake or flux. No visible damage occurred in plants which had the lowest stomatal conductance prior to and during ozone introduction, although ozone is known to induce a loss of chlorophyll in foliage without promoting visible injury (Tenga and Ormrod, 1990). Analysis of the chlorophyll content of leaves would have been useful to determine whether ozone affected the quantity of chlorophyll present. As stomatal conductance increased, interveinal chlorosis was the first visible symptom to develop, indicating that exposure to ozone had directly affected the chloroplasts. In plants with the highest conductance, damage appeared initially during the first 7 h exposure as patches of wilted tissue, particularly in the interveinal areas. This was accompanied by a general 'stickiness' of the leaves, which suggested that exposure to ozone had caused severe membrane disruption and liberation of cell contents. Despite the apparent recovery of turgidity between exposures, irrevocable cell damage led to cell death and the formation of necrotic lesions. The relationship between stomatal

conductance and visible damage in *B. campestris* appears unequivocal. A similar relationship between stomatal conductance and visible injury has been demonstrated for cultivars of tobacco (*Nicotiana tabacum*; MacDowall, 1965) and white clover (*Trifolium repens*; Fuhrer *et al.*, 1993). In these species, the more resistant cultivars had lower stomatal conductances than the sensitive cultivars thereby reducing ozone uptake. Environmental factors which reduce stomatal conductance have also been shown to decrease ozone injury. These include low relative humidity (McLaughlin and Taylor, 1981) and water stress (Olszyk and Tibbitts, 1981b). It is probable that the variable response of *B. campestris* to ozone exposure resulted from differences in environmental conditions between experiments which influenced stomatal conductance. Such changes in stomatal conductance would have altered the flux of ozone into the leaves since stomatal conductance is a primary determinant of ozone uptake (Heath, 1980). It is now commonly agreed that it is the flux of ozone into the leaf that is important in determining responses, rather than the concentration of ozone to which the plants are exposed (Heath, 1994). The flux of ozone into the leaf may be calculated as follows:

$$F_o = \frac{A_o}{R_s + R_b}$$

where F_o is the flux of ozone ($\mu g\ m^{-2}\ s^{-1}$), A_o is the atmospheric ozone concentration ($\mu g\ m^{-3}$), R_b is the boundary layer resistance ($s\ m^{-1}$) and R_s is the stomatal resistance ($s\ m^{-1}$). The internal mesophyll resistance is assumed to be zero (Laisk *et al.*, 1989).

Since no estimates of boundary layer resistance (R_b) were made in this study, it was not possible to determine the flux of ozone into the leaves of *B. campestris*. However, to provide an indication of the relative difference in flux between the plants from different experiments, calculations were made by assuming a value for R_b of 100 $s\ m^{-1}$. A_o was recorded in each experiment and g_s was converted into R_s. Resistance values were multiplied by 1.67 to correct for the different molecular diffusivity of ozone (Laisk *et al.*, 1989). By substituting the value for g_s recorded in each experiment prior to fumigation, the flux of ozone into the leaves of *B. campestris* was calculated as 0.410, 0.496 and 0.510 $\mu g\ m^{-2}\ s^{-1}$ in the plants from Experiments 1a, b and c respectively, thus, flux varied less between experiments than conductance. Although the flux of ozone was limited during the exposure period by the concurrent reduction in g_s, the difference in flux between control and ozone-treated plants in individual experiments was maintained, since conductance was reduced by a similar amount in each experiment. Assuming that the initial flux of ozone is important in

determining the sensitivity of the plants to ozone, the threshold flux for the development of visual damage in *B. campestris* appears to lie between 0.410 and 0.496 μg m^{-2} s^{-1}. Increasing the flux slightly from 0.496 to 0.510 μg m^{-2} s^{-1} had a significant effect on the type of damage expressed by leaves.

The loss of chlorophyll and photosynthetically active tissue following exposure to ozone would have been at least partly responsible for the irreversible reduction of photosynthesis. In *B. campestris*, a partial return of greenness in leaves exhibiting interveinal chlorosis was observed during the post-exposure period, although this effect was not quantified. The greening of the tissue may have been a mechanism to increase total photosynthesis and partially or completely offset the injurious effects of ozone. A previous study by Tenga and Ormrod (1990) showed that the chlorophyll content of leaves may recover post-exposure since these workers witnessed a recovery of chlorophyll content in the leaves of tomato (*Lycopersicon esculentum*) during the 10 day period following four days of exposure to 160 ppb ozone for 7 h d^{-1}.

The extent of foliar damage which develops in response to ozone is known to differ between species (Mortensen, 1992; Nebel and Fuhrer, 1994; Bergmann *et al.*, 1995; Warwick and Taylor, 1995). This was clearly demonstrated in the present study since, although the physiology of both *B. campestris* and *P. major* was significantly affected by two 7 h exposures to 70 ppb ozone, only *B. campestris* exhibited foliar damage. The difference between species in the expression of visible damage may result from differences in their ability to exclude ozone from the leaf through stomatal responses, and/or their ability to detoxify ozone or repair damage. A possible relationship between the formation of stress ethylene and the sensitivity of plants to ozone has been suggested by Mehlhorn and Wellburn (1987). Ethylene is normally produced by all plants in trace amounts to regulate various physiological and developmental events such as fruit ripening and leaf abscission. However, in stressful environments, plants respond by producing increased quantities of ethylene, in a phenomenon known as stress ethylene formation. Mehlhorn and Wellburn (1987) demonstrated that pea (*Pisum sativum*) plants exposed to 150 ppb ozone for 7 h d^{-1} for three weeks showed no visible damage and a low rate of ethylene production. In contrast, plants exposed for only one day to the same concentration of ozone showed substantial visible damage and enhanced rates of ethylene production. It was suggested that the reaction of ethylene with ozone initiates the formation of free radicals which promote tissue injury (Mehlhorn and Wellburn, 1987). It is known that rapid-cycling *B. campestris* can produce stress ethylene (Lentini *et al.*, 1988) and it is therefore possible that this plant hormone was involved in the development of visible damage. The leaves of *P. major* do not develop the characteristic symptoms of visible ozone damage, indicating that this

138

species may either be able to exclude toxic levels of the pollutant or possess the ability to detoxify or repair damage. A possible explanation may be that the decrease in stomatal conductance following exposure to ozone reduced the ozone flux sufficiently to protect the leaves from damage since stomatal conductance was consistently reduced to between 300-400 mmol m^{-2} s^{-1} within 3.5 h of the start of each fumigation, regardless of the prefumigation conductance (Figs. 3.17a-3.20a).

Visible damage also developed in *B. campestris* during a 10 day exposure to ozone; this appeared as interveinal chlorosis, particularly on the cotyledons and older leaves. Ozone injury has been related to the age of individual leaves at the time of exposure, with older leaves generally being more sensitive than younger leaves (Tingey *et al.*, 1973; Rajput and Ormrod, 1986; Krupa and Manning, 1988; Kasana, 1991; Sandelius *et al.*, 1995). It is thought that leaves go through a stage of development when they are most sensitive to ozone (Dugger and Ting, 1970) and that this stage corresponds with the phase of rapid leaf growth prior to full leaf extension (Ting and Mukerji, 1971; Tingey *et al.*, 1973). In some species, this sensitive stage coincides with the period of greatest stomatal conductance, although sensitivity may also be related to the capacity of the leaf to produce photosynthate and repair ozone-injured tissue (Sutton and Ting, 1977) or to other factors such as the formation of intercellular spaces which allow rapid penetration of ozone to the palisade cells (Tingey *et al.*, 1973; Evans and Ting, 1974). The greater damage apparent in the older leaves of *B. campestris* may have occurred because these leaves matured, became sensitive and were injured during the 10 day exposure period. Alternatively or in addition, there may have been a cumulative effect of ozone whereby the older leaves, which developed earlier during the exposure period, received a larger dose of ozone which may have exceeded the thresholds for repair mechanisms, thereby inducing greater damage.

In addition to chlorosis and necrosis, the leaves of *B. campestris* exhibited marginal leaf curl after exposure to ozone for 10 days. Similar symptoms were evident to some extent in plants of *P. major* which had been exposed for 14 days. Similar marginal leaf curl has been observed in *Vicia faba* following exposure to ozone (V.J. Black, pers. comm). There is little documentary evidence of similar responses in other species, although Feder (pers. comm., cited by Craker, 1971) reported that epinasty was characteristic of ozone injury while Mehlhorn and Wellburn (1987) noticed a slight curling of pea leaves (*Pisum sativum* L.) when plants were exposed for three weeks to 50-150 ppb ozone for 7 h d^{-1}. It is known that epinasty may occur in response to stress ethylene formation (Hewitt *et al.*, 1990) and that plants can produce stress ethylene in response to ozone (Craker, 1971; Mehlhorn and Wellburn, 1987; Wellburn and Wellburn, 1996). Leaf curl in response to ozone has not been reported previously

for *P. major,* although it is known that this species is capable of producing stress ethylene in response to ozone (Wellburn and Wellburn, 1996). In rapid-cycling *B. campestris,* ethylene has been shown to induce leaf curling when plants are grown in sealed containers (Lentini *et al.,* 1988). However, in this study, the proposed response to stress ethylene may have been exacerbated by the slower flow rate of air through the exposure chambers compared to other studies (Reiling and Davison, 1992d), which would have precluded the rapid dissipation of ethylene away from the leaf. Further investigation would be required to determine the cause of leaf curling observed in ozone-treated plants of *P. major* and *B. campestris* during the current study.

3.4.3. Effects of ozone on biomass and assimilate partitioning

The results indicate that total plant biomass was reduced in *B. campestris* (Table 3.8) and all populations of *P. major* (Table 3.17) following exposure to 70 ppb ozone for 10 and 14 days respectively, probably due to the reductions in net photosynthesis observed in both species. Although a six day exposure to 26 ppb ozone reduced photosynthesis in each population of *P. major,* there was no detectable reduction in total plant dry weight. This may have been due to the shorter duration of exposure and the smaller reduction in net photosynthesis at 26 as compared to 70 ppb ozone.

In addition to reducing total plant biomass, ozone altered biomass allocation. Following 10 days of exposure to ozone, root growth was more affected than shoot growth in *B. campestris,* decreasing the root:shoot ratio. For the above-ground organs, a higher proportion of the total biomass was allocated to the stem and a lower proportion to the leaves in ozone-treated plants, whereas the allocation to the inflorescences was unaffected. It has been widely reported for many species that ozone reduces root growth more than shoot growth (Tingey *et al.,* 1971; Cooley and Manning, 1987; Darrall, 1989; Reiling and Davison, 1992a; Fernandez-Bayon *et al.,* 1993). At present, the mechanisms responsible for this effect remain unclear, although there is evidence from carbon-labelling studies to suggest that ozone interferes with the processes of phloem loading and/or translocation (McCool and Menge, 1983; Darrall, 1989; Spence *et al.,* 1990). In the present study, an increased proportion of the total biomass was allocated to the stems of *B. campestris* following exposure to ozone. A similar response was witnessed in alfalfa (*Medicago sativa*) when plants were exposed to 60 ppb ozone for 6 h d^{-1}, 5 d wk^{-1} for several weeks (Cooley and Manning, 1988). These authors suggested that this response may have occurred because the stem was the first sink encountered by photosynthate after it was exported from the leaves. The reduction in leaf biomass in *B. campestris* resulted from a decrease in leaf area rather

than an effect on leaf number or specific leaf area. Leaf area was reduced most in the older leaves (Fig. 3.6), which also showed the greatest visible damage, and may have resulted either from a decrease in the rate of leaf expansion or a reduction in final leaf size. The proportion of total biomass allocated to the reproductive structures prior to first flowering was unaffected by ozone-treatment, suggesting that this allocation was fixed and independent of environmental conditions. As the plants matured, it is probable that the proportion of biomass allocated to reproduction increased in ozone-treated plants compared to the controls since final yield was unaffected by treatment even though there was a reduction in vegetative growth.

For each population of *P. major*, the relative growth rate ($\bar{R}$; growth rate per unit size) and the allometric root:shoot coefficient (K; an index of the balance of growth between root and shoot) were not significantly affected by either a six or 14 day exposure to ozone (Tables 3.16-3.17). These results contrast with those of Reiling and Davison (1992d) who screened 28 populations of *P. major* for their relative sensitivity to ozone. In their study, the $\bar{R}$ values for the High Low and Penicuik populations (identified in their study as ISP and Bush respectively) were significantly reduced following 14 days of exposure to 70 ppb ozone for 7 h d^{-1}. K was also significantly reduced in both populations, indicating that shoot growth was favoured over root growth. In both Lullington and Sibton, $\bar{R}$ and K were unaffected by ozone treatment. Of the 28 populations screened by Reiling and Davison (1992d), High Low was identified as the most sensitive, with an ozone resistance ($\bar{R}$ in ozone-treated plants expressed as a percentage of $\bar{R}$ in control plants) of 75.8 %. In the present study, $\bar{R}$ was reduced, although not significantly, and K was unaffected in the High Low, Lullington and Sibton populations following 14 days of exposure to 70 ppb ozone for 7 h d^{-1}. Unlike the study by Reiling and Davison (1992d), each of these three populations appeared to be sensitive to ozone, with High Low showing the greatest and Sibton the least ozone resistance. This decrease in growth corresponds to the reduction in photosynthesis recorded for each of these populations following a two day exposure to ozone. Even though the concentration and duration of ozone exposure were similar in the present study to those used by Reiling and Davison (1992d), differing responses were observed. This may have been due to differences in environmental variables such as temperature, humidity and light availability within the growth cabinets or exposure chambers since environmental conditions are known to affect plant responses to ozone (Heck, 1968; Mortensen, 1989). In the present study, higher relative growth rates and stomatal conductances were recorded for each population than those reported by Reiling and Davison (1992d, 1995); the latter may have facilitated ozone uptake, thereby increasing injury and decreasing total dry weight. Differences between studies in the response of plants to ozone are not uncommon. Knudson Butler *et al.* (1979), for

example, found that *Phaseolus vulgaris* cv. Tempo, which had previously been reported to be one of the most sensitive cultivars to ozone, was more resistant than most cultivars. Indeed, in the present study, *B. campestris* responded differently in each of the three, two day exposure experiments when plants were grown under different environmental conditions.

3.4.4. Effects of ozone on the reproduction of *Brassica campestris*

3.4.4.1. *Flowering*

Ozone has been shown to delay flowering in several species including carnation (*Dianthus caryophyllus*; Feder and Campbell, 1968), duckweed (*Lemna perpusilla*; Feder and Sullivan, 1969b) cotton (*Gossypium hirsutum*; Oshima *et al.*, 1979) and soybean (*Glycine max*; Amundson *et al.*, 1986). In rapid-cycling *B. campestris*, it has been shown that exposure to enhanced UV-B radiation increases the time to first flowering (Feldheim and Conner, 1996), although the time to first flowering was unaffected by treatment with ozone.

The two day exposure to ozone prior to first flowering did not to affect the number of reproductive sites which formed on the terminal raceme. It is probable that the reproductive sites had all been initiated by the start of exposure, such that ozone had little effect on this number. However, following 10 days of exposure to ozone, there was a significant reduction in the total number of reproductive sites present on the terminal raceme (Fig.3.7). This type of response has been recorded in other crop species; for example, Fernandez-Bayon *et al.* (1993) observed a significant reduction in flower production in two cultivars of watermelon (*Citrullus lanatus* cvs. Sugar Baby and De La Reina) and muskmelon (*Cucumis melo* cvs. Verde Tendral Tardio and Amarillo Temprano) following 21 days of exposure to 70 ppb ozone for 6 h d^{-1}. Feder and Campbell (1968) also noted that bud formation was depressed in carnations (*Dianthus caryphyllus* cv. White Sim) when exposed to an average of 75 ppb ozone for 10 days around the time of bud initiation. In the present study, a reduction in the number of floral sites on the terminal raceme of *B. campestris* may have resulted from a decrease in photosynthesis and the availability of assimilates for reproduction which limited either the number of sites initiated or the development of sites to a visually recognisable size. During plant development, the initiation of both vegetative and reproductive primordia occurs within a defined time period which is usually determined by genetic factors. During this time period, primordia are initiated at a rate which is primarily controlled by temperature, but which may be altered by other environmental

conditions (Squire, 1993). It is possible that treatment with ozone may have reduced the rate of primordia initiation without affecting the duration of the initiation phase, leading to fewer sites being produced on the terminal raceme.

The flowering period of *B. campestris* plants exposed to ozone for 10 days was completed before that of control plants since fewer flowers developed on the terminal raceme (Fig. 3.8). Although not statistically significant, fewer flowers opened on each day in ozone-treated plants, suggesting that the time interval between successive flower openings had been increased. Since the reproductive structures were not exposed to ozone during flower opening, flowering may have been slowed as a consequence of fewer reproductive sites being present on the terminal raceme or there may have been an indirect effect of ozone which slowed flower development.

3.4.4.2. *Pollen*

The germination of *B. campestris* pollen was significantly reduced after plants had been exposed for 10 days to 70 ppb ozone for 7 h d^{-1} (Table 3.9), even though the mature pollen was not directly exposed to ozone. Previous studies have shown that ozone can reduce pollen germination when pollen is exposed directly either *in vivo* (Feder, 1968) or *in vitro* (Feder, 1968; Hormaza *et al.*, 1996) although the mechanisms of damage are still unknown. Very few studies have examined the effect of ozone on pollen which is liberated post-exposure. Benoit *et al.* (1983) showed that the germination of *Pinus strobus* pollen was unaffected after 35 days of exposure to 100 ppb ozone for 8 h d^{-1}. However, in another long term exposure, Mumford *et al.* (1972) showed that the germination of corn pollen (*Zea mays*) was reduced by 90 % after 60 days of exposure to 120 ppb ozone for 5 h d^{-1}. Biochemical analyses indicated that ozone had increased free amino acid and decreased reducing and neutral sugar contents within the pollen. It is known that when plants are grown under low light levels, the carbohydrate content of mature pollen is decreased, probably as a direct effect of reduced photosynthesis (Stanley, 1971). In *B. campestris*, the reduction in photosynthesis recorded during exposure to ozone may have been sufficient to affect the quantity of carbohydrate accumulated within the pollen. As carbohydrates are the metabolic substrates used as an energy source by germinating pollen (Stanley, 1971) any reduction in their carbohydrate content may have consequences for germination. Future work involving ultrastructural studies or biochemical analysis of pollen may prove useful in determining the precise nature of the effect on germination.

As the quantity of pollen produced by each flower was not recorded in this study, it is not known whether exposure to ozone reduced pollen production in *B. campestris*.

However, since fewer flowers opened on ozone-treated plants, this would have probably reduced the total amount of pollen produced by each plant.

3.4.4.3. *Seed yield*

Many studies have reported reductions in the yield of crop species following exposure to ozone (Shannon and Mulchi, 1974; Reich and Amundson, 1985; Takemoto *et al.*, 1988; Kobayashi *et al.*, 1995). It has been suggested that these yield losses result from reductions in net photosynthesis because growth and yield are closely related to net photosynthesis (Zelitch, 1982). In contrast, many other species have been shown to tolerate substantial foliar injury and/or a significant loss of plant biomass without affecting yield (Clarke *et al.*, 1978; Oshima, 1977; Mulholland *et al.*, 1997a). In the present study, there was no significant effect of ozone on seed yield of *B. campestris* following either a two (table 3.5) or a 10 day exposure to ozone (Table 3.11), despite significant reductions in net photosynthesis and the development of visible foliar injury. Seed yield was maintained because the number of pods and the number and weight of mature seeds per pod were unaffected by treatment with ozone.

By virtue of its indeterminate reproductive habit, *B. campestris* has the ability to produce more floral sites than it can sustain to maturity. This helps to maximise the number of seeds set in an environment where pollination of every flower cannot be guaranteed. The reduction in the total number of floral sites on the terminal raceme observed following the 10 day exposure to ozone would almost certainly have reduced the plants flexibility to compensate for unpollinated sites, which may, in turn, have had consequences for seed yield. In the present study, all of the flowers which opened on the terminal raceme were hand-pollinated to ensure that the maximum seed set was achieved under the prevailing environmental conditions. Pod number was not significantly affected by either a two or 10 day exposure to ozone. Many studies suggest that pod set is regulated by the availability of assimilates to developing reproductive organs (Jeuffroy and Warembourg, 1991). Indeed, in *B. campestris* cvs. Arlo, Echo and Polar, yield was significantly correlated with total plant dry weight (Thurling, 1974a). It has been demonstrated that during the early stages of reproductive development in *B. campestris*, the pods rely primarily on the translocation of assimilates from the leaves but that during the later stages of development, the pod walls produce the majority of the assimilates required for seed growth (Khanna-Chopra and Sinah, 1976). This is in contrast to other species, particularly peas (*Pisum* spp.), in which the pods mainly receive assimilates from their subtending leaf as the pods themselves fix relatively little external CO_2 (Flinn and Pate, 1970). The ability of *B. campestris* to maintain pod number following ozone-exposure therefore suggests

that the initial assimilate supply from the leaves was relatively unaffected. Assimilate supply to the developing pods may have been sustained, especially since the younger leaves subtending the terminal raceme showed little visual damage.

Within each pod, the total number of seeds, including mature, germinated and aborted seeds, was significantly reduced following 10 days of ozone treatment (Table 3.11). This may have been a consequence of a reduced assimilate supply and/or a direct effect of ozone on ovule initiation or development. Of the total number of seeds in each pod, fewer seeds aborted and more seeds germinated prior to harvest in ozone-treated plants, with the result that the final number of mature seeds per pod was similar to control plants. In many species, particularly legumes, abortion of the developing embryo during the period of cell division prior to cell expansion is a common phenomenon, although the reasons for this have still to be established (Ney et al., 1993). In B. campestris cvs. Arlo, Echo and Polar, it has been demonstrated that a decrease in the number of pods per plant is compensated for by an increase in the number and weight of seeds per pod (Thurling, 1974b). Thus, any reduction in the number of seed sites per pod following exposure to ozone would reduce the plants ability to compensate when the number of pods set is low by retaining seeds within the existing pods which would have otherwise aborted. Significantly more seeds germinated within the pods of ozone-treated plants and, as these seeds did not exhibit dormancy, this suggests that ozone may have advanced maturation or affected seed structure, allowing premature uptake of water and germination. Individual mature seed weight was similar in ozone-treated and control plants, possibly because assimilate production within the pods was unaffected by ozone treatment. The similar biomass allocation to the pod walls in both treatments provides evidence to support this view.

Although photosynthesis was reduced and visible damage developed in B. campestris during both the 2 and 10 day exposures to ozone, significant effects on reproductive biology were only recorded after the 10 day exposure. The timing and dose of ozone is known to be important in determining the response of plants to ozone. For example, in P. major, Reiling and Davison (1992b) showed that the timing of a two week exposure to 70 ppb ozone for 7 h d $^{-1}$ during the first eight weeks of development was important in determining the effect on reproduction. The greatest reductions in inflorescence dry weight were recorded following exposure during the first four weeks of growth. In contrast, the greatest reduction in the number of spikes per plant occurred following later exposures. The number of capsules per spike appeared to be sensitive only when plants were exposed during 3-4 weeks after germination, while the number of seeds per capsule was not affected by any exposure. Seed yield was reduced irrespective of the timing of exposure, although the reductions were not always statistically significant.

Thus exposure to ozone at certain times during flowering and pod development may have consequences for final seed yield. Further work is required to establish the importance of ozone dose and timing of exposure on reproductive development in *B. campestris*.

3.4.4.4. *Seed colour*

Although the seeds of *B. campestris* usually have a light/dark brown seed coat, 10 days of exposure to 70 ppb ozone for 7 h d^{-1} resulted in 28 % of the seeds forming yellow, yellow/green or green seed coats (Table 3.12). It has been demonstrated that seed coat colour in *B. campestris* is primarily determined by the maternal genotype, which results in plants producing entirely yellow or entirely brown seeds, with brown seed colour being dominant over yellow (Ahmed and Zuberi, 1971; Chen and Heneen, 1992). The genetic control of seed coat colour in *B. campestris* has pleiotropic effects such that yellow seeds have a considerably higher oil and protein content, a reduced fibre content and thinner seed coat than brown seeds (Jonsson and Bengtsson, 1970, cited by Jonsson, 1977; Theander *et al.*, 1977). Yellow-seeded cultivars of rapeseed have been developed because of the economic value of a high oil and protein content as well as the decreased fibre content of the meal, which increases its digestibility and therefore value as an animal feed (Van Deynze and Pauls, 1994). While individual plants producing entirely yellow seeds were noted occasionally during the present study, this did not explain the difference in seed colour between ozone-treated and control plants since colour variation from yellow to brown occurred amongst the seeds from individual plants. If the differences in seed colour exhibited by ozone-treated plants had been due to genetic effects, the yellow seeds might have been expected to have a thinner seed coat than brown seeds. However, the seed coat structure was observed to be similar in both brown and yellow seeds, supporting the view that seed colour was not determined solely by genetic factors. In *Brassica* species, the seed pigments, which are composed primarily of condensed polyphenols (Leung *et al.*, 1979), are deposited in the epidermal and supporting layers which are derived from the outer integuments of the ovary (Vaughan, 1970). It is known that these pigments are more abundant in dark seeded than yellow seeded *B. campestris* genotypes (Theander *et al.*, 1977). The production of yellow seeds in ozone-treated plants may have occurred either because ozone affected pigment production or because it slowed the maturation process, resulting in some seeds being harvested before the maximum quantity of pigment was deposited within the seed coat. The latter suggestion may be more probable since a number of seeds with an unusually high chlorophyll content were recorded in ozone-treated plants, implying that these seeds had been harvested prematurely. However, the presence of a dark brown seed coat usually masks the

146

appearance of chlorophyll. In yellow seeds, the occurrence of chlorophyll is easier to determine (Jonsson, 1977) with the result that some of these seeds may have appeared green. The occurrence of seeds in ozone-treated plants with an uneven distribution of yellow and green colouration over the seed coat supports this view.

3.4.4.5. *Seed germination/ imbibition*

Two days of exposure to ozone had no significant effect on the timecourse of germination; however, when the exposure was extended to 10 days, the initial rate of germination was significantly reduced, although the overall ability of seeds to germinate was unaffected (Fig. 3.10). This effect on the initial rate of germination was probably due to the slower germination of yellow, yellow/green and green seeds from ozone-treated plants, which comprised a higher percentage of the total seed yield than the slower germinating brown/purple seeds produced by control plants.

Percentage germination during the first 48 h after sowing was similar for the brown seeds from both ozone-treated and control plants (Fig. 3.11). Germination was slower in all other seed categories, although differences were observed between categories. The rate of germination appeared to be inversely related to the rate of imbibition, with the seeds which imbibed faster generally germinating slower. Of all seed categories, the brown/purple seeds of control plants were slowest to germinate, with only 30 % of the seeds germinating within seven days. These seeds were the smallest and lightest of those produced and the structure of the seed coat was found to be irregular. This may have been an indication that seeds within this colour category had aborted before attaining full development, or that the development of the seeds was abnormal. These seeds also imbibed rapidly and absorbed more water than any other seed category; over 2.5 times their own weight of water within 2 h. The limited number of brown/purple seeds which germinated may have resulted from the proposed abnormal or aborted development of the seed or, alternatively, from rapid imbibition which is known to cause death of the cotyledonary tissue and substantial solute leakage (Powell *et al.*, 1986b). Rapid water uptake may result from cracking of the testa, as found in peas (*Pisum sativum*; Powell and Matthews, 1979), soybeans (*Glycine max*; Oliveira *et al.*, 1984) and long beans (*Vigna sesquipedalis*; Abdullah *et al.*, 1991) or the loose adherence of the testa to the cotyledons, which was shown to facilitate water uptake in white-coated dwarf French beans (*Phaseolus vulgaris*; Powell *et al.*, 1986a). Although the seed coat appeared to be complete in the brown/purple seeds, its abnormal development may have rendered it fragile, thereby facilitating rapid water uptake. In ozone-treated plants, the brown and yellow seeds germinated faster than yellow/green and green seeds, with the germination of green seeds being particularly slow. Since the

seeds of *B. campestris* are not photoblastic and dependent on specific wavelengths of light for germination, this process should not have been influenced directly by the colour of the testa. The rate of imbibition was greater in green and yellow/green seeds, possibly due to immaturity of the testa. Examination of seed sections showed that the outer layer of the testa in particular was not as well developed as in other categories of mature seeds, possibly allowing easier penetration of water. Higher rates of imbibition have previously been recorded for early-harvested kernels of wheat (*Triticum aestivum*; Clarke and DePauw, 1989) and corn (Dungan, 1924) compared to those harvested at full maturity. The germination of green seeds could have been inhibited by imbibition damage, but it is more likely that these seeds were immature and not viable.

3.4.5. Summary

The main physiological, visible injury and growth responses of *B. campestris* and *P. major* to ozone exposure recorded in the present study were as follows:

Physiology

- In both *B. campestris* and each population of *P. major,* stomatal conductance (g_s) and the net rate of photosynthesis (A) of leaves were significantly reduced within 3.5 h of the start of each exposure to 70 ppb ozone, and in general this decline continued until the end of the 7 h exposure period.
- The decline in g_s and A was similar in each population of *P. major,* in contrast to the work of Reiling and Davison (1995).
- The net rate of photosynthesis was reduced to a greater extent in plants of *B. campestris* which had higher g_s values prior to ozone exposure. g_s was reduced by a similar amount in all plants irrespective of the prefumigation value.
- In *B. campestris*, A and g_s did not recover fully either between or up to 24 h after two daily exposures to 70 ppb ozone for 7 h d^{-1}.
- In general, there was a partial or full recovery of A and g_s in each population of *P. major* outwith the exposure period, although notable differences between populations were observed.

Visible injury

- No symptoms of visible injury developed in any population of *P. major*.
- The leaves of *B. campestris* showed characteristic symptoms of ozone damage, including chlorosis and necrosis. The type and extent of damage varied between

experiments and could be related to the stomatal conductance of plants prior to exposure.

Growth

- The total biomass of *B. campestris* was reduced significantly by a 10 day exposure to ozone and root growth was more affected than shoot growth.
- In each population of *P. major*, $\overline{R}$ and K were not significantly affected by a two week exposure to ozone although $\overline{R}$ was reduced in each population. These results contrast to those of Reiling and Davison (1992d).

Reproduction in B. campestris

- A two day exposure to ozone had no significant effect on any aspect of reproduction despite the development of visible foliar injury.
- A 10 day exposure to ozone had various effects on reproduction. The number of flowers produced on the terminal raceme was significantly reduced, although this did not affect the total number of pods which developed on each plant or final seed yield.
- A range of seed colours was produced in all plants following a 10 day exposure to ozone; however, the range of colours differed between treatments, with ozone-treated plants producing yellow, yellow/green and green seeds which were not found in control plants. The different seed colour categories exhibited differing rates of imbibition and germination.
- Pollen germination was significantly reduced and pollen tube length was unaffected for pollen released following a 10 day exposure to ozone.

CHAPTER FOUR - THE DIRECT EFFECTS OF OZONE ON THE REPRODUCTIVE DEVELOPMENT OF *BRASSICA CAMPESTRIS* L.

4.1. INTRODUCTION

In the previous chapter, it was demonstrated that exposure to ozone for 10 d prior to first flowering can affect both the vegetative growth and reproductive development of *Brassica campestris*. Despite a significant reduction in the number of flowers borne on the terminal raceme, seed yield was maintained in these plants because a similar number of pods developed to maturity. However, significant effects on seed colour and seed germination were observed in the harvested seeds. Since both the vegetative and reproductive structures were exposed simultaneously to ozone, it was not possible to establish to what extent the effects observed on seed colour and germination were due to indirect effects on the vegetative structures or direct effects on the reproductive structures themselves.

A recent study of oilseed rape (*Brassica napus* L.) has shown that reproductive development can be affected directly by exposure to ozone. Bosac (1992) showed that exposure of the flowering racemes of two cultivars, Tapidor and Libravo, to 100 ppb ozone for 6 h significantly increased bud abortion and abscission. However, the indeterminate reproductive habit of this species permitted at least partial compensation for these losses by replacing some or all of the lost reproductive sites. Despite this compensation for the loss of fertile sites, significant effects on pod length, grain yield and seed oil content were observed at final harvest (Bosac *et al.,* 1993), and seed germination and seedling vigour were also depressed (Bosac, 1992).

This chapter presents data from three experiments undertaken to establish whether the reproductive structures of *B. campestris* can be affected directly by exposure to ozone and whether the response, in terms of damage and compensation, is similar to that of the related species, *B. napus*. Unlike *B. napus*, *B. campestris* is self-incompatible; thus by controlling pollination, direct effects on the maternal structures may be examined. In addition, pollen from *B. campestris* was exposed to ozone both *in vivo* and *in vitro*, in order to examine its effects on pollen germination and pollen tube growth.

4.2. MATERIALS AND METHODS

4.2.1. Exposure of the terminal raceme to ozone

In *Brassica campestris*, flowering of the terminal raceme begins 16-17 days after sowing (DAS) and continues for 10-14 days. After a further 10-15 days, the seed has matured and is ready for harvest. During reproductive development, the reproductive structures of plants growing under field conditions may come into direct contact with elevated concentrations of ozone over a variable number of days and at different times during the reproductive phase. Experiments were therefore designed to assess whether specific stages of floral development such as buds, flowers or pods are particularly sensitive to ozone received either as a single exposure or as a multiple exposure, and whether the timing of multiple exposures is important in determining the impact on final seed yield.

4.2.1.1. *Single exposure to 100 ppb ozone for 6 h*

In order to assess the direct impact of a single exposure to ozone on different stages of floral development and seed yield, the inflorescences of 10 plants were exposed to filtered air (control treatment) or 100 ppb ozone for 6 h at 20 DAS, when all stages of floral development from buds to developing pods were present on the terminal raceme (Plate 4.1). This particular dose of ozone was chosen since it is within the limits of a 'natural ozone episode' (UKPORG, 1993) and would permit comparison with data obtained in a similar study using *Brassica napus* (Bosac, 1992).

4.2.1.2. *Multiple exposure to 100 ppb ozone for 6 h on four consecutive days*

During the spring and summer months in the UK, daily ozone episodes may occur over a number of days (UKPORG, 1993). A daily exposure to ozone on four consecutive days was therefore applied at two different times during the main phase of flowering which occurred 17-23 DAS. In two separate experiments, the inflorescences of 10 plants were exposed to filtered air (control treatment) or 100 ppb ozone for 6 h on four consecutive days between 17-20 DAS or 20-23 DAS; on each day of exposure, all stages of floral development from buds to developing pods were present on the terminal raceme with the sole exception of the first day of flowering at 17 DAS, when only flowers and buds were present.

151

Plate 4.1. Plants of *Brassica campestris* showing isolation of the terminal raceme within the exposure chamber. Note that developing pods, open flowers and buds were all present on the terminal raceme during exposure.

4.2.2. Cross-pollination

In all three experiments, newly opened flowers were cross-pollinated by hand at 24 h intervals using pollen from unexposed plants, such that each flower was pollinated only once. Since it is known that direct exposure of pollen to ozone can affect pollen germination and tube growth (Feder, 1968; Benoit *et al.*, 1983), pollination was performed each day at the end of ozone introduction so that pollen germination would not be affected by direct exposure to ozone and the effect of ozone on the maternal reproductive structures could be investigated. For multiple exposure experiments, the plants remained within the exposure system between successive fumigations to provide consistent environmental conditions. After exposure, the plants were returned to the controlled environment growth cabinet to complete their life cycle.

4.2.3. Non-destructive measurements of reproductive biology

For each plant the following variables were recorded in all three experiments;

Terminal raceme development

The length of the terminal raceme was measured daily throughout the period of flowering as the distance between the points where the first and last floral sites were initiated.

Number and development of floral sites

The total number of floral sites present on the terminal raceme was recorded daily, starting at 16-17 DAS and continuing until completion of flowering at around day 30. In addition, the stage of floral development was recorded for each site at 24 h intervals. The following five stages of floral development were identified for *Brassica campestris*:

1. **Bud** - >1 mm in diameter.
2. **Open flower** - either opening or fully opened.
3. **Developing pod** - flowers with petals wilting and reflexing forward or petals, sepals and anthers abscinding, ovary enlarging.
4. **Aborted post-flowering** - flowers with petals wilting and reflexing forward or petals, sepals and anthers abscinding, ovary not enlarging.
5. **Aborted buds** - non-abscinding yellow buds which failed to open.

153

This classification provided a record of the stage of development of each floral site during the single exposure to ozone and the stage or stages of development passed through during multiple exposures. In addition, the number of reproductive sites on the terminal raceme at each stage of floral development was calculated daily to determine whether treatment with ozone affected the number of sites aborting or progressing through to each stage of floral development.

Pod development

For each plant, the total number of pods was counted and the length of each fully elongated ovary measured on day 30.

4.2.4. Seed yield

4.2.4.1. *Single exposure to 100 ppb ozone for 6 h*

For each plant, individual pods were identified as having developed from floral sites which were exposed to ozone or filtered air as:

a) Developing pods.
b) Open flowers.
c) Buds.

After the ripening-off period (Section 2.4.1.1), the number of mature, germinated and aborted seeds, as defined in Section 3.2.1.2, were counted for each pod. For each plant, the mature seeds were bulked for sites exposed at each of the three stages of development described above, before being weighed to calculate total and individual seed weight per plant.

4.2.4.2. *Multiple exposure to 100 ppb ozone for 6 h on four consecutive days*

Seed number was recorded for each pod as described for the single exposure above. However, for multiple exposures, the pods were identified as having developed from floral sites which were exposed to ozone or filtered air as:

a) Developing pods - present throughout the four day exposure.
b) Open flowers (1) - flowers open on the first day of exposure.
c) Open flowers (2) - flowers open on the second day of exposure.
d) Open flowers (3) - flowers open on the third day of exposure.

e) Open flowers (4) - flowers open on the fourth day of exposure.

f) Buds - formed during or present throughout the four day exposure.

For plants exposed from 17-20 DAS, no sites were exposed for four days as developing pods (a). However, sites were exposed at all stages of development (a-f) when exposure occurred between 20-23 DAS. For each plant, the mature seeds were bulked for sites exposed at the various stages of development defined above before being weighed to calculate total seed weight per plant and mean individual seed weight.

After the seeds had been harvested, the remainder of the pods was oven-dried at 60 °C for 72 h and weighed.

4.2.5. Germination of seeds

For both the single and multiple exposures to ozone, the mature seeds from each of the 10 plants per treatment were bulked for sites exposed at specific stages of development, prior to being tested for germination. Within seven days of being harvested, 80 seeds were randomly selected from each developmental category to give four replicates of 20 seeds; these were then placed in petri dishes containing 1 % agar. The seeds were germinated within the controlled environment growth cabinet under the conditions set for *B. campestris* and germination was recorded at 24 h intervals over a seven day period, as detailed in Section 3.2.2.2.

To examine the long term viability and germination of seeds, the germination tests were repeated using seed from plants exposed on four consecutive days between 20-23 DAS, after these had been stored for two years at 4 ° C. Germination tests were only carried out for the pod categories for which sufficient seeds were available i.e., those exposed as developing pods through to those which were open flowers on the third day of exposure.

Imbibition of seeds

The rate of imbibition of seeds from floral sites exposed to ozone as developing pods on four consecutive days (20-23 DAS) was calculated to determine whether there was any relationship between this factor and the rate of germination. For each treatment, three replicates of 10 randomly chosen seeds were weighed before being placed in separate beakers containing 20 ml of deionised water. The seeds were removed from the water at one hour intervals and lightly towel-dried to remove surface water before being weighed and replaced in fresh deionised water. The measurements continued

155

until there was no more weight gain, i.e. after 4 h. Water uptake per seed was calculated as a percentage of the initial seed weight.

4.2.6. Seed colour

At seed harvest, it was noted that a range of seed colours was produced when plants were exposed to ozone or filtered air on four consecutive days between 17-20 DAS or 20-23 DAS. After completion of the germination tests, the seeds from the oldest pods which had been flowers on the first day of exposure at 17-20 DAS or present during exposure at 20-23 DAS were sorted into three or four colour categories. Colours varied from dark brown through to yellow, with the range of colours being particular to each treatment. The seeds from each colour category were counted and weighed to calculate the average weight of individual seeds of different colours.

4.2.7. Exposure of pollen to ozone

4.2.7.1. In vivo *exposure*

For each exposure *in vivo*, 20 plants were selected at random at 17 DAS, 10 as treatment and 10 as control. In two separate experiments, the terminal racemes of 10 plants were exposed to filtered air (control treatment) or to either 100 ppb or 120 ppb ozone for 6 h. Pollen from the most newly dehiscent flower of each plant was then collected and transferred onto a 50 µl drop of pollen germination medium (Section 3.2.2.2.). The pollen was germinated within the controlled environment growth cabinet for 24 h and pollen germination and pollen tube length were recorded as detailed in Section 3.2.2.2.

4.2.7.2. In vitro *exposure*

For exposure *in vitro*, the most newly dehiscent flower from each of 10 plants was collected in the morning 17 DAS and the pollen was dusted onto one of 10 microscope slides, five as treatment and five as control. Each slide was then placed onto moist filter paper in an open petri dish to maintain a high relative humidity during exposure, before placing the petri dishes on the base of each chamber and exposing the pollen to filtered air (control treatment) or 120 ppb ozone for 6 h. The pollen was subsequently transferred onto 50 µl drops of germination medium and germinated within the controlled environment growth cabinet for 24 h. Pollen germination and pollen tube growth were recorded as detailed in Section 3.2.2.2.

4.2.8. Data Analysis

Non-destructive measurements of reproductive biology

The following measurements were recorded daily throughout the period of flowering:

1. Terminal raceme length.
2. Total number of reproductive sites on the terminal raceme.
3. The number of reproductive sites present as buds, open flowers, developing pods, sites which aborted post-flowering and aborted buds.

Using the data for the number of reproductive sites and terminal raceme length, the average distance between each floral site (intersite length) was calculated for each day during flowering.

With two treatments and a sample size of 10, the means for all of the above parameters were analysed separately for each day of measurement using a paired, two tailed t-test to establish significance ($P<0.05$).

Seed yield parameters

For all experiments, the following variables were recorded for each plant.

1. Number, length and weight (minus seeds) of pods.
2. Number of mature, germinated and aborted seeds per pod.
3. Total weight of mature seeds.

Using these data, the total numbers of mature, germinated and aborted seeds, the average number of mature seeds per pod and average individual mature seed weight were calculated for each plant. Means for the total number of pods and mature seeds, average number of mature seeds per pod, total mature seed weight and average individual mature seed weight were analysed for ozone-treated and control plants using a paired, two tailed t-test to establish significance ($P<0.05$).

In addition, the length and weight (minus seeds) of pods and the number of mature, germinated and aborted seeds per pod for all plants from each treatment were analysed for sites exposed at different stages of floral development (Sections 4.2.4.1 and 4.2.4.2). By using each pod as a replicate, this increased the sample number ($n>30$),

and so to test for significant differences between ozone-treated and control means d, the 'standardized normal deviate' was calculated (cf. Section 3.2.4.2).

Averages for individual seed weight and seed weight per pod were calculated for each plant for sites exposed at different stages of development and the values obtained for ozone-treated and control plants were analysed using a paired, two tailed t-test, to identify significant effects ($P<0.05$).

Seed germination and imbibition

Prior to analysis, the means for percentage germination were transformed using an arc sine transformation (Gomez and Gomez, 1984), while those for percentage water uptake were untransformed. The mean percentage germination values for seeds from sites exposed at different stages of floral development were analysed independently. A comparison of mean percentage germination and percentage water uptake for seeds from treated and control plants was made at each time interval using a paired, two tailed t-test.

Pollen germination and tube length

For each exposure of pollen *in vitro* or *in vivo*, the number of germinated pollen grains in each sample was converted to a percentage and then transformed using an arc sine transformation. The sample means for germination were then compared using a paired, two-tailed t-test to identify significant differences ($P<0.05$). The 20 measurements of pollen tube length from each sample were used collectively for analysis. Means for pollen tube length were analysed by calculating d, the standardised normal deviate, as for seed yield above.

4.3. RESULTS

4.3.1. **Exposure of the terminal raceme to ozone**

4.3.1.1. *Single exposure to 100 ppb ozone for 6 h*

Environmental conditions

During the 6 h exposure to ozone or filtered air, the average temperature and relative humidity were 22.7 ± 0.03 °C and 61.1 ± 0.18 % in the control chamber and

158

22.8 ± 0.02 °C and 58.0 ± 0.16 % in the treatment chamber. The concentration of ozone within the treatment chamber was slightly greater than the target of 100 ppb, and averaged 109.9 ± 10.3 ppb. In these and other experiments, the target concentrations will be referred to hereafter for the sake of clarity.

Terminal raceme and floral site development

After exposure to 100 ppb ozone for 6 h at 20 DAS, there was no significant effect on the total number of reproductive sites which formed on the terminal raceme (Table 4.1). Ozone-treated plants appeared to produce shorter terminal racemes than the controls, although this difference was already evident at 17 DAS, three days prior to exposure, and was not statistically significant (Fig. 4.1). Since there was no effect of ozone on the number of reproductive sites or terminal raceme length, intersite length was also not significantly affected (Table 4.1).

In both ozone-treated and control plants, most of reproductive sites were present on the terminal raceme before the majority of plants began to flower at 17 DAS, but the maximum number of sites was not attained until 23 DAS (Figs. 4.2a, 4.2b). Between 17-23 DAS, an average of four flowers opened every 24 h in both ozone-treated and control plants, after which progressively fewer flowers opened each day until flowering stopped at 30 DAS. From 20 DAS, sites began to abort after flowering, particularly towards the top of the terminal raceme in both ozone-treated and control plants. In addition, buds began to abort 21-22 DAS at the apex of the terminal raceme as the maximum seed number was established for each plant. During the period of flowering between 17-30 DAS, there was no significant effect of ozone on the total number of sites at each stage of floral development and therefore no apparent effect on the timing of floral development or the abortion of reproductive sites.

Of the total number of sites produced on the terminal raceme of both ozone-treated and control plants, approximately 66 % formed pods (Table 4.2.) while the remainder either aborted post-flowering (28 %) or aborted as buds (5 %).

Pod development

On each plant, an average of 15 developing pods, four open flowers and nine buds were exposed to filtered air or 100 ppb ozone for 6 h at 20 DAS (Fig. 4.3). Of these sites, 98.4 % of control and 99.5 % of ozone-treated pods developed fully, while 98.3 % of control and 80.0 % of ozone-treated flowers formed pods. Fewer pods developed towards the apex of the terminal raceme, with only 23.9 % of control

Figure 4.1. Increase in length of the terminal raceme of *Brassica campestris* during flowering when the inflorescences were exposed to filtered air (control) or 100 ppb ozone for 6 h at 20 DAS. Solid circles, control; open circles, ozone-treated. Single standard errors are shown; n=10. There was no significant difference between treatments between 17-30 DAS.
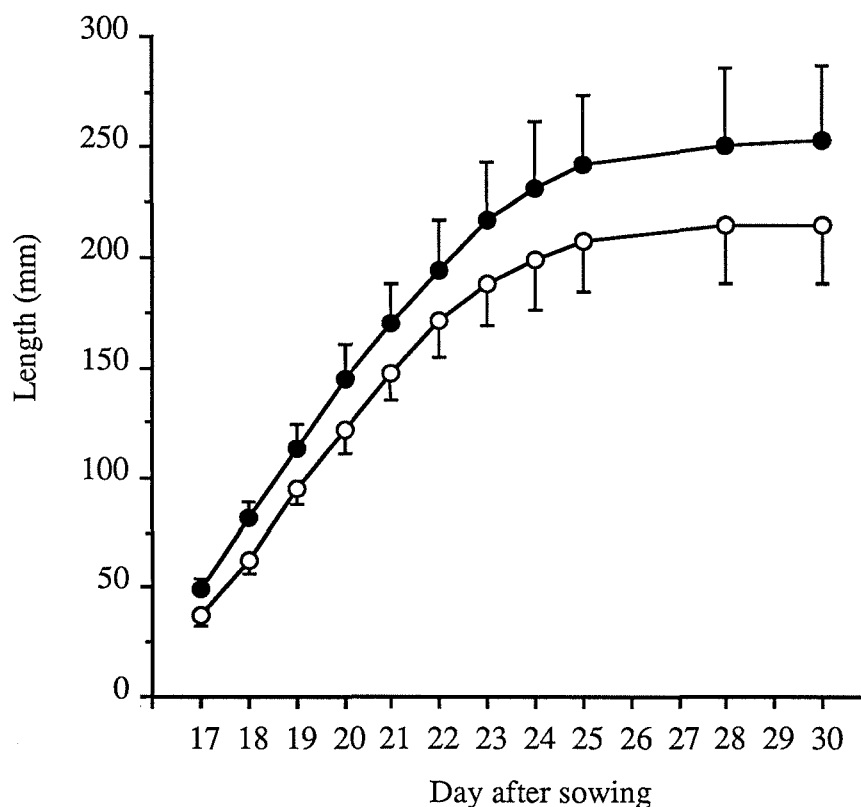
Table 4.1. Final length, number of reproductive sites and intersite length of the terminal raceme of *Brassica campestris* at 30 DAS, following exposure to filtered air (control) or 100 ppb ozone for 6 h at 20 DAS. Means and standard errors are shown. NS, non-significant; n=10.

	Control	Ozone	Significance
Raceme length (mm)	252.6 ± 34.41	214.7 ± 26.16	NS
No. reproductive sites	28.9 ± 2.57	30.0 ± 1.73	NS
Intersite length (mm)	8.5 ± 0.89	7.1 ± 0.71	NS

Figure 4.2. Mean numbers of floral sites on the terminal raceme of *Brassica campestris* at each stage of development throughout the flowering period in plants exposed to a) filtered air (control) or b) 100 ppb ozone for 6 h at 20 DAS. n=10. There was no significant difference between treatments in the total number of sites at each stage of development between 17-30 DAS.
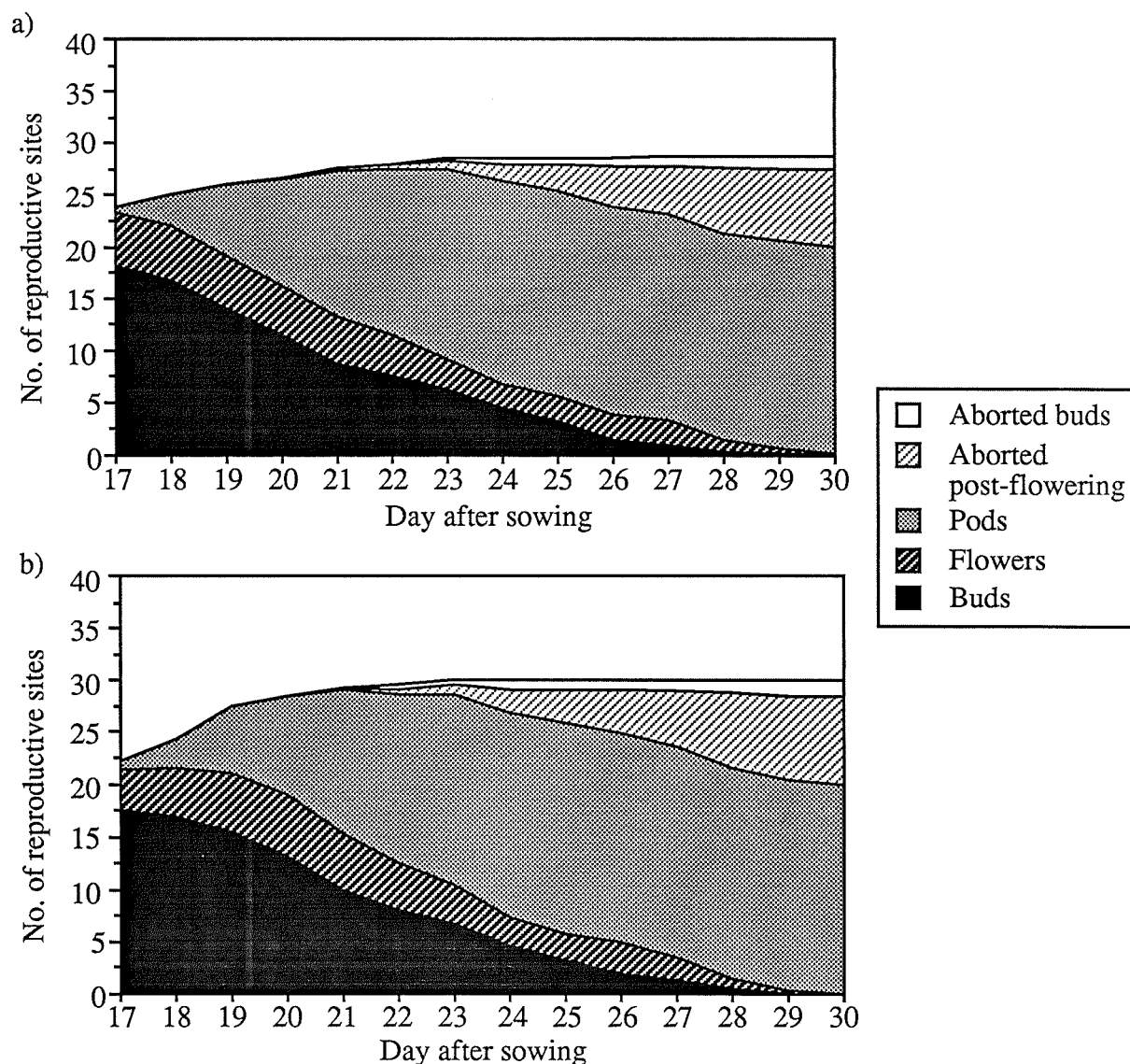
Table 4.2. Mean numbers of floral sites on the terminal raceme of *Brassica campestris* which had aborted or developed into pods by the time of harvest at 42 DAS, following exposure to filtered air (control) or 100 ppb ozone for 6 h at 20 DAS. Standard errors are shown; n=10. NS, non-significant.

Number of sites	Control	Ozone	Significance
Total	28.9 ± 2.57	30.0 ± 1.73	NS
Aborted	9.2 ± 1.62	10.0 ± 1.48	NS
Pods	19.7 ± 1.12	20.0 ± 1.09	NS

Figure 4.3. Mean number of floral sites on the terminal raceme of *Brassica campestris* exposed to a) filtered air (control) or b) 100 ppb ozone for 6 h at 20 DAS as developing pods, open flowers or buds and the number of these sites which formed pods. Single standard errors are shown. Number of pods formed as a percentage of floral sites exposed is shown in brackets. *** indicates significance at $P<0.001$; $n=10$.

a)

Number of reproductive sites (y-axis, 0 to 20)

(98.4)

(98.3)

***
(23.9)

Developing pods | Open flowers | Buds

Stage of floral development at time of exposure

b)

Number of reproductive sites (y-axis, 0 to 20)

(99.5)

(80.0)

***
(15.8)

Developing pods | Open flowers | Buds

Stage of floral development at time of exposure

■ Sites exposed
▨ Pods formed
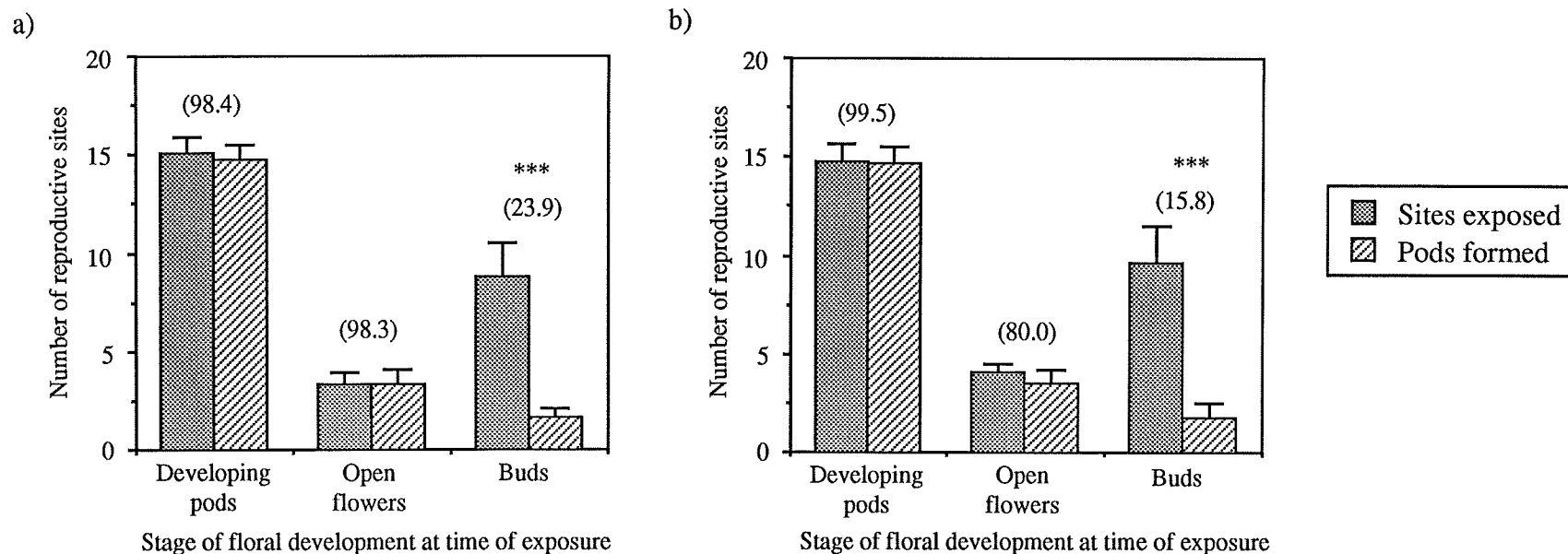
Table 4.3. Mean individual length and weight (minus seeds) at maturity of pods from the terminal raceme of *Brassica campestris* for sites exposed to filtered air (control) or 100 ppb ozone for 6 h at 20 DAS as developing pods, open flowers or buds. Standard errors are shown. n= number of pods. Means for each stage of floral development do not differ significantly between treatments.

Stage of floral development during exposure	Developing pods		Open flowers		Buds	
	Control	Ozone	Control	Ozone	Control	Ozone
Pod length (mm)	36.5 ± 0.50	35.2 ± 0.50	34.1 ± 0.92	33.0 ± 0.86	26.5 ± 1.78	32.3 ± 1.33
Pod weight (mg)	21.4 ± 1.94	17.1 ± 1.16	15.2 ± 2.23	13.6 ± 1.10	11.4 ± 2.60	11.8 ± 1.47
n	148	147	31	35	19	18

(Fig. 4.3a; P<0.001) and 15.8 % of ozone-treated buds (Fig. 4.3b; P<0.001) forming pods. Ozone had no significant effect on the number of pods formed from sites exposed at any of the three stages of development examined.

The pods (minus seeds) became progressively shorter and lighter towards the apex of the terminal raceme in both control and ozone-treated plants (Table 4.3). This is typical of racemose inflorescences where the youngest and smallest flowers are located nearest the apex (Clapham *et al.*, 1989). Ozone had no significant effect on the length or weight of pods developing from sites exposed as developing pods, open flowers or buds.

Seed yield

Data for the number and proportion of mature, germinated and aborted seeds per pod for sites exposed to ozone or filtered air as developing pods, open flowers or buds, are shown in Figure 4.4 and Table 4.4. In this study, the numbers of mature, germinated and aborted seeds per pod were used to estimate the total number of ovules (seed sites) produced per pod. However, it was impossible to establish whether early-aborted seeds or unfertilised ovules were visible at harvest and therefore included as aborted seeds. The total number of mature, germinated and aborted seeds per pod may therefore provide only an approximate estimate of initial ovule number, a factor which will have to be considered when drawing conclusions from the data obtained.

The results show that there were between 12.4-19.2 mature, germinated and aborted seeds present in each pod of ozone-treated and control plants, with more seeds being produced in the earlier formed pods which had been present during exposure or developed from exposed flowers (Table 4.4). Ozone had no significant effect on the total number of seeds per pod although fewer seeds developed in sites exposed as pods and flowers than in control plants, while the converse applied for sites exposed as buds (Table 4.4).

In control plants, the number and proportion of aborted seeds per pod increased acropetally from 5.0 % in the earlier formed pods to 10.5 % in the pods which had been present as buds at the time of treatment (Table 4.4). The number of aborted seeds per pod in ozone-treated plants did not differ significantly from controls (Fig. 4.4a), although more seeds were aborted in sites exposed as buds (Table 4.4).

The seeds of *B. campestris* have no dormancy period and may germinate within the pod if there is sufficient water available. In this experiment, a small number of seeds

163

Figure 4.4. Mean number of a) aborted, b) germinated and c) mature seeds per pod in *Brassica campestris* for floral sites exposed to filtered air (control) or 100 ppb ozone for 6 h at 20 DAS as developing pods, open flowers or buds. ☐, control; ☒, ozone-treated. Single standard errors are shown. * indicates significance at P<0.05; n for each stage of floral development as in Table 4. 3.
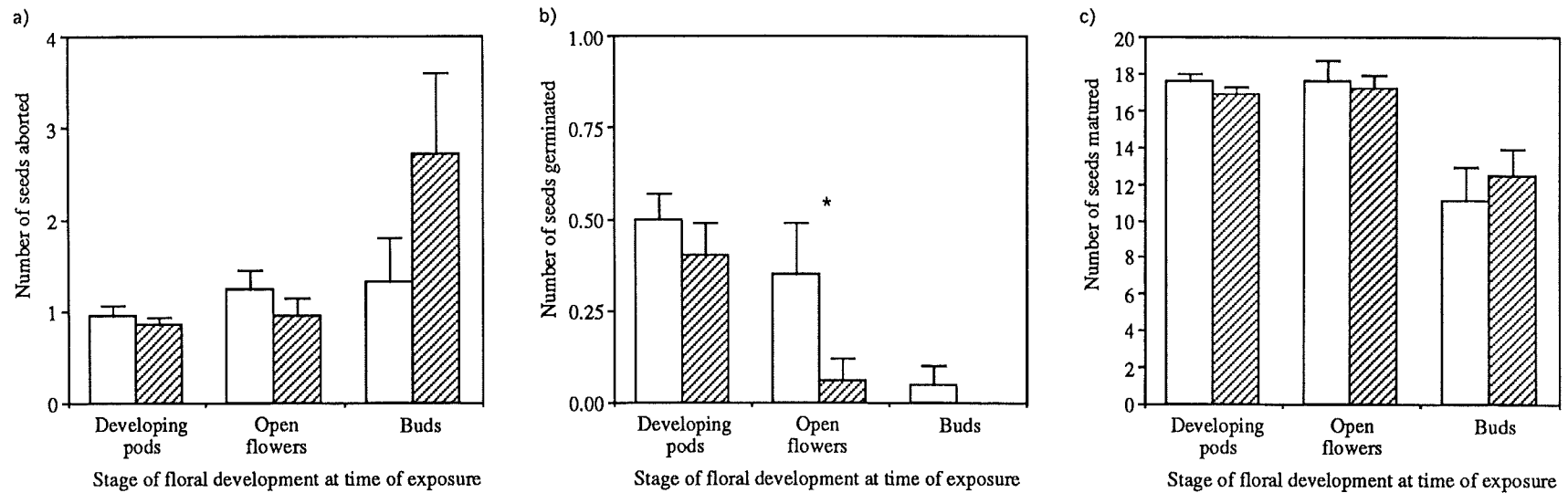
Table 4.4. Mean total number of seeds and percentages of aborted, germinated and mature seeds per pod in *Brassica campestris* for floral sites which were exposed to filtered air (control) or 100 ppb ozone for 6 h at 20 DAS as developing pods, open flowers or buds. Standard errors are shown. The total number of seeds per pod did not differ significantly between treatments for any stage of floral development.

Stage of floral development during exposure	Developing pods		Open flowers		Buds	
	Control	Ozone	Control	Ozone	Control	Ozone
Per pod;						
Total number of seeds	19.0 ± 0.46	18.1 ± 0.39	19.2 ± 1.15	18.2 ± 0.76	12.4 ± 1.94	15.2 ± 1.38
Aborted seeds (%)	5.0	4.6	6.4	5.2	10.5	18.0
Germinated seeds (%)	2.6	2.2	1.8	0.3	0.4	0.0
Mature seeds (%)	92.4	93.2	91.8	94.5	89.1	82.0

germinated within the pods of control plants (Fig. 4.4b), which suggests that watering should have been discontinued prior to 30 DAS to avoid germination. Fewer seeds germinated than aborted in both treatments; however, in contrast to the number of aborted seeds, more seeds germinated in the older pods present during exposure than in the younger pods which had been exposed as flowers or buds. Treatment with ozone reduced the number of germinated seeds per pod and no germinated seeds were found in sites exposed as buds; however, this reduction was only significant for sites exposed as open flowers ($P<0.05$; Fig. 4.4b).

In control plants, the proportion of seeds which developed to maturity without aborting or germinating prematurely, was similar for all pods and varied between 89.1-92.4 % (Table 4.4), although fewer seeds matured in the later formed pods which had been exposed as buds (Fig. 4.4c). Treatment with ozone had no significant effect on the number or proportion of mature seeds per pod.

The individual weight of mature seeds decreased acropetally in both ozone-treated and control plants, with the lightest seeds being produced in pods which had been exposed as buds (Fig. 4.5a). Total mature seed weight per pod followed a similar pattern in both ozone-treated and control plants, with fewer and lighter seeds being produced in the smaller pods towards the apex of the terminal raceme (Fig. 4.5b). Ozone had no significant effect on either variable. Since there was no significant effect of a single exposure to 100 ppb ozone for 6 h on the number of pods, the average number of mature seeds per pod or individual seed weight, the total number and weight of seeds per plant were not significantly affected by treatment with ozone (Table 4.5).

Seed germination

Germination began within 24 h of sowing for all seeds from ozone-treated and control plants and by 48 h the majority of seeds (85-96 %) had germinated (Fig. 4.6). By the end of the seven day germination period, only a few seeds (0.5-4.5 %) had not germinated. Ozone had no significant effect on the percentage of germinated seeds at any time during the germination period (Fig. 4.6).

4.3.1.2. Multiple exposure to 100 ppb ozone for 6 h on four consecutive days between 17-20 DAS

As one of the 20 plants which had been selected for this experiment exhibited abnormal growth characteristics prior to the first day of exposure, this and another similar plant were removed, reducing the sample size for each treatment to nine plants.

165

Figure 4.5. Mean a) individual seed weight and b) seed weight per pod in *Brassica campestris* for floral sites exposed to filtered air (control) or 100 ppb ozone for 6 h at 20 DAS as developing pods, open flowers or buds. Single standard errors are shown; n=10.
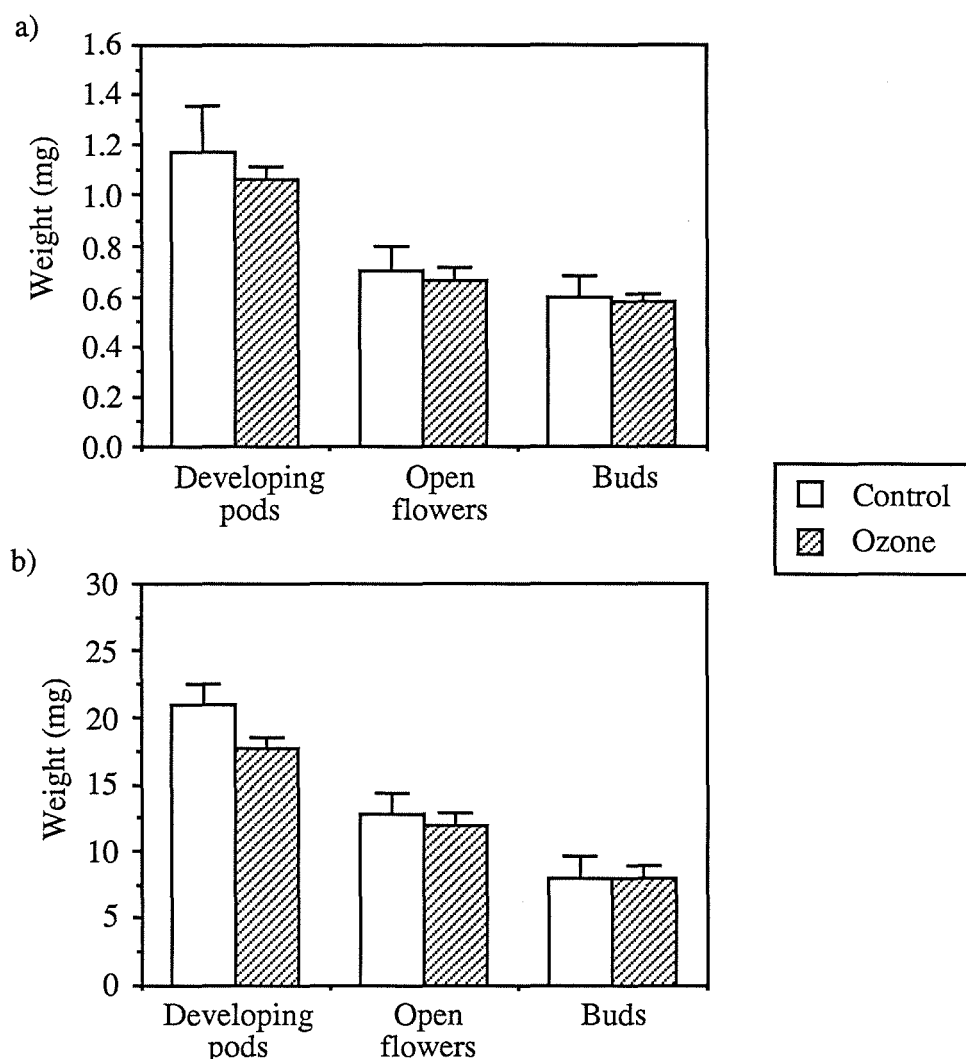
Table 4.5. Summary of seed yield from the terminal raceme of *Brassica campestris* after exposure to filtered air (control) or 100 ppb ozone for 6 h at 20 DAS. Standard errors are shown; n=10. NS, non-significant.

	Control	Ozone	Significance
Number of pods	19.7 ± 1.12	20.0 ± 1.09	NS
Mature seeds per pod	16.9 ± 0.45	16.5 ± 0.35	NS
Mature seeds per plant	334.5 ± 37.18	330.8 ± 24.37	NS
Individual seed weight (mg)	1.06 ± 0.154	0.96 ± 0.052	NS
Seed weight per plant (g)	0.35 ± 0.021	0.31 ± 0.015	NS

166

Figure 4.6. Timecourse of germination for fresh seeds of *Brassica campestris* from floral sites exposed to filtered air (control) or 100 ppb ozone for 6 h at 20 DAS as a) developing pods, b) open flowers and c) buds. Solid circles, controls; open circles, ozone-treated. Double standard errors are shown. n=4.
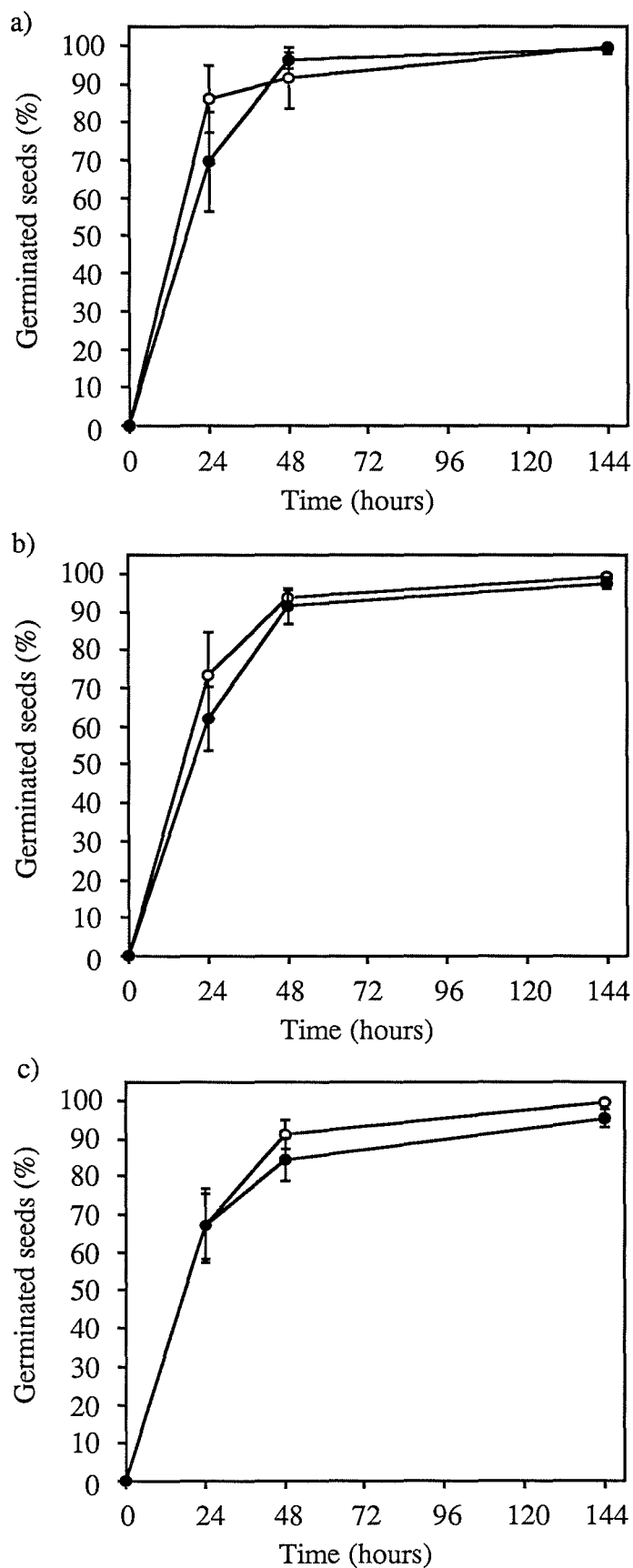
Environmental conditions

During each of the 6 h exposures to ozone or filtered air, the average values for temperature and relative humidity were 23.8 ± 0.05 °C and 45.8 ± 0.06 % in the control chamber and 24.0 ± 0.05 °C and 50.4 ± 0.06 % in the treatment chamber. The average concentration of ozone within the treatment chamber was 100.4 ± 1.16 ppb. Outwith the ozone introduction period, the average temperature and relative humidity were 24.0 ± 0.02 °C and 45.7 ± 0.06 % in the control chamber and 24.2 ± 0.02 °C and 50.1 ± 0.06 % in the treatment chamber.

Terminal raceme and floral site development

There was no significant effect of exposure to 100 ppb ozone for 6 h on four consecutive days between 17-20 DAS on either the length of the terminal raceme (Fig. 4.7) or the total number of reproductive sites produced (Table 4.6). Intersite length was therefore not significantly affected by ozone (Table 4.6).

In both ozone-treated and control plants, the majority of sites were present on the terminal raceme before first flowering at 17 DAS (Fig. 4.8). As seen for the single exposure experiment (Section 4.3.1.1), the maximum number of reproductive sites on control plants was attained by 23 DAS (Fig. 4.8a). This was also the case for the majority of ozone-treated plants, although two plants continued to produce buds up to 26 DAS (Fig. 4.8b). In control plants, an average of five flowers opened every day between 17-19 DAS, after which progressively fewer flowers opened each day until flowering stopped at 26 DAS. When compared to control plants, fewer flowers opened in ozone-treated plants between 17-19 DAS, but more opened between 20-27 DAS, with significantly more flowers open 26 DAS (P<0.05). From the first day of flowering, sites began to abort post-flowering in both ozone-treated and control plants, and the number aborting increased as flowering continued towards the apex of the terminal raceme. Between 19-20 DAS buds also began to abort towards the apex of the terminal raceme as maximum seed number was established for each plant. The total number of reproductive sites and the number of sites aborted either post-flowering or as buds were not significantly affected by the four days of exposure to ozone any time during the period of flowering (Fig. 4.8b).

Of the total number of sites produced on the terminal raceme of ozone-treated and control plants, approximately 50 % formed pods, while the remainder either aborted post-flowering (26 %) or aborted as buds (24 %; Table 4.7).

Figure 4.7. Increase in length of the terminal raceme of *Brassica campestris* during the period of flowering when exposed to filtered air (control) or 100 ppb ozone for 6 h d^{-1} on four consecutive days between 17-20 DAS. Solid circles, control; open circles, ozone-treated. Double standard errors are shown; n=9. There was no significant difference between treatments between 17-27 DAS.
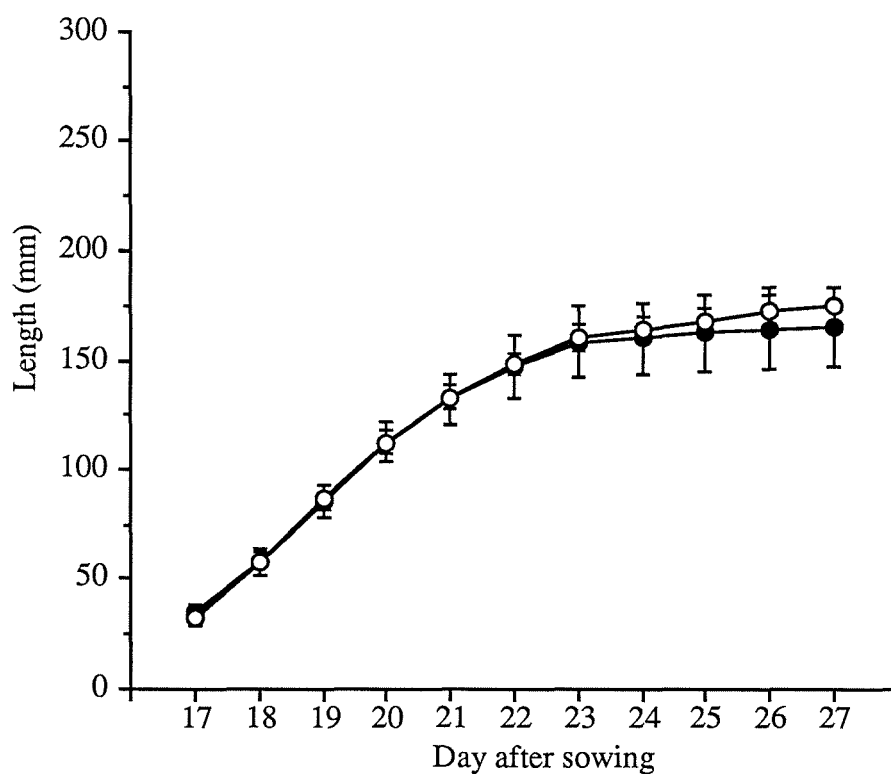
Table 4.6. Final length, number of reproductive sites and intersite length for the terminal raceme of *Brassica campestris* at 27 DAS following exposure to filtered air (control) or 100 ppb ozone for 6 h d^{-1} on four consecutive days between 17-20 DAS. Means and standard errors are shown. NS, non-significant; n=9.

	Control	Ozone	Significance
Raceme length (mm)	164.7 ± 18.17	174.8 ± 8.69	NS
No. reproductive sites	26.7 ± 2.41	27.6 ± 3.28	NS
Intersite length (mm)	6.2 ± 0.40	6.8 ± 0.51	NS

169

Figure 4.8. Mean numbers of floral sites on the terminal raceme of *Brassica campestris* at each stage of development throughout the flowering period following exposure to a) filtered air (control) or b) 100 ppb ozone for 6 h d^{-1} on four consecutive days between 17-20 DAS. n=9. Ozone-treated plants had significantly more flowers at 26 DAS (P<0.05).
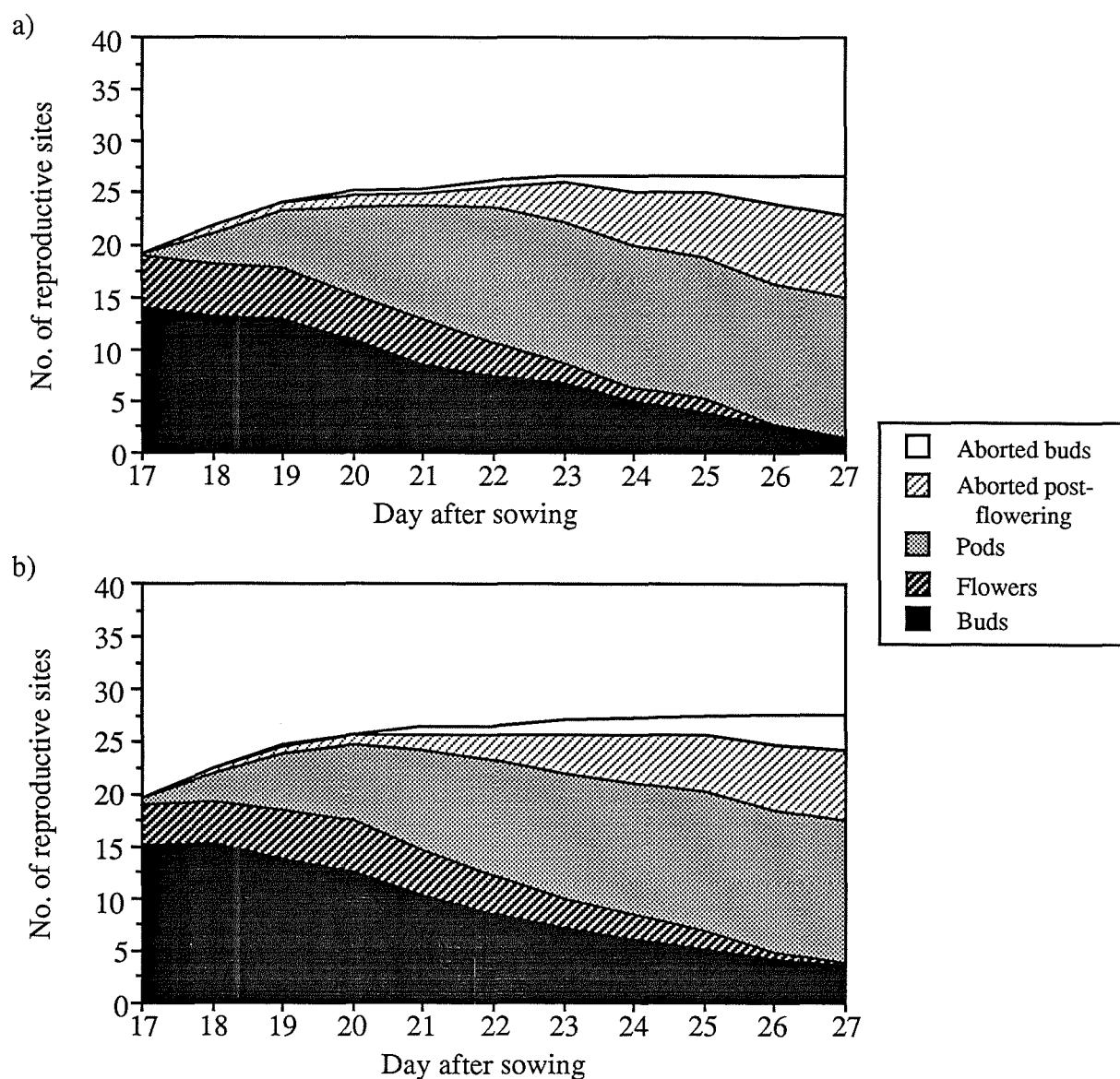
Table 4.7. Mean numbers of reproductive sites on the terminal raceme of *Brassica campestris* which had aborted or developed into pods by the time of harvest at 49 DAS, following exposure to filtered air (control) or 100 ppb ozone for 6 h d^{-1} on four consecutive days between 17-20 DAS. Standard errors are shown; n=9. NS, non-significant.

Number of sites	Control	Ozone	Significance
Total	26.7 ± 2.41	27.6 ± 3.28	NS
Aborted	13.1 ± 1.57	14.0 ± 2.27	NS
Pods	13.6 ± 1.43	13.6 ± 1.21	NS

Pod development

Between 2-5 flowers opened on the terminal raceme during each of the four days of exposure to ozone or filtered air (Fig. 4.9). As well as being exposed on the day of flowering, these sites were exposed as buds and/or developing pods during three of the four days of exposure. In addition, approximately 14 sites per plant were exposed for four consecutive days as buds. In both ozone-treated and control plants, more pods developed from flowers which opened on the second and third days of exposure (85.2-100 %) than from flowers which opened on the first and fourth days of exposure (66.7-82.2 %). Fewer pods developed towards the apex of the terminal raceme, with only 8.7 % of control (Fig. 4.9a; P<0.001) and 15.7 % of ozone-treated (Fig. 4.9b; P<0.01) buds forming pods. The total number of sites exposed at each stage of development and the number of these which formed pods were not significantly affected by treatment with ozone.

As shown in Table 4.8, pod length and pod weight were both significantly affected by four days of exposure to ozone. The pods of ozone-treated plants were significantly longer (11.9 %; P<0.05) for sites which flowered on the first day of exposure and significantly shorter for sites which flowered on the third (16.3 %; P<0.05) and fourth (29.0 %; P<0.01) days of exposure, as well as for sites exposed as buds (24.9 %; P<0.01). Despite the effects of ozone on pod length, pod weight was similar to the controls for all sites except those which flowered on the second day of exposure, for which pod weight was a significantly increased (38.6 %; P<0.05; Table 4.8).

Seed yield

Figure 4.10 shows that a total of 11-20 mature, germinated and aborted seeds developed in each pod of control plants, with more being produced in the older pods than in the younger pods. Ozone reduced the total number of seeds per pod, suggesting an effect on ovule number, but this was only significant for sites which flowered on the first, third and fourth days of exposure (P<0.01;P<0.001 and P<0.05).

In control plants, the percentage of aborted seeds per pod varied between 2.3-6.2 % (Table 4.9) but, unlike the single exposure experiment (Section 4.3.1.1), the number of aborted seeds per pod did not increase acropetally; but instead varied non-systematically (Fig. 4.11a). Ozone increased both the number and percentage of aborted seeds in all pods (Fig. 4.11a, Table 4.9), although this was only significant for sites which

Figure 4.9. Mean number of floral sites on the terminal raceme of *Brassica campestris,* exposed on four consecutive days between 17-20 DAS to a) filtered air (control) or b) 100 ppb ozone for 6 h d^{-1} as open flowers (open on day 1, 2, 3 or 4 of exposure as indicated) or buds, and the number of these sites which formed pods. Single standard errors are shown. Number of pods formed as a percentage of floral sites exposed is shown in brackets. ** and *** indicate significance at $P<0.01$ and $P<0.001$; n=9.

a)

b)

| ▦ | Sites exposed |
| ▨ | Pods formed |

Table 4.8. Mean individual length and weight (minus seeds) at maturity of pods from the terminal raceme of *Brassica campestris* for floral sites exposed on four consecutive days between 17-20 DAS to filtered air (control) or 100 ppb ozone for 6 h d^{-1} as open flowers (open on day 1, 2, 3 or 4 of exposure as indicated) or buds. Standard errors are shown. * and ** indicate significance at P<0.05 and P<0.01. NS, non-significant; n= number of pods.

Stage of floral development at time of exposure	Treatment	n	Pod length (mm)	Significance	Pod weight (mg)	Significance
Flowers (1)	Control	36	32.6 ± 0.62	*	25.2 ± 1.72	NS
	Ozone	32	36.5 ± 1.65		30.7 ± 3.25	
Flowers (2)	Control	33	33.5 ± 0.84	NS	20.2 ± 1.13	*
	Ozone	25	32.8 ± 2.29		28.0 ± 2.94	
Flowers (3)	Control	21	32.5 ± 1.54	*	20.7 ± 1.64	NS
	Ozone	25	27.2 ± 1.94		23.4 ± 3.20	
Flowers (4)	Control	19	32.4 ± 2.22	**	16.3 ± 2.43	NS
	Ozone	15	23.0 ± 2.72		13.0 ± 1.59	
Buds	Control	12	29.3 ± 1.61	**	11.1 ± 2.27	NS
	Ozone	23	22.0 ± 1.92		13.9 ± 3.07	

173

Figure 4.10. Mean total number of seeds per pod in *Brassica campestris* for floral sites exposed on four consecutive days between 17-20 DAS to filtered air (control) or 100 ppb ozone for 6 h d-1 as open flowers (open on day 1, 2, 3 or 4 of exposure as indicated) or buds. ☐ control; ▨ ozone-treated. Single standard errors are shown. *, ** and *** indicates significance at P<0.05. P<0.001 and P<0.001. n= number of pods for each stage of floral development as shown in Table 4.8.
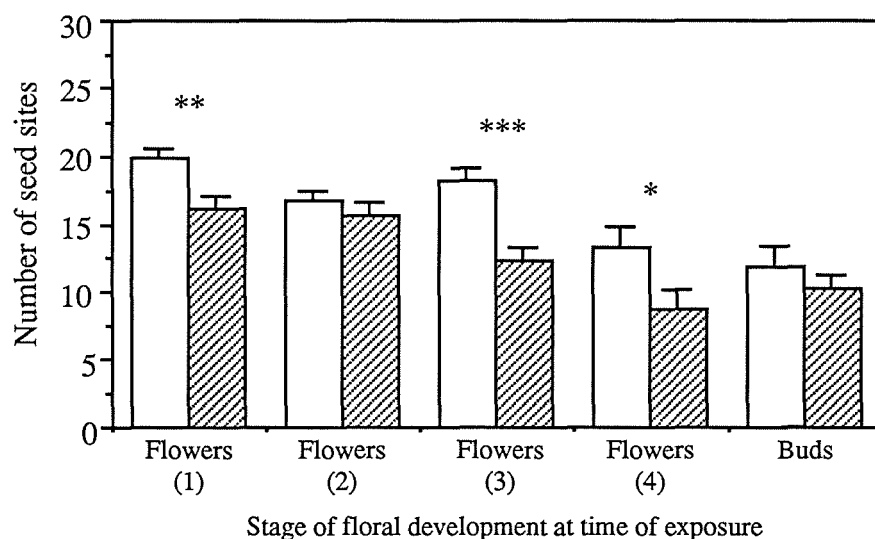
Table 4.9. Mean percentage of aborted, germinated and mature seeds per pod in *Brassica campestris* for floral sites which were exposed on four consecutive days between 17-20 DAS to filtered air (control) or 100 ppb ozone for 6 h d-1 as open flowers (open on day 1, 2, 3 or 4 of exposure as indicated) or buds.

Stage of floral development at time of exposure	Treatment	% Aborted seeds	Germinated seeds	Mature seeds
Flowers (1)	Control	6.2	1.2	92.6
	Ozone	11.8	13.6	74.6
Flowers (2)	Control	2.3	0.4	97.3
	Ozone	10.7	5.1	84.2
Flowers (3)	Control	4.7	0.0	95.3
	Ozone	12.3	1.7	86.0
Flowers (4)	Control	5.2	0.4	94.4
	Ozone	12.3	0.0	87.7
Buds	Control	4.3	0.0	95.7
	Ozone	24.0	3.2	72.8

174

Figure 4.11. Mean number of a) aborted, b) germinated and c) mature seeds per pod in *Brassica campestris* for floral sites exposed on four consecutive days between 17-20 DAS to filtered air (control) or 100 ppb ozone for 6 h d^{-1} as open flowers (open on day 1, 2, 3 or 4 of exposure as indicated) or buds. □, control; ▨, ozone-treated. Single standard errors are shown. *, ** and *** indicate significance at P<0.05, P<0.01 and P<0.001. n=pod number for each stage of development as shown in Table 4.8.
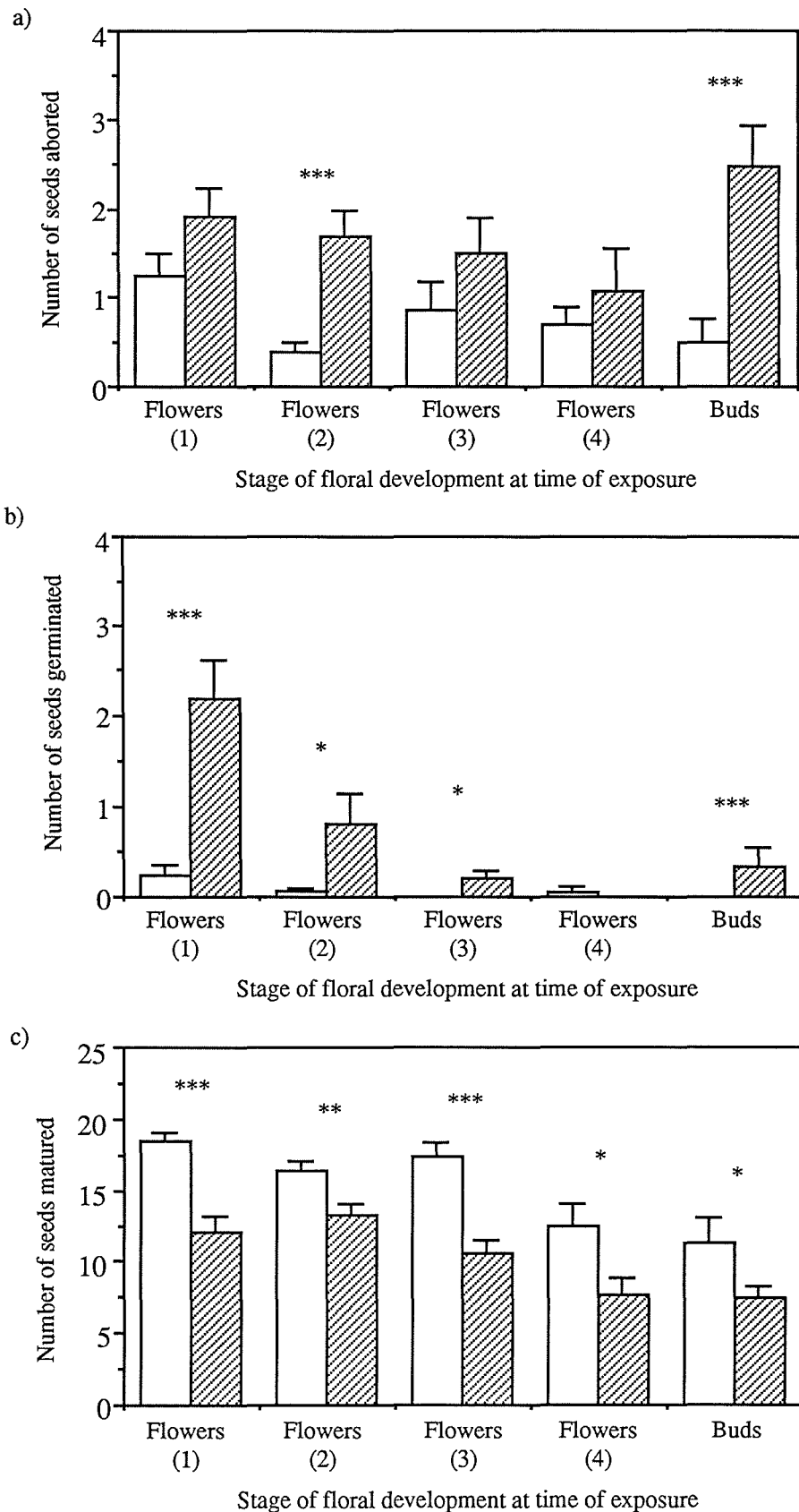
flowered on the second day of exposure and sites which were exposed as buds (P<0.001).

Very few seeds germinated within the pods of control plants (Fig. 4.11b), with a maximum 1.2 % of seeds germinating in the oldest pods which had been flowers on the first day of exposure (Table 4.9). Treatment with ozone significantly increased the number of germinated seeds within all pods except those which had been flowers on the fourth day of exposure (Fig. 4.11b). The greatest increase in the number of germinated seeds was in the oldest pods which had been flowers on the first day of exposure.

For control plants, the total number of seeds per pod which developed to maturity without aborting or germinating prematurely decreased acropetally (Fig. 4.11c), although the proportion of mature seeds was similar in all pods varying between 92.6-97.3 % (Table 4.9). Significantly fewer seeds matured in all pods of ozone-treated plants (Fig. 4.11c) and the percentage of mature seeds per pod was reduced to between 72.8-68.0 % (Table 4.9).

The numbers of mature, germinated and aborted seeds per pod were also recorded in a repeat of this experiment (Fig. 1.1, Appendix 1). However, in this experiment the number of reproductive sites present on the terminal raceme was significantly greater in the plants selected as controls plants prior to exposure. Fewer pods formed on ozone-treated plants, possibly reflecting the initial difference in the number of reproductive sites, which might have influenced seed yield in the absence of ozone treatment. The results from this experiment could therefore not be used to draw any meaningful conclusions about the effects of ozone. However, it is useful to note that, although there was no significant difference between control and ozone-treated plants in the total number of seeds per pod (Fig. 1.2, Appendix 1), the numbers of aborted and germinated seeds were increased in the majority of pods on ozone-treated plants, although these differences were usually not significant (Figs. 1.1a, 1.1b, Appendix 1). The number of mature, non-germinated seeds per pod was also reduced in ozone-treated plants, significantly for sites which flowered on the second and fourth days of exposure (P<0.05; Fig. 1.1c, Appendix 1). Similar effects on the proportion of mature, germinated and aborted seeds were therefore seen in both experiments in which the terminal raceme was exposed on four consecutive days between 17-20 DAS to 100 ppb ozone for 6 h a day (Table 4.9; Table 1.1, Appendix 1).

The individual weight of mature seeds decreased acropetally in both ozone-treated and control plants, with the lightest seeds being produced in the later formed pods which

had been exposed as buds (Fig. 4.12a). Although not significant, individual seed weight was greater in ozone-treated plants, perhaps because there were fewer seeds in each pod. In control plants, the total mature seed weight per pod decreased acropetally as the number of mature seeds per pod and individual seed weight also decreased. Although there were significantly fewer mature seeds per pod in ozone-treated plants, individual seed weight was slightly greater than controls, resulting in there being no significant effect on total mature seed weight per pod (Fig. 4.12b).

Table 4.10 summarises seed yield in *Brassica campestris* after four consecutive 6 h exposures to ozone between 17-20 DAS. The results show that the average number of mature seeds per pod was significantly reduced by exposure to ozone ($P<0.01$). This was due to an increase in the numbers of aborted and germinated seeds per pod and a reduction in the total number of seeds per pod, suggesting a decrease in ovule number. The number of pods per plant was unaffected by treatment and although the number of mature seeds per pod was reduced by ozone, a reduction in the number of mature seeds per plant was not significant due to high variability. However because of the smaller number of mature seeds, the total weight of seeds per plant was significantly reduced by ozone ($P<0.05$) even though the seeds of ozone-treated plants were slightly, although not significantly, heavier.

Seed germination

As seen in previous germination experiments (Section 4.3.1.1), germination began within 24 h of sowing for all seed categories from ozone-treated and control plants, and by 48 h the majority of seeds (93-99 %) had germinated (Fig. 4.13). Ozone had no significant effect on the timecourse of germination for seeds from sites which flowered on the first three days of exposure, but significantly increased the percentage germination within 24 h of sowing for seeds from sites which were flowering on the fourth day of exposure (Fig. 4.13d; $P<0.05$) and sites which were exposed as buds (Fig. 4.13e; $P<0.01$).

Seeds from the repeat experiment showed a similar effect of ozone on germination (Fig. 1.3, Appendix 1), although in this instance the percentage germination of seeds from all sites was significantly increased 24 h after sowing, suggesting that ozone had a stimulatory effect on germination.

Figure 4.12. Mean a) individual seed weight and b) seed weight per pod in *Brassica campestris* for floral sites exposed on four consecutive days between 17-20 DAS to filtered air (control) or 100 ppb ozone for 6 h d^{-1} as open flowers or buds. Single standard errors are shown. n=9. No significant effects were detected.

a)

b)

Stage of floral development at time of exposure

Table 4.10. Summary of seed yield from the terminal raceme of *Brassica campestris* after exposure on four consecutive days between 17-20 DAS to filtered air (control) or 100 ppb ozone for 6 h d^{-1}. Means and standard errors are shown. * and ** indicate significance at P<0.05 and P<0.01. NS, non-significant; n=9.

	Control	Ozone	Significance
Number of pods	13.6 ± 1.44	13.6 ± 1.21	NS
Mature seeds per pod	16.2 ± 0.72	10.8 ± 1.27	**
Mature seeds per plant	217.6 ± 23.91	141.3 ± 14.03	NS
Individual seed weight (mg)	1.20 ± 0.072	1.33 ± 0.152	NS
Seed weight per plant (g)	0.25 ± 0.015	0.18 ± 0.022	*

178

Figure 4.13. Timecourse of germination for fresh seeds of *Brassica campestris* collected from floral sites exposed on four consecutive days between 17-20 DAS to filtered air (control) or 100 ppb for 6 h d^{-1} as open flowers (open on day 1, 2, 3 or 4 day of exposure as indicated) or buds. Solid circles, controls; open circles, ozone-treated. Double standard errors are shown. * and ** indicate significance at P<0.05 and P<0.01; n=4.

a) Open flowers (1)

b) Open flowers (2)

c) Open flowers (3)

d) Open flowers (4)

e) Buds
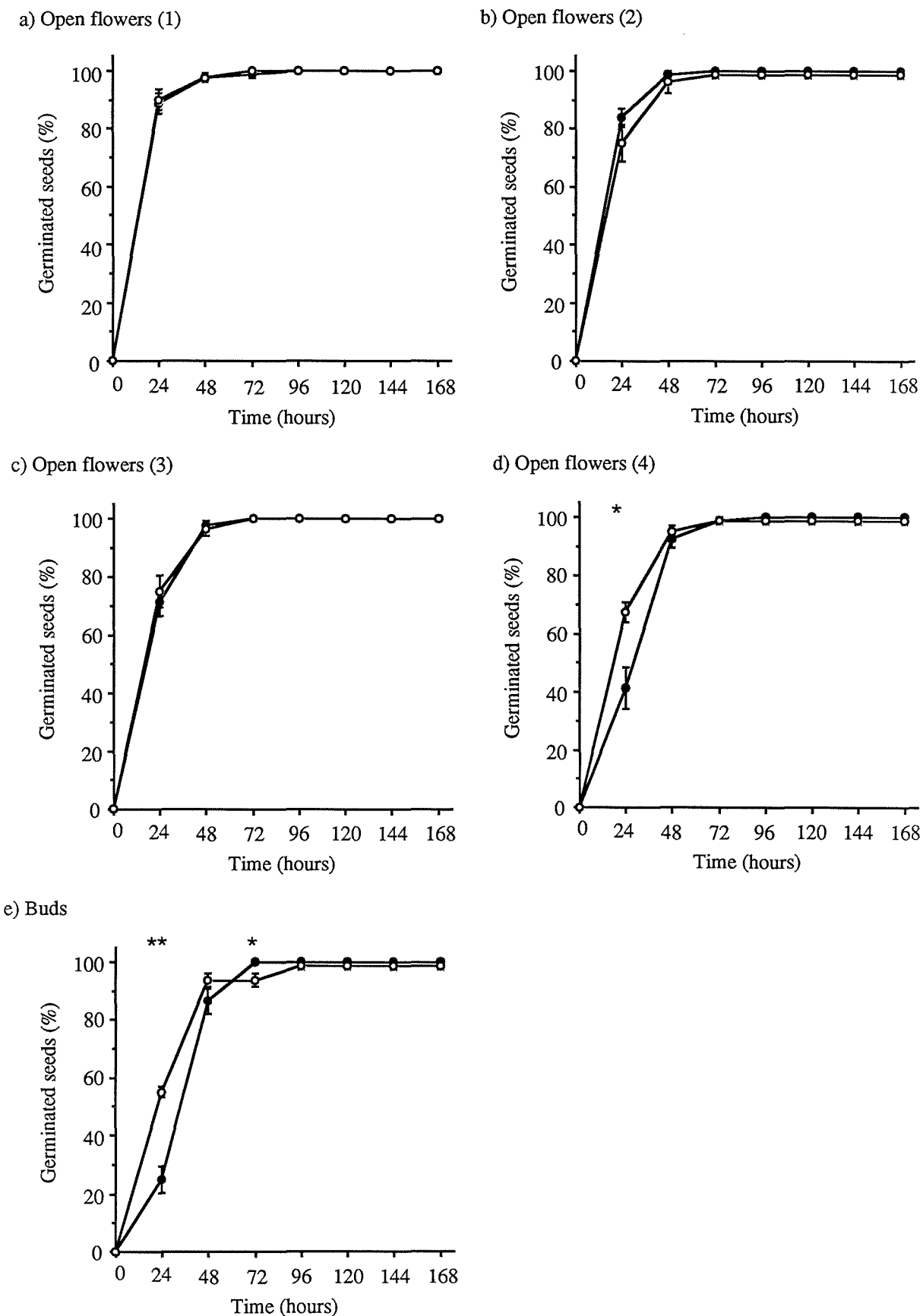
Seed colour

Mature seeds from the earliest formed pods from each treatment were separated into the colour categories shown in Plate 4.2. The colour of seeds obtained differed between treatments; whilst both treatments produced a number of brown and light brown seeds, the control plants also produced yellow/brown seeds and the ozone-treated plants produced green and yellow seeds. This production of green and yellow seeds was also recorded for *Brassica campestris* after exposure of the entire plant to 70 ppb ozone for 7 h d^{-1} for 10 d prior to first flowering (Section 3.3.2.2). Table 4.11 shows that the majority of seeds in control plants were brown, with less than 20 % each of light brown and yellow/brown seeds, while in ozone-treated plants approximately 30 % of the seeds were brown or yellow and 19 % were light brown or green. The individual weight of each seed type varied but all seeds were invariably heavier following exposure to ozone. The brown seeds of ozone-treated plants were heaviest, almost three times greater than the brown seeds from control plants. The individual weight of seeds from control plants was similar for each of the three colour categories.

4.3.1.3. Multiple exposure to 100 ppb ozone for 6 h on four consecutive days between 20-23 DAS

Environmental conditions

During each of the 6 h exposures to ozone or filtered air, the average temperature and relative humidity were 22.7 ± 0.03 °C and 61.1 ± 0.18 % in the control chamber and 22.8 ± 0.02 °C and 58.0 ± 0.16 % in the treatment chamber. The average concentration of ozone within the treatment chamber was 100.1 ± 0.02 ppb. Outwith ozone introduction, the average temperature and relative humidity were 22.7 ± 0.02 °C and 60.7 ± 0.11 % in the control chamber and 22.8 ± 0.01 °C and 57.6 ± 0.10 % in the treatment chamber.

Terminal raceme and floral site development

There was no significant effect of exposure to 100 ppb ozone for 6 h on four consecutive days between 20-23 DAS on the length of the terminal raceme (Fig. 4.14), although the difference between control and ozone-treated plants increased progressively from 17-29 DAS. The number of reproductive sites on the terminal raceme was similar to that recorded in previous experiments and was unaffected by treatment with ozone (Table 4.12). Since the terminal raceme of ozone-treated plants
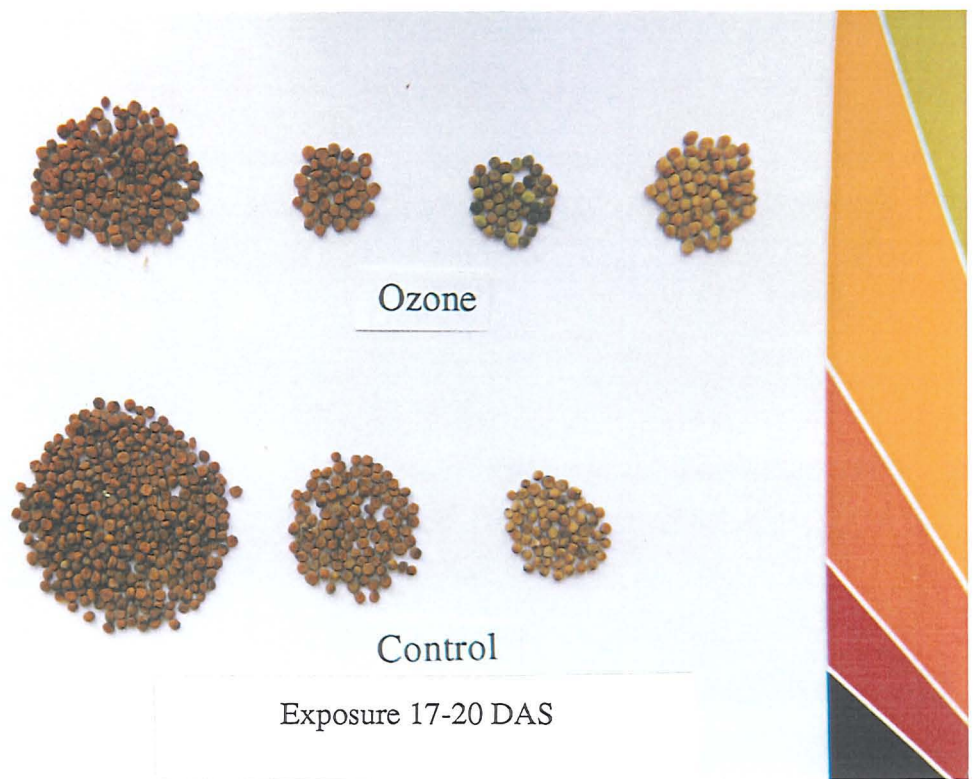
Plate 4.2. Seed yield within various colour categories in *Brassica campestris*. The seeds were harvested from floral sites exposed on four consecutive days between 17-20 DAS to filtered air (control treatment) or 100 ppb ozone for 6 h d^{-1} as open flowers on the first day of exposure. Seed colours from left to right: ozone, brown, light brown, green, yellow; control, brown, light brown, yellow/brown.

Table 4.11. Summary of mature seed yield within various colour categories in *Brassica campestris*. The seeds were harvested from floral sites exposed on four consecutive days between 17-20 DAS to filtered air (control treatment) or 100 ppb ozone for 6 h d^{-1} as open flowers on the first day of exposure.

Treatment	Colour category				
Control	Brown	Light Brown	Yellow/ Brown		Total, all colours
Number of seeds	412	113	65		590
Percentage of total	69.8	19.2	11.0		
Total weight of seeds (g)	0.517	0.140	0.089		0.746
Mean individual seed weight (mg)	1.255	1.239	1.369		
Ozone	Brown	Light Brown	Yellow	Green	Total, all colours
Number of seeds	62	40	70	40	212
Percentage of total	29.2	18.9	33.0	18.9	
Total weight of seeds (g)	0.221	0.062	0.132	0.065	0.480
Mean individual seed weight (mg)	3.565	1.550	1.886	1.625	

Figure 4.14. Increase in length of the terminal raceme of *Brassica campestris* during the period of flowering when exposed to filtered air (control) or 100 ppb ozone for 6 h d^{-1} on four consecutive days between 20-23 DAS. Solid circles, control; open circles, ozone-treated. Double standard errors are shown; n=10. There was no significant difference between treatments between 17-29 DAS.
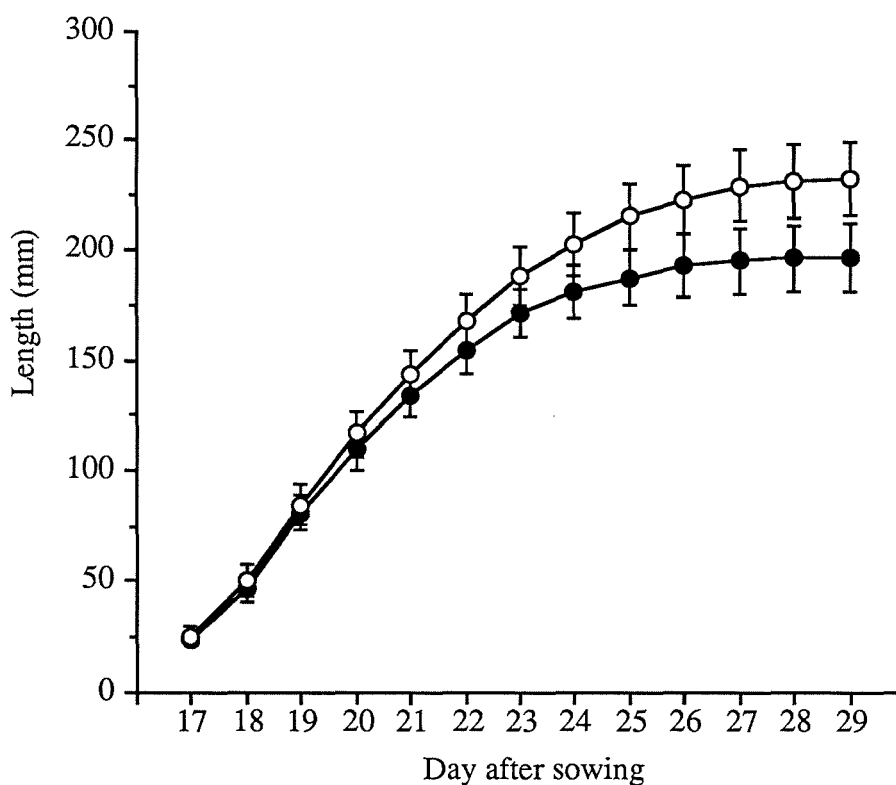
Table 4.12. Final length, number of reproductive sites and intersite length of the terminal raceme of *Brassica campestris* at 29 DAS following exposure to filtered air (control) or 100 ppb ozone for 6 h d^{-1}, on four consecutive days between 20-23 DAS. Means and standard errors are shown. NS, non-significant; n=10.

	Control	Ozone	Significance
Raceme length (mm)	196.2 ± 34.41	232.7 ± 16.65	NS
No. reproductive sites	29.2 ± 2.06	30.4 ± 2.19	NS
Intersite length (mm)	6.9 ± 0.48	7.9 ± 0.67	NS

was slightly longer than controls, the intersite length was also longer, although not significantly (Table 4.12).

The majority of sites were present on the terminal racemes of both control and ozone-treated plants before first flowering at 17 DAS (Fig. 4.15), with the maximum number being attained by 23-24 DAS. At no time between 17-29 DAS was the total number of reproductive sites significantly different between treatments. The number of flowers which opened each day during the period of flowering was also unaffected by treatment with ozone. Sites began to abort post-flowering from 22-23 DAS in both control and ozone-treated plants, and the number increased as flowering progressed towards the apex of the terminal raceme. By the end of the flowering period at 30 DAS, the number of sites which had aborted post-flowering was greater in ozone-treated plants, although not significantly (Fig. 4.15b). Buds began to abort from 22 DAS in control plants and from 25 DAS in ozone-treated plants, with the latter having significantly fewer aborted buds at both 25 and 29 DAS ($P < 0.05$). These results suggest that four consecutive daily exposures to 100 ppb ozone for 6 h between 20-23 DAS had no significant effect on the abortion of floral sites or the timing of floral development, although there is some evidence that more buds developed into flowers before aborting.

Approximately 73 % of all sites on the terminal raceme formed pods irrespective of treatment (Table 4.13). In control and ozone-treated plants, 17.8 and 23.4 % respectively of sites aborted post-flowering while 7.9 and 3.9 % of sites aborted as buds.

Pod development

Between 2-4 flowers opened on the terminal raceme on each day during the four consecutive days of exposure to ozone or filtered air (Fig. 4.16). As well as being exposed on the day of flowering, these sites were exposed as buds and/or developing pods during the rest of the four day exposure period. In addition, 6-7 buds and 9-10 developing pods on each plant were exposed for four consecutive days. For both ozone-treated and control plants, the number sites which developed into pods decreased acropetally in both ozone-treated and control plants (Fig. 4.16), with more pods developing from sites pollinated prior to or on the first day of exposure (100 %) than from sites exposed as buds (22.0-25.6 %). The total number of sites exposed at each stage of development and the number of these which formed pods was not significantly affected by exposure to ozone.

184

Figure 4.15. Mean numbers of floral sites on the terminal raceme of *Brassica campestris* at each stage of development throughout the flowering period following exposure to filtered air (control) or 100 ppb ozone for 6 h d^{-1} on four consecutive days between 20-23 DAS. n=10. Ozone-treated plants had significantly fewer aborted buds at 25 and 29 DAS (P<0.05).
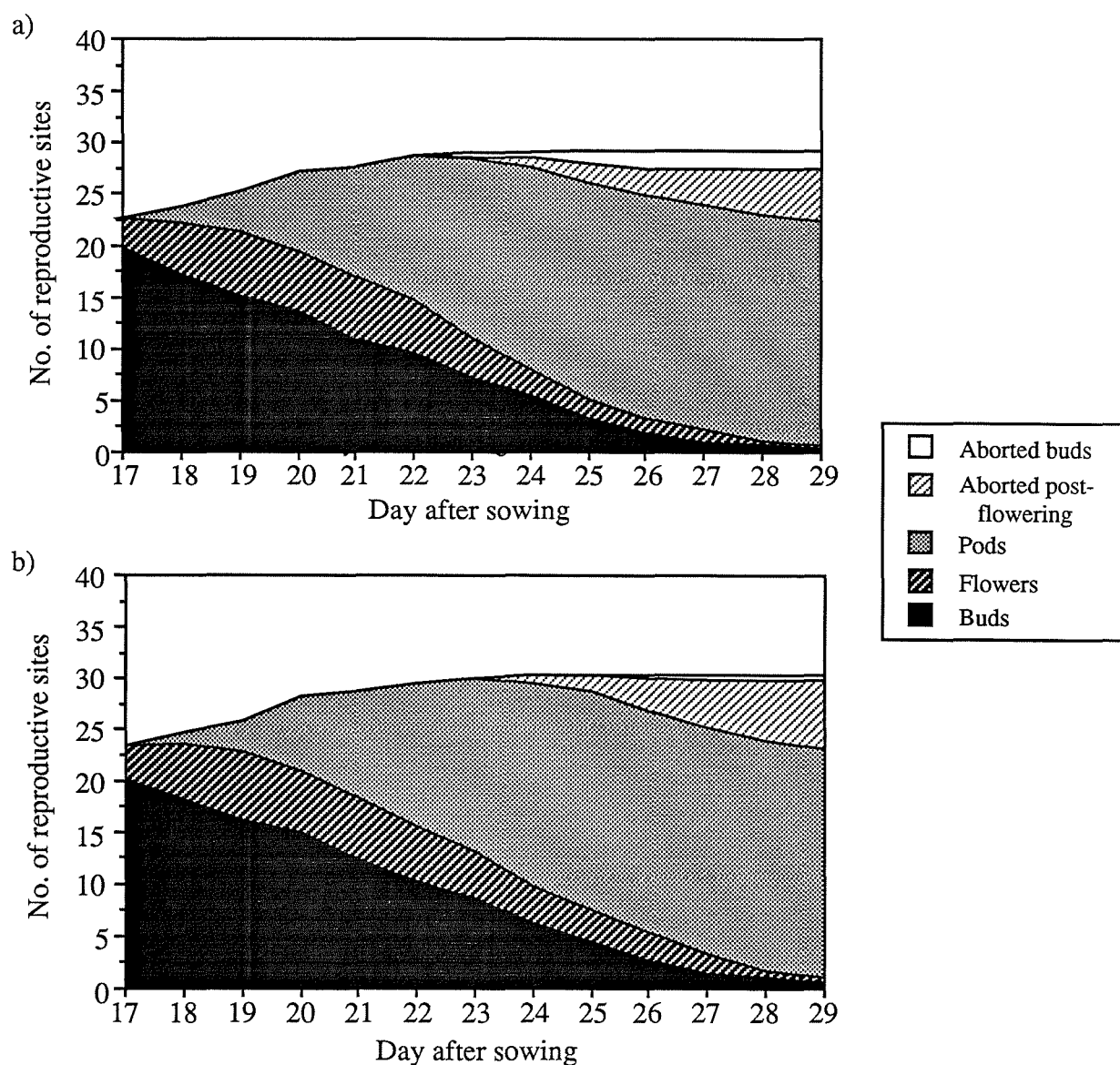
Table 4.13. Mean numbers of floral sites on the terminal raceme of *Brassica campestris* which had aborted or developed into pods by the time of harvest at 47 DAS, following exposure to filtered air (control) or 100 ppb ozone for 6 h d^{-1} on four consecutive days between 20-23 DAS. Standard errors are shown; n=10. NS, non-significant.

Number of sites	Control	Ozone	Significance
Total	29.2 ± 2.06	30.4 ± 2.19	NS
Aborted	7.5 ± 1.28	8.3 ± 1.13	NS
Pods	21.7 ± 1.59	22.1 ± 1.29	NS

Figure 4.16. Mean number of floral sites on *Brassica campestris,* exposed on four consecutive days between 20-23 DAS to a) filtered air (control) or b) 100 ppb ozone for 6 h d^{-1} as developing pods, open flowers (open on day 1, 2, 3 or 4 as indicated) or buds, and the number of these sites which formed pods. Single standard errors are shown. Number of pods formed as a percentage of floral sites exposed is shown in brackets. * and *** indicate significance at P<0.05 and P<0.001; n=10.
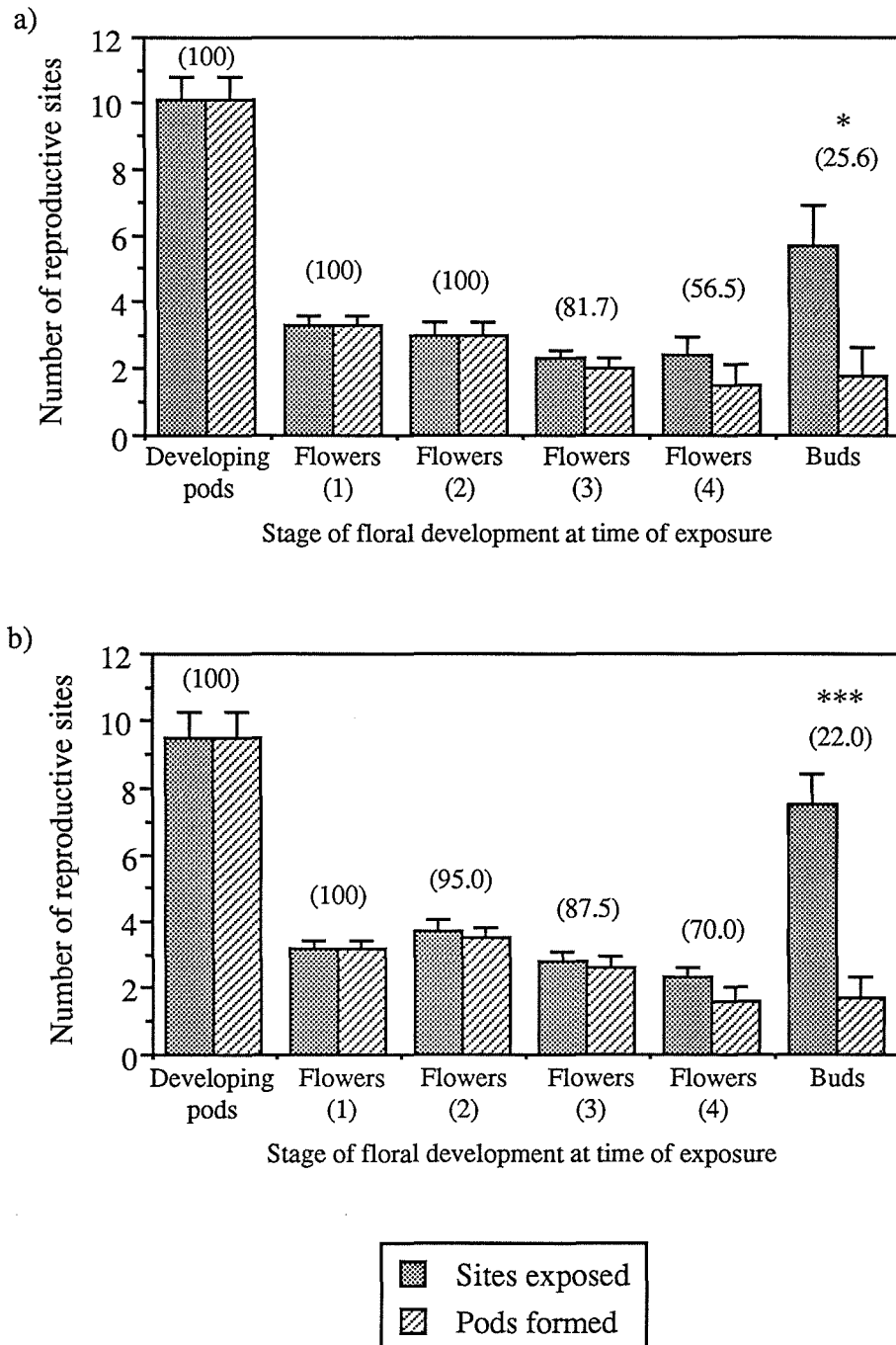
Table 4.14 shows that pod length and pod weight decreased acropetally in both ozone-treated and control plants. Ozone had no effect on these variables except for a significant reduction (P<0.05) in the weight of pods from sites exposed entirely as buds.

Seed yield

In control plants, a total of 15-22 mature, germinated and aborted seeds were present in each pod, with more seeds being produced in older pods than in the younger pods (Fig. 4.17). Unlike the multiple exposure between 17-20 DAS, in which ozone decreased the total number of seeds per pod, exposure between 20-23 DAS increased seed number; however this effect was only significant for those sites which had been exposed as developing pods (P<0.001).

The percentage of aborted seeds increased acropetally in control plants from 7.2 % in sites exposed as developing pods to 42.5 % in sites exposed as buds (Table 4.15). The latter was the highest recorded value for control plants in any of the three exposure experiments. Ozone significantly reduced the number and percentage of aborted seeds from sites which were exposed entirely as developing pods (P<0.05) but increased these values for all other categories, significantly for sites which flowered on the third and fourth days of exposure and sites which were exposed entirely as buds (Fig. 4.18a, Table 4.15).

Less than 0.4 % of seeds germinated within the pods of control plants (Table 4.15) although, as seen in previous experiments, this percentage decreased acropetally such that no seed germination was observed for sites which flowered after the second day of exposure to filtered air (Fig. 4.18b). In ozone-treated plants there was also very little germination within the pods and no seeds germinated in pods produced from sites which flowered after the first day of exposure to ozone. Ozone did, however, significantly increase (P<0.05) the number of seeds which germinated in the oldest pods which had been exposed for four days during their development (Fig. 4.18b).

The number and percentage of mature seeds per pod decreased acropetally in both control and ozone-treated plants (Fig. 4.18c) as the percentage of aborted seeds increased (Table 4.15). Four days of exposure to ozone between 20-23 DAS appeared to increase the number of mature seeds per pod in sites which flowered before the third day of exposure and decreased the number thereafter, with the increase in seed number being significant (P<0.001) for those sites which had been exposed as developing pods (Fig. 4.18c).
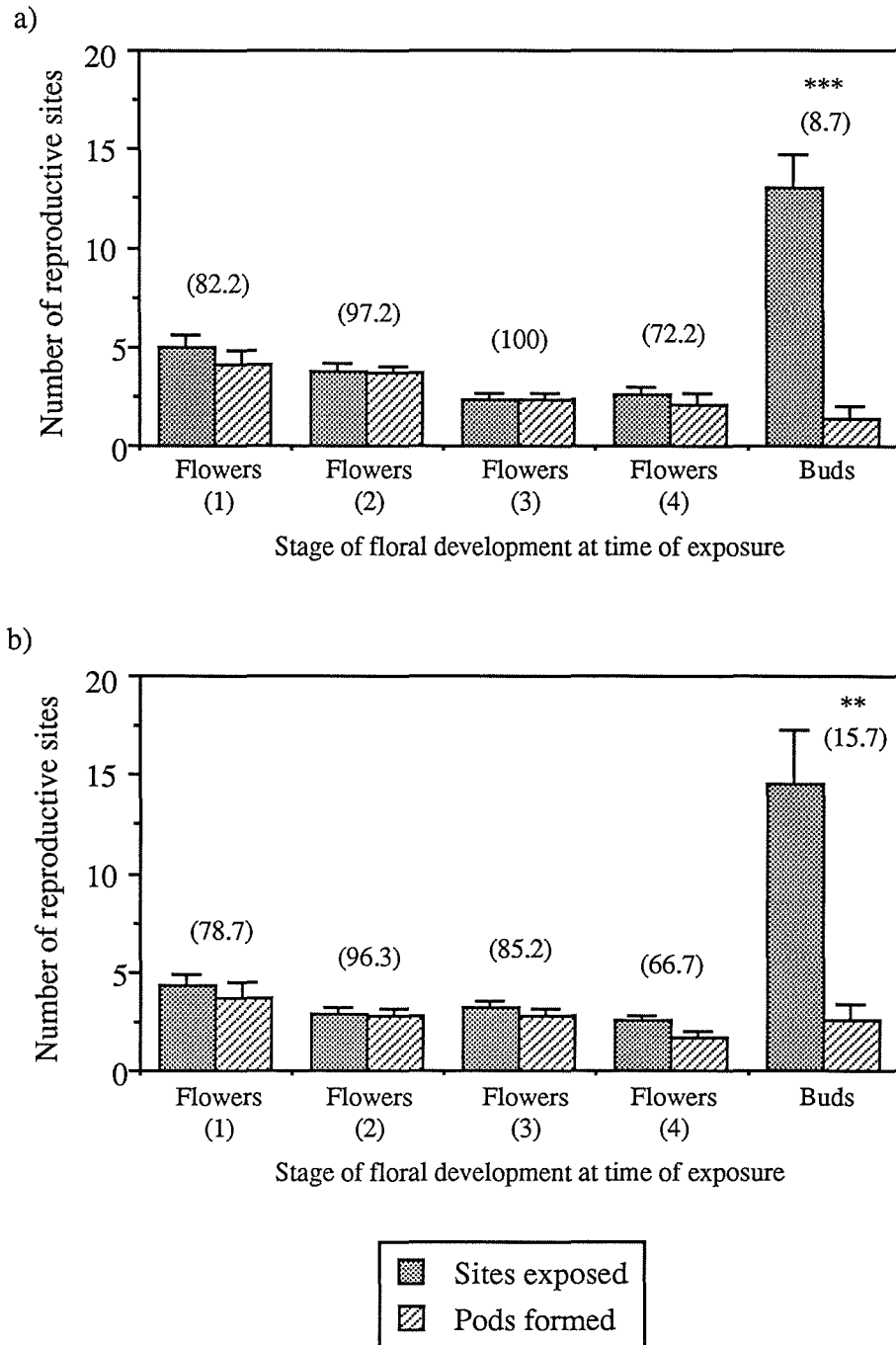
Table 4.14. Mean individual length and weight (minus seeds) at maturity of pods from the terminal raceme of *Brassica campestris* for floral sites exposed on four consecutive days between 20-23 DAS to filtered air (control) or 100 ppb ozone for 6 h d^{-1} as developing pods, open flowers (open on day 1, 2, 3 or 4 of exposure as indicated) or buds. Standard errors are shown. * indicates significance at P<0.05. NS, non-significant; n=number of pods.

Stage of floral development at time of exposure	Treatment	n	Pod length (mm)	Significance	Pod weight (mg)	Significance
Developing pods	Control	101	47.1 ± 0.71	NS	26.4 ± 1.85	NS
	Ozone	95	45.4 ± 0.63		25.5 ± 1.79	
Flowers (1)	Control	33	43.5 ± 0.97	NS	19.9 ± 1.38	NS
	Ozone	32	42.0 ± 1.07		18.0 ± 1.37	
Flowers (2)	Control	30	40.8 ± 1.02	NS	15.7 ± 1.22	NS
	Ozone	35	38.8 ± 1.03		14.3 ± 1.28	
Flowers (3)	Control	20	37.4 ± 1.27	NS	12.8 ± 1.24	NS
	Ozone	26	36.0 ± 1.49		10.3 ± 1.05	
Flowers (4)	Control	15	35.3 ± 0.88	NS	9.2 ± 0.78	NS
	Ozone	16	34.3 ± 1.67		9.1 ± 1.26	
Buds	Control	18	27.6 ± 2.37	NS	8.2 ± 1.31	*
	Ozone	17	28.3 ± 1.61		5.2 ± 0.79	

Figure 4.17. Mean total number of seed sites per pod for floral sites of *Brassica campestris* exposed on four consecutive days between 20-23 DAS to filtered air (control) or 100 ppb ozone for 6 h d^{-1} as developing pods, open flowers (open on day 1, 2, 3 or 4 of exposure as indicated) or buds. □, control; ▨, ozone-treated. Single standard errors are shown. *** indicates significance at P<0.001. n=pod number for each stage of floral development as shown in Table 4.14.

Stage of floral development at time of exposure
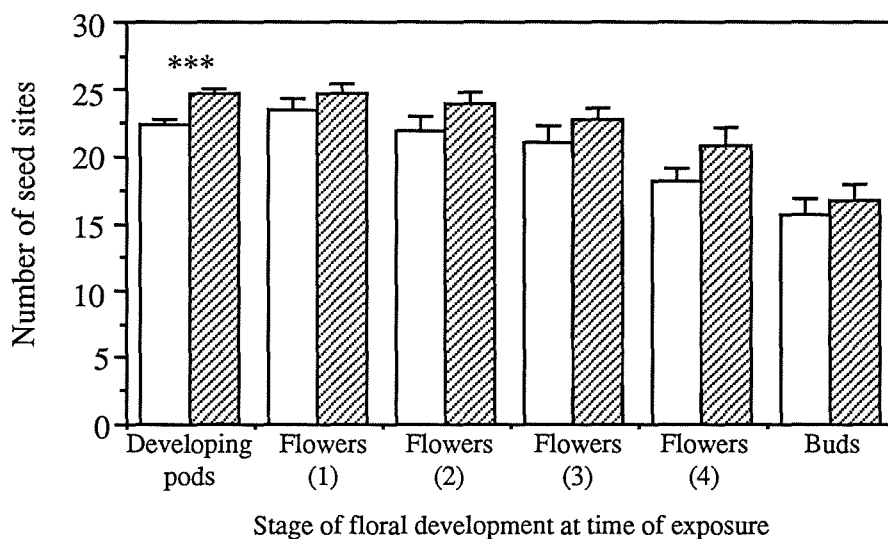
Table 4.15. Mean percentage of aborted, germinated and mature seeds per pod in *Brassica campestris* for floral sites which were exposed on four consecutive days between 20-23 DAS to filtered air (control) or 100 ppb ozone for 6 h d^{-1} as developing pods, open flowers (open on day 1, 2, 3 or 4 of exposure as indicated) or buds.

Stage of floral development at time of exposure	Treatment	% Aborted seeds	% Germinated seeds	% Mature seeds
Developing pods	Control	7.2	0.4	92.4
	Ozone	4.2	1.3	94.5
Flowers (1)	Control	7.3	0.3	92.4
	Ozone	8.1	0.1	91.8
Flowers (2)	Control	12.3	0.2	87.5
	Ozone	13.1	0.0	86.9
Flowers (3)	Control	22.6	0.0	77.4
	Ozone	43.4	0.0	56.6
Flowers (4)	Control	28.6	0.0	71.4
	Ozone	52.3	0.0	47.7
Buds	Control	42.5	0.0	57.7
	Ozone	68.7	0.0	31.3

Figure 4.18. Mean number of a) aborted, b) germinated and c) mature seeds per pod in *Brassica campestris* for floral sites exposed on four consecutive days between 20-23 DAS to filtered air (control) or 100 ppb ozone for 6 h d^{-1} as developing pods, open flowers (open on day 1, 2, 3 or 4 of exposure as indicated) or buds. □, control; ▨, ozone-treated. Single standard errors are shown. *, ** and *** indicate significance at P<0.05, P<0.01 and P<0.001. n=number of pods for each stage of floral development as shown in Table 4.14.
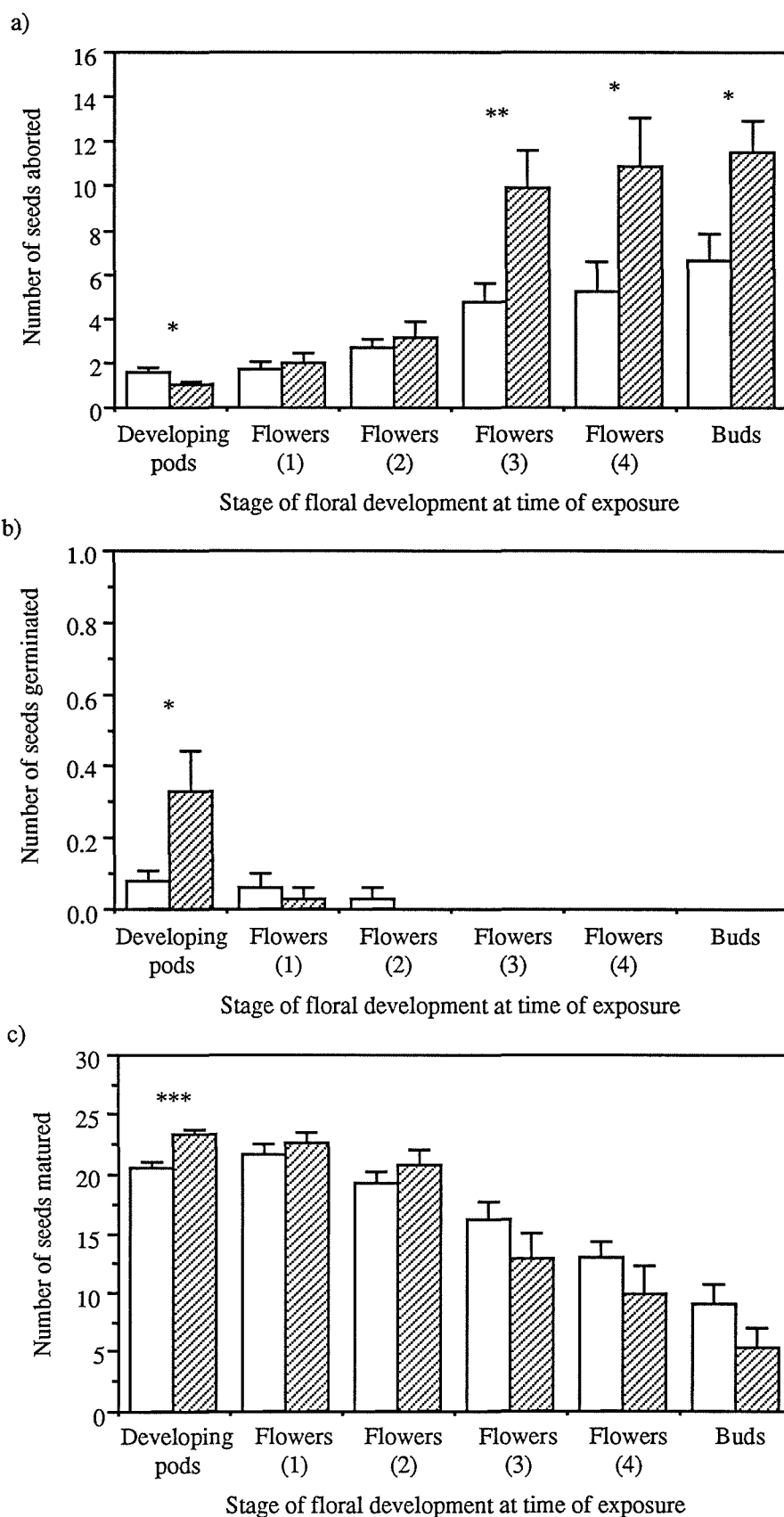
a)

b)

c)

The individual weight of mature seeds decreased acropetally in both ozone-treated and control plants, with the lightest seeds being produced in the youngest pods which had been exposed as buds (Fig. 4.19a). Ozone had no significant effect on individual seed weight except for a reduction in seeds from sites exposed as buds (P<0.001). The total weight of mature seeds per pod also decreased acropetally in both ozone-treated and control plants due to reductions in both the number of mature seeds per pod and individual seed weight (Fig 4.19b). Despite the existence of some significant effects of ozone on individual seed weight and the number of mature seeds per pod, there was no significant effect of ozone on the total weight of mature seeds per pod.

When averaged over the entire plant, exposure to ozone had no significant effect on either the number of mature seeds per pod or the total number of seeds per plant (Table 4.16). Since there was no significant effect of ozone on the number of pods per plant to influence seed number, the significant abortion of seeds observed in the later formed pods must have been offset by a corresponding reduction in abortion and an increase in the number of mature seeds produced in the earlier formed pods, particularly for those sites which had been exposed to ozone as developing pods. As there was no significant effect of ozone on the total number of mature seeds per plant or individual seed weight, the total weight of seeds per plant was also not significantly affected (Table 4.16).

Seed germination

For control plants, the timecourse of germination was similar for the fresh seeds harvested from sites which flowered before the fourth day of exposure; germination began within 24 h of sowing and by 72 h the seeds had all germinated (Figs. 4.20a-d). Germination was slower for seeds which developed in the later formed pods, although the majority of seeds had germinated by 168 h (Figs. 4.20e and 4.20f). Treatment with ozone increased percentage germination at 24 and 48 h for seeds from all sites except the buds at 24 h; this effect was significant at various times for all seed categories except the oldest pods which had been present during exposure. Seeds from the younger pods of ozone-treated plants achieved 100 % germination 72-96 h after sowing, up to 48 h before the control seed (Figs. 4.20e and 4.20f).

Seeds from all sites which flowered before the last day of exposure were stored at 4 °C for two years and then germinated to establish whether the timecourse of germination had changed (Fig 4.21). As seen for the fresh seeds from both ozone-treated and control plants, germination began within 24 h of sowing and was virtually complete within 72 h; however, the percentage of germinated seeds was greater after

191

Figure 4.19. Mean a) individual seed weight and b) seed weight per pod in *Brassica campestris* for floral sites exposed on four consecutive days between 20-23 DAS to filtered air (control) or 100 ppb ozone for 6 h d^{-1} as developing pods, open flowers or buds. Single standard errors are shown. *** indicates significance at P<0.001; n=10.

a)
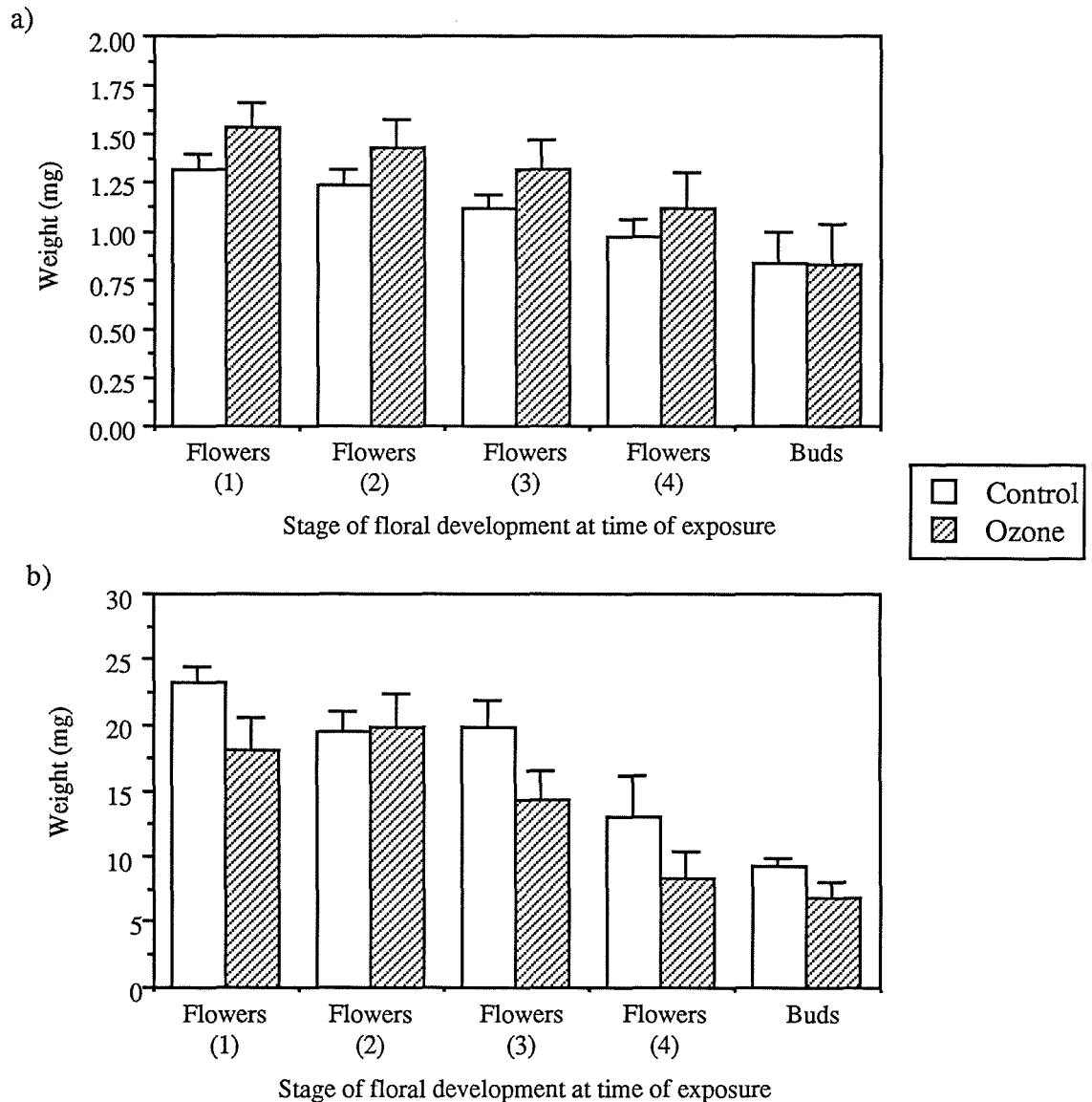
b)

Stage of floral development at time of exposure
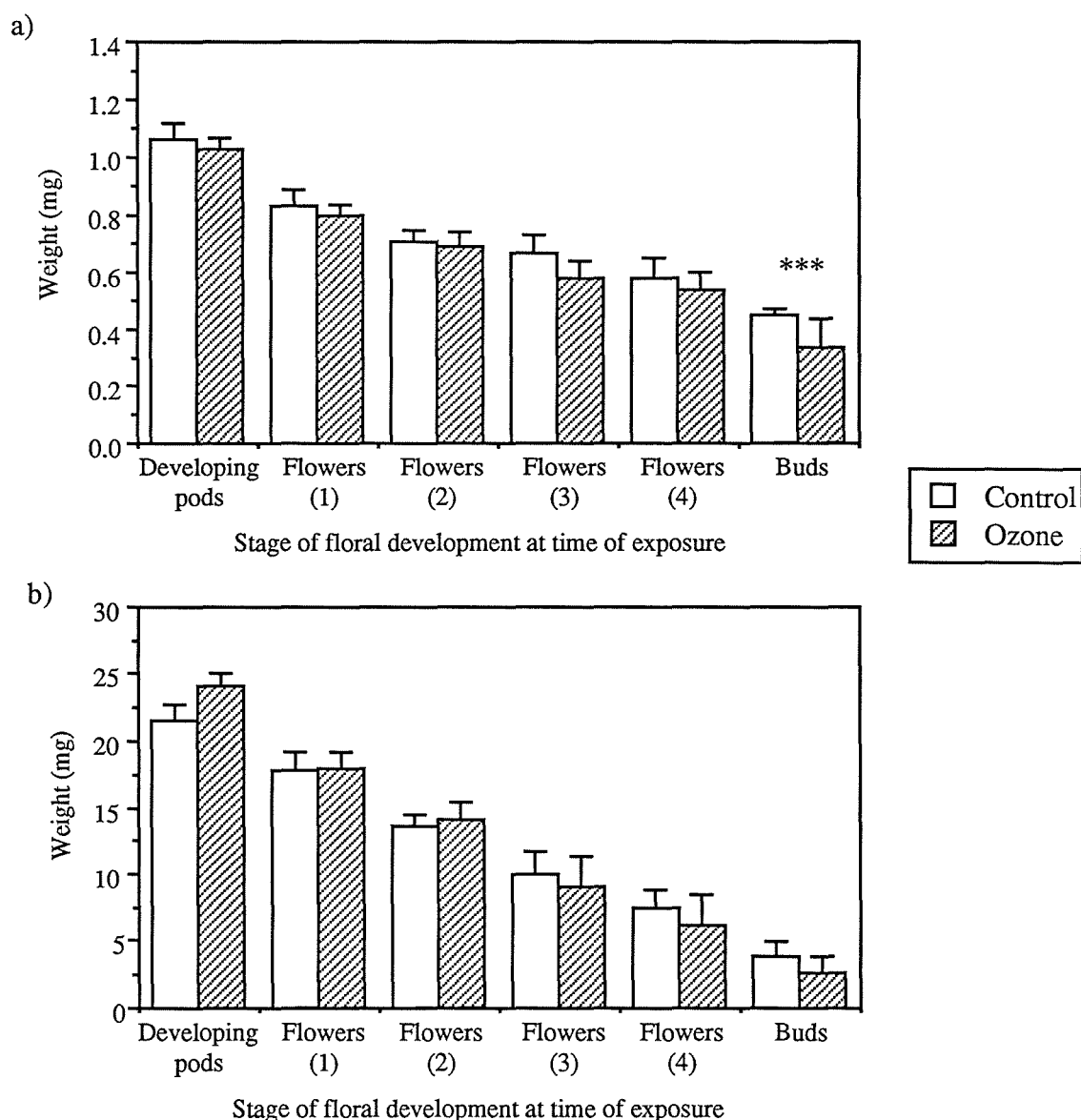
Table 4.16. Summary of seed yield from the terminal raceme of *Brassica campestris* after exposure on four consecutive days between 20-23 DAS to filtered air (control) or 100 ppb ozone for 6 h d^{-1}. Means and standard errors are shown; n=10. NS, non-significant.

	Control	Ozone	Significance
Number of pods	21.7 ± 1.59	22.1 ± 1.29	NS
Mature seeds per pod	18.9 ± 1.25	19.5 ± 1.32	NS
Mature seeds per plant	402.7 ± 27.72	425.1 ± 30.11	NS
Individual seed weight (mg)	0.88 ± 0.041	0.87 ± 0.035	NS
Seed weight per plant (g)	0.35 ± 0.027	0.36 ± 0.016	NS

Figure 4.20. Timecourse of germination for fresh seeds of *Brassica campestris* collected from floral sites exposed on four consecutive days between 20-23 DAS to filtered air (control) or 100 ppb for 6 h d^{-1} as developing pods, open flowers (open on day 1, 2, 3 or 4 of exposure as indicated) or buds. Solid circles, controls; open circles, ozone-treated. Double standard errors are shown. * and ** indicate significance at $P<0.05$ and $P<0.01$; n=4.

a) Developing pods

b) Open flowers (1)

c) Open flowers (2)

d) Open flowers (3)

e) Open flowers (4)

f) Buds

193
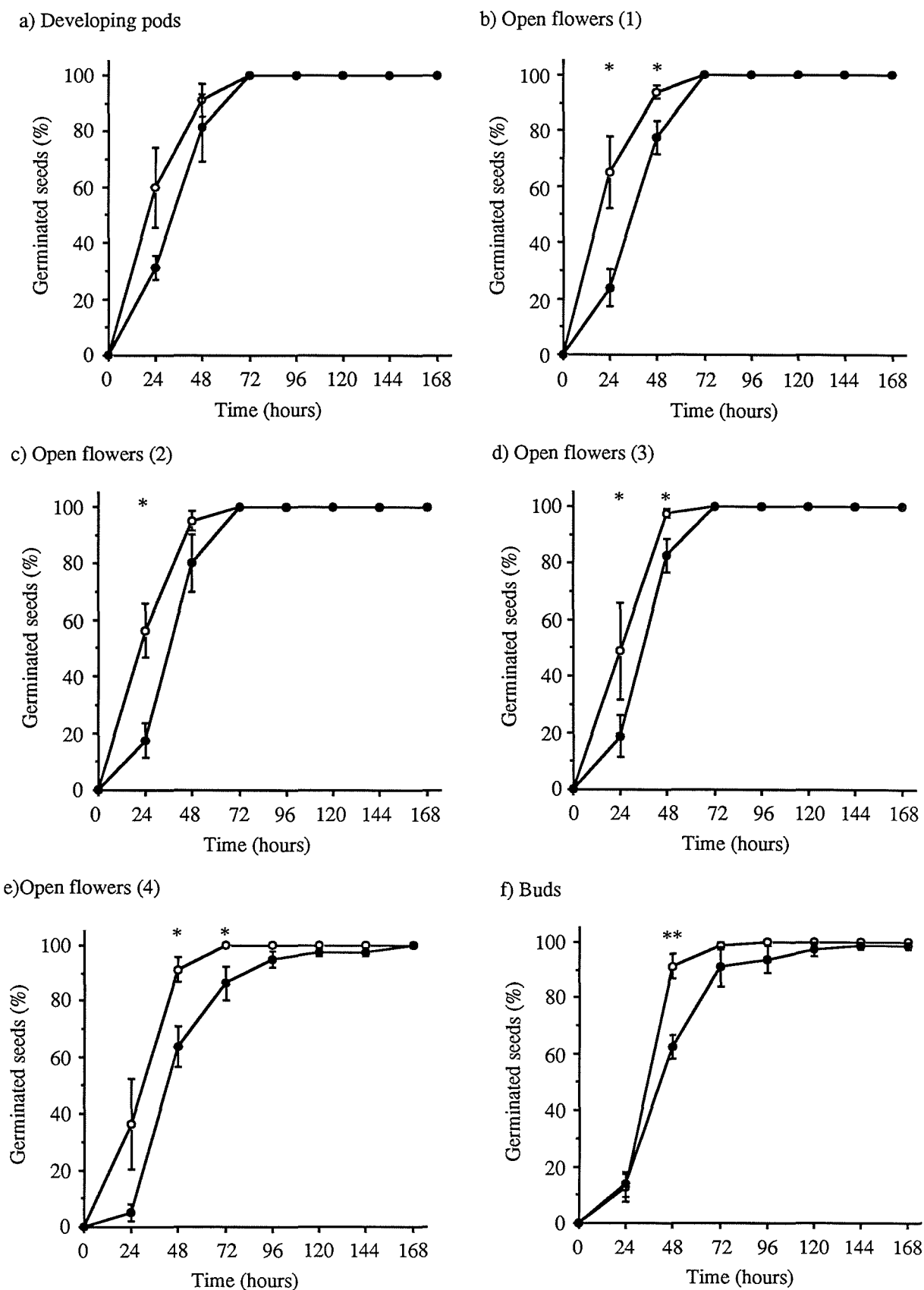
Figure 4.21. Timecourse of germination after two years of storage for seeds of *Brassica campestris* harvested from floral sites exposed on four consecutive days between 20-23 DAS to filtered air (control) or 100 ppb for 6 h d^{-1} as developing pods and open flowers (open on day 1, 2 and 3 of exposure as indicated). Solid circles, controls; open circles, ozone-treated. Double standard errors are shown. * indicates significance at P<0.05; n=4.

a) Developing pods
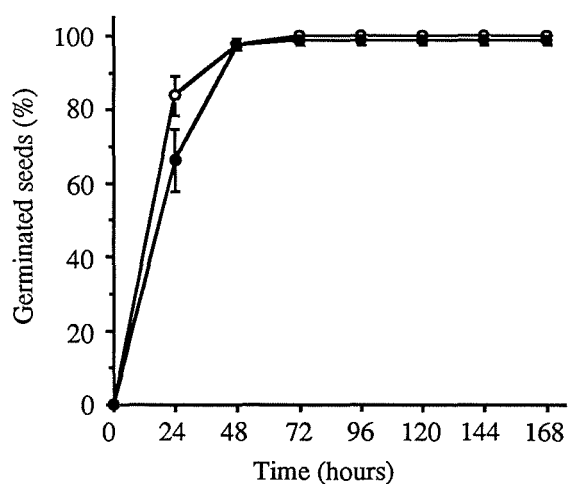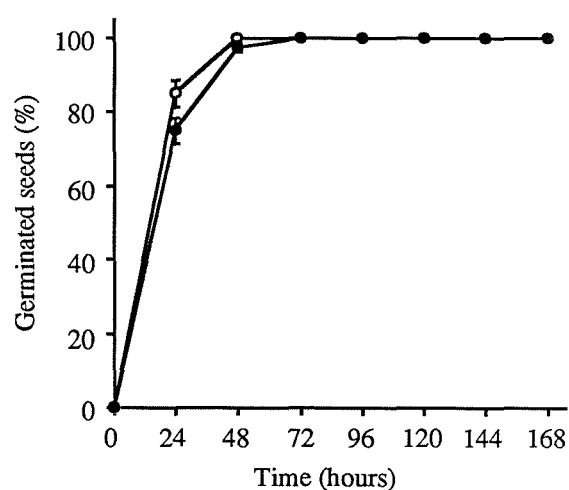
b) Open flowers (1)
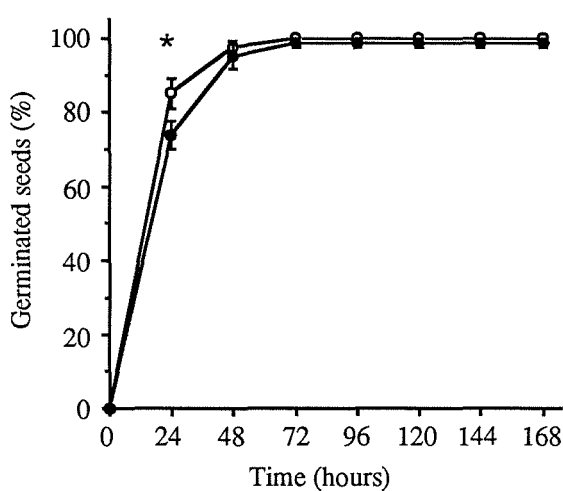
c) Open flowers (2)

d) Open flowers (3)
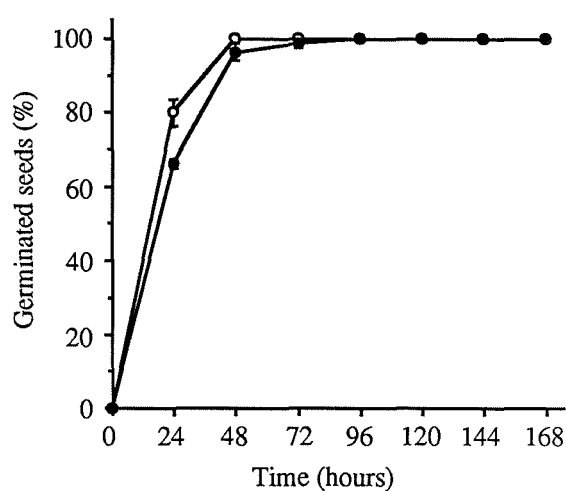
24 and 48 h than in fresh seeds. The stimulation of germination in seeds from ozone-treated plants was still evident at 24 h after sowing, although this effect was only significant for seeds from sites which had flowered on the second day of exposure (Fig. 4.21c).

Imbibition of seeds

Since there were significant effects of ozone on the rate of germination, the rate of imbibition was investigated to help determine the mechanisms underlying this response. Figure 4.22 shows that water uptake was rapid in seeds from both ozone-treated and control plants, with seed weight almost doubling within two hours. Although the final amount of water taken up after 4 h was not significantly different between treatments, the amount of water taken up by seeds from ozone-treated plants was significantly greater than controls during the first two hours of imbibition (P<0.05).

Seed colour

The mature seeds from sites which had been exposed to ozone or filtered air as developing pods were separated into colour categories as shown in Plate 4.3. Both treatments produced brown, light brown and dark brown seeds, but in addition, ozone-treated plants produced a number of yellow seeds. Table 4.17 shows that over 60 % of the seeds in both ozone-treated and control plants were brown. Of the remainder, more were light brown than dark brown or yellow. Individual seed weight varied between colour categories but in general the brown seeds from ozone-treated plants were heavier than those of the control plants. Yellow seeds were the lightest of all categories from ozone-treated plants and were similar in weight to the dark brown seeds, which were the lightest category in control plants.

4.3.1.4. Summary of the effects of single or multiple exposures to ozone on reproductive development

Direct exposure of the terminal raceme of *Brassica campestris* to 100 ppb ozone for 6 h either as a single fumigation 20 DAS or on four consecutive days between 17-20 DAS or 20-23 DAS had no significant effect on the development of the terminal raceme, the total number of floral sites produced, the timing of floral development or the number of floral sites which aborted. None of the stages of floral development present on the terminal raceme at 20 DAS was sensitive to a single 6 h exposure to 100 ppb ozone since no significant treatment effects were observed for the number of

Figure 4.22. Imbibition of *Brassica campestris* seeds collected from floral sites exposed on four consecutive days between 20-23 DAS to filtered air (control) or 100 ppb ozone for 6 h d^{-1} as developing pods. Solid circles, controls: open circles, ozone-treated. Double standard errors are shown. * indicates significance at $P<0.05$; n=3.
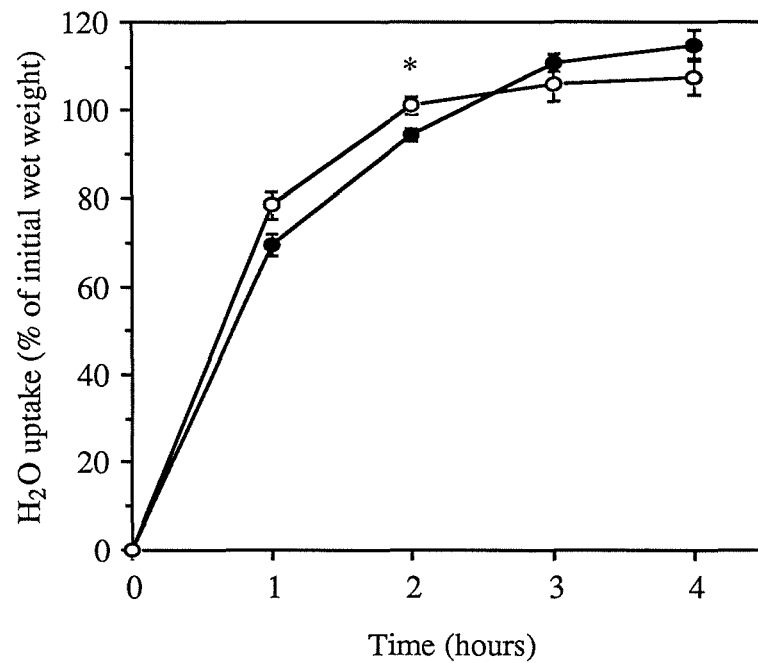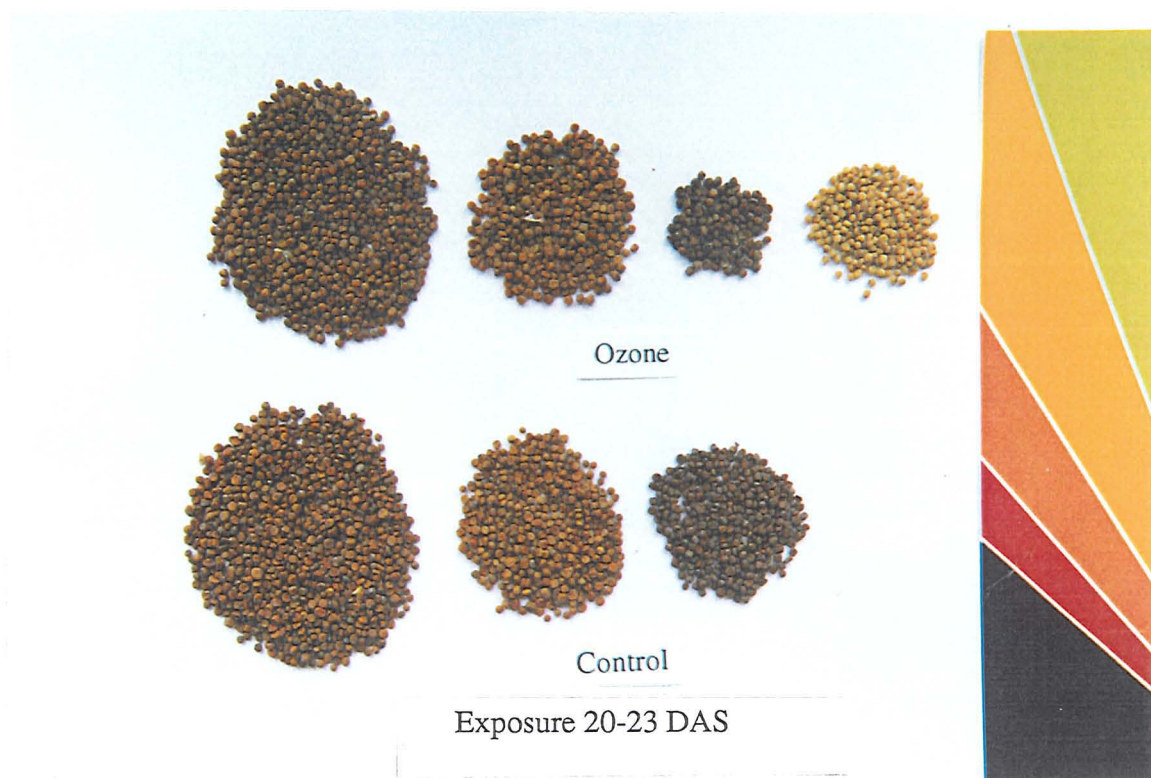
Plate 4.3.   Seed yield within various colour categories in *Brassica campestris*. The seeds were harvested from floral sites exposed on four consecutive days between 20-23 DAS to filtered air (control treatment) or 100 ppb ozone for 6 h d^{-1} as developing pods. Seed colours from left to right: ozone, brown, light brown, dark brown, yellow; control, brown, light brown, dark brown.

Table 4.17. Summary of mature seed yield within various colour categories in *Brassica campestris*. The seeds were harvested from floral sites exposed as devloping pods on four consecutive days between 20-23 DAS to filtered air (control treatment) or 100 ppb ozone for 6 h d^{-1}.

Treatment	Colour category				
Control	Brown	Dark Brown	Light Brown		Total, all colours
Number of seeds	1598	391	489		2478
Percentage of total	64.5	15.8	19.7		
Total weight of seeds (g)	2.000	0.441	0.630		3.071
Mean individual seed weight (mg)	1.251	1.128	1.288		
Ozone	Brown	Dark Brown	Light Brown	Yellow	Total, all colours
Number of seeds	1649	140	432	279	2500
Percentage of total	66.0	5.6	17.3	11.1	
Total weight of seeds (g)	2.349	0.217	0.620	0.315	3.186
Mean individual seed weight (mg)	1.424	1.550	1.435	1.129	

pods produced, the number and proportion of mature, germinated or aborted seeds per pod, individual seed weight or total seed weight per plant.

However, when the exposure period was extended to four days, there were significant effects on seed yield, although the nature and extent of these effects differed depending on the timing of ozone application. When the terminal raceme was exposed from 17-20 DAS, the total number of seed sites per pod was reduced and the numbers of germinated and aborted seeds per pod were increased, significantly reducing the number of mature seeds per pod. This effect occurred for all reproductive sites irrespective of their stage of floral development during exposure. Although individual seed weight was not significantly affected by this treatment, a reduction in the number of mature seeds per pod significantly decreased the total weight of seeds per plant. When the terminal raceme was exposed to ozone from 20-23 DAS, the total number of seed sites per pod was increased. As seen following exposure from 17-20 DAS, the number of aborted seeds per pod was increased under ozone, particularly towards the apex of the terminal raceme, and the number of germinated seeds was also increased in the oldest pods. However, the loss of seeds through abortion in later formed pods was compensated for by a reduction in the abortion of seeds in earlier formed pods, such that there was no overall effect on the number of mature seeds per plant. There was also no significant effect on individual seed weight after exposure between 20-23 DAS and thus no effect on total seed weight per plant.

The germination of seeds was unaffected by a single exposure but after a multiple exposure, either between 17-20 or 20-23 DAS, ozone increased the percentage of seeds which germinated within 24 h of sowing, particularly in seed from the younger pods. This effect on germination was still evident after the seeds had been stored for two years. Ozone had no effect on the extent of germination, which was usually 100 % for all pod categories. Tests showed that seeds from plants exposed to ozone between 20-23 DAS imbibed water more rapidly during the first two hours, but that the total amount of water absorbed was not significantly different from control seeds.

Seed colour was unaffected by a single exposure to ozone, with all plants producing brown seeds. A number of yellow seeds were also produced after both multiple exposures to ozone, while exposure from 17-20 DAS resulted in the production of a limited number of green seeds in ozone-treated plants.

4.3.2. Exposure of pollen to ozone

4.3.2.1. In vivo *exposure*

The percentage germination of pollen was lower, although not significantly, when pollen was germinated *in vitro* after *in vivo* exposure to 100 ppb ozone for 6 h (Table 4.18). This exposure also had no significant effect on pollen tube growth. When the concentration of ozone was increased to 120 ppb, ozone significantly reduced both pollen germination ($P<0.001$) and pollen tube growth ($P<0.001$). These results suggest that, for *in vivo* exposure of *Brassica campestris* pollen, the 6 h threshold concentration for damage lies between 100-120 ppb ozone.

4.3.2.2. In vitro *exposure*

Percentage pollen germination was not significantly affected following *in vitro* exposure to 120 ppb ozone, although germination was much lower than when pollen had been exposed *in vivo* (Table 4.18). Pollen tube length was also unaffected by ozone but was similar to that recorded following exposure in *vivo*.

4.4. DISCUSSION

Using a novel fumigation chamber design, the inflorescences of *B. campestris* were exposed to ozone in isolation from the vegetative structures, thus permitting the direct effects of ozone on reproductive development to be ascertained. In three separate experiments, the inflorescences of 10 plants were exposed to 100 ppb ozone for 6 h either in a single exposure or daily over four consecutive days during flowering. Exposure occurred when all stages of floral development were present on the terminal raceme, in order to determine whether particular stages of floral development were sensitive to treatment with ozone. By pollinating flowers with pollen from unexposed donors, the effects of ozone on the maternal structures could be established. In addition, pollen from *B. campestris* was exposed to ozone both *in vivo* and *in vitro*, in order to examine its effects on pollen germination and pollen tube growth.

The results showed that exposure to an elevated concentration of ozone can affect both the maternal and paternal structures. Although a single exposure of the inflorescence to 100 ppb ozone for 6 h had little effect on any of the aspects of reproductive development examined, daily exposure on four consecutive days between 17-20 DAS

Table 4.18. Mean percentage germination and tube length for pollen collected from *Brassica campestris* after exposure to filtered air (control) or air containing 100 or 120 ppb ozone for 6 h. Standard errors are shown. n=sample number. *** indicates significance at P<0.001. NS, non-significant.

		Pollen germination (%)				Pollen tube length (μm)			
Ozone concentration (ppb)	Exposure	n	Control	Ozone	Significance	n	Control	Ozone	Significance
100	*In vivo*	10	74.6 ± 4.34	66.6 ± 6.66	NS	200	91.3 ± 3.15	91.1 ± 2.10	NS
120	*In vivo*	10	75.6 ± 2.94	44.7 ± 5.18	***	200	83.6 ± 1.50	76.0 ± 1.36	***
120	*In vitro*	5	33.2 ± 8.69	30.2 ± 9.77	NS	100	87.4 ± 3.73	89.8 ± 4.88	NS

and 20-23 DAS had significant effects on seed yield, seed colour and germination, although the effects differed between exposures.

4.4.1. Pollen

In order to obtain normal seed set, it is necessary to have an adequate number of germinating pollen grains and growth of the pollen tube to the ovule. Pollen may be exposed directly to atmospheric pollutants during one or more stages of reproduction: pollen maturation in the anthers, pollen transfer in the air or by other vectors, and pollen germination and tube growth on the stigma and in the style (Wolters and Martens, 1987). Many studies have indicated that pollen germination and/or pollen tube growth can be adversely affected by air pollutants including sulphur dioxide (SO_2) (Masaru et al., 1976; Varshney and Varshney, 1981; O'Conner et al., 1987), fluorides (Sulzbach and Pack, 1972; Facteau and Rowe, 1977) and ozone (Feder 1968; Feder and Sullivan, 1969a; Masaru et al., 1976; Feder and Shrier, 1990; Hormaza et al., 1996). Most studies to date have considered the effects of SO_2 on pollen exposed either in vitro or in vivo, and have demonstrated that SO_2 can reduce pollen germination and/or pollen tube growth, although the sensitivity of pollen can vary between species and cultivars (Masaru et al., 1976; Varshney and Varshney, 1981; Dubay and Murdy, 1983; O'Conner et al., 1987). Fewer studies have been concerned with the effects of ozone on pollen; however, these have indicated that elevated concentrations of ozone can reduce pollen germination and/or pollen tube growth when pollen is exposed either in vivo on the anthers (Feder, 1968) or in vitro (Feder, 1968; Masaru et al., 1976; Hormaza et al., 1996). In the present study with B. campestris, exposure of pollen on the anthers to 100 ppb ozone for 6 h had no significant effect on pollen germination or tube growth in vitro (Table 4.18). A similar study by Bosac et al. (1993) also showed the pollen of Brassica napus cv. Libravo to be unaffected by treatment with 100 ppb ozone when exposed for 6 h either in vitro or in vivo. While there was little effect of 100 ppb ozone on the pollen of B. campestris, there was a significant reduction in both germination and pollen tube length when the concentration of ozone was raised to 120 ppb, suggesting that there is a 6 h threshold concentration for in vivo damage which lies between 100 and 120 ppb ozone. The effect of different ozone concentrations on pollen has been demonstrated previously by Hormaza et al. (1996), who exposed the pollen of various fruit (apricot, apple, nectarine, peach and pear) and nut (almond) tree species in vitro to 20, 40 or 60 ppb ozone for 4 h. Results showed that pollen germination and pollen tube growth were both reduced in the majority of species, and that the reduction was greater at the higher ozone concentrations. Feder (1981) also noted a similar type of response to increasing ozone concentrations in

pollen from tobacco and petunia cultivars and suggested that pollen from these species could be used as a bioassay for air quality.

Exposure of *B. campestris* pollen to 120 ppb ozone *in vivo* reduced both pollen germination and pollen tube growth (Table 4.18). Both processes are commonly affected by air pollutants (Cela Renzoni *et al.*, 1990) including ozone (Feder and Sullivan, 1969a). However, in other species, ozone may affect only pollen germination (Benoit *et al.*, 1983) or pollen tube growth (Riley and Feder, 1974). There are also reports of pollen being insensitive to ozone, even at high concentrations. For example, following *in vitro* exposure of lily (*Lilium longiflorum*) pollen to 2090 ppb ozone (2.09 ppm) there was little effect on germination or pollen tube growth (Masaru *et al.*, 1976). Similarly, Krause *et al.* (1975) showed that the pollen of *Lycopersicum esculentum* and *Petunia hybrida* was unaffected by an *in vivo* exposure to 800 ppb ozone for 6 h. This difference between species may reflect differences in ozone tolerance and/or the concentration of ozone applied and length of treatment (Hormaza *et al.*, 1996). The sites(s) of ozone action and the changes to pollen responsible for the observed effects on germination and pollen tube growth are unclear. In pollen exposed to SO_2, the reduction in germination and tube growth is thought to occur because absorption of SO_2 leads to acidification of stigmatal exudates or the artificial germination media used (Karnosky and Stairs, 1974). Increasing acidity has been shown to decrease pollen germination and pollen tube growth in several species (Cox, 1983; Van Ryn *et al.*, 1986; DuBay, 1988; Paoletti and Raddi, 1995). Ozone, however, does not acidify the germination medium (Bosac *et al.*, 1993) and in the present experiments, its effect on pollen was clearly direct, since the pollen was exposed on the anthers and not in a germination medium. Feder and Shrier (1990) suggested that ozone may interact with cell membranes. In a study by Harrison and Feder (1974), the germination of *Petunia hybrida* pollen was reduced by 80 % in an ozone-sensitive cultivar (White Bountiful) and by 15 % in an ozone-tolerant cultivar (Blue Lagoon) following exposure to 500 ppb ozone for 3 h. Detailed examination of the pollen revealed that over 50 % of the ozonated pollen from the ozone-sensitive cultivar had a peripheral band of cytoplasm which was free of all organelles except ribosomes. This contrasted with the pollen from the ozone-tolerant cultivar where fewer grains showed the cytoplasmic change. The authors suggested that the organelles migrate away from the plasma membrane in response to ozone and that this, in turn, affects germination and cell wall development of the pollen tube.

Despite significant effects on the pollen of *B. campestris* exposed to 120 ppb ozone *in vivo*, there was no significant effect of this concentration on pollen exposed *in vitro*, although the percentage germination of both ozone-treated and control pollen was

greatly reduced (Table 4.18). This reduction in germination may have resulted from high temperature stress or dehydration when the pollen was exposed in a dry state, since both are known to affect pollen germination and pollen tube growth (Corbet and Plumridge, 1985; Rao *et al.*, 1992). Rao *et al.* (1992) showed that exposure of *Brassica juncea* cv. Pusa Bold pollen to temperatures above 45 °C for 4 or 24 h significantly reduced germination and pollen tube growth when compared to pollen exposed at 22 °C. Corbet and Plumridge (1985) also reported that the germination success of *Brassica napus* pollen depended upon the humidity experienced by the grains *in vitro*, before contact with the germination medium. During exposure of pollen *in vivo*, the anthers may afford protection from particular environmental stresses (Krause *et al.*, 1975; Heslop-Harrison, 1992); this may explain why the number of germinating pollen grains was greater for *B. campestris* pollen exposed *in vivo* than for pollen exposed *in vitro*. The anthers may also protect some pollen grains from direct contact with ozone, thus preventing damage. Indeed, evidence suggests that ozone has a greater effect on pollen when it is exposed *in vitro* outside the protective environment of the anthers (Krause *et al.*, 1975). For *B. campestris* pollen exposed *in vitro*, the damage induced by potentially stressful environmental conditions may have reduced pollen germination to a greater extent than ozone exposure, such that the effects of ozone were not evident. Alternatively, dry pollen may be less sensitive to ozone exposure, as suggested by Riley and Feder (1974).

The ability of pollen to germinate is commonly used as an indication of pollen viability (Heslop-Harrison, 1992). Ideally this would be assessed on the stigma, but it is generally assumed that pollen which germinates and produces a pollen tube *in vitro* is likely to do so *in vivo* (Heslop-Harrison, 1992). For germination *in vitro*, an artificial medium is used to mimic that secreted by the stigma. It has been noted that the chemical constitution of the medium is an important factor in obtaining successful germination, and that individual species have specific chemical requirements which increase germination (Brewbaker and Kwack, 1963; Chiang, 1974). It cannot be guaranteed, therefore, that germination *in vitro* will reflect what occurs *in vivo* since both the physical and chemical environments will differ between the stigma and the artificial germination medium. A difference between *in vitro* and *in vivo* germination of pollen has been demonstrated for *B. juncea* cv. Pusa Bold following exposure of pollen to 75 °C for 4 h. Germination *in vitro* was decreased by 25 %, while pollen failed to germinate on the stigma and consequently to set seed (Rao *et al.*, 1992). A more realistic study of the effects of ozone on pollen germination and pollen tube growth in *B. campestris* would therefore require *in vivo* exposure of pollen and germination on the stigma. Studies are also required to assess the effects of a loss of viable pollen on final seed set. Indications from studies with other pollutants suggest that there may or

may not be a significant effect of pollen loss on seed set. For example, a reduction in the germination of pollen from *Lepidium virginatum* when exposed to SO_2 was seen to have little effect on seed set (DuBay and Murdy, 1983). Conversely, pollen from corn (*Zea mays*) exposed to simulated acid rain showed a decrease in germination and pollen tube growth and, as a consequence, a reduction in seed yield (Craker *et al.*, 1988).

4.4.2. Single exposure to 100 ppb ozone for 6 h

A single direct exposure of the terminal inflorescence of *B. campestris* to 100 ppb ozone for 6 h on 20 DAS had no significant effect on any of the measured parameters. This contrasts with a previous study by Bosac (1992) using the related species, oilseed rape (*Brassica napus*), in which a number of significant effects on reproductive development were recorded in two cultivars, Tapidor and Libravo, following exposure of the inflorescences to 100 ppb ozone for 6 h. In *B. napus*, an increase in bud abortion and abscission was evident 2 d after exposure in cv. Tapidor and 5 d after exposure in cv. Libravo. Despite a long term trend for the loss of reproductive sites in both cultivars following ozone exposure, the indeterminate reproductive growth habit of *B. napus* allowed for the production of new fertile sites to compensate for at least some of the ozone-induced losses (Bosac *et al.*, 1994). In *B. campestris*, there was no increase in bud abortion following exposure to ozone, although this occurred naturally in all plants towards the apex of the terminal raceme as the pod carrying capacity of each plant was established. In contrast to *B. napus*, the buds of *B. campestris* did not abscind, suggesting that this was an inherent difference between the two species. Ozone treatment had no significant effect on floral development (Fig. 4.2), the number of pods (Table 4.2), the number of seeds which matured, germinated and aborted in each pod (Fig. 4.4), mean or total seed weight (Fig. 4.5) or seed germination (Fig. 4.6) in *B. campestris*. This is in contrast to *B. napus*, in which, despite compensation for the loss of fertile sites, a single exposure to ozone resulted in significant effects on seed yield and seed quality in cv. Libravo (Bosac, 1992).

These results suggest that the terminal inflorescence of *B. campestris* is less sensitive than that of *B. napus* to a 6 h exposure to 100 ppb ozone. However, it is possible that the inflorescence of *B. campestris* was exposed at a particularly insensitive stage of development. In some species, the vegetative structures go through a stage when they are maximally sensitive to ozone (Dugger and Ting, 1970) and this may also be true of the reproductive structures. It is also known that some species can respond differently to various exposure regimes and the apparent insensitivity of *B. campestris* may have resulted from the exposure period being too short or the ozone concentration being too low. Indeed, when the exposure period was extended to four days, significant effects

on various aspects of reproduction were recorded, although these differed depending on the timing of exposure.

4.4.3. Exposure to 100 ppb ozone for 6 h on four consecutive days

During reproductive development, plants may be exposed repeatedly to elevated concentrations of ozone since ozone episodes can occur over a number of days during the spring and summer months (UKPORG, 1993). It is therefore important to understand how plants respond to repeated exposures. In this study, the inflorescences of *B. campestris* were exposed during flowering to 100 ppb ozone for 6 h d^{-1} on four consecutive days (approximately one quarter of the total flowering period) between 17-20 or 20-23 DAS. The results are discussed for each aspect of reproductive development examined.

4.4.3.1. *Inflorescence and reproductive site development*

The length of the terminal raceme was unaffected by four consecutive daily exposures to ozone between 17-20 or 20-23 DAS (Figs. 4.7 and 4.14). For many species, exposure to ozone may decrease plant height because of deleterious effects on plant growth, although there have also been reports of ozone stimulating plant height in tomato (*Lycopersicon esculentum*; Neil *et al.*, 1973) and carrot (*Daucus carota*; Bennett and Oshima, 1976). Bosac *et al.* (1994) noted that, following a direct exposure of the inflorescences of *B. napus* to 100 ppb ozone for 6 h, cv. Tapidor tended to develop a longer total raceme length and a greater number of raceme branches, and cv. Libravo a greater number of raceme branches, although these effects were not statistically significant. It was suggested that these responses may be methods whereby plants produce new fertile sites to compensate for those lost through increased bud abortion (Bosac *et al.*, 1994).

Neither of the multiple exposures to ozone had any significant impact on the number of reproductive sites initiated on the terminal raceme (Figs. 4.8 and 4.15). This contrasts with an exposure of the whole plant for 10 d prior to first flowering, in which the number of reproductive sites was significantly reduced (Section 3.3.2.2). This difference in response may have been due to different parts of the plant being exposed in these experiments or to differences in the timing and duration of exposure. The importance of the timing of exposure on reproductive development was demonstrated by Amundson *et al.* (1987), who showed that exposure of entire plants of winter wheat (*Triticum aestivum* L.) prior to anthesis can reduce kernel number, while exposure at anthesis, after kernel primordia have formed, tends to have a greater effect on kernel

206

weight. It is possible that, due to the rapid development of *B. campestris*, all of the floral initials were initiated prior to a direct exposure of the inflorescence and therefore the final number of reproductive sites was unaffected by treatment.

In addition to the natural abortion of buds which occurs in *B. campestris*, 18-28 % of the total number of reproductive sites aborted post-flowering, leading to the failure of pods to elongate and increase in girth (Figs. 4.8 and 4.15). This occurred on all plants irrespective of treatment, particularly towards the end of the flowering period when the maximum number of pods which each plant could sustain had been established. The proportion of reproductive sites which aborted, including buds and flowers, varied between 27-50 %, comparable to values reported for other *Brassica* species. For example, the percentage abortion of reproductive sites has been recorded as 40-50 % in *B. juncea* (Winfield, 1962), 55 % in *B. napus* cv. Zollerngold (Tayo and Morgan, 1975), 38 % in *B. napus* cv. Tapidor, and 51.5 % in cv. Libravo (Bosac, 1992). Treatment with ozone had no significant effect on bud or post-flowering abortion in *B. campestris*, in contrast to *B. napus*, where a single exposure was sufficient to increase abortion in cvs. Tapidor and Libravo and to sustain this loss until 25 d after exposure in cv. Tapidor (Bosac *et al.*, 1994).

The timing and duration of flowering are important aspects of reproduction since any alteration of these may affect the likelihood of successful pollination. In *B. campestris*, flowering had started before exposure began and therefore the time to first flowering could not be influenced by treatment with ozone. Previous research has shown that exposure to ozone can delay flowering in a number of species. Amundson *et al.* (1986) exposed plants of soybean (*Glycine max* cv. Hodgson) for 6.8 h d^{-1} to 10, 50, 90 or 130 ppb ozone for eight weeks. The results showed that flowering started 37 d after seedling emergence in plants exposed to 10 ppb ozone, while exposure to 50, 90 and 130 ppb ozone progressively delayed initial flowering until 40, 42 and 44 d after emergence respectively. Similarly, flowering in duckweed (*Lemna perpusilla*) was delayed by 4 d following a two week exposure to 100 ppb for 5 h d^{-1} (Feder and Sullivan, 1969b). Treatment with ozone appeared to have no visible effect on the morphology of *B. campestris* flowers, although this aspect was not investigated in detail. Analyses revealed that the flowers progressed through the normal pattern of opening at a similar rate to that of the control plants (Figs. 4.8 and 4.15). In *B. napus*, the pattern of bud and flower opening was also unaffected by direct exposure to ozone (Bosac *et al.*, 1994). The absence of an effect on flower opening in *B. campestris* contrasts to that observed when this species was exposed to ozone for 10 d prior to first flowering (Section 3.3.2.2), when fewer flowers opened on each day in ozone-treated

than in control plants, although this effect was not statistically significant. The possible reasons for this were discussed in Section 3.4.4.1.

Treatment with ozone had no significant effect on the initiation or abortion of reproductive sites and hence no effect on the total number of sites which developed into pods (Figs 4.9 and 4.16). This contrasts with exposure of whole plants for 10 d prior to first flowering, which reduced pod number, although not significantly (Section 3.3.2.2). The differing responses observed in these experiments may have been due to differences in the timing and duration of ozone exposure, although the effect on pod number may have been at least partly attributable to different effects on the supply of assimilates from the leaves to the inflorescence. Evidence that assimilate supply from the leaves is important in determining pod number comes from a study by Sharma and Ghildiyal (1992). These authors showed that, in *Brassica juncea* cv. Pusa Bold, defoliation decreased seed yield by decreasing the number of pods present, but had little effect on seed number per pod or seed size. In contrast, the shading of pods decreased seed yield by 95 % relative to control plants by decreasing seed number per pod and seed size. The origin of these responses is that the pods of *Brassica* species go through two phases of development. During the first phase, the pods are heterotrophic and their development depends on assimilate supplies from other parts of the plant. During the second phase, the pods become autotrophic as photosynthesis occurs in the pod wall (De Bouille *et al.*, 1989). When whole plants of *B. campestris* were exposed for 10 d prior to first flowering, fewer pods were produced on ozone-treated than on control plants, although this reduction was not statistically significant. This effect may have resulted from a decrease in assimilate supply from the leaves since ozone treatment was shown to reduce the net rate of photosynthesis (Fig. 3.5). Although fewer pods developed, the length and weight of individual pods was unaffected by treatment and seed number per pod was reduced only slightly. Therefore, the significant reduction in seed number per plant was mainly a consequence of the decrease in pod number. In contrast, direct exposure of the terminal raceme between 17-20 or 20-23 DAS did not affect the number of pods produced (Tables 4.7 and 4.13), possibly because assimilate supplies from the unexposed leaves were unaffected by treatment, although this was not verified. When the terminal raceme was exposed to ozone between 17-20 DAS, there was, however, a significant reduction in pod length of 16.3-29.0 %, particularly in sites which flowered after the first day of exposure (Table 4.8). This reduction in pod length may have resulted from a direct effect of ozone on pod growth or have been a consequence of these pods producing fewer mature seeds. Evidence that pod length may be at least partly determined by the number of seeds present, was reported by Pechan *et al.*, (1980), who showed that pod wall growth in *B. napus* only occurs adjacent to developing seeds and that the wall contracts in areas where seeds abort.

When the inflorescence of B. campestris was exposed to ozone between 20-23 DAS the effect upon seed number was smaller and there was no significant effect on pod length (Table 4.14). Despite the differing effects on pod length, individual pod weight was maintained, suggesting that the timing of ozone treatment had no significant effect on the supply of assimilates for pod growth.

4.4.3.2. Seed yield parameters

Although the number of pods was unaffected by treatment with ozone, the total weight of mature seeds per plant was significantly reduced following exposure between 17-20 DAS (Table 4.10). This was due to a reduction in the number of seeds per pod since individual seed weight was maintained. Exposure between 20-23 DAS had no significant effect on either the total number or weight of mature seeds per plant (Table 4.16). However, detailed analyses of the seed yield from both experiments revealed that ozone exposure significantly affected the number of mature, germinated and aborted seeds per pod at different positions on the terminal raceme (Figs. 4.11 and 4.18). The response was complex and differed between the two experiments.

In B. campestris, a number of seeds germinated within the pods prior to harvest, particularly in the older, more mature pods. Precocious germination has been recorded previously in B. campestris cv. Marco and B. napus cv. Lair (Brown and Dyer, 1991). When grown under field conditions, 0.8 % of the seeds in both of these cultivars germinated prematurely, a value similar to that observed in the present study with B. campestris. Four consecutive daily exposures to ozone between 17-20 DAS or 20-23 DAS significantly increased the number of germinated seeds per pod, with the increase being greater following exposure from 17-20 DAS (Fig. 4.11b) than exposure from 20-23 DAS (Fig. 4.18b). There have been no previous reports that ozone treatment may promote precocious seed germination, although V.J. Black (pers. comm.) noted an increase in the number of germinated seeds in B. napus following direct exposure of the inflorescence to 100 ppb ozone for 6 h. An increase in precocious germination from 0.8 % to 36.2 % was recorded by Brown and Dyer (1991) following pollination of B. campestris cv. Marco with pollen which had been stored at -20 °C for one year. In the present study, B. campestris was pollinated using fresh pollen collected from plants which had not been exposed to ozone, indicating that the increase in seed germination within the pod could not have resulted from effects on pollen. Since there is no dormancy mechanism in the seeds of B. campestris (Williams and Hill, 1986), it is possible that an increase in precocious germination occurred as a result of the seeds maturing more rapidly. Environmental factors are known to influence the rate of embryo growth; for example, Ahuja (1955) showed that the rate of

embryo development in *B. campestris* cv. Toria differed depending on the time of year when the plants were sown, with embryo development being more rapid when conditions were warmest and day length was at its longest. In *B. campestris*, the increased germination of seeds within the pod may have been due to a more rapid development of the embryo brought about by the slightly warmer conditions (0.1-0.2 °C) of the ozone-treatment chamber during exposure, although it is unlikely that such a small difference in temperature would have significant effects. Alternatively, there may have been an indirect effect of ozone on seed development. The hormone, ethylene, is thought to have a regulatory role in seed maturation and germination (Johnson-Flanagan and Spencer, 1994; Petruzzelli *et al.*, 1994; Ward *et al.*, 1995) and ozone treatment has been shown to stimulate the production of ethylene from plant tissues (Mehlhorn and Wellburn, 1987). This may provide a possible link between ozone treatment and the increase in seed germination within the pods of *B. campestris*.

The greater increase in precocious seed germination following exposure from 17-20 DAS suggests that the timing of exposure may be important in determining plant responses to ozone treatment. The different responses observed following exposure at 17-20 or 20-23 DAS may have been due to differing effects of ozone on seed maturation. Alternatively, ozone may have had similar effects on maturation in both experiments, but exposure from 17-20 DAS allowed more time for seed germination to occur prior to harvest.

A number of seeds aborted before maturity in each pod, with the proportion increasing acropetally. Abortion of both ovules and seeds is a natural process in *Brassica* species and is most prevalent in distal reproductive sites on earlier racemes and on the lower axillary branches (Tayo and Morgan, 1975; McGregor, 1981). Although this loss of seeds can cause considerable reductions in yield, little is known about the mechanisms which determine seed number per pod as previous research has suggested that competition for assimilates (Morgan, 1974) and changes in hormone levels (Bouttier and Morgan, 1992b) may both be involved. Following both single (Fig 4.4a) and multiple exposures (Figs. 4.11a and 4.18a), it was apparent that treatment with ozone increased the number of aborted seeds per pod, although this was only significant following repeated exposures. Following both multiple exposures, the increase in abortion was greatest in those sites which had been exposed during the bud phase of development, indicating that this stage may be particularly sensitive to ozone exposure. An increase in the number of aborted seeds was also evident for sites exposed as flowers during the multiple exposure experiments, although these increases were not always significant. Following exposure between 17-20 and 20-23 DAS, it was noted that the greatest significant increase in seed abortion was for sites which flowered on or

after 22 DAS irrespective of their stage of development during exposure. Thus, those sites showing the greatest abortion of seeds were exposed as buds between 17-20 DAS, and as open flowers and buds between 20-23 DAS. The position of the reproductive site on the terminal raceme may therefore be more important in determining the sensitivity of individual reproductive sites to ozone, than the particular stage(s) of development at which the sites are exposed. In support of this proposal that ozone sensitivity is determined by the position of the reproductive site on the terminal raceme rather than the stage of development at the time of exposure, a significant increase in the number of germinated seeds per pod was recorded for sites which flowered on or before 19 DAS in both multiple exposure experiments. These sites had been exposed as developing pods during exposure between 20-23 DAS and as flowers/ developing pods during exposure between 17-20 DAS. It may be possible that, irrespective of the timing of exposure, ozone affected the inflorescence in such a way that the amount of assimilates transported to younger pods was reduced and the amount to older pods increased, resulting in an increase in the abortion and germination of seeds respectively.

Clearly, when the terminal raceme of *B. campestris* is exposed directly to 100 ppb ozone for 6 h over four consecutive days, ozone can affect seed development by stimulating germination and abortion, particularly within the older and younger pods respectively. There are three ways in which ozone treatment may potentially affect seed development. Firstly, ozone could damage the embryo directly, either by damaging the embryo itself or the pollen and ovule which form the embryo. Secondly, ozone may stress the maternal plant e.g. through the disruption of photosynthesis or respiration, adversely affecting the type and quantity of resources provided to the seed. Thirdly, ozone may affect the synthesis and distribution of hormones, which are required for the successful development and maturation of the seed.

It is known that exposure to ozone can adversely affect pollen germination and pollen tube growth (Feder, 1968; Feder, 1981; Benoit *et al.*, 1983); indeed, in this chapter, pollen germination and pollen tube growth were shown to be reduced in *B. campestris* following exposure of pollen to 120 ppb ozone for 6 h *in vitro* (Table 4.18). Such a decrease in pollen germination could potentially reduce the chances of successful fertilisation and therefore seed set. In addition, treatment with ozone could bring about changes within pollen which may induce seed abortion, as has been recorded following exposure of *B. campestris* pollen to UV-B radiation (Demchik and Day, 1996). In the current investigation, *B. campestris* was pollinated with fresh pollen from unexposed plants so that the impact of ozone treatment on the maternal structures could be

investigated. Thus, the observed effect of ozone treatment upon seed development must have been mediated via effects on the maternal structures.

During development, the ovules and embryos of *B. campestris* are enclosed within the tissues of the ovary and are therefore shielded from direct contact with ozone. Because of the high reactivity of ozone with plant tissues (Runeckles and Chevone, 1992), it is unlikely that ozone could penetrate unreacted to the developing ovules and seeds. However, it is possible that the reaction products of ozone with plant metabolites may be transported within the plant and interfere with ovule and/or embryo development.

The supply of assimilates to the flowers and young pods is considered to be a major factor in regulating the number of pods and seeds which are retained in *Brassica* species, although assimilate supply from the pods may be more important for seed development than the supplies from the leaves (Khanna-Chopra and Sinha, 1976). Indeed, Singal *et al.* (1987), suggested that in *Brassica* species, almost 100 % of the seed dry matter comes from photosynthetic CO_2 assimilation within the pod. Experiments have shown that the number of seeds per pod and seed size can be reduced in various *Brassica* species by shading the developing pods, indicating that they are a major source of photosynthate for the developing seeds (Sheoran *et al.*, 1991; Sharma and Ghildiyal, 1992). In addition to fixing internal CO_2 respired from the developing seeds, the pods of *Brassica* species have stomata which allow them to assimilate external CO_2. Using radio-labelled carbon, Sheoran *et al.* (1991), showed that about ten times more CO_2 is fixed from the external environment in the pods of *B. campestris* than is assimilated internally, and that assimilates are translocated to the seeds within 30 s of being produced. Ozone could potentially affect seed survival in *B. campestris* by affecting the net photosynthesis of pods, either directly through effects on stomatal conductance or photosynthesis, or indirectly through adverse effects on the buds and flowers and hence the photosynthetic capacity of pods which subsequently develop. Ozone may also reduce net photosynthesis by stimulating respiration; for example, Todd (1956) showed a significant increase in the respiration of lemon fruit following repeated exposure to 100 ppb ozone for 10-15 h. Since pod weight was similar in ozone-treated and control plants of *B. campestris,* this suggests that the photosynthetic capacity of the pod was unaffected by treatment. However, it is thought that pod walls may compete more successfully than developing seeds for available assimilates (Mendham *et al.*, 1981), suggesting that pod weight may be maintained at the expense of seed growth when resources are limited.

There is evidence to suggest that pod number, seed number per pod and seed weight depend not only on nutritional factors but also on hormonal factors (Bouttier and

Morgan, 1992b). De Bouille *et al.* (1989) showed that abscisic acid (ABA), cytokinins and auxin are present at relatively high concentrations in the pods of *B. napus*, and that these fluctuate greatly depending on the developmental stage of the pod. In apices, buds and open flowers, ABA levels increase and those of cytokinins decrease during the later stages of flowering; these changes may contribute to the lower pod and seed set found in late-formed flowers (De Bouille *et al.*, 1989). Although there is little evidence to suggest that ozone can affect the levels of these hormones, several studies have shown that ozone may influence the production of ethylene (Mehlhorn and Wellburn, 1987; Mehlhorn *et al.*, 1991). Further studies are required to establish whether ozone can affect the synthesis of other plant hormones, their distribution within the plant or the sensitivity of particular plant tissues to these hormones, as well as how they may influence reproductive processes.

For plants exposed to ozone between 17-20 DAS, the total number of mature seeds per plant was reduced (Table 4.10), although not significantly, due to increases in both seed abortion and seed germination within the pod. Following exposure between 20-23 DAS, the increased abortion of seeds from sites exposed as open flowers and buds was compensated for by lower abortion and increased retention of seeds to maturity in sites which were fertilised prior to exposure. Thus, although effects on seed number per pod were observed at particular sites, the overall seed production of the plant was maintained (Table 4.16). It would appear therefore that *B. campestris* has the ability to compensate for a loss of seeds, but that this response is dependent upon the timing of exposure.

The indeterminate flowering habit of *Brassica* species provides the potential to recover from a loss of reproductive sites early in flowering, caused by severe environmental conditions or pests (Winfield, 1962; McGregor, 1981). Compensation can occur in one or more ways, for example, through the production of more flowers and hence more pods on existing racemes, new axillary racemes or secondary branches, more seeds per pod or by increasing seed weight (McGregor, 1981). Following exposure of *B. napus* to 100 ppb ozone for 6 h, Bosac *et al.* (1994) noted that new fertile sites were produced to compensate for at least some of the reproductive sites lost through increased bud abortion. Evidence from the current investigation suggests that the type of damage and mechanism by which compensation is achieved in *B. campestris* occurs at the level of seed sites rather than floral sites, as in *B. napus*. This difference between the two species may reflect their differing patterns of growth and development. Since *B. campestris* develops rapidly, producing about 30 reproductive sites in 14 days, this may limit the plant's ability to compensate for seed losses by producing additional fertile sites on the inflorescence. In contrast, *B. napus* has a longer reproductive period

and larger inflorescences, and therefore has the flexibility to compensate for the losses of reproductive sites by producing additional racemes or fertile sites on existing racemes.

Previous studies have suggested that *Brassica* species have a substantial capacity to recover from damage which occurs earlier rather than later during the flowering period (Winfield, 1962). This contrasts with *B. campestris*, in which compensation for the loss of seeds in the apical pods only occurred following the later exposure between 20-23 DAS. This difference in the ability to compensate was reflected by total seed weight being maintained following exposure between 20-23 DAS (Table 4.10) but being reduced, although not significantly, following exposure between 17-20 DAS (Table 4.16), with this reduction in total seed weight being the result of a decrease in seed number rather than mean seed weight. This reduction in total seed weight may have resulted from a decrease in the quantity of assimilates available to support seed production. Thus, with limited resources, compensation for a loss of seeds in apical pods, by retaining seeds which would otherwise have aborted naturally, may not have been possible. In contrast, the later exposure between 20-23 DAS may have had less impact on assimilate production, perhaps because reproductive development was more advanced, with the result that the available assimilates could have been redirected away from aborted seeds to support seed growth in the lower pods. Although compensation may have been mediated by resource availability, it may also have involved the transmission of chemical signals between pods; such effects might include increased synthesis and distribution of hormones within the inflorescence.

There has been very little work to examine the performance of seeds harvested from plants which have been exposed to ozone, although some studies on crop plants have examined the effects on seed quality because of their importance as human food source (Kress and Miller, 1983; Grunwald and Endress, 1988; Mulchi *et al.*, 1988; Fuhrer *et al.*, 1990; Scotti *et al.*, 1994). Bosac (1992) showed that direct exposure of the inflorescences to 100 ppb ozone for 6 h could affect seed quality, germination and seedling vigour in *B. napus* cvs. Tapidor and Libravo. Although effects on seed quality were not assessed in the present study, significant effects on seed colour and seed germination were recorded for *B. campestris* following four consecutive days of exposure between 17-20 and 20-23 DAS.

4.4.3.3. *Seed colour*

A single exposure to ozone at 20 DAS had no significant effect on seed colour, with brown seeds being produced by all plants. However, direct exposure of the terminal

raceme over four days resulted in plants producing a number of green and yellow seeds (Tables 4.11 and 4.17). Green seeds are recorded frequently in *Brassica* species; indeed, they are an economically significant problem since the high chlorophyll content of these immature seeds affects both the colour and flavour of the oil extract (Ward *et al.*, 1995). The chlorophyll levels in harvested rape seed are known to be affected both by the genotype involved and the environment in which it is grown (Ward *et al.*, 1995). In the present study, green seeds were produced only in those plants exposed to ozone between 17-20 DAS (Table 4.11). Since no green seeds were produced following either the single exposure or exposure between 20-23 DAS, this suggests that the duration and/or timing of exposure was important in influencing seed maturation. It is possible that earlier multiple exposure to ozone slowed the maturation process, although the presence of green seeds does not necessarily reflect slower maturation. As the seeds of *Brassica* species mature, seed chlorophyll is degraded and the moisture content of the seed falls (Johnson-Flanagan and Spencer, 1996). Ward *et al.* (1995) showed that there is a highly significant positive correlation between the moisture content of ripening rape seed and its chlorophyll level. Drought during hot, dry weather can lead to rapid dehydration, and with moisture unavailable to support normal metabolic processes, the seeds fail to ripen fully, resulting in a high seed chlorophyll content at harvest. In rapid-cycling *B. campestris*, green seeds may have resulted from a more rapid drying of ozone-treated inflorescences, although there was no evidence to suggest that desiccation of ozone-treated plants was more rapid than in control plants. The de-greening of *Brassica* seeds has been correlated with the activity of chlorophyllase and peroxidase enzymes (Johnson-Flanagan and Spencer, 1996) as well as with endogenous ethylene evolution (Ward *et al.*, 1995; Johnson-Flanagan and Spencer, 1994, 1996). Although the relationship between these enzymes and ethylene has not been clearly established, ozone could potentially influence seed de-greening by affecting either ethylene evolution or enzyme activity.

As observed following exposure of whole plants for 10 days prior to first flowering (Section 3.3.2.2), a number of yellow seeds were produced following direct exposure of the inflorescence between 17-20 or 20-23 DAS (Tables 4.11 and 4.17). If the mechanisms behind such a response were similar for each experiment, this would suggest that the response was mediated via effects on the reproductive structures. As already discussed in Chapter 3 (Section 3.4.4.4), the production of yellow seeds may have resulted from an effect of ozone on pigment production or seed maturation, such that seeds were harvested before pigment deposition within the seed coat had been completed.

4.4.3.4. *Seed germination and imbibition*

Although there was no significant effect of ozone exposure on the ability of seeds to germinate, there was a consistent stimulation of germination at 24-48 h after sowing following both single (Fig. 4.6) and multiple exposures (Figs. 4.13 and 4.20), and this effect was evident for up to two years after harvest (Fig. 4.21). This is in contrast to seeds from plants in which the vegetative and reproductive structures were both exposed to ozone for 10 d prior to first flowering (Section 3.3.2.2), for which germination was significantly delayed by 24-48 h after sowing. Similarly Bosac (1992) noted that germination was delayed relative to the controls in *B. napus* following ozone exposure. Although the rate of germination was shown previously to differ between seed colour categories in *B. campestris* (Section 3.3.2.2), this could not adequately explain the advancement of germination observed in the present experiments since there was no effect on seed colour following a single exposure to ozone. This advancement of seed germination may be related to the increase in precocious seed germination observed within the pod. The seeds of *B. campestris* have no dormancy mechanism which can be affected by ozone-treatment and imbibition studies showed that water uptake was similar for a random sample of seeds from both ozone-treated and control plants (Fig. 4.22), suggesting that the effects were not due to differences in the rate or extent of water uptake. It has been shown for a number of species that treatment with ozone can affect seed quality by altering the relative amounts of protein, carbohydrates and oils within the seed (Kress and Miller, 1983; Grunwald and Endress, 1988; Mulchi *et al.*, 1988; Bosac, 1992), which may have consequences for seed germination. Further studies would be required to determine the impact of ozone treatment on seed quality in *B. campestris*.

4.4.4. Summary

This study clearly shows that the reproductive structures of *Brassica campestris* are sensitive to ozone. The results showed that while a single 6 h exposure to 100 ppb ozone had no significant effect on reproductive development, multiple exposures over four consecutive days increased the abortion of seeds in apical pods and the precocious germination of seeds in older pods. However, the impact on final seed yield was dependent upon the timing of exposure; thus seed yield was significantly reduced following exposure during the early flowering phase, but was unaffected following exposure during the later stages of reproductive development. The compensation for seed losses in more mature plants resulted from their ability to retain naturally aborting seeds in older pods. Nonetheless, single and multiple exposures to ozone both reduced the rate of germination of harvested seeds and multiple exposures also increased the

proportion of yellow and green coloured seeds produced. The pollen from *B. campestris* was also shown to be sensitive to ozone since a 6 h *in vivo* exposure of pollen to 120 ppb ozone significantly reduced both pollen germination and pollen tube growth.

CHAPTER FIVE - THE DIRECT EFFECTS OF OZONE ON THE REPRODUCTIVE DEVELOPMENT OF *PLANTAGO MAJOR* L.

5.1. INTRODUCTION

The previous chapter demonstrated that the reproductive development of *Brassica campestris* may be affected directly by exposure to ozone. The main effects were increases in seed abortion and germination within the pods and in the rate of germination of harvested seeds. Depending upon the timing of exposure, the plants possessed the capacity to compensate for seed losses resulting from abortion and precocious germination. However, compensation was not brought about by the production of additional floral sites on the indeterminate inflorescence, but rather by the plants being able to regulate the number of seeds which aborted in older pods. Thus seed yield was maintained without any alteration in the number of pods produced. To investigate whether similar responses occur in species which exhibit less flexibility, the experiments reported in this chapter examined the direct effects of ozone on the reproductive structures of *Plantago major*.

Plantago major differs to *B. campestris* in that the reproductive structures are more determinate in their growth habit. That is to say, once the floral initials have been established and the reproductive spike has begun to elongate, the plants lose their ability to produce additional floral sites because the apical meristem is transformed into the terminal reproductive site. Thus, it can be hypothesised that ozone treatment may have a greater effect on the seed yield of *P. major,* at least within individual spikes, than in *B. campestris* because the former has a more limited capacity to compensate for damage to the reproductive structures. Using the purpose-designed exposure chambers described in Chapter 2, individual spikes were exposed to ozone during flowering or seed filling. The impact of ozone on the reproductive structures of *P. major* was determined by examining in detail the responses of the different seed yield parameters within individual spikes.

Previous studies have shown that populations of *P. major* differ in their vegetative sensitivity to ozone (Reiling and Davison, 1992d; cf. Chapter 3). Reiling and Davison (1992d) suggested that this variation in sensitivity may be related to the concentrations of ozone encountered in the field, since populations which routinely experienced high concentrations appeared to be more resistant than those which had experienced low concentrations. Further studies demonstrated that the seed production of various *P. major* populations was reduced following exposure to ozone (Reiling and Davison,

218

1992b; Pearson *et al.*, 1996), although the responses in terms of reproductive effort (seeds produced per gramme dry weight) differed between populations (Pearson *et al.*, 1996). Since whole plants were exposed, these studies did not allow the direct effects of ozone on the reproductive structures to be separated from those mediated via damage to the vegetative parts. The present study therefore aimed to investigate whether the direct effects of ozone on reproductive development differed between populations. The studies reported here of four populations with differing vegetative sensitivities to ozone, were also intended to establish whether reproductive sensitivity could be linked to the vegetative sensitivity of each population, and whether these populations differed in their ability to compensate for ozone-induced injury to the reproductive structures.

The impact of ozone on pollen germination and pollen tube growth has been examined in a number of species, both *in vitro* (Masaru *et al.*, 1976; Hormaza *et al.*, 1996) and *in vivo* (Feder, 1968). The limited number of studies which have investigated the effects of ozone on pollen from different cultivars suggest that the sensitivity of pollen to ozone may differ within individual species (Harrison and Feder, 1974; Hormaza *et al.*, 1996). The present study provided the ideal opportunity to investigate whether the sensitivity to ozone of pollen from *P. major* differed between populations.

5.2. MATERIALS AND METHODS

5.2.1. Ozone exposure of reproductive spikes

5.2.1.1. *Exposure during flowering*

The flowers of *Plantago major* are small (2-4 mm diam.), densely packed on spikes and open in acropetal succession, with the stigma of each flower emerging before the anthers. For the purpose of this study, the flowering period for each spike was defined as the time taken from emergence of the stigma in the first developed flower to anther emergence in the last developed flower; thus by the end of the flowering period, the earliest formed flowers on the spike were undergoing seed filling.

The first reproductive spike to develop on each plant was exposed to ozone throughout the flowering period in order to assess the impact of a direct exposure to ozone during flowering on subsequent seed yield. Each of the four populations of *P. major* was exposed separately to a particular dose of ozone as shown in Table 5.1. The plants from each population selected for exposure were those in which the first spike began to flower on the same day (first spike flowering). The number of plants chosen for each exposure varied from 5 to 9 because of variability between plants in the

Table 5.1. Summary of each fumigation undertaken to establish the direct effects of ozone on the first reproductive spike during flowering in four populations of *Plantago major*. n=number of plants.

Fumigation	Population	Days after sowing when fumigation started	Ozone dose (ppb * h d^{-1} * d)	n
1	High Low	35	70 * 7 * 7	5
2	Lullington	50	120 * 7 * 9	5
3	Penicuik	58	120 * 7 * 9	5
4	Sibton	52	120 * 7 * 9	6
5 a b c	High Low	37 51 60	120 * 7 * 9	5 9 6

timing of flowering. The first spike of each plant was placed within one of two exposure chambers as shown in Plate 5.1 and exposed to filtered air (control treatment) or to ozone for the duration of the flowering period. The stage of spike development at the start and end of the flowering period is illustrated in Plates 5.2a and 5.2b. The spikes remained within the chambers between daily exposures to provide consistent environmental conditions and prevent cross-pollination of treated and untreated flowers. The duration of flowering of individual spikes varied from seven to nine days; however, to ensure that all spikes experienced a similar number of days of exposure to ozone, exposure continued until flowering of the first spike had been completed in all plants. The plants were then returned to the controlled environment growth cabinet until harvest.

Exposure to 70 ppb ozone

In a preliminary study, the first spikes of 10 plants from the High Low population, 5 as treatment and 5 as control, were exposed for seven days to a target concentration of 70 ppb ozone for 7 h d^{-1} from 35 DAS (Fumigation 1, Table 5.1). This exposure regime had been used in earlier experiments with vegetative plants (Section 3.2.3). At the end of the seven day exposure, flowering had been completed in all plants.

Exposure to 120 ppb

Additional fumigations were carried out for each population of *P. major*, in which the first reproductive spikes were exposed for nine days to 120 ppb ozone for 7 h d^{-1}. The concentration of ozone was increased from the target concentration of 70 ppb to 120 ppb due to initial problems with the ozone generation system. Because the period of flowering varied between 7-9 days for each spike, the exposure period was extended to 9 days to ensure that all spikes experienced similar ozone exposures.

For each population, exposure started from a different number of days after sowing (DAS) due to variability in the timing of flowering both between and within populations. In order to assess whether timing of first spike flowering, with respect to days after sowing, influences the response to ozone, the first reproductive spikes of plants from High Low were exposed for nine days to a target concentration of 120 ppb ozone for 7 h d^{-1} from the start of flowering at 37, 51 and 60 DAS (Fumigations 6a-c, Table 5.1).
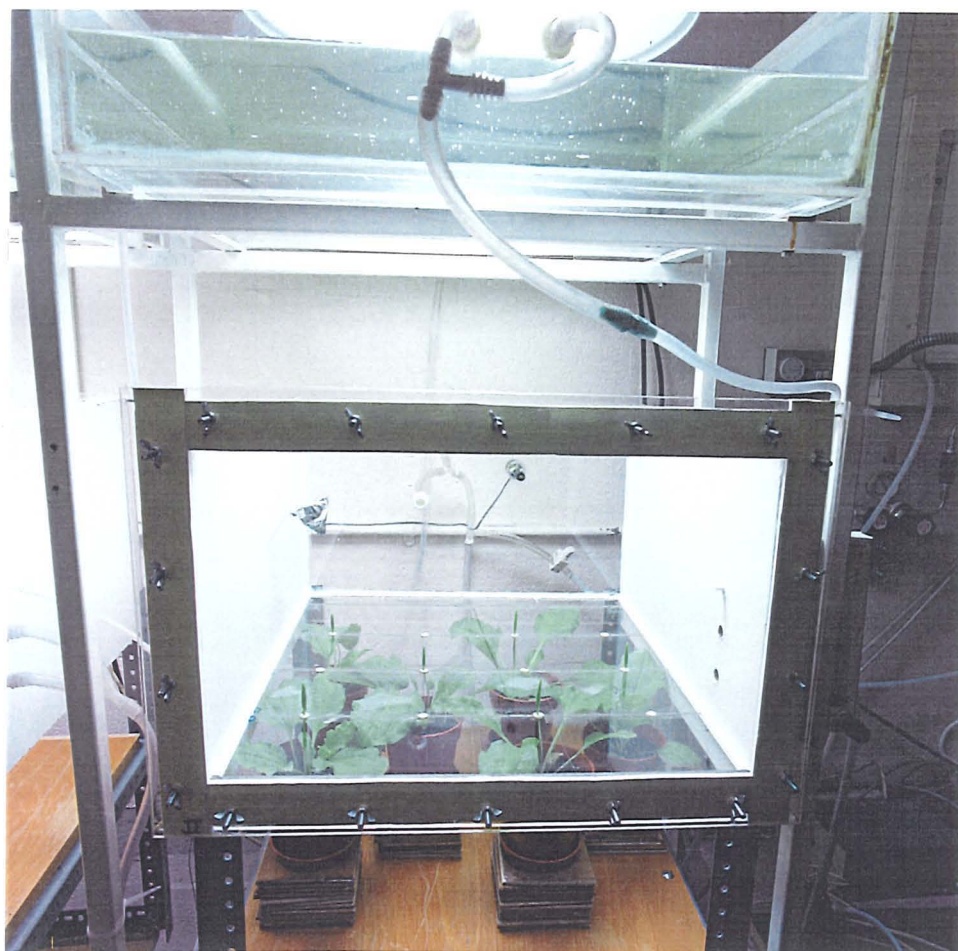
Plate 5.1. Spikes of *Plantago major* being introduced into the exposure chamber.
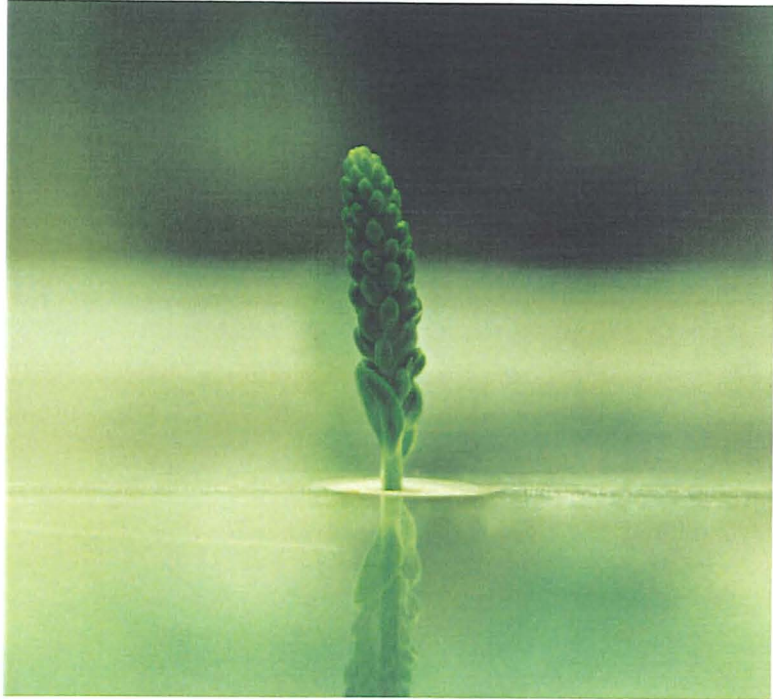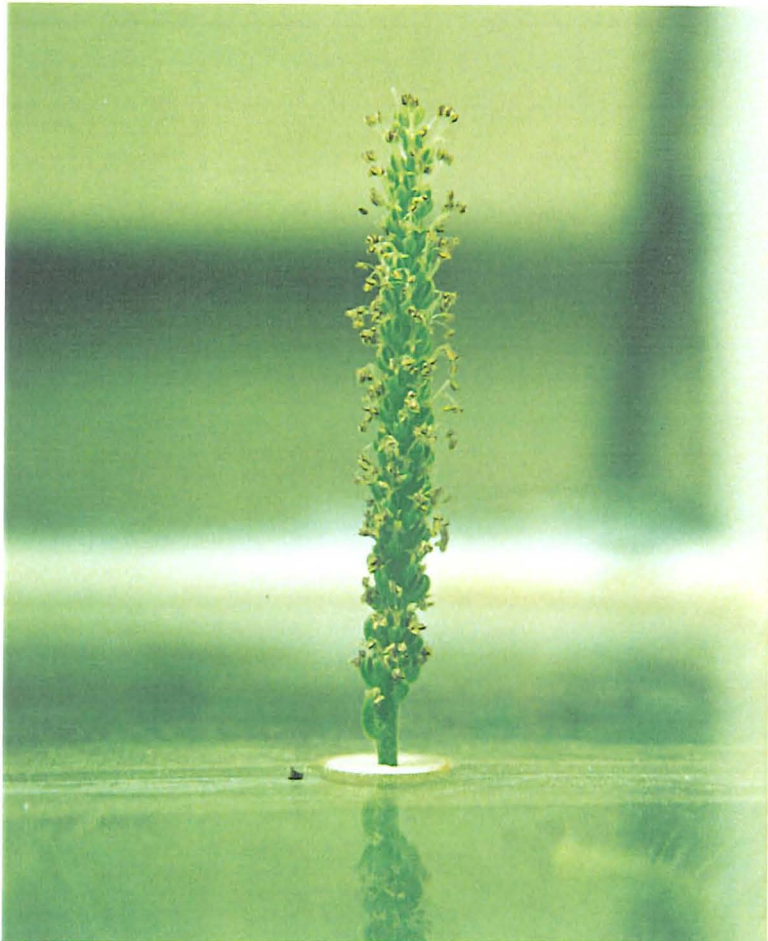
Plate 5.2. The stage of spike development in *Plantago major* at a) the start and b) the end of the flowering period.

5.2.1.2. Exposure during seed filling

Under the controlled environment growth cabinet conditions experienced by *P. major*, the period between the end of flowering and seed maturity lasted approximately 35 to 40 days in each population. In order to determine the effect on final seed yield of exposure to ozone during the period of seed development, the first spikes of five plants from the High Low population were exposed for 14 days to filtered air (control treatment) or to a target concentration of 120 ppb of ozone for 7 h d^{-1} from 56 DAS, after the completion of flowering. The plants were then returned to the growth cabinet for a further 21-26 days until the seeds reached maturity.

5.2.1.3. Exposure of the entire plant during flowering

In order to assess the combined direct and indirect effects of ozone on seed production, 6 plants from the High Low population were placed within each chamber so that the whole plant, comprising the vegetative and reproductive components, would be exposed. The plants were exposed from first spike flowering (35 DAS) for nine days to filtered air (control treatment) or to a target concentration of 120 ppb ozone for 7 h d^{-1}.

5.2.2. Seed harvest

After exposure, the plants were returned to the controlled environment growth cabinet to allow seed filling and ripening. The first (exposed) and second (unexposed) spikes to develop on each plant were harvested when the seed matured. Maturity was visually recognisable when both the spike and scape became desiccated and changed colour from green to brown. The fruit of *P. major* is a capsule containing a variable number of seeds from one to twenty. Starting from the base of the spike, each capsule was opened in turn and the number of seeds present was counted. Seeds collected from each group of 20 capsules along the spike were stored in brown paper envelopes before being weighed to determine the total weight of seeds per spike and the average individual seed weight per spike.

5.2.3. Seed germination

To determine whether ozone affected the germination of seeds which had developed at different positions along the spike, seeds from the first 20 (lower) capsules and the last 20 or less (upper) capsules of both exposed and unexposed spikes were tested for germinability. Directly after harvest, 20 randomly chosen seeds from both the lower

and upper capsules of each spike were placed in a petri dish containing 1 % agar. The petri dishes were then placed within the controlled environment growth cabinet under the conditions set for *P. major* (Section 2.4.2.1), and the number of seeds which germinated were counted at 24 h intervals over the following 10 days. Seeds were regarded as having germinated if the radicle had emerged through the testa.

5.2.4. Exposure of pollen to ozone

5.2.4.1. In vitro *exposure*

For each of the four populations of *P. major*, pollen was collected from c. 20 of the most newly dehiscent flowers on the first spike of at least five plants, mixed together and then dusted onto 10 dry microscope slides (5 treatment and 5 control). To allow comparison with the response of pollen from *Brassica campestris*, pollen from each population of *P. major* was exposed in a single fumigation to either 100 ppb (2 replicates) or 120 ppb ozone for 6 h (1000-1600 h) or to filtered air in the control treatment. After exposure, the pollen on each slide was transferred onto 50 μl drops of pollen germination medium (Brewbaker and Kwack, 1963) consisting of;

10 % sucrose
100 ppm H_3BO_3
300 ppm $Ca(NO_3)_2.4H_2O$
200 ppm $MgSO_4.7H_2O$
100 ppm KNO_3
All made up in deionised water.

The microscope slides were then placed on moist filter paper in petri dishes to maintain a high relative humidity and placed in the CEGC under the conditions set for *P. major,* where the pollen was allowed to germinate for 24 h. A permanent slide of the germinated pollen was made as described in Section 3.2.2.2 and 200 pollen grains from a central transect through the slide were scored for germination. Pollen was regarded as having germinated if the pollen tube was equal or greater in length to the diameter to the pollen grain (Benoit *et al.*, 1983). The length of the pollen tube was also measured for 20 randomly chosen grains per slide.

5.2.4.2. In vivo *exposure*

The first spikes of 5 plants from the High Low population were exposed to filtered air (control treatment) or to 120 ppb ozone for 6 h (1000-1600 h) during flowering at 40 DAS. After exposure, pollen was collected from the most recently dehiscent anthers of each spike. The pollen from each spike was germinated and measurements were made as described above.

5.2.5. Data analysis

Seed yield parameters

For all experiments, the following variables were recorded for both the first and second spikes of each plant at harvest:

1. Number of capsules per spike.
2. Number of seeds per capsule.
3. Total seed weight per spike.

From these data, the total number of seeds per spike and mean individual seed weight were calculated. For each spike, the total number of capsules was divided equally into three ($\pm$ 1 capsule) to facilitate comparison of seed number per capsule from the lower (earliest flowering), middle and upper thirds of each spike.

Since each population was exposed independently to ozone, direct statistical comparison was not possible; a separate analysis was therefore made between the treated and control plants of each population. For both the exposed and unexposed spikes of each population, sample means for the total number of capsules, total number of seeds, total seed weight and individual seed weight were compared using an unpaired two tailed t-test to determine significant effects ($P<0.05$). A comparison was made between treatments of the number of seeds per capsule, both for the entire spike and for each of the three sections along the spike, using the value from each capsule on each plant as a single replicate. This made the sample number large ($n>30$), and to test for significant differences between treated and control means d, the 'standardized normal deviate' was calculated (cf. Section 3.2.4.2).

Seed germination

The mean percentage germination of seeds from the lower and upper capsules of both exposed and unexposed spikes was analysed independently for each population. A comparison of mean percentage germination for seeds from treated and control plants was made at each time interval using an unpaired, two tailed *t*-test, after transforming the percentage values using an arc sine transformation (Gomez and Gomez, 1984). From each timecourse of germination, the mean time taken for 50 % of the seeds to germinate (T_{50}) was calculated.

Pollen germination and tube length

In each of the experiments in which pollen was exposed *in vitro*, all four populations of *Plantago major* were examined simultaneously to allow statistical comparison of pollen germination and tube growth for all populations. The following analyses were undertaken for each experiment. For each of the five samples per population, the number of germinated pollen grains was converted to a percentage and then transformed using an arc sine transformation. The 20 measurements of pollen tube length from each of the five samples per population were used collectively for analysis to give n=100. The transformed percentage germination data and the pollen tube length data were both tested for significance using a two-factor Analysis of Variance (ANOVA). The sample means were then compared using Duncan's Multiple Range Test to identify significant differences (P<0.05) between all possible pairs of means (Gomez and Gomez, 1984).

For the *in vivo* exposure of pollen from the High Low population, percentage germination data were transformed using an arc sine transformation before comparing means using an unpaired, two tailed *t*-test. Pollen tube length data were analysed collectively and differences between means were tested by calculating *d*, as for the seed yield parameters above.

227

5.3. RESULTS

5.3.1. Ozone exposure of reproductive spikes

Environmental conditions

Table 5.2 summarises the environmental conditions experienced within the treatment and control chambers for each experiment. For all experiments, the average day and night-time temperature ranged between 22.9-25.0 °C and 21.5-22.8 °C in both chambers. Relative humidity also varied between chambers and experiments, averaging between 43.1-72.1 % during the day and 49.3-80.1 % at night. A consistently higher relative humidity was recorded within the treatment chamber which may have been due to problems encountered with the dewpoint hygrometer used to monitor the airstream passing through this chamber. In each experiment, the mean concentration of ozone within the treatment chamber was close to the target concentration except when whole plants of High Low were exposed during first spike flowering, when a mean of 53.9 ppb was achieved instead of the target value of 120 ppb. This may have been due, in part, to the large surface area of plant, pot and soil material within the exposure chamber, which may have reacted with and depleted ozone faster than it could be replaced within the chamber. With the exception of the latter exposure of High Low, the target concentration of ozone for each experiment will be referred to hereafter for the sake of clarity.

5.3.1.1. *Exposure during flowering*

Exposure to 120 ppb ozone

In this section, the results obtained for the four populations of *Plantago major* are presented together for each individual aspect of seed yield. The seed yield data for each population are shown in Tables 5.3-5.8.

Seed number per capsule

Exposure to 120 ppb ozone for 7 h d^{-1} affected the number of seeds per capsule in the exposed (first) and unexposed (second) spikes differently in each population. Table 5.9 shows the percentage increase or decrease in the mean number of seeds per capsule relative to control plants for the exposed and unexposed spikes of each population. Ozone significantly increased (P<0.001) seed number per capsule in both spikes of

Table 5.2. Summary of environmental conditions within the control and treatment chambers during exposure of spikes of *P. major* populations to ozone during flowering or seed filling. Means and standard errors are shown.

Type of exposure	Population	Chamber	Ozone Concentration within treatment chamber (ppb)		Temperature (°C)			Relative Humidity (%)		
			Target	Actual	Day during O$_3$ introduction [1]	Day outwith O$_3$ introduction [2]	Night	Day during O$_3$ introduction [1]	Day outwith O$_3$ introduction [2]	Night
First spike exposed during flowering	High low	Control	70	69.1 ± 5.43	22.3 ± 0.79			54.2 ± 6.77		
		Ozone			22.6 ± 0.29			54.3 ± 7.40		
	Lullington	Control	120	123.9 ± 0.09	23.3 ± 0.05	22.9 ± 0.06	21.6 ± 0.05	52.5 ± 0.16	54.0 ± 0.20	57.2 ± 0.17
		Ozone			23.5 ± 0.05	23.1 ± 0.06	21.7 ± 0.05	52.2 ± 0.15	53.1 ± 0.19	57.3 ± 0.15
	Penicuik	Control	120	116.3 ± 0.63	23.5 ± 0.03	23.4 ± 0.05	21.7 ± 0.04	45.6 ± 0.15	45.5 ± 0.08	50.3 ± 0.08
		Ozone			23.6 ± 0.03	23.5 ± 0.06	21.5 ± 0.04	50.4 ± 0.14	50.5 ± 0.12	56.5 ± 0.11
	Sibton	Control	120	109.1 ± 1.84	24.7 ± 0.03	24.5 ± 0.06	22.8 ± 0.05	54.5 ± 0.23	54.5 ± 0.20	60.6 ± 0.17
		Ozone			25.0 ± 0.03	24.9 ± 0.07	22.6 ± 0.06	61.7 ± 0.42	62.6 ± 0.28	71.6 ± 0.28
	High Low 37 DAS	Control	120	118.5 ± 1.29	23.6 ± 0.02	23.4 ± 0.06	21.7 ± 0.04	45.8 ± 0.06	46.2 ± 0.12	51.5 ± 0.13
		Ozone			23.8 ± 0.02	23.6 ± 0.06	21.5 ± 0.05	50.3 ± 0.07	50.9 ± 0.15	57.6 ± 0.15
	High Low 51 DAS	Control	120	121.9 ± 0.48	23.7 ± 0.01	23.5 ± 0.04	22.7 ± 0.09	47.3 ± 0.04	47.7 ± 0.12	50.5 ± 0.29
		Ozone			23.9 ± 0.01	23.8 ± 0.05	22.7 ± 0.11	52.2 ± 0.04	52.7 ± 0.14	56.2 ± 0.36
	High Low 60 DAS	Control	120	118.8 ± 0.89	23.2 ± 0.07	23.2 ± 0.04	22.3 ± 0.06	60.7 ± 0.15	54.4 ± 0.09	54.4 ± 0.07
		Ozone			23.9 ± 0.01	23.9 ± 0.03	22.8 ± 0.10	54.4 ± 0.17	60.7 ± 0.11	60.7 ± 0.12
First spike exposed during seed filling	High Low	Control	120	119.5 ± 0.76	24.7 ± 0.02	24.6 ± 0.07	22.2 ± 0.08	51.8 ± 0.19	52.2 ± 0.12	80.1 ± 0.15
		Ozone			24.3 ± 0.02	24.2 ± 0.06	22.2 ± 0.05	71.7 ± 0.11	72.1 ± 0.16	59.2 ± 0.18
Whole plant exposed at first spike flowering	High Low	Control	120	53.9 ± 0.64	23.9 ± 0.03	23.5 ± 0.07	21.5 ± 0.06	43.1 ± 0.06	44.2 ± 0.22	49.3 ± 0.21
		Ozone			24.4 ± 0.03	24.0 ± 0.09	21.5 ± 0.08	45.8 ± 0.06	47.1 ± 0.29	54.5 ± 0.28

[1] Ozone introduction between 1000-1700 h during the 15 h photoperiod.

[2] Period during the 15 h photoperiod outwith ozone introduction; 0600-1000 h and 1700-2100 h.

Table 5.3. Summary of seed yield in *Plantago major* population Lullington for a) exposed and b) unexposed spikes when the first spike was exposed to 120 ppb ozone for 7 h d⁻¹ for 9 d during flowering. Means and standard errors are shown. ** and *** indicate significance at P<0.01 and P<0.001; NS, non-significant; n=5 unless otherwise shown in brackets.

a) EXPOSED SPIKE

	Control		Ozone		Significance
Total number of capsules	61.6 ± 10.07		62.6 ± 8.18		NS
Total number of seeds	391.4 ± 87.60		446.0 ± 84.82		NS
Number of seeds per capsule					
Per plant	6.4 ± 0.12	(308)	7.1 ± 0.11	(313)	***
Lower third	7.1 ± 0.22	(104)	7.4 ± 0.29	(105)	NS
Middle third	6.5 ± 0.19	(103)	7.3 ± 0.15	(105)	**
Upper third	5.4 ± 0.18	(101)	6.7 ± 0.13	(103)	***
Total seed weight (g)	144.9 ± 20.56		139.2 ± 30.05		NS
Individual seed weight (mg)	0.35 ± 0.007	(16)	0.34 ± 0.014	(16)	NS

b) UNEXPOSED SPIKE

	Control		Ozone		Significance
Total number of capsules	75.2 ± 18.85		90.8 ± 20.80		NS
Total number of seeds	491.0 ± 153.31		724.0 ± 195.36		NS
Number of seeds per capsule					
Per plant	6.5 ± 0.13	(376)	8.0 ± 0.09	(454)	***
Lower third	7.5 ± 0.22	(127)	8.3 ± 0.15	(153)	**
Middle third	6.7 ± 0.21	(126)	8.4 ± 0.11	(151)	***
Upper third	5.3 ± 0.18	(123)	7.2 ± 0.16	(150)	***
Total seed weight (g)	157.3 ± 46.08		220.7 ± 42.66		NS
Individual seed weight (mg)	0.33 ± 0.010	(20)	0.32 ± 0.013	(24)	NS

Table 5.4. Summary of seed yield in *Plantago major* population Penicuik for a) exposed and b) unexposed spikes when the first spike was exposed to 120 ppb ozone for 7 h d^{-1} for 9 d during flowering. Means and standard errors are shown. ** and *** indicate significance at P<0.01 and P<0.001; NS, non-significant; n=5 unless otherwise shown in brackets.

a) EXPOSED SPIKE

	Control		Ozone		Significance
Total number of capsules	98.4 ± 9.09		91.8 ± 6.55		NS
Total number of seeds	756.4 ± 53.91		648.2 ± 68.06		NS
Number of seeds per capsule					
Per plant	7.7 ± 0.13	(493)	7.1 ± 0.15	(457)	**
Lower third	8.3 ± 0.26	(166)	8.4 ± 0.26	(154)	NS
Middle third	7.7 ± 0.24	(163)	6.8 ± 0.25	(153)	**
Upper third	7.1 ± 0.17	(164)	6.0 ± 0.22	(152)	***
Total seed weight (g)	203.7 ± 24.41		204.3 ± 23.44		NS
Individual seed weight (mg)	0.27 ± 0.009	(25)	0.31 ± 0.008	(24)	***

b) UNEXPOSED SPIKE

	Control		Ozone		Significance
Total number of capsules	147.8 ± 16.28		138.2 ± 10.86		NS
Total number of seeds	1279.8 ± 159.00		1288.8 ± 113.69		NS
Number of seeds per capsule					
Per plant	8.6 ± 0.09	(743)	9.5 ± 0.49	(691)	***
Lower third	9.2 ± 0.20	(250)	10.6 ± 0.15	(233)	***
Middle third	8.8 ± 0.12	(248)	9.8 ± 0.14	(229)	***
Upper third	7.8 ± 0.11	(245)	8.0 ± 0.15	(229)	
Total seed weight (g)	288.1 ± 37.26		335.1 ± 34.32		NS
Individual seed weight (mg)	0.22 ± 0.005	(36)	0.26 ± 0.005	(33)	***

231

Table 5.5. Summary of seed yield in *Plantago major* population Sibton for a) exposed and b) unexposed spikes when the first spike was exposed to 120 ppb ozone for 7 h d^{-1} for 9 d during flowering. Means and standard errors are shown. *, ** and *** indicate significance at P<0.05, P<0.01 and P<0.001; NS, non-significant; n=6 unless otherwise shown in brackets.

a) EXPOSED SPIKE

	Control		Ozone		Significance
Total number of capsules	64.8 ± 4.24		80.7 ± 5.17		*
Total number of seeds	527.8 ± 43.94		642.3 ± 59.69		NS
Number of seeds per capsule					
Per plant	8.1 ± 0.09	(389)	8.0 ± 0.09	(485)	NS
Lower third	8.7 ± 0.17	(132)	9.1 ± 0.14	(163)	NS
Middle third	8.3 ± 0.13	(129)	8.1 ± 0.14	(163)	NS
Upper third	7.3 ± 0.15	(128)	6.6 ± 0.15	(159)	***
Total seed weight (g)	198.2 ± 19.1		234.4 ± 21.64		NS
Individual seed weight (mg)	0.38 ± 0.008	(25)	0.36 ± 0.007	(21)	NS

b) UNEXPOSED SPIKE

	Control		Ozone		Significance
Total number of capsules	103.7 ± 8.67		119.7 ± 6.30		NS
Total number of seeds	826.5 ± 81.79		976.3 ± 62.98		NS
Number of seeds per capsule					
Per plant	8.0 ± 0.07	(662)	8.1 ± 0.07	(717)	NS
Lower third	8.7 ± 0.09	(209)	8.6 ± 0.10	(242)	NS
Middle third	8.3 ± 0.09	(207)	8.7 ± 0.08	(239)	***
Upper third	6.9 ± 0.15	(206)	7.1 ± 0.15	(236)	NS
Total seed weight (g)	263.4 ± 23.06		315.4 ± 25.03		NS
Individual seed weight (mg)	0.32 ± 0.006	(31)	0.32 ± 0.008	(36)	NS

232

Table 5.6. Summary of seed yield in *Plantago major* population High Low for a) exposed and b) unexposed spikes when the first spike was exposed from 37 DAS to 120 ppb ozone for 7 h d^{-1} for 9 d during flowering. Means and standard errors are shown. *** indicates significance at P<0.001; NS, non-significant; n=5 unless otherwise shown in brackets.

a) EXPOSED SPIKE

	Control		Ozone		Significance
Total number of capsules	83.2 ± 12.21		90.6 ± 16.54		NS
Total number of seeds	673.6 ± 99.68		581.2 ± 111.00		NS
Number of seeds per capsule					
Per plant	8.1 ± 0.14	(415)	6.4 ± 0.12	(452)	***
Lower third	8.2 ± 0.28	(139)	6.2 ± 0.26	(154)	***
Middle third	8.9 ± 0.24	(139)	6.9 ± 0.19	(150)	***
Upper third	7.3 ± 0.19	(137)	6.2 ± 0.16	(148)	***
Total seed weight (g)	239.3 ± 31.66		203.9 ± 44.10		NS
Individual seed weight (mg)	0.36 ± 0.007	(22)	0.35 ± 0.006	(23)	NS

b) UNEXPOSED SPIKE

	Control		Ozone		Significance
Total number of capsules	130.8 ± 16.09		122.6 ± 11.56		NS
Total number of seeds	1027.4 ± 197.35		963.6 ± 98.20		NS
Number of seeds per capsule					
Per plant	7.9 ± 0.12	(653)	7.8 ± 0.08	(615)	NS
Lower third	8.6 ± 0.24	(219)	8.6 ± 0.12	(207)	NS
Middle third	8.4 ± 0.18	(218)	8.0 ± 0.11	(206)	NS
Upper third	6.6 ± 0.15	(216)	6.8 ± 0.13	(202)	NS
Total seed weight (g)	336.1 ± 46.93		305.8 ± 42.37		NS
Individual seed weight (mg)	0.34 ± 0.008	(32)	0.32 ± 0.007	(31)	NS

Table 5.7. Summary of seed yield in *Plantago major* population High Low for a) exposed and b) unexposed spikes when the first spike was exposed from 51 DAS to 120 ppb ozone for 7 h d^{-1} for 9 d during flowering. Means and standard errors are shown. *** indicates significance at P<0.001; NS, non-significant; n=9 unless otherwise shown in brackets.

a) EXPOSED SPIKE

	Control		Ozone		Significance
Total number of capsules	61.6 ± 9.00		76.6 ± 8.19		NS
Total number of seeds	485.4 ± 73.85		499.1 ± 53.98		NS
Number of seeds per capsule					
Per plant	7.9 ± 0.10	(553)	6.5 ± 0.11	(688)	***
Lower third	8.1 ± 0.20	(220)	7.0 ± 0.20	(200)	***
Middle third	8.4 ± 0.14	(216)	7.4 ± 0.16	(198)	***
Upper third	6.9 ± 0.15	(213)	5.2 ± 0.21	(195)	***
Total seed weight (g)	150.7 ± 21.84		169.0 ± 13.15		NS
Individual seed weight (mg)	0.31 ± 0.011	(30)	0.34 ± 0.009	(36)	NS

b) UNEXPOSED SPIKE

	Control		Ozone		Significance
Total number of capsules	93.7 ± 7.48		102.6 ± 12.86		NS
Total number of seeds	816.0 ± 82.23		840.3 ± 104.86		NS
Number of seeds per capsule					
Per plant	7.7 ± 0.07	(839)	7.9 ± 0.06	(923)	NS
Lower third	8.3 ± 0.13	(282)	8.1 ± 0.13	(311)	NS
Middle third	8.0 ± 0.09	(281)	8.0 ± 0.11	(308)	NS
Upper third	7.3 ± 0.09	(276)	7.0 ± 0.12	(304)	NS
Total seed weight (g)	205.2 ± 18.40		240.2 ± 24.86		NS
Individual seed weight (mg)	0.28 ± 0.010	(41)	0.31 ± 0.008	(48)	*

Table 5.8. Summary of seed yield in *Plantago major* population High Low for a) exposed and b) unexposed spikes when the first spike was exposed from 60 DAS to 120 ppb ozone for 7 h d^{-1} for 9 d during flowering. Means and standard errors are shown. ** and *** indicate significance at P<0.01 and P<0.001; NS, non-significant; n=5 unless otherwise shown in brackets.

a) EXPOSED SPIKE

	Control		Ozone		Significance
Total number of capsules	97.3 ± 11.94		82.7 ± 12.03		NS
Total number of seeds	714.5 ± 104.74		548.3 ± 132.2		NS
Number of seeds per capsule					
Per plant	7.3 ± 0.13	(586)	6.6 ± 0.18	(495)	**
Lower third	7.1 ± 0.27	(197)	7.2 ± 0.31	(167)	NS
Middle third	7.4 ± 0.23	(196)	6.6 ± 0.33	(166)	***
Upper third	7.5 ± 0.16	(193)	6.1 ± 0.27	(162)	***
Total seed weight (g)	234.4 ± 27.41		163.1 ± 23.25		NS
Individual seed weight (mg)	0.34 ± 0.015	(32)	0.33 ± 0.022	(25)	NS

b) UNEXPOSED SPIKE

	Control		Ozone		Significance
Total number of capsules	119.3 ± 16.64		109.7 ± 17.35		NS
Total number of seeds	917.2 ± 141.63		858.5 ± 170.46		NS
Number of seeds per capsule					
Per plant	7.7 ± 0.10	(714)	7.8 ± 0.09	(659)	NS
Lower third	7.7 ± 0.19	(241)	8.0 ± 0.20	(222)	NS
Middle third	8.3 ± 0.15	(237)	8.3 ± 0.13	(219)	NS
Upper third	7.2 ± 0.15	(236)	7.1 ± 0.13	(218)	NS
Total seed weight (g)	229.5 ± 32.43		203.8 ± 27.56		NS
Individual seed weight (mg)	0.25 ± 0.009	(33)	0.25 ± 0.016	(37)	NS

235

Table 5.9. Mean percentage difference in the number of seeds per capsule relative to control plants for spikes 1 and 2 of four populations of *P. major* after spike 1 was exposed for 9 d to 120 ppb ozone for 7 h d^{-1} during flowering. ** and *** indicate significance at P<0.01 and P<0.001; NS, non-significant.

Population	Spike 1 (exposed)	Significance	Spike 2 (unexposed)	Significance
Lullington	+ 10.9	***	+ 23.1	***
Penicuik	- 7.8	**	+ 10.5	***
Sibton	- 1.2	NS	+ 1.3	NS
High Low 37 DAS	- 21.0	***	- 1.3	NS
High Low 51 DAS	- 17.7	***	+ 2.6	NS
High Low 60 DAS	- 9.6	**	+ 1.3	NS

Lullington. For Penicuik and High Low, seed number per capsule was significantly decreased by ozone in the exposed spike (P<0.01 and 0.001 respectively); however, the unexposed spike responded differently in these two populations, with a significant increase in seed number per capsule being observed in Penicuik (P<0.001) and no change in High Low. Although the number of seeds per capsule in the exposed spike was significantly less than in control plants in all three experiments involving High Low (P<0.01-0.001), the reduction became progressively smaller as first spike flowering and exposure started later after sowing. The Sibton population exhibited no effect of ozone on seed number per capsule in either spike.

In all populations of *Plantago major,* the number of seeds per capsule on each spike of control plants decreased acropetally from ~7-8 seeds at the bottom (first flowers to open) to ~6-7 seeds at the top (Tables 5.3-5.8). Figures 5.1 and 5.2 show that the effect of ozone on seed number per capsule varied in each population depending on the position of the capsules on the spike. For the exposed spike of Lullington and Penicuik, the effect of ozone on seed number was greatest (P<0.001) in the upper (top) third of the spike with an increase in Lullington and decrease in Penicuik, and least in the lower (bottom) third, where the differences were not significant (Fig. 5.1a). In Sibton, there was a significant decrease in seed number per capsule following ozone exposure in the upper third of the exposed spike, but this was compensated for by a non-significant increase in the lower third, such that there was no effect on seed number per capsule averaged over the whole spike (Table 5.9). Ozone exposure increased the number of seeds per capsule along the entire length of the unexposed spike of Lullington, particularly towards the top of the spike (Fig. 5.1b). A significant increase in the number of seeds per capsule was observed in the lower section of spikes from ozone-treated plants of Penicuik. This increase in the number of seeds per capsule in ozone-treated plants decreased acropetally, such that the difference was no longer significant in the upper third of the spike. In Sibton, there was little variation in seed number along the length of the unexposed spike of ozone-treated plants, although there was a significant increase in seed number in the middle third of the spike.

When flowering and exposure of High Low commenced at 37 and 51 DAS, ozone significantly reduced the number of seeds per capsule throughout the length of the exposed spike (Fig. 5.2a). However, when flowering and exposure began at 60 DAS, a reduction in seed number per capsule occurred only in the middle and upper thirds of the spike (Fig. 5.2a). Ozone had no significant effect on the number of seeds per capsule of the unexposed spike for any exposure of High Low (Fig. 5.2b).

Figure 5.1. Mean percentage difference in seed number per capsule relative to control plants for *P. major* populations Lullington, Penicuik and Sibton after exposing the first spike to 120 ppb O_3 for 7 h d^{-1} for 9 d during flowering. Results are shown for the lower, middle and upper thirds of a) exposed and b) unexposed spikes. ** and *** indicate significance at P<0.01 and P<0.001.
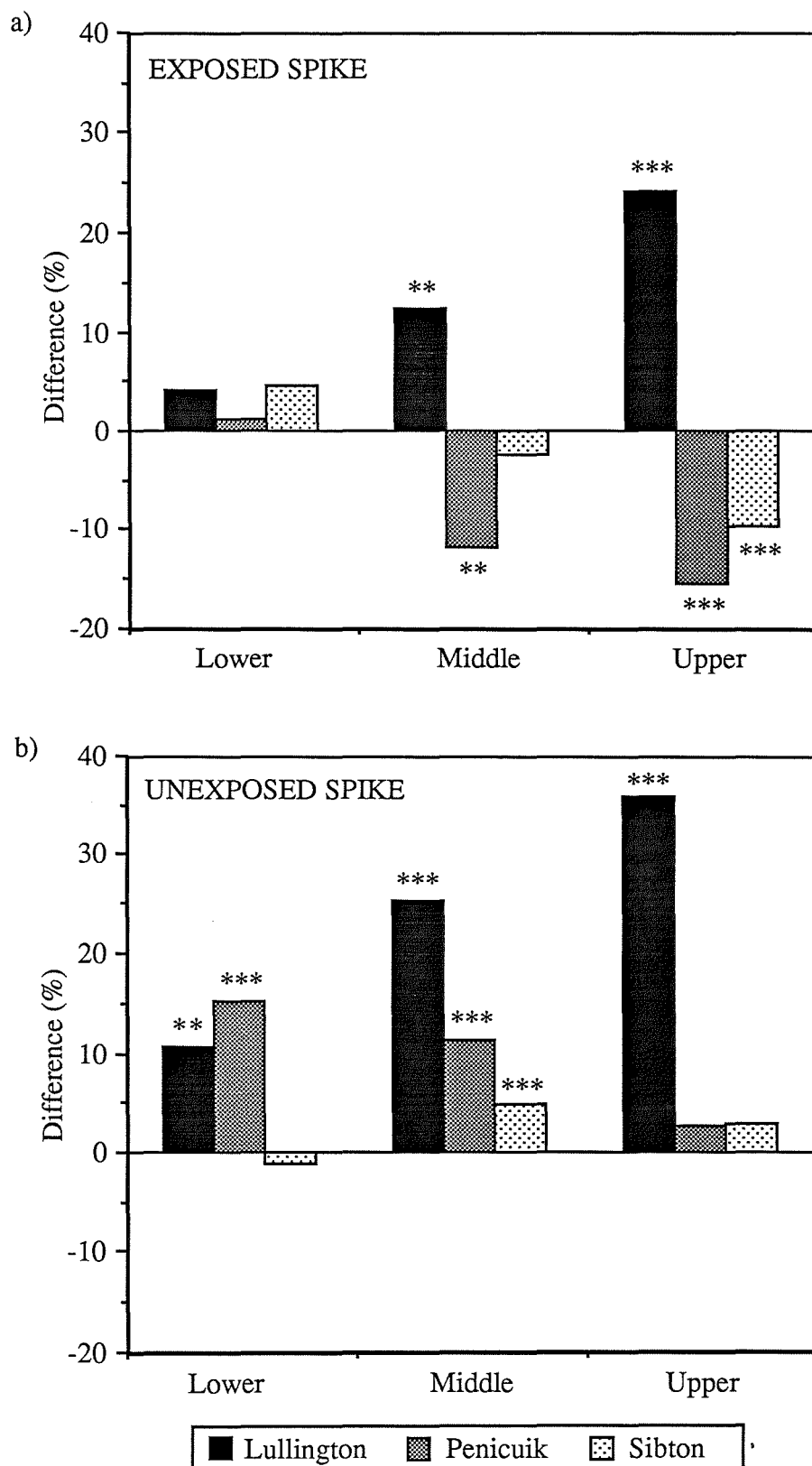
Figure 5.2. Mean percentage difference in seed number per capsule relative to control plants for *P. major* population High Low after exposing the first spike to 120 ppb O_3 for 7 h d^{-1} for 9 d during flowering when flowering started at 37, 51 and 60 DAS. Results are shown for the lower, middle and upper thirds of a) exposed and b) unexposed spikes. *** indicates significance at $P<0.001$.

a)

EXPOSED SPIKE

Difference (%)

10

0

-10

-20

-30

*** *** *** *** *** *** *** ***

Lower   Middle   Upper

b)

UNEXPOSED SPIKE

Difference (%)

10

0

-10

-20

-30

Lower   Middle   Upper

■ 37 DAS   ▨ 51 DAS   ▧ 60 DAS
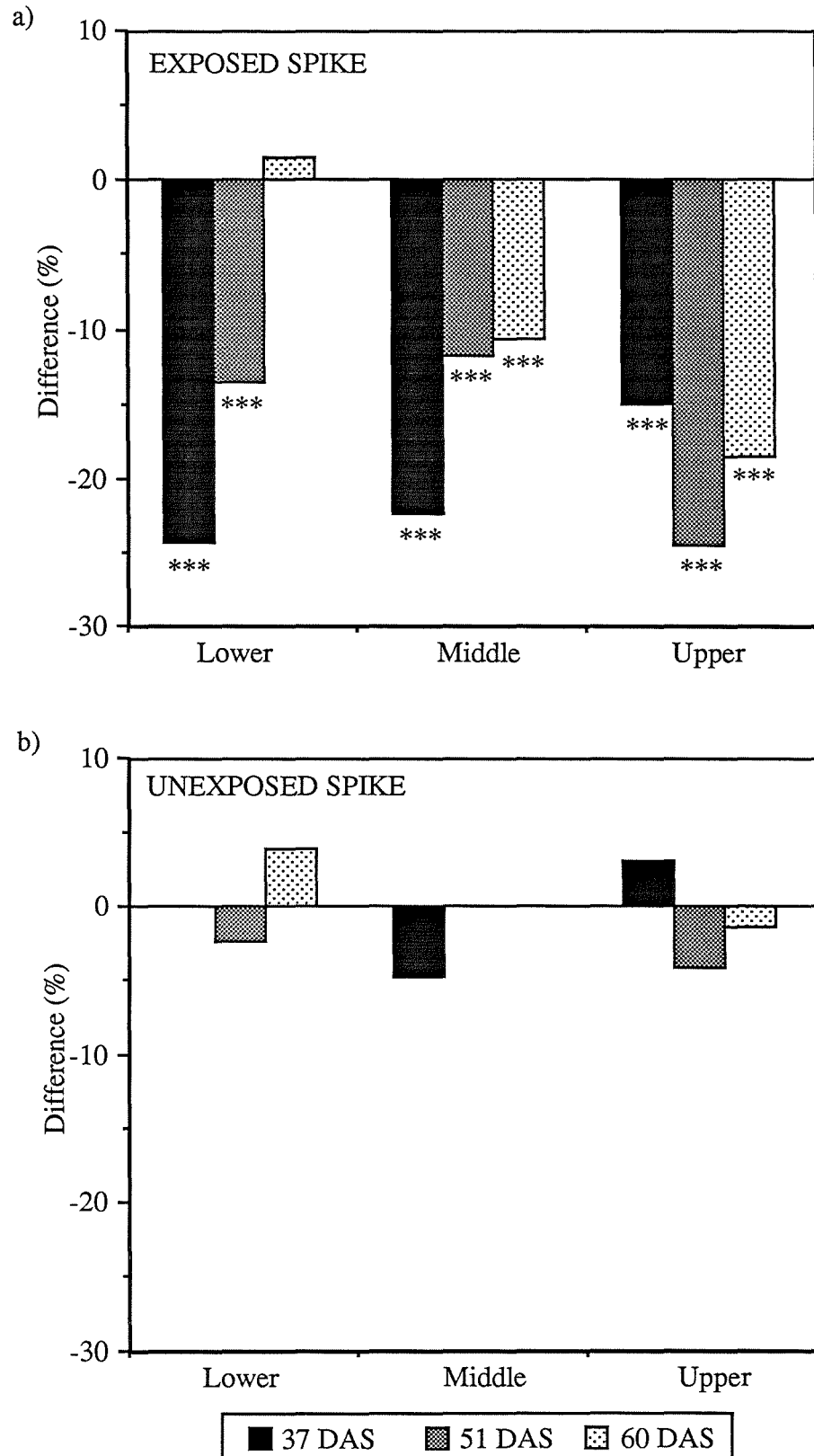
When seed number per capsule was averaged over the exposed and unexposed spikes (Table 5.10), it was shown that Penicuik was able to compensate for the loss of seeds in the exposed spike by increasing seed number in the next spike to develop. Indeed, this population appeared to overcompensate for the loss, resulting in an overall increase in seed number per capsule under ozone. In High Low, the loss of seeds per capsule of the exposed spike was not compensated for by an increase in the unexposed spike, except when flowering and exposure started 60 DAS. The similar effects of ozone on both the exposed and unexposed spikes of Lullington resulted in a significant increase in seeds per capsule when averaged over both spikes. Seed number per capsule was unaffected in both spikes of the Sibton population and remained similar to that of control plants.

Seed weight

For the first spike of control plants exposed to filtered air, mean individual seed weight varied between populations, with Penicuik and Sibton producing the lightest and heaviest seeds respectively (Table 5.11). After exposure to ozone, the Penicuik population still produced the lightest seeds on spike 1 despite a significant increase (14.8 %, $P<0.001$) in individual seed weight. Ozone exposure had no significant effect on individual seed weight for the first spike of other populations.

For the control plants of all populations, mean individual seed weight was greater for the first than for the second spike (Tables 5.3-5.8). The difference in individual seed weight between spikes 1 and 2 as a percentage of spike 1 varied between populations, with the plants of High Low which flowered from 60 DAS showing the greatest difference and those from this population which flowered from 37 DAS the least difference between spikes (Fig. 5.3). Notably, for High Low, the difference in individual seed weight between spikes 1 and 2 increased as flowering started later after sowing. The difference in individual seed weight between the spikes of ozone-treated plants was similar to that for the control plants of each population and did not differ significantly. Following exposure of the first spike to ozone, a significant increase in the individual seed weight of spike 2 was observed in both Penicuik (18.2 %, $P<0.001$, Table 5.4) and High Low exposed from 51 DAS (10.7 %, $P<0.05$, Table 5.7).

Capsules per inflorescence

The number of capsules on the first spike of control plants varied between populations of *Plantago major* with Penicuik producing the most and Lullington and High Low

Table 5.10. Mean number of seeds per capsule averaged over spikes 1 (exposed) and 2 (unexposed) for four populations of *P. major* when the first spike was exposed to filtered air (control treatment) or 120 ppb ozone for 7 h d^{-1} for 9 d during flowering. * and *** indicate significance at $P<0.05$ and $P<0.001$; NS, non-significant.

Population	Control	Ozone	Significance
Lullington	6.5 ± 0.09	7.6 ± 0.07	***
Penicuik	8.2 ± 0.08	8.5 ± 0.09	*
Sibton	8.0 ± 0.06	8.1 ± 0.06	NS
High Low 37 DAS	8.0 ± 0.09	7.2 ± 0.07	***
High Low 51 DAS	7.9 ± 0.06	7.2 ± 0.06	***
High Low 60 DAS	7.5 ± 0.08	7.3 ± 0.09	NS

Table 5.11. Mean individual seed weight (mg) for the first spike of each population of *Plantago major*, after exposing the first spike to filtered air (control treatment) or 120 ppb O_3 for 7 h d^{-1} for 9 d during flowering. Standard errors are shown. *** indicates significance at P<0.001; NS, non-significant.

Population	Control	Ozone	Significance
Lullington	0.35 ± 0.007	0.34 ± 0.014	NS
Penicuik	0.27 ± 0.009	0.31 ± 0.008	***
Sibton	0.38 ± 0.008	0.36 ± 0.007	NS
High Low 37 DAS	0.36 ± 0.007	0.35 ± 0.006	NS
High Low 51 DAS	0.31 ± 0.011	0.34 ± 0.009	NS
High Low 60 DAS	0.34 ± 0.015	0.33 ± 0.022	NS

Figure 5.3. Mean difference in individual seed weight between spikes 1 and 2 as a percentage of spike 1, for each population of *Plantago major* following exposure of the first spike to filtered air (control treatment) or 120 ppb O_3 for 7h d^{-1} for 9 d during flowering. Single standard errors are shown.
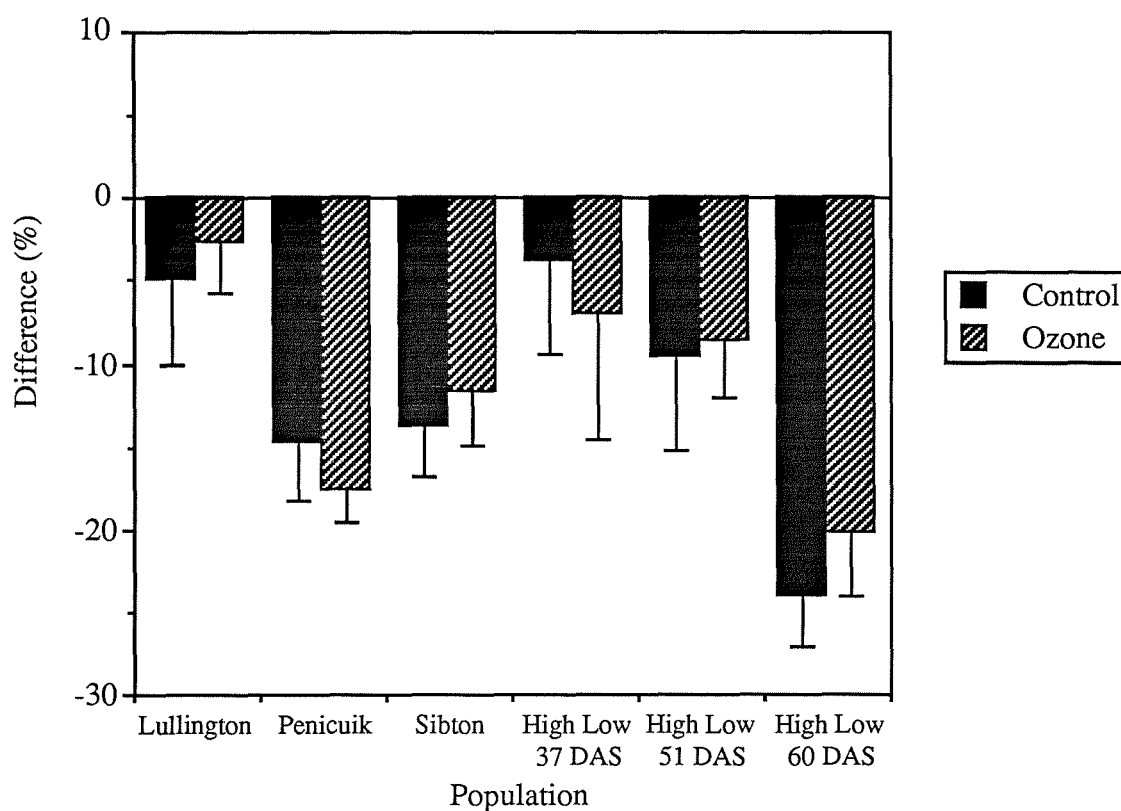
flowering from 51 DAS the fewest capsules (Table 5.12). The only significant effect of ozone on the number of capsules for spike 1 was an increase (24.5 %, P<0.05) in the Sibton population.

For the control plants of all populations, more capsules were produced on the second than on the first spike (Tables 5.3-5.8). The difference in the number of capsules between spikes 1 and 2 as a percentage of spike 1 varied between populations, with High Low which flowered from 37 DAS showing the greatest difference and Lullington the least difference between spikes under control conditions (Fig 5.4). Although not significant, the difference in the number of capsules between spikes 1 and 2 was increased under ozone in Lullington, Penicuik and High Low which flowered from 37 and 60 DAS, and decreased in Sibton and High Low which flowered from 51 DAS. In addition, the difference in the number of capsules between spikes 1 and 2 was less for the control and ozone-treated plants of High Low when flowering started later after sowing. Following exposure of the first spike to ozone, there was no significant effect on the number of capsules produced on the second spike in any of the populations examined, due to the high variability observed between plants.

Seed number per spike

The total number of seeds produced on the first spike of control plants varied between populations in relation to the number of capsules per spike, with Penicuik producing the most and Lullington the fewest seeds per spike (Table 5.13). Exposure to ozone had no effect on the total number of seeds on the first spike of any population.

Primarily as a consequence of having more capsules, the total number of seeds was greater on the second than on the first spike of control plants. The difference in the total number of seeds between spikes 1 and 2 as a percentage of spike 1 varied between populations with Penicuik having the greatest and Lullington the least difference between spikes (Fig. 5.5). This difference between spikes 1 and 2 was greater for ozone-treated than for control plants of each population, with the exception of Sibton and High low which flowered from 51 DAS, however these differences were not significant. Following exposure of the first spike to ozone there was no significant effect on the total number of seeds produced on the second spike of any population. Despite the significant effects of ozone on the number of seeds per capsule, the total number of seeds on spikes 1 and 2 was not significantly affected by ozone in any population due to the high variability between plants; however, all significant effects of

Table 5.12. Mean number of capsules on the first spike of each population of *Plantago major* after exposing the first spike to filtered air (control treatment) or 120 ppb O_3 for 7 h d^{-1} for 9 d during flowering. Standard errors are shown. * indicates significance at P<0.05; NS, non-significant.

Population	Control	Ozone	Significance
Lullington	61.6 ± 10.07	62.6 ± 8.18	NS
Penicuik	98.4 ± 9.09	91.8 ± 6.55	NS
Sibton	64.8 ± 4.24	80.7 ± 5.17	*
High Low 37 DAS	83.2 ± 12.21	90.6 ± 16.54	NS
High Low 51 DAS	61.6 ± 9.00	76.6 ± 8.19	NS
High Low 60 DAS	97.3 ± 11.94	82.7 ± 12.03	NS

Figure 5.4. Mean difference in the number of capsules between spikes 1 and 2 as a percentage of spike 1, for each population of *Plantago major* following exposure of the first spike to filtered air (control treatment) or 120 ppb O_3 for 7h d^{-1} for 9 d during flowering. Single standard errors are shown.
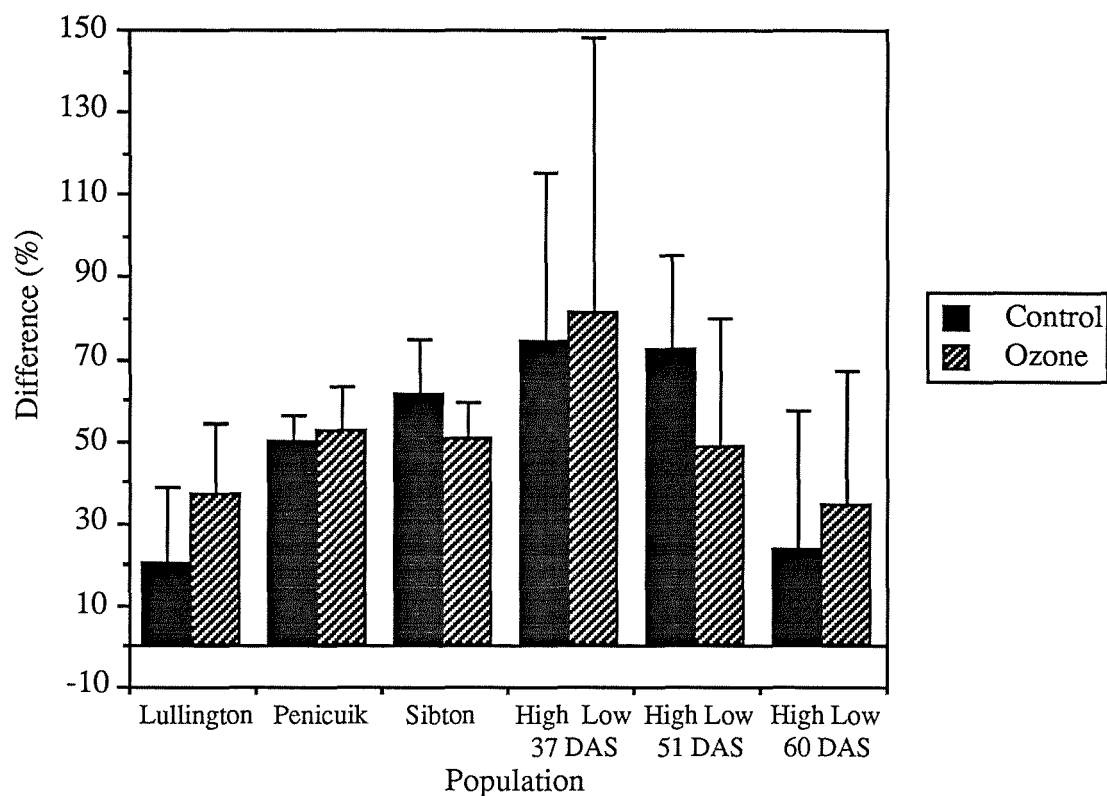
Table 5.13. Mean total number of seeds from the first spike of each population of *Plantago major* after exposing the first spike to filtered air (control treatment) or 120 ppb O_3 for 7 h d^{-1} for 9 d during flowering. Standard errors are shown. NS, non-significant.

Population	Control	Ozone	Significance
Lullington	391.4 ± 87.60	446.0 ± 84.82	NS
Penicuik	756.4 ± 53.91	648.2 ± 68.06	NS
Sibton	527.8 ± 43.94	642.3 ± 59.69	NS
High Low 37 DAS	673.6 ± 99.68	581.2 ± 111.00	NS
High Low 51 DAS	485.4 ± 73.85	499.1 ± 53.98	NS
High Low 60 DAS	714.5 ± 104.74	548.3 ± 132.2	NS

Figure 5.5. Mean difference in the total number of seeds between spikes 1 and 2 as a percentage of spike 1, for each population of *Plantago major* following exposure of the first spike to filtered air (control treatment) or 120 ppb O_3 for 7h d^{-1} for 9 d during flowering. Single standard errors are shown.
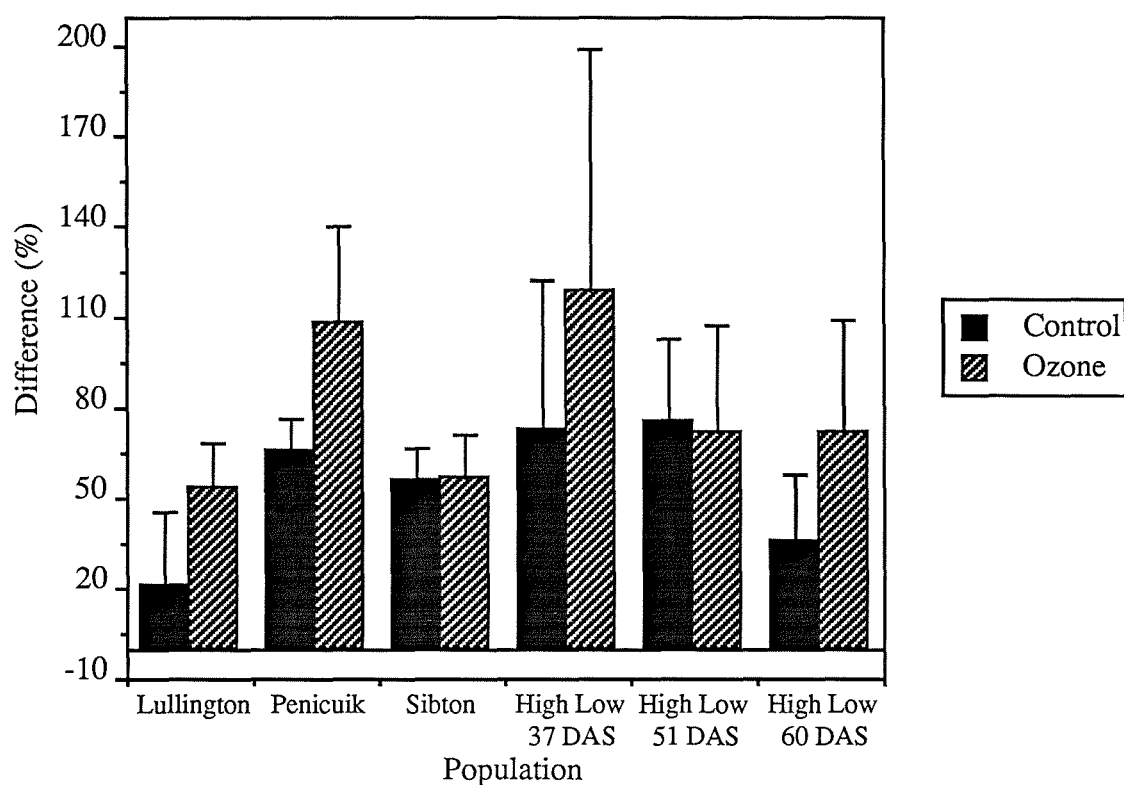
ozone on the number of seeds per capsule were reflected to some extent by differences in the total number of seeds per spike (Tables 5.3-5.8).

Exposure to 70 ppb ozone

The number of seeds per capsule was the only component of seed yield which was significantly affected by ozone after exposure of population High Low to 70 ppb for 7 d during flowering (Table 5.14). Ozone significantly reduced seed number per capsule by 19.5 % in the exposed spike (P<0.001) and 2.5 % in the unexposed spike (P<0.01), reductions which were similar to those for this population when exposed from 37 DAS to 120 ppb for 9 d (Table 5.9). For the exposed spike, the significant decrease (P<0.001) in seed number per capsule was 15.6, 31.6 and 11.6 % for the lower, middle and upper thirds respectively, while the only significant reduction for the unexposed spike was 5.9 % (P<0.01) in the lower third of the spike. As seen for previous experiments involving High Low, individual seed weight was greater in the first than in the second spike, and there was no significant effect of ozone on the individual seed weight of either spike. In addition, there was no significant effect of ozone on the number of capsules, total number of seeds or total seed weight for either the exposed or unexposed spikes.

5.3.1.2. *Exposure during seed filling*

When the first spike of High Low was exposed to ozone during seed filling, the only significant effect was a 9 % increase in seed number per capsule in the unexposed spike (Table 5.15); this increase occurred throughout the length of the unexposed spike, but was greatest (11.2 %) in capsules from the top third of the spike. There was no significant effect of ozone on individual seed weight, number of capsules, total number of seeds or total seed weight for either the exposed or unexposed spikes.

5.3.1.3. *Exposure of the entire plant during flowering*

When entire plants of High Low were fumigated for 9 d with 54 ppb ozone the first and second spikes were both exposed directly to ozone. Ozone significantly increased the number of seeds per capsule, by 18 % in the first spike and 11 % in the second spike (Table 5.16). This increase in seed number per capsule occurred throughout the length of both spikes, with the largest increase being 21 % in the lower third of each spike. In previous experiments, in which the first spike of High Low was exposed during flowering or seed filling, individual seed weight was greater in the first

Table 5.14. Summary of seed yield in *Plantago major* population High Low for a) exposed and b) unexposed spikes when the first spike was exposed to 70 ppb of ozone for 7 h d^{-1} for 7 d during flowering. Means and standard errors are shown. ** and *** indicate significance at P<0.01 and P<0.001; NS, non-significant ; n=5 unless otherwise shown in brackets.

a) EXPOSED SPIKE

	Control		Ozone		Significance
Total number of capsules	90.8 ± 17.68		95.2 ± 12.62		NS
Total number of seeds	699.4 ± 154.91		587.6 ± 70.90		NS
Number of seeds per capsule					
Per plant	7.7 ± 0.09	(454)	6.2 ± 0.11	(476)	***
Lower third	8.3 ± 0.16	(152)	7.0 ± 0.17	(159)	***
Middle third	7.9 ± 0.13	(152)	5.4 ± 0.20	(158)	***
Upper third	6.9 ± 0.14	(150)	6.1 ± 0.16	(158)	***
Total seed weight (g)	242.2 ± 49.49		207.7 ± 28.21		NS
Individual seed weight (mg)	0.35 ± 0.018	(25)	0.36 ± 0.019	(26)	NS

b) UNEXPOSED SPIKE

	Control		Ozone		Significance
Total number of capsules	130.6 ± 16.70		107.6 ± 14.55		NS
Total number of seeds	1069.2 ± 125.62		845.6 ± 103.62		NS
Number of seeds per capsule					
Per plant	8.2 ± 0.07	(653)	8.0 ± 0.05	(538)	**
Lower third	8.5 ± 0.14	(218)	8.0 ± 0.17	(180)	**
Middle third	8.4 ± 0.10	(218)	8.2 ± 0.09	(180)	NS
Upper third	7.7 ± 0.12	(217)	7.4 ± 0.09	(178)	NS
Total seed weight (g)	308.9 ± 17.95		255.9 ± 25.41		NS
Individual seed weight (mg)	0.29 ± 0.008	(33)	0.30 ± 0.009	(27)	NS

Table 5.15. Summary of seed yield in *Plantago major* population High Low for a) exposed and b) unexposed spikes when the first spike was exposed from 56 DAS to 120 ppb ozone for 7 h d^{-1} for 14 d during seed filling. Means and standard errors are shown. ** and *** indicate significance at P<0.01 and P<0.001; NS, non-significant; n=5 unless otherwise shown in brackets.

a) EXPOSED SPIKE

	Control		Ozone		Significance
Total number of capsules	84.0 ± 17.01		81.4 ± 10.78		NS
Total number of seeds	694.6 ± 141.03		690.8 ± 101.75		NS
Number of seeds per capsule					
Per plant	8.3 ± 0.10	(419)	8.5 ± 0.14	(407)	NS
Lower third	9.0 ± 0.19	(141)	9.5 ± 0.27	(138)	NS
Middle third	8.8 ± 0.14	(140)	9.0 ± 0.22	(135)	NS
Upper third	7.1 ± 0.12	(138)	6.8 ± 0.14	(134)	NS
Total seed weight (g)	193.7 ± 35.15		176.8 ± 17.37		NS
Individual seed weight (mg)	0.28 ± 0.009	(22)	0.26 ± 0.011	(22)	NS

b) UNEXPOSED SPIKE

	Control		Ozone		Significance
Total number of capsules	121.8 ± 21.39		114.6 ± 16.93		NS
Total number of seeds	925.4 ± 159.39		952.0 ± 127.53		NS
Number of seeds per capsule					
Per plant	7.6 ± 0.06	(609)	8.3 ± 0.10	(573)	***
Lower third	8.1 ± 0.14	(204)	8.7 ± 0.18	(193)	**
Middle third	7.6 ± 0.08	(204)	8.4 ± 0.14	(191)	***
Upper third	7.1 ± 0.10	(201)	7.9 ± 0.15	(189)	***
Total seed weight (g)	237.9 ± 36.64		242.2 ± 19.64		NS
Individual seed weight (mg)	0.25 ± 0.008	(31)	0.26 ± 0.008	(27)	NS

Table 5.16. Summary of seed yield in *Plantago major* population High Low for a) first and b) second spikes when whole plants were exposed from 35 DAS to 54 ppb ozone for 7 h d^{-1} for 9 d during flowering. Means and standard errors are shown. *, ** and *** indicate significance at P<0.05, P<0.01 and P<0.001; NS, non-significant; n=6 unless otherwise shown in brackets.

a) FIRST SPIKE

	Control		Ozone		Significance
Total number of capsules	92.2 ± 6.61		97.3 ± 6.25		NS
Total number of seeds	567.5 ± 61.06		701.3 ± 60.18		NS
Number of seeds per capsule					
Per plant	6.1 ± 0.09	(552)	7.2 ± 0.08	(582)	***
Lower third	6.4 ± 0.16	(188)	7.8 ± 0.16	(196)	***
Middle third	6.6 ± 0.15	(183)	7.3 ± 0.12	(195)	***
Upper third	5.4 ± 0.15	(183)	6.6 ± 0.13	(191)	***
Total seed weight (g)	214.8 ± 18.16		250.5 ± 40.00		NS
Individual seed weight (mg)	0.39 ± 0.019	(29)	0.36 ± 0.017	(30)	NS

b) SECOND SPIKE

	Control		Ozone		Significance
Total number of capsules	101.8 ± 12.63		121.8 ± 14.06		NS
Total number of seeds	761.5 ± 110.45		714.8 ± 167.58		NS
Number of seeds per capsule					
Per plant	6.3 ± 0.08	(732)	7.0 ± 0.10	(611)	***
Lower third	6.2 ± 0.16	(246)	7.5 ± 0.18	(205)	***
Middle third	6.3 ± 0.14	(243)	7.3 ± 0.18	(203)	***
Upper third	5.7 ± 0.14	(243)	6.3 ± 0.17	(203)	*
Total seed weight (g)	310.7 ± 44.74		240.0 ± 46.88		NS
Individual seed weight (mg)	0.42 ± 0.011	(38)	0.36 ± 0.016	(31)	**

(exposed) than in the second (unexposed) spike of both ozone-treated and control plants. In the present experiment, in which the entire plant was exposed, individual seed weight was greater in the second than in the first spike of control plants. The individual seed weight of the second spike was reduced significantly (P<0.01) in ozone-treated plants such that it was similar to that of the first, exposed spike (0.36 mg). There was no significant effect of ozone on the number of capsules, total number of seeds or total seed weight for either the exposed or unexposed spikes.

Summary of exposure of reproductive spikes

When the reproductive structures of the various *Plantago major* populations were exposed to ozone, either in isolation or together with the rest of the plant, seed number per capsule proved to be the aspect of seed yield which was most affected.

Table 5.17 summarises, for each of the four populations of *Plantago major* used in this study, the effects of exposure to 120 ppb ozone during flowering on the number of seeds per capsule for both the first (exposed) and second (unexposed) spikes. The data show that the exposed and unexposed spikes may both be affected by ozone, and that this effect can differ between spikes and populations. The combined effect of ozone on the two harvested spikes was an increase in the number of seeds per capsule in Lullington and Penicuik and a decrease in High Low; there was no discernible effect in the Sibton population. This decrease in seed number per capsule in High Low occurred following exposure to both 70 ppb ozone for 7 d and 120 ppb ozone for 9 d during flowering. At 120 ppb ozone, fewer seeds per capsule were lost on the first spike of the High Low population when flowering and exposure started later after sowing. Only with exposure from 60 DAS was the loss of seeds on the first spike compensated for by increased seed production on the second spike.

Table 5.18 shows that the response to ozone of the High Low population differed between spikes and the type of exposure involved. The combined effect of ozone on the first two spikes was a significant reduction in seed number per capsule following a direct exposure of the first spike during flowering, and an increase in seed number following exposure of the first spike during seed filling or of the entire plant during first spike flowering.

Table 5.17. Effect of ozone on the mean number of seeds per capsule when the first spike was exposed to 120 ppb ozone for 7 h d^{-1} for 9 d during flowering; + = increased by ozone, - = decreased by ozone, o = unaffected by ozone. *, ** and *** indicate significance at P<0.05, P<0.01 and P<0.001. NS, non-significant.

Population	Vegetative Sensitivity (A.W. Davison, pers. comm.)	Spike 1 - exposed	Spike 2 - unexposed	Overall effect = spike 1 + spike 2
High Low	Very sensitive	- (***)	o (NS)	- (***)
Penicuik	Sensitive	- (**)	+ (***)	+ (*)
Sibton	Resistant	o (NS)	o (NS)	o (NS)
Lullington	Very resistant	+ (***)	+ (***)	+ (***)

Table 5.18. Effect of ozone on the mean number of seeds per capsule in plants of *P. major* population High Low after direct exposure of the first spike at flowering or during seed filling, or after exposure of entire plants during flowering of the first spike. + = increased by ozone, - = decreased by ozone, o = unaffected by ozone. *** indicates significance at P<0.001. NS, non-significant.

Stage of reproductive development when exposed	Organs exposed to ozone	Spike 1	Spike 2	Overall effect = spike 1 + spike 2
Flowering	First spike	- (***)	o (NS)	- (***)
Seed filling	First spike	o (NS)	+ (***)	+ (***)
Flowering	Entire plant	+ (***)	+ (***)	+ (***)

5.3.2. Seed germination

Timecourses showing percentage germination of seeds from the lower and upper capsules of the exposed (first) and unexposed (second) spikes of each population are presented in Appendix 1.

5.3.2.1. *Exposure during flowering*

Exposure to 120 ppb ozone

For both spikes of ozone-treated and control plants, germination began within 24-72 h of seeds being placed onto agar for the Lullington, Sibton and High Low populations and within 72-96 h for the Penicuik population (Appendix 1). The timecourses for percentage germination varied between populations but were generally sigmoidal (Appendix 1). For control plants of Lullington, Sibton and High Low, the mean time taken for 50 % of the seeds to germinate (T_{50}) varied between 56-81 h for the first spike (Table 5.19a) and 59-89.5 h for the second spike (Table 5.19b). T_{50} was appreciably greater for control plants of Penicuik, at 165 h and 229 h for seeds from the first and second spikes respectively, suggesting that germination was inherently slower in this population. For Sibton and High Low, 95-100 % of all seeds from control plants germinated within 240 h. Final germination was lower for seeds from both spikes of control plants of the Lullington (69-83 %) and Penicuik (56-77 %) populations. For Penicuik, this may have been due to the slower germination of the seeds.

The timing of first spike flowering and exposure of the High Low population had little effect on total percentage germination at 240 h, whereas the mean T_{50} values for both the exposed and unexposed spikes apparently reached a maximum at around 51 DAS in both treatments.

Although not significant, it appeared that ozone increased T_{50} and reduced the percentage germination of seeds from the upper and lower capsules of the exposed and unexposed spikes of the Lullington population (Tables 5.19a, 5.19b). Ozone had no significant effect on the percentage germination of seeds from the lower and upper capsules of the exposed and unexposed spikes of the Penicuik, Sibton and High Low populations at any time interval up to 240 h (Figs. 1.1-1.6, Appendix 1).

Table 5.19a. Summary of the germination of seeds from the lower and upper capsules of spike 1 of four populations of *Plantago major* after spike 1 had been exposed to filtered air (control treatment) or to 120 ppb ozone for 7 h d^{-1} for 9 d during flowering. Results show the time taken for 50 % of the seed population to germinate (T_{50}) and percentage germination after 240 h. For seeds from the lower and upper capsules of each population, means for $T_{(50)}$ and germination do not differ significantly between treatments.

Population	SPIKE 1 (EXPOSED)							
	Lower				Upper			
	Control		Ozone		Control		Ozone	
	T_{50} (h)	Germination after 240 h (%)	T_{50} (h)	Germination after 240 h (%)	T_{50} (h)	Germination after 240 h (%)	T_{50} (h)	Germination after 240 h (%)
Lullington	58.5	83	108	71	65	69	153	59
Penicuik	165	77	142	93	185	66	181	79
Sibton	64	100	63.5	100	71.5	98	85.5	66
High Low 37 DAS	56	97	55	99	59	93	62	100
High Low 51 DAS	81	100	83.5	100	80	100	82	100
High Low 60 DAS	61	99	60	100	68	100	67	100

253

Table 5.19b. Summary of the germination of seeds from the lower and upper capsules of spike 2 of four populations of *Plantago major* after spike 1 had been exposed to filtered air (control treatment) or to 120 ppb ozone for 7 h d^{-1} for 9 d during flowering. Results show the time taken for 50 % of the seed population to germinate (T_{50}) and percentage germination after 240 h. For seeds from the lower and upper capsules of each population, means for $T_{(50)}$ and germination do not differ significantly between treatments.

Population	SPIKE 2 (UNEXPOSED)							
	Lower				Upper			
	Control		Ozone		Control		Ozone	
	T_{50} (h)	Germination after 240 h (%)	T_{50} (h)	Germination after 240 h (%)	T_{50} (h)	Germination after 240 h (%)	T_{50} (h)	Germination after 240 h (%)
Lullington	59	80	106	61	60	74	141.5	60
Penicuik	229	56	223.5	67	203	75	204	67
Sibton	85.5	100	84.5	100	89.5	95	98	79
High Low 37 DAS	68	99	63	100	66.5	100	62.5	95
High Low 51 DAS	78	100	83.5	100	77	100	82	100
High Low 60 DAS	67.5	100	67	97	72	100	79	100

For ozone-treated and control plants of High Low, seeds from both the first and second spikes started to germinate after 144 h (Fig. 1.7, Appendix 1). The respective mean T_{50} values for seeds from the lower and upper capsules of control plants were 167.5 and 165.5 h for the first spike and 167 and 181 h for the second spike. The total percentage germination recorded at 240 h varied between 59-74 % for all seeds from control plants (Fig. 1.7, Appendix 1). Exposure to ozone reduced the percentage of seeds from the upper and lower capsules of both spikes which had germinated at each time interval up to 240 h, thus increasing the T_{50} value. This reduction in percentage germination was only significant ($P<0.05$) for seeds from the upper capsules of the exposed spike between 168-240 h (Fig. 1.7, Appendix 1).

5.3.2.2. *Exposure during seed filling*

Less than 50 % of all seeds germinated within 240 h for both the control and ozone-treated plants of High Low, with a maximum of 1 % germination being observed in spike 2 (Table 5.20). Ozone increased the percentage of seeds which germinated at all time intervals, but this effect was not significant (Fig. 1.8., Appendix 1).

5.3.2.3. *Exposure of the entire plant during flowering*

The results presented in Table 5.20 for population High Low show that 50 % of the seeds from control plants germinated within 44-61 h of the start of the germination period and that almost 100 % of these seeds had germinated by 240 h. Ozone increased percentage germination by 240 h and also decreased the T_{50} value for seeds from the upper capsules of both the exposed and unexposed spikes; however, this effect on percentage germination was only significant for the exposed spike at 48 h (Fig. 1.9, Appendix 1).

5.3.3. Exposure of pollen to ozone

Environmental conditions

Within each exposure chamber, the average temperature and relative humidity during the 6 h exposure period varied between 23.4-23.7 °C and 56.8-64.0 %, for all exposures of pollen. During *in vitro* exposure of pollen to a target concentration of 100 ppb ozone, mean values of 106.8 ± 3.32 ppb and 97.2 ± 2.24 ppb ozone were achieved in replicates 1 and 2 respectively. For experiments with a target concentration

Table 5.20. Summary of the germination of seeds from the lower and upper capsules of spikes 1 and 2 of *Plantago major* population High Low after spike 1 had been exposed to filtered air (control treatment) or to 120 ppb ozone for 7 h d^{-1} for 9 d during seed filling or 54 ppb ozone for 7 h d^{-1} during flowering (entire reproductive plant exposed). Results show the time taken for 50 % of the seed population to germinate (T$_{50}$) and percentage germination at 240 h. For seeds from the lower and upper capsules of each spike, means for T$_{(50)}$ and germination do not differ significantly between treatments.

	Lower				Upper			
	Control		Ozone		Control		Ozone	
Exposure at seed filling	T$_{50}$ (h)	Germination after 240 h (%)	T$_{50}$ (h)	Germination after 240 h (%)	T$_{50}$ (h)	Germination after 240 h (%)	T$_{50}$ (h)	Germination after 240 h (%)
Spike 1	>240	26	>240	42	>240	7	>240	22
Spike 2	>240	0	>240	1	>240	1	>240	0

	Lower				Upper			
	Control		Ozone		Control		Ozone	
Exposure of entire plant at flowering	T$_{50}$ (h)	Germination after 240 h (%)	T$_{50}$ (h)	Germination after 240 h (%)	T$_{50}$ (h)	Germination after 240 h (%)	T$_{50}$ (h)	Germination after 240 h (%)
Spike 1	58	100	52.5	100	61	96	46	99
Spike 2	55.5	100	51.5	100	44	95	41	99

of 120 ppb ozone, the respective mean concentrations were 120.9 ± 1.80 ppb and 124.0 ± 4.73 ppb ozone during the *in vitro* and *in vivo* exposures of pollen.

5.3.3.1. In vitro *exposure*

Exposure to 100 ppb ozone for 6 h

After *in vitro* exposure to filtered air or 100 ppb ozone for 6 h, subsequent germination of pollen *in vitro* varied between populations and replicates. Of control pollen, the Sibton population had the highest percentage germination in both replicates and the values obtained were significantly greater than those recorded for the Lullington and Penicuik populations in replicate one (Table 5.21a) and Lullington in replicate two (Table 5.21b). In replicate two, the germination of control pollen was greater than in replicate one for all populations. Ozone caused a significant 28.3 % (P<0.05) increase in the germination of pollen from Lullington in the first replicate, but had no significant effect on any other population. As with germination, mean pollen tube length varied between populations and replicates. For control pollen, there was a significant difference (P<0.05) in both replicates between population High Low, which produced the longest pollen tubes, and Sibton, which produced the shortest. The only effect of ozone was a significant increase (P<0.05) in pollen tube length of Sibton in replicate two.

Exposure to 120 ppb ozone for 6 h

For control pollen exposed to filtered air, there was no significant difference between populations in percentage germination *in vitro* (Table 5.22). Ozone significantly reduced germination by 24.2 % in Lullington and by 21.2 % in Sibton (P<0.05). There were significant differences in the tube length of control pollen between populations, however, unlike both replicate exposures to 100 ppb ozone, there was no significant difference between the High Low and Sibton populations observed in this experiment. Ozone significantly increased (P<0.05) tube length of pollen from the High Low and Lullington populations.

5.3.3.2. In vivo *exposure*

Following *in vivo* exposure of pollen from the High Low population to filtered air or 120 ppb ozone for 6 h, 14.6 ± 4.30 % of control and 12.3 ± 3.23 % of ozone-treated pollen germinated *in vitro*, a difference which was not significant. Ozone significantly reduced pollen tube length from 378.4 ± 19.26 μm to 314.9 ± 16.89 μm (P<0.05).

Table 5.21a. Mean percentage germination and tube length for pollen collected from four populations of *Plantago major* after *in vitro* exposure to 100 ppb ozone for 6 h. Standard errors are shown. Means followed by a common letter are not significantly different at the 5 % level when compared using Duncan's multiple range test.

Population	Germination (%) n=5		Pollen tube length (μm) n=100	
	Control	Ozone	Control	Ozone
High Low	55.0 ± 7.50 *bcd*	59.2 ± 5.46 *abcd*	349.3 ± 22.07 *a*	317.4 ± 25.80 *ab*
Lullington	46.9 ± 7.26 *cd*	75.2 ± 2.20 *a*	300.8 ± 20.63 *ab*	312.7 ± 23.72 *ab*
Penicuik	44.4 ± 4.42 *d*	56.2 ± 2.02 *bcd*	292.9 ± 16.77 *ab*	261.4 ± 20.67 *bc*
Sibton	58.9 ± 5.80 *ab*	64.9 ± 9.38 *abc*	263.7 ± 20.72 *bc*	204.6 ± 14.00 *c*

Table 5.21b. Mean percentage germination and tube length for pollen collected from four populations of *Plantago major* after *in vitro* exposure to 100 ppb ozone for 6 h. Standard errors are shown. Means followed by a common letter are not significantly different at the 5 % level when compared using Duncan's multiple range test.

Population	Germination (%) n=5		Tube length (μm) n=100	
	Control	Ozone	Control	Ozone
High Low	64.2 ± 6.47 *ab*	59.1 ± 6.77 *ab*	212.2 ± 15.28 *bc*	192.1 ± 14.26 *b*
Lullington	49.4 ± 7.04 *b*	52.8 ± 8.38 *b*	187.5 ± 12.94 *bc*	192.0 ± 16.24 *bc*
Penicuik	66.0 ± 6.15 *ab*	59.1 ± 6.81 *ab*	181.1 ± 11.64 *bc*	169.5 ± 7.28 *bc*
Sibton	76.9 ± 3.17 *a*	76.9 ± 3.81 *a*	165.5 ± 10.13 *a*	279.6 ± 21.3 *c*

Table 5.22. Mean percentage germination and tube length for pollen collected from four populations of *Plantago major* after *in vitro* exposure to 120 ppb ozone for 6 h. Standard errors are shown. Means followed by a common letter are not significantly different at the 5 % level when compared using Duncan's multiple range test.

| Population | Germination (%) n=5 | | Tube length (μm) n=100 | |
	Control	Ozone	Control	Ozone
High Low	39.5 ± 6.88 *bc*	47.3 ± 8.78 *bc*	159.4 ± 10.33 *cde*	225.5 ± 15.41 *b*
Lullington	56.6 ± 1.96 *ab*	32.4 ± 5.72 *c*	193.1 ± 13.38 *bc*	301.3 ± 18.81 *a*
Penicuik	63.2 ± 4.46 *ab*	67.5 ± 2.31 *a*	124.0 ± 4.62 *e*	144.1 ± 7.04 *de*
Sibton	57.0 ± 3.07 *ab*	35.8 ± 8.46 *c*	136.9 ± 7.62 *de*	167.5 ± 16.50 *cd*

<u>5.4. DISCUSSION</u>

The results presented in this chapter show that both the maternal and paternal structures of *P. major* can be directly affected by exposure to ozone, but that the responses differ between populations. Direct exposure of the first reproductive spike during flowering had significant effects on seed number in three of the four populations examined. For two populations, there were also significant effects on seed number in the second spike of each plant which was not exposed to ozone. In addition, significant effects on seed number were recorded following direct exposure of the first spike during seed filling and also following exposure of whole plants during flowering, although these effects differed from those observed when only the reproductive structures were exposed to ozone. The following discussion considers separately the different aspects of reproduction which were examined.

5.4.1. Pollen

In vitro exposure of pollen to 100 ppb ozone for 6 h significantly increased pollen germination in Lullington and pollen tube length in Sibton, but there were no significant effects on the germination or growth of pollen from either the Penicuik or High Low populations (Tables 5.21a, b). These results show that there can be direct effects of ozone on pollen, and that there was differential sensitivity between populations, with pollen from Lullington and Sibton being more sensitive to ozone exposure than pollen from Penicuik and High Low. Differential sensitivities of pollen to ozone exposure have been reported previously for cultivars of petunia (*Petunia hybrida*; Harrison and Feder, 1974) and almond (*Prunus dulcis*; Hormaza et al., 1996). For Lullington, pollen germination was stimulated at 100 ppb (Table 5.21a) but inhibited at 120 ppb ozone (Table 5.22). There are no previous studies which suggest that ozone treatment may stimulate pollen germination, but there have been reports that adverse effects on the germination of pollen from almond (*Prunus dulcis* cvs. Butte, Carmel, Mission and Nonpareil), nectarine (*Prunus persica* cv. Fantasia) and peach (*Prunus persica* cv. O'Henry) increase with increasing ozone concentrations (Hormaza et al., 1996). The stimulation of pollen germination in Lullington was only observed in one of the two replicate exposures and this may be due, in part, to the environmental conditions of *in vitro* exposure, since the germination of pollen is known to be sensitive to variables such as light, temperature and relative humidity, as well as the chemical composition of the germination medium (Wolters and Martens, 1987). More detailed studies would be required to clarify the effects of *in vitro* exposure to ozone on the pollen of these four populations.

In general, exposure to ozone has been shown to affect both pollen germination and pollen tube growth (Feder, 1968; Feder and Sullivan, 1969a), although these processes may differ in their responses even though they are interdependent (Searcy and Mulcahy, 1985). Such a differential response was demonstrated by the Lullington population since germination was significantly increased following *in vitro* exposure to 100 ppb ozone, while pollen tube growth was unaffected. In Sibton, exposure to 100 ppb ozone had no significant effect on germination but appeared to stimulate pollen tube growth, while, in contrast, exposure to 120 ppb ozone reduced pollen germination without a concomitant effect on pollen tube growth (Table 5.22). This strongly suggests that there are independent effects of ozone on germination and pollen tube extension and that the threshold exposure for damage for germination is lower than that for tube growth. Previous studies have shown that treatment with ozone can significantly reduce pollen germination without affecting pollen tube growth (Benoit *et al.*, 1983) and *vice versa* (Riley and Feder, 1974); however, as noted for Sibton, different responses of pollen germination and pollen tube growth at different ozone concentrations have not been previously reported.

Although not always the case (Feder, 1968), it is generally accepted that pollen germination and pollen tube growth are more sensitive to *in vitro* than *in vivo* exposure to ozone because the anthers and stigmatic surfaces afford protection to the pollen. Krause *et al.* (1975), for example, showed that, although *in vitro* exposure of pollen from petunia (*Petunia hybrida*) cultivar 'White Bountiful' and tomato (*Lycopersicon esculentum*) cultivar 'Tiny Tim' to 10 or 15 ppb ozone for 3 h significantly reduced pollen tube growth, pollen was unaffected when exposed to 80 ppb ozone for 6 h either inside the anther or upon the stigmatic surface. In the present study, neither *in vitro* nor *in vivo* exposure to 120 ppb ozone had any significant effect on the germination of pollen from the High Low population. In contrast, pollen tube length was significantly reduced by exposure to 120 ppb *in vivo* but not *in vitro,* suggesting that the sensitivity of pollen from this population, with regards to pollen tube growth, was greater when exposed on the anther. This compares with the response of *Brassica campestris* (Section 4.3.2) in which pollen germination and pollen tube growth were significantly reduced by a 6 h *in vivo* exposure to 120 ppb ozone but not by exposure to this concentration *in vitro*.

For many species, the sensitivity of pollen to ozone can be positively correlated with the sensitivity of the foliage of the pollen parent (Feder, 1981). Feder and Sullivan (1969a), for example, showed that exposure of pollen to 100 ppb ozone for 5.5 h significantly reduced both pollen germination and pollen tube growth by about 50 % in two sensitive varieties of Bel W-3 tobacco, while pollen from two resistant varieties

was unaffected by treatment. Hormaza *et al.* (1996) also found a correlation between the vegetative and pollen sensitivities to ozone of various fruit and nut tree species and suggested that ozone may affect metabolic steps common to both the sporophyte and gametophyte. For *Pinus strobus,* however, Benoit *et al.* (1983) found no correlation between foliar symptom expression and pollen sensitivity. Although foliar symptoms can be used to assess sensitivity to ozone, effects on growth are more useful since the growth of many species can be reduced by exposure to ozone without the development of visible symptoms (Reiling and Davison, 1992a). In the present study, there was no visible damage to *P. major* following ozone treatment, but significant effects on physiological processes and growth were demonstrated which differed between populations (Section 3.3.3). By collating results from the two experiments undertaken to determine the effects of ozone on growth (Experiments 3b and 3c, Section 3.3.3), it appeared that ozone reduced growth to the greatest extent in Sibton followed, in turn, by the Lullington, High Low and Penicuik populations. Thus, following ozone treatment within the present exposure system, both vegetative growth and pollen development were affected to the greatest extent in Sibton and Lullington, suggesting that there may be a correlation between the vegetative and pollen sensitivities of these populations. More detailed research, however, would be required to substantiate this hypothesis and to elucidate the underlying mechanisms.

The results presented here show that ozone may directly affect germination and pollen tube growth when pollen is germinated *in vitro*. Further studies would be required to assess whether similar effects occur when pollen is germinated *in vivo* and whether this would ultimately affect seed set. In this study, there was no investigation of the effects of ozone on the stigmatal surface. Any alteration to the structure or chemical composition of the germination medium secreted by the stigma could influence the ability of pollen to germinate or penetrate the stigmatal surface or stylar tissues. As demonstrated with *Brassica campestris* (Section 3.3.2.2), pollen development may also be affected indirectly by ozone treatment, perhaps due to an alteration in assimilate supply to the reproductive structures. Therefore, investigations would also be required to assess whether ozone could indirectly affect the quantity or quality of pollen produced by *P. major*, and whether the effects induced depend upon the population being examined.

5.4.2. Exposure of reproductive spikes

In *P. major*, seed yield is determined by a number of components; the number of inflorescences per plant, the number of capsules per inflorescence, seed number per capsule and mean seed weight. In the present study, the first reproductive spike of

each plant was exposed to ozone during flowering or seed filling in order to assess the direct effects on seed yield components. Long-term studies to assess the impact of ozone exposure on the total number of inflorescences were not possible, but seed yield in the second spike of each plant was recorded to determine whether there were indirect effects of ozone treatment on the yield of seeds in subsequent spikes, either in terms of damage or compensation for any seed losses from the exposed spike.

The results indicate that the primary effect of direct exposure to ozone during flowering or seed filling, was upon seed number per capsule. As well as being dependent upon the timing of exposure, the response of seed number per capsule to ozone treatment was shown to differ between populations, between the spikes of each population and also within individual spikes.

5.4.2.1. *Exposure during flowering*

Floral development

For both the exposed (first) and unexposed (second) spike of each population there appeared to be no significant effect of ozone treatment on either floral morphology or development, and the duration of the flowering period was similar in both the ozone-treated and control plants, although these aspects of reproduction were not investigated in detail. Unlike *Brassica napus*, where direct exposure of the inflorescence to ozone increased the abortion of floral sites (Bosac *et al.*, 1994), there was no increase in floral abortion in any population of *P. major,* although in both ozone-treated and control plants a small number of sites aborted towards the apex of each spike once the maximum number of capsules had been established.

Capsule number

The number of capsules per spike was highly variable between spikes and individual plants, confirming a report by Primack (1978) that capsule number per spike is the most variable component of seed yield in *P. major*. The number of capsules produced by the first spike was unaffected by exposure to 120 ppb ozone during flowering, except in Sibton for which a significant increase was observed (Table 5.12). This increase in capsule number may have occurred because more flowers developed on the spike as a result of changes in the timing or rate of floral initiation, or because proportionally more flowers developed into capsules. The latter suggestion may be more plausible since it was commonly observed that a limited number of floral sites aborted towards the apex of the spike and therefore it may have been possible for

264

spikes to exhibit an increased number of capsules if more sites were retained to maturity. The results for Sibton contrast with those from previous studies which showed that ozone treatment generally decreases rather than increases fruit production (Feder and Sullivan, 1969b; Feder, 1970; Fernandez-Bayon *et al.*, 1993). In particular, Reiling and Davison (1992b) have shown that ozone treatment may decrease capsule number in *P. major*. In their study, 5 d old seedlings of the ISP (High Low) population were exposed to 70 ppb ozone for 7 h d^{-1} either for 8 wks or for 2 wk periods during weeks 1+2, 3+4, 5+6 or 7+8. A significant reduction in the number of capsules per spike was observed following exposure during weeks three and four. In the current study it was also shown that direct exposure of the first spike of High Low had no significant effect on capsule number, suggesting that this aspect of reproductive development is not particularly sensitive to ozone.

For each population, the number of capsules was greater on the second than on the first spike of both ozone-treated and control plants; this may have been due to more assimilates being available and allocated to reproductive development as the plants matured (Fig. 5.4). Treatment of the first spike with ozone had no significant effect on the number of capsules produced on the second spike in any of the populations examined.

Seeds per capsule - exposed spike

The number of seeds per capsule recorded in each of the four populations was within the range of 6-14 recorded by Kuiper and Bos (1992) for field grown plants of *P. major*. For the Penicuik and High Low populations, treatment with ozone significantly decreased the number of seeds per capsule in the exposed spike (Table 5.9), suggesting that ozone treatment may have increased the abortion of developing ovules or seeds. In contrast, there was a significant increase in the number of seeds per capsule in the exposed spike of Lullington. Since ovule number is primarily determined by genetic factors, it is unlikely that the increase in seed number observed in Lullington was the result of an increase in ovule initiation induced by ozone treatment. A more plausible explanation would be, at least for this population, that ozone acts by increasing the number of fertilised ovules which are retained to maturity. Detailed microscopic examinations of floral structures would be required, however, to detect any ozone-induced changes in ovule or seed abortion. Clearly these results suggest that ozone can have a direct effect on the reproductive development of *P. major*, but that the type of response is dependent upon the population examined. The increase in seed number noted for Lullington suggests that a mechanism exists by which plants could compensate for seed losses.

The response of seed number per capsule in the ozone-treated spikes of Penicuik and Lullington can be compared with the results of a previous study by Pearson *et al.* (1996), which examined the effect of ozone exposure on the growth and reproduction of three *P. major* populations, Great Dun Fell, Lullington and Penicuik (in their study referred to as Bush). Plants from each of these populations were exposed for 10 wks to filtered air, 35 ppb ozone for 24 h d^{-1}, 70 ppb ozone for 7 h d^{-1}, filtered air followed by three episodes each week of 70 ppb ozone for 7 h, or 35 ppb ozone continuously plus three 7 h episodes each week of 70 ppb ozone. Seed number per plant was significantly reduced in response to all ozone treatments in Penicuik whereas in Lullington and Great Dun Fell, seed number was significantly reduced in all treatments except the continuous exposure to 35 ppb ozone. Data were not presented to permit clarification as to whether these reductions were due to decreased numbers of capsules or seeds per capsule. However, the reproductive effort expressed in terms of the number of seeds produced per gramme of vegetative dry weight was increased in all ozone treatments for Lullington, but was decreased for Penicuik. In the present study, since the vegetative structures were maintained in clean air throughout the exposure and vegetative dry weight was unaffected by treatment, the observed increases and decreases in seed number reflect similar changes in reproductive effort to those reported by Pearson *et al.* (1996) for Penicuik and Lullington. The current study therefore highlights that effects on reproductive effort may occur independently of direct effects of ozone on foliar tissue; thus ozone-induced changes in foliar carbon assimilation and/or partitioning may not have been the primary factors responsible for the change in seed yield observed in the work of Pearson *et al.* (1996).

It could be suggested that the increase in reproductive effort observed for Lullington occurred at the expense of either root or shoot growth, or total spike production. However, although vegetative dry weight appeared to be maintained following exposure of the first spike to ozone (data not shown), relatively small changes in dry weight may have been difficult to detect; in addition, the plants were harvested before the final number of spikes had been produced, thus long-term changes in assimilate partitioning could not be investigated fully.

For each spike, it was observed that seed number per capsule decreased acropetally. As noted for other species (Bouttier and Morgan, 1992a), this may have been due to fewer ovules being produced in apical sites and/or a greater abortion of ovules or seeds in the later formed sites as a consequence of resource limitation. In each population, ozone treatment affected seed number per capsule at all positions along the exposed spike, but the effects were generally greater and more significant in the apical third of the spike (Figs. 5.1a, 5.2a). Interestingly, in Sibton, seed number per capsule was

266

significantly decreased in the upper third of the spike even though there was no significant effect on mean seed number per capsule averaged over the entire spike. These results suggest that all stages of floral development, and in particular the later formed sites may be sensitive to direct exposure to ozone during flowering. The greater effect in capsules from the upper third of the spike may have resulted from these sites being exposed at a more sensitive stage of development. Alternatively, ozone treatment may have decreased the amount of assimilates available within the spike through effects on photosynthesis and/or changes in translocation. Under stressful conditions where the supply of essential assimilates is limited, young flower buds constitutes weak sinks in comparison to developing fruits, and compete poorly for the available assimilates (Halevy, 1984). Thus greater abortion of ovules or seeds may have occurred in the apical sites as a result of resource limitation. The results for *P. major* compare favourably with those recorded for *B. campestris* following direct exposure of the terminal raceme. These showed that ozone treatment also increased seed abortion in *B. campestris*, but particularly so in the later formed sites towards the apex (Section 4.3.1.3).

It is particularly interesting that the responses to ozone exposure differed between populations and is possible that genetic variability or differences in the degree of phenotypic plasticity may explain these responses. Populations may become resistant to ozone as a result of genetic adaptation through selection for or development of ozone tolerance. It has been well established that genetic variation occurs between populations of *P. major* (Groot and Boscuhhuizen, 1970; Kuiper and Bos, 1992) and this has been suggested by Reiling and Davison (1992d) as a possible reason for the observed differences between populations in vegetative sensitivity to ozone. Alternatively, or in addition, the plants from each population may exhibit a different degree of phenotypic plasticity. Having plasticity means that plants have some capacity to alter certain aspects of their morphology or physiology in order to maintain themselves in a habitat which experiences fluctuating environmental conditions (Kuiper and Bos, 1992) *P. major* is a species which has been shown to exhibit a high degree of phenotypic plasticity. Trivedi and Tripathi (1982), for example, demonstrated plasticity in the response of *P. major* to various treatments of moisture and soil type. In their study, relatively more seeds were produced in plants grown in garden soil than in those grown in sandy soil, due primarily to an increased number of seeds per capsule. In addition, seed yield was shown to increase with increasing moisture stress although this was the result of an increase in the number of capsules per plant rather than seed number per capsule. The results of their study indicate that the adjustment of investment in reproduction may occur at more than one stage of development and that the specific changes depend upon the environmental stress to which the plants are

exposed. Similarly, the plastic responses in all of the seed yield components of *P. major* except single seed weight have been shown to vary between plants from different microhabitats (Lotz and Spoormakers, 1988). In an investigation of the degree of physiological plasticity in four genotypes of *P. major*, Kuiper (1983) noted that not all genotypes responded in the same way to alterations in the supply of mineral nutrients, with both highly plastic and marginally plastic genotypes being identified. In subsequent studies involving two of these genotypes, Poorter and Lambers (1986) demonstrated that, when grown in competition, a highly plastic genotype was able to perform better than a marginally plastic line as the frequency of fluctuations in nutrient availability increased. This response was thought to occur because the highly plastic genotype was able to react more rapidly to change. It may be possible, therefore, that the different responses of the four *P. major* populations examined in the present investigation were due to differences in their degree of physiological or morphological plasticity. Such differences may influence which aspects of seed yield respond to ozone stress and how rapidly these responses occur. Further investigations would be required to determine the importance of genetic variability and phenotypic plasticity in the response of individual populations to ozone treatment.

Seeds per capsule - unexposed spike

Despite being maintained in clean air throughout their development, an increase in seed number per capsule was recorded for the second spike of each population, although this was only significant for Lullington and Penicuik (Table 5.9). These responses contrast with the decrease in seed number per capsule generally observed in the exposed spike, but indicate that there may be an indirect effect of ozone treatment on seed number per capsule in subsequent spikes, the extent of which depended upon the population examined.

Ozone affected seed number per capsule throughout the length of the second spike in both Lullington and Penicuik, with the greatest effect being observed in the upper capsules of Lullington and the lower capsules of Penicuik (Fig. 5.1b). Compared with control plants, the response of seed number in ozone-treated plants was similar for the exposed and unexposed spikes of both populations, with seed number increasing progressively towards the apex in Lullington and decreasing towards the apex in Penicuik. In High Low and Sibton there was no significant effect on seed number per capsule when averaged over the whole of the unexposed spike and in general there was no significant effect on seed number at any position along this spike in either population (Figs. 5.1b, 5.2b).

The variability between populations in the response of the unexposed spike may have resulted from the different responses of the exposed spike. Thus changes in seed number or physiology within the exposed spike may have altered the availability and partitioning of assimilates to subsequent spikes. Alternatively, the variable response of the second spike may indicate differences between populations in their ability to compensate for damage to reproductive structures or the mechanisms involved in such compensatory responses.

Seeds per capsule - the influence of the time to first flowering

In order to determine whether natural genetic variability within populations could alter the response of *P. major* to ozone, plants of High Low which initiated flowering at three different dates after sowing were exposed for 9 d to 120 ppb for 7 h d^{-1} during flowering. The results showed that, although there was a consistent reduction in seed number per capsule in the exposed spike of High Low, this reduction became progressively less the later flowering started after sowing (Table 5.9). The time to first flowering had no significant effect on the response of the second spike in which seed number per capsule was similar in both ozone-treated and control plants. These results show conclusively that direct exposure of the inflorescences of High Low to ozone during flowering reduces seed number per capsule, although the extent of the reduction appears to depend upon the time to first flowering. It could be suggested that there was less effect on seed number in later flowering plants because more resources were available from the larger leaf area present to support repair processes and/or compensation mechanisms. Plants which are able to delay the initiation of flowering may therefore be able to limit the impact of ozone stress on reproductive output.

The reduction in seed number per capsule recorded for the first spike of High Low following exposure from 35 DAS to 70 ppb ozone for 7 h d^{-1} for 7 d during flowering was similar to that observed following a 9 d exposure from 37 DAS to 120 ppb ozone for 7 h d^{-1}. Although the duration of exposure and concentration of ozone differed between these two experiments, the responses of seed number were similar, substantiating the hypothesis that the time to first flowering is an important factor in determining plant responses to ozone. If responses are dependent upon the time of flowering then this could have particular consequences not only for individual plants but also for populations, since the time to first flowering is known to vary between individuals and also between populations of *P. major* as a result of genetic variability (Lotz and Blom, 1986; Lotz, 1990).

Seeds per spike and seed weight

Since the number of capsules was generally unaffected by exposure to ozone, the significant effects of ozone on seed number per capsule were reflected in the total number of seeds per spike although because of the variability between plants, the effect of ozone was not significant for any population (Table 5.13). Mean seed weight was similar in the ozone-treated and control plants of each population except for both spikes harvested from ozone-treated plants of Penicuik (Table 5.4), and the second spike of ozone-treated plants of High Low exposed from 51 DAS (Table 5.7), in which seed weight was significantly increased. These effects may have occurred because fewer seeds developed on the exposed spikes of these two populations. For those populations in which mean seed weight was maintained, the total weight of seeds reflected the total number of seeds per spike and as such was not significantly different between the spikes of ozone-treated and control plants. In a study by Reiling and Davison (1992b), it was shown that there was no significant effect on mean seed weight of either two or eight week exposures of the *P. major* population High Low (ISP) to 70 ppb ozone for 7 h d^{-1}, and it was suggested that this was because seed weight was under strong genetic rather than environmental control. It has also been noted that seed size is the least plastic character of *P. major* under various conditions of moisture stress, soil type (Trivedi and Tripathi, 1982), nutrient availability and competition (Palmbald, 1968). However, in the present study, as well as variability in seed weight between populations, it was noted that mean seed weight was greater in the first than in the second spike of each population irrespective of treatment. This may have occurred because the greater number of seeds produced by the second spike reduced the amount of resources available to support the growth of individual seeds. Although no data have been presented, it was also noted that individual seed weight tended to decrease towards the apex of the spike, possibly due to resource or space limitation for growth. Any future studies of the impact of ozone on the reproductive output of *P. major* should be aware of such variability within the plant if the precise nature of responses is to be detected.

Compensation for seed losses?

It has been shown that many species can compensate for the loss of floral structures following environmental stresses such as herbivory (Lowenberg, 1994; Obeso and Grubb, 1994) by increasing assimilate allocation to reproduction although the degree of compensation depends upon the timing of the stress (Escarre *et al.*, 1996) and the type and severity of damage (Lowenberg, 1994). Once all of the floral initials have been established on the spikes of *P. major*, the plants have little capacity to compensate for

seed loss by producing additional floral sites since the production of reproductive sites within individual spikes is essentially determinate as in wheat. However, *P. major* may be able to compensate for seed loss through the production of more capsules or seeds per capsule on subsequent spikes, an increase in seed weight, or the production of additional spikes. For each population, seed number per capsule was averaged over the two harvested spikes to determine whether compensation for any seed loss occurred following ozone treatment (Table 5.10). These calculations revealed that the significant decrease in seed number per capsule in the fumigated spike of Penicuik was offset by a corresponding increase in the unexposed spike; indeed, the overall effect was a significant increase in seed number per capsule. In contrast, there was no apparent compensation for seed loss in the fumigated spike of High Low since seed number was maintained unchanged in the unexposed spike. Seed number per capsule may have been maximised in this population such that the number of seeds could not be increased in later spikes by retaining ovules and/or seeds which might otherwise have aborted naturally. A decrease in ovule and/or seed abortion may however explain the increased number of seeds in Lullington and Penicuik following ozone treatment. In a number of species, decreased seed abortion has been shown to occur as a compensatory mechanism to maintain seed yield following the loss of inflorescences through herbivory (Lowenberg, 1994; Lehtila and Syrjanen, 1995). Although there appeared to be no compensation for ozone-induced seed loss in the first spike of High Low, longer-term studies would be required to determine whether compensation may have occurred over time, for example, through the production of additional spikes.

The predominant way that plants compensate for stress is through shifts in carbon allocation (Pell *et al.*, 1994); for example, the shoot:root ratio tends to increase in ozone-treated plants in response to damage to above-ground plant tissues (Deveau *et al.*, 1987; Held *et al.*, 1991). The mechanisms responsible for the induction of compensatory shifts are not known although it is likely that hormonal changes may lead to altered genetic regulation of protein synthesis, which may in turn modify metabolism within individual tissues or organelles, leading to an increase in sink strength (Pell *et al.*, 1994).

5.4.2.2. *Exposure during seed filling*

Although exposure of the first spike of High Low to 120 ppb ozone for 7 h d^{-1} for 14 d during seed filling had no significant effect on any of the measured seed parameters, there was a significant increase in seed number per capsule in the unexposed spike. This is in contrast to the response observed following exposure during flowering, when seed number per capsule was significantly decreased in the

exposed spike but was unaffected in the unexposed spike. It is probable that, by the time the seed filling stage was reached, seed number per capsule had been determined and the number of seeds was therefore unaffected by treatment. However, an increase in seed number was observed in the second spike, a response which was not observed following exposure during flowering. Although the dose of ozone applied differed between the exposures during flowering and seed filling, it could be suggested that the timing of exposure may be important in determining the direct effect of ozone on seed yield.

5.4.2.3. *Exposure of whole plants during flowering*

In a previous study by Reiling and Davison (1992b), the number of spikes produced by plants of *P. major* population ISP (High Low) was significantly reduced following exposure of 5 d old seedlings to 70 ppb ozone for 7 h d^{-1} over an 8 wk period or for 2 wk periods during weeks 1+2, 3+4, 5+6 or 7+8 of this 8 wk period, with the greatest effects being observed following the 8 wk treatment or exposure after week five. Their study also showed that seed number per capsule was unaffected by ozone treatment and that the number of capsules per spike was only significantly reduced following exposure in the third and fourth weeks; thus the total number of seeds per plant was reduced primarily as a consequence of a reduction in spike number. In the present study, exposure of High Low to 54 ppb ozone for 7 h d^{-1} for 9 d during flowering significantly increased the number of seeds per capsule in both the first and second spikes, in contrast to the results of Reiling and Davison (1992b) which showed seed number per capsule to be unaffected by ozone treatment. Evidence from the current study that seed number per capsule was increased when whole plants were exposed to ozone, but was decreased when only the inflorescence was exposed suggests that indirect positive effects on seed number were mediated via the vegetative structures. However, as the plants were harvested before the maximum number of spikes had been produced, the potential effects of ozone on the total number of inflorescences or final seed yield could not be assessed.

5.4.2.4. *Possible mechanisms of ozone action*

The results of the present study clearly indicate that exposure to ozone primarily affects seed number per capsule and suggest that ozone treatment may either promote or inhibit seed maturation depending upon the population and timing of exposure. As already suggested in Chapter 4 for *B. campestris*, ozone may influence seed development in three ways. Firstly, ozone could damage the embryo directly, either by damaging the embryo itself or the pollen and ovule which form the embryo. Secondly

ozone may stress the maternal plant, affecting the quantity or quality of resources provided to the seed. Thirdly, ozone may affect the synthesis or distribution of hormones required for the successful development and maturation of the seed. Potentially, the direct effects of ozone on the exposed spike may have originated from one or more of the above, whereas the effects on the unexposed spike are clearly indirect, suggesting the existence of a signalling mechanism between spikes, perhaps involving changes in hormone levels or a redirection of assimilates from damaged towards healthy tissues.

Since *P. major* is largely self-pollinating, it is possible that ozone may have directly affected the number of seeds produced on the exposed spike by influencing pollen germination and development. Studies by Hawthorn (1974) have shown that, as an adaptation to wind pollination, each anther of *P. major* may produce as many as 600 pollen grains or around 2400 grains per flower. In the present study, it appeared that, of the four populations examined, pollen from Lullington was the most sensitive to ozone since germination was reduced by 24 % following a 6 h *in vitro* exposure to 120 ppb ozone. Since a copious amount of pollen is produced by the anthers and only 6-9 seeds per flower are produced on average, a 24 % reduction in the amount of viable pollen would still leave sufficient pollen to guarantee maximum seed set. Indeed, for Lullington, a significant increase in seed number was observed following exposure to 120 ppb ozone during flowering, suggesting that the potential reduction in pollen germinability did not adversely affect seed yield. Nonetheless, as repeated exposures to ozone over a number of days may have had a greater effect on pollen than a single 6 h exposure, it is possible that the decrease in seed number recorded for Penicuik and High Low was the result of adverse effects on pollen germination or development. The observation that seed number was maintained in High Low when exposed to ozone after the completion of seed set, may support this hypothesis. Although it is possible that direct effects of ozone on pollen could affect seed number per capsule in the exposed spike, the pollen produced by the unexposed spike was never exposed to ozone, therefore effects on pollen are unlikely to explain the increased seed number in the unexposed spike recorded for Lullington and Penicuik.

There is no evidence in the literature to suggest whether the spikes of *P. major* are able to carry out photosynthesis or if they are a major source of assimilates for the developing seeds. However, previous investigations have demonstrated the importance of ear photosynthesis in supplying assimilates for grain growth in wheat (Evans *et al.*, 1972) while inflorescence have been shown to be effective assimilatory organs in grasses (Ong *et al.*, 1978). Using radio-labelled carbon, Lacey and Marshall (1992) studied the pattern of CO_2 uptake in *Plantago virginica*, a relative of *P. major* with a

similar morphology. They noted that the spikes were able to assimilate CO_2, but that this accounted for only 5.5 % of the total CO_2 uptake by the plant. The primary source of assimilates for the developing spikes came from the subtending leaf, and from the time of spike appearance to the time of seed development the amount of assimilate moving from this leaf to the spike approximately doubled to 66 %. Between 38-50 % of assimilates were also exported from mature leaves to younger leaves and their associated spikes, but there was no movement of assimilates from the spikes to any other part of the plant. It appears probable that, in a similar way to *P. virginica*, the leaves of *P. major* are the major source of photosynthate to the developing seeds, since the photosynthetic area of the developing capsule is small relative to that of the leaves. If this is the case, it is unlikely that an effect of ozone on spike photosynthesis would be sufficient to affect ovule or seed development by limiting resource availability. Therefore, a more direct effect of ozone on ovule or seed development, perhaps acting through cellular damage, may have decreased seed number per capsule in High Low and Penicuik. These two populations may have a more limited capacity to tolerate and/or repair ozone damage than Sibton and Lullington, possibly explaining, at least in part, the different responses of each population. To attain the increase in seed number per capsule recorded for Lullington, ozone treatment may either have promoted seed maturation or increased the sink strength of the exposed spike such that more assimilates were transported to this spike. As shown by Rigney (1995), increased availability of resources decreases ovule abortion, thereby allowing more seeds to develop to maturity.

As already discussed in Section 4.4.3.2., reproductive development depends not only upon resource availability but also upon hormones (Bouttier and Morgan, 1992b). Changes in the levels of hormones in response to ozone treatment may occur for several reasons. Firstly, ozone may alter hormone production directly. Studies have shown that ozone can affect the production of ethylene (Mehlhorn and Wellburn, 1987; Mehlhorn *et al.*, 1991), although further research is required to establish whether the levels of other hormones are also affected, particularly within the reproductive structures. Secondly, changes in resource partitioning in response to ozone stress may alter the transport of hormones within the plant (Halevy, 1987). Hormones may affect ovule or seed development directly, although it is also thought that the level of endogenous growth substances may regulate the import of assimilates into sinks by influencing sink activity (Gifford and Evans, 1981; Halevy, 1987). Changes to hormone levels could therefore indirectly alter the quantity of assimilates received by the developing ovules and seeds.

The results presented here, indicate that the effect of direct exposure to ozone during flowering on seed number per capsule may be associated with the vegetative sensitivity of each population as defined by Reiling and Davison (1992d). Following their classification, direct exposure to ozone caused a significant reduction in seed number per capsule in the two sensitive populations, High Low and Penicuik, had no effect on seed number in the resistant population, Sibton, and significantly increased seed number per capsule in the very resistant population, Lullington. These results might suggest that physiological or biochemical processes common to both the vegetative and reproductive structures are affected by exposure to ozone. Alternatively, it may be possible that the vegetative and reproductive structures of plants have a similar ability to respond to ozone stress and compensate for damage. Thus the increase in seed yield in Lullington observed in the present study and the relative insensitivity to ozone of the vegetative structures noted by Reiling and Davison (1992d) may indicate a high level of adaptability in this population. In contrast, a low level of adaptability in High Low may have precluded compensation for the significant reduction in seed yield which occurred following direct exposure to ozone and the corresponding decrease in growth observed for this population (ISP) by Reiling and Davison (1992d). The vegetative sensitivity of each population could not be verified within the present exposure system and therefore the suggestion that vegetative and reproductive sensitivity could be linked may not be valid. The indirect effect of ozone treatment on the second spike of each plant could not be linked to vegetative sensitivity, although similar responses of the exposed and unexposed spikes were observed in the two resistant populations, Sibton and Lullington.

5.4.3. Seed germination

The percentage germination of *P. major* seeds varied between 60-100 % but was generally over 90 % (Appendix 2). These values are typical of those recorded for *P. major* in other laboratory studies (Sagar and Harper, 1960) and also in the field (Kuiper and Bos, 1992). Within populations, the timecourse of germination was similar for seeds from the upper and lower capsules of each spike and there also appeared to be little variability between spikes. Between populations, however, there were inherent differences in the timecourse of germination and it appeared from the data obtained that the rate of germination was lowest in Penicuik, possibly because the seeds were the smallest of those produced by all four populations. As suggested by Karssen (1970), smaller seeds have relatively thicker seed coats than larger seeds, and this forms a mechanical barrier that has to be overcome by the embryo. Although ozone

275

treatment during flowering appeared to increase mean seed weight in Penicuik, there was no effect on the rate or extent of germination. Previous studies have shown that larger seeds tend to produce larger seedlings and that this leads to an increase in competitive ability (Salonen and Suhonen, 1995). Further studies would be required to determine whether the observed increase in seed weight in Penicuik resulted in an increase in seedling vigour. Ozone treatment reduced the rate and extent of germination in all of the seed fractions harvested from Lullington (Fig. 2.1, Appendix 2) and High Low following exposure to 70 ppb ozone for 7 h d^{-1} for 7 d during flowering (Fig. 2.7, Appendix 2), although the decrease was only significant for seeds from the upper capsules of the exposed spike of High Low. Surprisingly perhaps, the effect of ozone on the germination of seeds from High Low was greater following a 7 d exposure to 70 ppb than a 9 d exposure to 120 ppb. Although exposure of High Low to ozone during seed filling had no significant effect on subsequent seed germination, the percentage germination of seeds from both ozone-treated and control plants was very low (Fig. 2.8, Appendix 2). This may have resulted from the extended storage period prior to germination, since it is known that germinability can decrease with time (Kuiper and Bos, 1992). It was also shown for High Low that germination was unaffected by exposure of entire plants during flowering, although germination was greater for seeds from the upper capsules of both the exposed and unexposed spikes (Fig. 2.9, Appendix 2).

Although numerous studies have shown that elevated concentrations of ozone may affect seed yield and seed quality, the effects of ozone on germination have rarely been investigated. In a previous study, Bosac (1992) reported that direct exposure of the flowering racemes of *Brassica napus* L. cvs. Tapidor and Libravo to 100 ppb ozone for 6 h slightly delayed germination relative to the controls, although this effect was not significant. In contrast, the current investigation showed that seed germination was stimulated in *B. campestris* following direct exposure of the terminal raceme to 100 ppb ozone for 6 h over four consecutive days (Sections 4.3.1.2 and 4.3.1.3). *P. major* is a rather short lived perennial species, which propagates only occasionally by vegetative means (Sagar and Harper, 1964). Survival of populations therefore depends considerably upon continued establishment from seeds. Although this species exhibits a high percentage germination, populations are reduced in size through heavy mortality, particularly as a result of infection by powdery mildew (*Erysiphe pisi*; Palmbald, 1968). Thus any significant reduction in the rate or ability of seeds to germinate may have consequences for the establishment and survival of populations. The seeds of *Plantago* are also important constituents of seed banks and are known to remain viable for up to 40 years (Crocker, 1938, cited by Hawthorn, 1974). Any effect of ozone treatment on the structure or physiology of seeds held within seed banks, leading to

changes, for example, in seed dormancy, may also lead to positive or negative effects on seed germination or mortality and therefore to alterations in the demographic structure of populations.

5.4.4. Summary

This study demonstrates that elevated concentrations of ozone can affect the reproductive structures of *P. major* directly, primarily by altering seed number per capsule. The responses, however, appear to be complex, with interactions being observed between individual spikes and also between the vegetative and reproductive structures. Furthermore, responses were shown to vary within populations and also with the timing of exposure to ozone. Both positive and negative effects on seed number per capsule were apparent, suggesting that ozone may influence the extent of ovule or seed abortion. Such effects may have resulted from direct damage to individual cells or tissues, leading to changes in membrane function or cell physiology, although changes in resource assimilation and/or partitioning or the levels of endogenous hormones may also be implicated, particularly in the compensation mechanisms which may have been operating. In particular, this study has demonstrated that responses to ozone stress can vary between populations, with both significant increases and decreases in seed yield being observed. Such variability between populations may have originated from differences in the ability of plants to tolerate and/or repair ozone damage to the reproductive structures or compensate for seed losses. It is conceivable that genetic variability or differences in the degree of phenotypic plasticity exhibited by individual populations may explain these diverse responses. The damage and compensation mechanisms which occur following direct exposure of the reproductive structures to ozone may or may not be apparent following whole plant exposures, possibly explaining the differing responses observed in this work and that of Reiling and Davison (1992b).

CHAPTER 6 - CONCLUDING DISCUSSION.

This study has clearly demonstrated that the vegetative and reproductive structures of *B. campestris* and *P. major* are sensitive to ozone. In particular, the reproductive development of both species was shown to be affected directly by exposure to ozone (Chapters 4 and 5), confirming the observation of Bosac (1992) that the reproductive structures themselves may be sensitive to ozone. The following discussion brings together the results for both species and considers the extent to which the observed effects on reproductive development may have been influenced by the reproductive growth habit. The implications for plants growing in the field and future research requirements are also discussed.

6.1. IMPACT OF OZONE ON PHYSIOLOGY AND GROWTH

The data presented in Chapter 3 showed that exposure of plants to 70 ppb ozone significantly reduced the net rate of photosynthesis (A) and stomatal conductance (g_s) of both *B. campestris* and *P. major*. These effects generally occurred within 3.5 h of the start of exposure and both A and g_s continued to decline until the end of the 7 h exposure period. An ozone-induced decline in g_s and/or A has been previously reported for many species (Darrall, 1989). However, the present study is the first to demonstrate that physiological processes in *B. campestris* are sensitive to ozone and also confirms previous reports that the physiology of *P. major* is sensitive to ozone (Reiling and Davison, 1994; Reiling and Davison, 1995; Pearson *et al.*, 1996), although for three of the four populations examined, the responses differed to those reported by Reiling and Davison (1995). In particular, Reiling and Davison (1995) noted that g_s and A were more adversely affected in populations which previous investigations of the effects of ozone on vegetative growth (Reiling and Davison, 1992d) had shown to be ozone-sensitive, and less affected in populations which were more ozone-tolerant. In the present study, the decline in g_s was similar for all populations and, with the exception of Penicuik, this was accompanied by a generally synchronous decline in A. The differences between the two studies may have resulted from the plants having experienced different environmental conditions during growth and/or exposure or being exposed at different stages of development. These factors may have been responsible for the larger g_s values observed for in the present study, which may have affected ozone uptake and therefore damage to the foliar tissues. The reasons for the differing responses between populations are not apparent from the present study, but Reiling and Davison (1995) have suggested that there may be different mechanisms of damage and resistance in different populations.

278

The generally synchronous decline in A and g_s in both *B. campestris* and *P. major* suggests that the decrease in A was caused by the reduction in stomatal conductance which limited the availability of CO_2 for photosynthesis, although evidence was presented in Chapter 3 which suggests that ozone may damage both the stomatal and photosynthetic apparatus directly. In the *P. major* population, Penicuik, exposure to 70 ppb ozone reduced stomatal conductance to a greater extent than A, and the intercellular CO_2 concentration (C_i) was also reduced, implying that ozone may have affected the stomata directly, with little effect on CO_2 fixation. By contrast, in *B. campestris*, A was reduced to a greater extent than g_s; in addition, the development of visible damage and an increase in the intercellular CO_2 concentration of these plants indicated a direct effect of ozone on the photosynthetic system. This work is important because it adds to the increasing quantity of literature documenting the possibility of direct effects of ozone on stomatal conductance (Aben *et al.*, 1990) and photosynthesis (Reich and Amundson, 1985; Temple, 1986; Myhre *et al.*, 1988; Farage *et al.*, 1991) in both native and crop species at low concentrations and dosages of ozone.

The results of three separate experiments with *B. campestris* suggested that the effect of ozone on A was influenced by the stomatal conductance of the plants immediately prior to exposure and therefore the flux of ozone into the leaves (Section 3.3.1.1.). In each experiment, exposure to 70 ppb ozone reduced g_s by a similar degree, but in plants with larger stomatal conductances the decline in A was greater and visible injury in the form of chlorosis and necrosis developed on the leaves. When stomatal conductance was greatest, extensive necrotic lesions developed after only a single 7 h exposure to 70 ppb ozone. It is probable that initial g_s varied between experiments because the plants had experienced different environmental conditions (temperature, humidity, light) prior to exposure. Indeed, environmental factors such as humidity and water availability which affect stomatal conductance have been shown in previous studies to alter the extent of visible foliar damage induced by ozone (McLaughlin and Taylor, 1981; Olszyk and Tibbitts, 1981b). The present study highlights the importance of considering pre-exposure environmental conditions as potentially influential factors in the response of plants to ozone in controlled chamber studies.

The observed decreases in net photosynthesis were probably responsible for the reductions in total plant biomass recorded for *B. campestris* (Table 3.8) and *P. major* (Table 3.17) following 10 and 14 consecutive days of exposure respectively to 70 ppb ozone for 7 h d^{-1}, although these decreases were not significant in either species. For *B. campestris,* this may have been due to the repair of damaged tissues or an acclimation to ozone stress since A was returned to control levels by the start of the tenth day of exposure (Fig 3.5). Such a response may have been facilitated by the

increased allocation of assimilates towards shoot growth in this species. In *P. major*, root:shoot ratio was unaffected by exposure to ozone but the greater ability for A to recover between exposures may have helped maintain relative growth rates. As with the physiological responses to ozone, the effects on growth were shown to vary between the populations of *P. major* examined in a manner which contrasted with the results of a similar study by Reiling and Davison (1992d). From calculations of ozone resistance ($\overline{R}$ in ozone-treated plants expressed as a percentage of $\overline{R}$ in control plants), Reiling and Davison (1992d) identified Lullington and Sibton as ozone-tolerant populations and High Low (ISP) as the most ozone-sensitive of the 28 populations which they examined. The present investigation, however, showed each of these three populations to be sensitive to ozone, with sensitivity increasing in the sequence High Low, Lullington and Sibton (Table 3.17). This disparity between comparable studies suggests that the response of $\overline{R}$ may not be a reliable indication of ozone sensitivity. A number of factors including environmental variables and the stage of plant development are known to influence $\overline{R}$ and it is possible that these may interact with the effects of ozone. In addition, plants may acclimatise to ozone stress or compensate for damage to the vegetative organs, particularly during the longer exposure periods required to assess the impact of ozone on growth. This ability to acclimatise or compensate for ozone damage may vary under different environmental conditions, with possible implications for $\overline{R}$. Clearly the results indicate that further research is required to verify the relative sensitivities of the *P. major* populations examined in this study.

For crop plants, a decrease in vegetative growth is important particularly when the vegetative structures are the harvestable components. Although not always the case (e.g. Oshima *et al.*, 1975; Mulholland, 1997a), an ozone-induced decrease in growth may also reduce the reproductive output of seed or fruit crops (Cooley and Manning, 1987). For native species, deleterious effects on reproductive output could have serious implications for the maintenance and dispersal of populations, while a reduction in biomass may place the plants at a competitive disadvantage such that they are less able to compete with more ozone-tolerant species for resources such as nutrients, water and light. In biennial and perennial species, a reduction in shoot growth could diminish the supply of assimilates available for storage in the roots and this might result in a poor start in growth the following season. If this cycle of decline were to continue over successive growing seasons, more susceptible species might easily be replaced by more pollutant-tolerant species. Smaller plants, however, may be less apparent to herbivores, increasing their chances of survival. In both crop and native species, decreases in root:shoot ratio which commonly occur in response to ozone (Darrall, 1989) may also predispose the plants to stresses such as drought.

6.2. IMPACT OF OZONE ON REPRODUCTIVE DEVELOPMENT

Numerous studies have shown that the yield of many plant species (cf. Section 1.3.2), including *P. major* (Reiling and Davison, 1992b; Pearson *et al.*, 1996), may be adversely affected by exposure to ozone. Indeed, the present study showed that the floral production and seed yield of *B. campestris* was reduced following a 10 day exposure to 70 ppb ozone for 7 h d^{-1} (Fig. 3.8, Table 3.11). Although it is generally considered that effects on yield may arise from damage to the vegetative structures and consequent decreases in the availability of assimilates for reproductive development, Bosac (1992) reported that the reproductive structures of *Brassica napus* were damaged directly by exposure to ozone. In the present study, the direct effects of ozone on the growth of pollen and other aspects of reproductive development in *B. campestris* and *P. major* were examined using two purpose-designed exposure chambers which allowed the reproductive structures of up to 12 plants to be exposed simultaneously to ozone or charcoal-filtered air. While this study showed conclusively, and for the first time, that the reproductive structures of both species were sensitive to ozone in terms of effects on pollen development and seed yield, the timing of exposure proved to be an important factor in determining the nature of the responses, while intraspecific variation in ozone-sensitivity was also demonstrated in *P. major*. These effects are now discussed.

6.2.1. Pollen

An understanding of the effects of air pollutants on seed yield requires a knowledge of the responses of factors which determine seed number and seed quality. Of fundamental importance to sexual reproduction is the availability of viable pollen. In a limited number of previous studies, it has been shown that exposure of pollen to SO_2 (Masaru *et al.*, 1976; Varshney and Varshney, 1981; O'Conner *et al.*, 1987) and/or ozone (Feder, 1968; Feder and Sullivan, 1969a; Hormaza *et al.*, 1996) may adversely affect pollen germination and pollen tube growth. These early investigations were the first to suggest a possible direct effect of air pollutants upon reproductive processes. The present study confirmed a direct effect of ozone on the pollen of *B. campestris* and *P. major*. As well as being dependent upon the concentration of ozone to which pollen was exposed, the effects on germination and pollen tube growth were shown to vary between species, populations of *P. major* and *in vitro* and *in vivo* exposure of pollen to ozone.

Pollen from *B. campestris* was affected both directly and indirectly by exposure to ozone. Pollen germination was significantly reduced following a 10 d exposure of

plants to 70 ppb ozone for 7 h d⁻¹ (Table 3.9), even though the mature pollen did not come into direct contact with ozone, confirming the suggestion by Mumford et $al.$ (1972) that pollen may be affected indirectly by exposure to ozone. While pollen from $B.$ $campestris$ was unaffected by in $vitro$ exposure to ozone, a 6 h in $vivo$ exposure of pollen to 120 ppb ozone significantly reduced in $vitro$ germination and pollen tube growth (Table 4.18). Similarly, pollen from $P.$ $major$ population High Low was shown to be more sensitive to 120 ppb ozone when exposed in $vivo$ on the anthers than when exposed in $vitro$ (Section 5.3.3). These results contrast with the study by Krause et $al.$ (1975) which showed pollen from tomato and petunia to be more sensitive to ozone when exposed outside the protective environment of the anthers. The current investigation also established that the 6 h threshold concentration for damage to $B.$ $campestris$ pollen lies between 100 ppb and 120 ppb ozone when exposure occurs in $vivo$. This compares favourably with previous studies by Feder (1981) and Hormaza et $al.$ (1996) which have shown pollen to be more sensitive when exposed to higher concentrations of ozone.

Since plants generally produce substantially more pollen grains than ovules, it could be argued that small ozone-induced reductions in the number of germinable pollen grains would be insufficient to limit the likelihood of successful fertilisation. However, most of the pollen which plants produce either fails to leave the anthers, especially when pollinator activity is low or when the weather is inclement for wind-pollination, or fails to reach receptive stigmata (Howe and Westley, 1991). Importantly, therefore, pollen dispersal may be limited during the anticyclonic weather conditions which favour ozone formation. Even if a pollen grain is successful in reaching a stigma, it may either be rejected by the maternal tissues or may have to compete with other pollen grains to reach the ovule (Willson and Burley, 1983). Thus, ozone-induced reductions in pollen tube growth may decrease the competitive ability of the pollen, thereby increasing the likelihood of fertilisation by more ozone-tolerant pollen or pollen which has not been exposed to ozone. If more ozone-tolerant pollen achieves successful fertilisation, this may have consequences for genetic variability within natural populations and therefore the potential for species to colonise different habitats or survive environmental change.

Although this study did not investigate the effects of ozone on the quantity of pollen produced by individual flowers, the observed reduction in the number of flowers produced by $B.$ $campestris$ following a 10 d exposure to ozone (Fig 3.8) is likely to have reduced the amount of pollen available for outcrossing. This decrease in the number of pollen grains would reduce the chances of pollen from individual plants being carried over long distances before deposition. A decrease in the number of flowers would also be reflected by reduced plant stature, which may, by decreasing

plant apparency, affect the number, duration and frequency of visits by pollinator species and hence the transfer of pollen between plants.

The sensitivity of pollen to ozone was shown to differ between the four populations of *P. major* examined. Following *in vitro* exposure to 100 ppb ozone for 6 h, pollen germination was increased and pollen tube length decreased in the Lullington and Sibton populations respectively (Tables 5.21a, b), while pollen from Penicuik and High Low was unaffected. These results confirm the existence of intraspecific variability in pollen sensitivity previously reported for cultivars of petunia (*Petunia hybrida*; Harrison and Feder, 1974) and almond (*Prunus dulcis*; Hormaz *et al.*, 1996). By examining the response of ozone-sensitive and ozone-tolerant cultivars of tomato, petunia and tobacco, Feder (1981) suggested that the ozone-sensitivity of pollen may be correlated with the ozone-sensitivity of the pollen parent. The current study substantiates this hypothesis since the vegetative and pollen sensitivities of Lullington and Sibton were both greater than those of Penicuik and High Low. Although only a single population of *B. campestris* was examined, the pollen and vegetative organs were both shown to be sensitive to ozone, thereby supporting the hypothesis of Feder (1981).

Because of the difficulty of studying pollen germination on the stigma, most studies, including the present one, have examined the impact of ozone on *in vitro* pollen germination using an artificial medium. However, the ability of pollen to germinate *in vitro* does not necessarily reflect its ability to germinate on the stigma (Rao *et al.*, 1992). Further studies are required to examine the effects of air pollutants on *in vivo* pollen germination so that the potential effects of ozone on final seed yield can be established more precisely.

In the field, pollen grains may be exposed to ozone repeatedly over a number of days and, although not investigated in the present study, this may be more damaging to pollen development than a single exposure. For asynchronously flowering species such as *B. campestris* and *P. major,* repeated exposures to ozone during the extended phase of flower opening would affect a higher proportion of pollen than a single exposure. In contrast, all of the pollen produced by synchronously flowering species could be damaged by a single exposure to ozone if this occurred when the pollen was particularly susceptible to damage.

The ways in which pollen may be damaged by ozone are still relatively unknown, although biochemical (Mumford *et al.*, 1972) and ultrastructural changes (Harrison and Feder, 1974) have been recorded. Further investigations are required to establish the

nature of the direct and indirect effects of ozone on pollen and the possible reasons for differences in the ozone sensitivity of pollen between species and cultivars.

6.2.2. Other aspects of reproductive development

In order to determine the direct effects of ozone on reproductive biology, the reproductive structures of both *B. campestris* and *P. major* were exposed to ozone during the flowering phase of development and the responses of various seed yield parameters in both species and the timing of floral opening in *B. campestris* were investigated. Most previous studies which have examined the effects of ozone on seed yield have done so by weighing the seed mass or by counting a representative sample of seeds. This study made a more detailed examination of the responses of seed yield to ozone stress by recording the number of seeds in each individual fruit and weighing smaller samples of seeds in order to obtain a more accurate measure of individual seed weight at different positions on the inflorescence. Since flowering is asynchronous in both species, it was hoped that this detailed examination would allow the most sensitive stage(s) of floral development to be identified.

The data presented in Chapters 4 and 5 showed that seed yield in both *B. campestris* and *P. major* was affected by ozone and that the nature of the responses varied between sites at different positions on the inflorescence. This suggests not only that direct injury may have been at least partly responsible for the effects of ozone on reproduction observed in previous studies with *P. major* (Reiling and Davison, 1992b; Pearson *et al.*, 1996) and other species (e.g. Cooley and Manning, 1987; Jäger *et al.*, 1993), but also that the data collected using subsamples may not provide a reliable indication of seed yield responses to ozone.

6.2.2.1. *Seed yield*

B. campestris

Various aspects of reproductive development in *B. campestris*, including floral development and seed yield, were shown to be unaffected by a single 6 h exposure to 100 ppb ozone on 20 DAS (Chapter 4). This contrasts with a previous study of the related species *B. napus* by Bosac (1992), in which a number of significant effects on reproductive development, including an increase in bud abortion, were recorded in two cultivars, Tapidor and Libravo, following exposure of the inflorescences to 100 ppb ozone for 6 h. The different responses of these two related species suggest inherent differences in sensitivity to ozone, although it is possible that *B. campestris* may have

been exposed at a relatively insensitive stage of development. When the exposure period was extended to four consecutive days, a realistic exposure period during anticyclonic weather conditions, there were significant alterations in the seed yield of *B. campestris*, although the nature of the responses varied depending upon the timing of exposure. From this investigation it was not possible to establish whether the differing effects induced by single and multiple exposures were due to differences in the exposure period, the dose of ozone, or the stage(s) of development at which the plants were exposed, although it is probable that multiple exposures were more damaging because the plants received a greater dose of ozone.

One of the major findings of the present study was that multiple exposures of inflorescences over four consecutive days between 17-20 and 20-23 DAS significantly increased both the abortion of seeds in apical pods and the precocious germination of seeds in older pods (Figs. 4.11, 4.18). Comparisons between experiments revealed that responses were similar for individual sites which flowered at certain times after sowing, suggesting that the position of sites on the terminal raceme was more important in determining responses to ozone than the stages of floral development at which individual sites were exposed. Because the plants were pollinated with pollen from unexposed donor plants, these responses were clearly the result of damage to the maternal structures. It was suggested in Chapter 4 that the increased abortion of seeds in apical pods may have resulted from a general decrease in assimilate availability within the inflorescence and that available resources were diverted preferentially to the seeds in older pods which had already begun the grain-filling and maturation process. Unlike other crop species such as pea (*Pisum* spp.), in which the reproductive organs depend predominantly on assimilates from subtending leaves (Flinn and Pate, 1970), seed development in *B. campestris* is supported mainly by assimilates produced by the pod walls (Singal *et al.*, 1987). Since the physiology of the leaves of *B. campestris* appeared to be particularly sensitive to ozone, it is not unreasonable to suggest that net photosynthesis in the pods may have been reduced by exposure to ozone. Damage may have also occurred to other photosynthetic tissues within the inflorescence, including the sepals, pedicels and stem, reducing still further the availability of assimilates to support reproductive processes. Clearly further studies would be required to determine whether ozone affects the production and translocation of photosynthetic assimilates within reproductive structures and whether such changes would be sufficient to alter seed yield.

Although the precocious germination of seeds is not uncommon in *Brassica* species (Brown and Dyer, 1991), the numbers in *B. campestris* were increased by exposure to ozone, particularly in the older pods and by exposure between 17-20 DAS. This may

285

have resulted either from a more rapid maturation of seeds due to an increase in resource allocation to individual seeds or from a more direct effect on seed maturation. However, it is possible that observed increases in both seed abortion and germination may have resulted from ozone-induced changes in the levels or translocation of the hormones which regulate seed maturation and germination, although these aspects were not examined in the present study and would require further investigation.

Despite the significant effects of ozone on the abortion and germination of seeds within the pods of B. campestris, the impact on final seed yield was shown to be dependent upon the timing of exposure, with seed yield being significantly reduced following exposure during the early flowering phase (17-20 DAS) due to a reduction in the number of mature seeds per pod, but unaffected by exposure during the later stages of reproductive development (20-23 DAS). These results suggest that, following exposure during the later stages of development, B. campestris has the ability to compensate for the increased abortion of seeds in apical pods by retaining more seeds to maturity in older pods. This ability to maintain seed yield may reflect a greater tolerance to ozone or increased assimilate availability to support compensatory processes in more mature plants. Thus, in common with B. napus (Bosac et al., 1994), B. campestris appears to have a limited capacity to compensate for reproductive site losses, although the nature of the causal and compensatory mechanisms involved differ between the two species, occurring at the level of the seed site in B. campestris and at the floral site in B. napus (Table 6.1). The different type of compensation mechanism exhibited by each species may have been due, at least in part, to differences in their growth and development; for example, the rapid life cycle of B. campestris may have limited the ability of plants to compensate for seed losses through the production of additional fertile sites or racemes, as was seen to occur in B. napus (Bosac et al., 1994).

P. major

This study demonstrated that seed number per capsule in P. major was the main determinant of seed yield which was affected by direct exposure of the reproductive structures to ozone. However, the nature of the effects on seed number varied not only between and within populations and individual spikes, but also with the timing of exposure to ozone.

Following exposure of the first reproductive spike to 120 ppb ozone for 7 h d^{-1} for 9 d during flowering, seed number per capsule was decreased in Penicuik and High

Table 6.1. Effect of O_3 on various stages of reproductive development in *Brassica napus* and *Brassica campestris*. ✓, affected by O_3; X, not affected by O_3.

Aspect of Reproductive Development	*Brassica napus* cv.Tapidor	*Brassica campestris*
Bud abortion	✓	X
Bud abscission	✓	X
Seed abortion	X	✓
Seeds per pod	X	✓
Seeds per plant	X	X

Low, increased in Lullington and unaffected in Sibton (Table 5.9). For High Low, seed number per capsule was decreased irrespective of the date at which flowering started after sowing, but was unaffected following exposure of the first spike to 120 ppb ozone for 7 h d^{-1} for 14 d during seed filling, suggesting that seed number was more sensitive to ozone exposure during the flowering stage(s) of development. Detailed analyses revealed that, for each population, seed number was affected at all positions along the reproductive spike, although the effects were generally greater and more significant in the apical sites (Figs. 5.1, 5.2).

In Chapter 5, it was suggested that the effects of ozone on seed number may have resulted from changes in the extent of ovule or seed abortion caused by alterations in the production or translocation of assimilates or endogenous hormones. The greater abortion of seeds in apical sites following exposure to ozone may have occurred as a result of resource limitation; however, since seed development in *P. major* relies primarily upon assimilates produced in the leaves, any decrease in assimilation within the capsules would be expected to be insufficient to affect seed development. Alternatively, the observed increases and decreases in seed yield in different populations may have resulted from alterations in the transport of assimilates from the leaves caused by direct effects on phloem transport within the spike, changes in photosynthesis within the capsules, and hence sink strength, or changes in the rates of production or transport of hormones which regulate development and photosynthate partitioning (Setter, 1990). Since *P. major* is a self-pollinating species, it is possible that the effects on seed number resulted, at least in part, from adverse effects on pollen germination and development; however, the investigations of the effects of ozone on *in vitro* pollen development suggested that this was not the case. Further studies would be required to establish the mechanisms by which seed number is determined in *P. major* and how these may be affected by treatment with ozone.

For two of the four populations, Penicuik and Lullington, seed number per capsule was increased significantly in the second spike of each plant even though it had not been exposed to ozone (Table 5.9). For Penicuik, this increase was sufficient to offset the loss of seeds from the exposed spike (Table 5.10); thus, in a similar manner to *B. campestris*, this population appears to have the ability to compensate for ozone damage to the reproductive structures by retaining more seeds to maturity. However, whereas seeds were retained in older sites fertilised prior to exposure in *B. campestris*, seed number in *P. major* was increased in the younger spikes. From the results obtained, it was not possible to establish whether these increases in seed number in Penicuik and Lullington originated from changes in assimilate partitioning following alterations to seed yield in the exposed spikes, or whether ozone affected seed number

indirectly by modifying other biochemical processes important for seed development, for example, hormone production or translocation. Since the responses of seed yield in the later-formed spikes and spike production were not recorded, the present study did not allow the impact of ozone treatment on final seed yield at the plant level to be ascertained. Longer term studies would be required to determine whether final seed yield is significantly affected following exposure of individual spikes to ozone, and whether there is long-term compensation for seed losses in High Low, for example, through an increase in spike production.

The significant increase in seed production observed in Lullington following exposure during flowering suggests that resources were diverted away from vegetative growth. Although the long-term effects of ozone exposure on reproductive performance in this population were not examined, a sustained increase in seed yield throughout the growing season could have consequences for plant growth. Law (1979), for example, showed that the size of the short-lived perennial grass, Poa annua, in the second season of growth was inversely related to reproductive effort during the first growing season. If such a decrease in plant size were to occur in Lullington, this could affect the competitive ability of individual plants. In addition, although the vast majority of seeds and seedlings may be destroyed by insects, vertebrates or pathogens, or succumb to desiccation, frost-heave, shade or competition (Watkinson, 1991), increased seed production may increase the chances of at least some individuals surviving to develop to maturity, while simultaneously increasing competition between individuals of the same or different species. For High Low, the observed decrease in seed yield may have contrasting effects on seedling establishment and survival.

The only significant effects of ozone on seed weight recorded in this study were increases in Penicuik and High Low following exposure during flowering. The impact of ozone on individual seed weight has rarely been considered in previous studies despite the importance of seed weight in influencing the size and competitive ability of the resulting seedlings (Black, 1957; Crawley and Nachapong, 1985). Bosac et al. (1998), for example, showed that the root and shoot growth of seedlings of B. napus cv. Libravo were reduced following a single 6 h exposure of the terminal inflorescence of the parent plants to 100 ppb ozone, which they suggested may have resulted from the observed decrease in seed weight and storage reserves. Further studies would be required to assess the impact of ozone on seedling vigour in B. campestris and P. major.

If the change in seed yield following ozone exposure during flowering can be used as an indication of ozone sensitivity, it appears that the sensitivity of the reproductive

structures may be similar to that of the vegetative organs in each of the populations examined (Reiling and Davison, 1992d). Such a correlation would suggest that similar damage and resistance mechanisms may occur within the vegetative and reproductive structures. In Chapter 5, it was suggested that the variable responses of seed yield to ozone stress may have been due to populations exhibiting different degrees of phenotypic plasticity. Thus, for Lullington, seed yield was significantly increased following exposure to ozone because this population had the ability to alter certain aspects of its physiology rapidly in order to limit ozone damage or initiate repair or compensation mechanisms. In contrast, the occurrence of a low level of plasticity in High Low may explain the observed significant decrease in seed yield. Since phenotypic plasticity is genotypically determined (Kuiper and Bos, 1992), it is possible that there is natural selection for a high level of phenotypic plasticity in response to ozone stress. Evidence for the evolution of tolerance to ozone already exists for the vegetative growth of *P. major* (Reiling and Davison, 1992d; Davison and Reiling, 1995).

6.2.2.2. *Seed colour*

While treatment with ozone had no significant effect on the colour of seeds produced by *P. major*, the proportion of yellow, yellow/green and green seeds produced by *B. campestris* was increased following direct exposure of the inflorescence to ozone. A number of green, immature seeds was produced following exposure between 17-20 DAS; since the germination of these seeds was also depressed, this represents an important loss to seed yield. Green seeds are also a problem in *Brassica* species because they reduce the quality of the oil extract (Ward *et al.*, 1995). The increased production of yellow seeds following exposure of both whole plants and the terminal inflorescence suggests that this particular response was due primarily to direct effects of ozone on the inflorescence. Since the germination of yellow seeds was similar to that of brown seeds, this change in seed colour may not have any serious implications for growth in the field. More detailed investigations of seed quality and seedling vigour would be required, however, to assess whether the production of yellow seeds had any major consequences for seedling establishment, survival and productivity in both agricultural and natural ecosystems.

6.2.2.3. *Seed germination*

Direct treatment of the reproductive structures with ozone had relatively little effect on the germination of seeds harvested from *P. major*, but the germination of seeds from *B. campestris* was increased 24-48 h after sowing following both single and multiple

exposures to ozone (Figs. 4.6, 4.13, 4.20). This response occurred in seeds harvested from all positions on the terminal raceme, suggesting a general effect of ozone on seed development. Further investigations showed that the difference in germination could not be explained by differences in the rates of imbibition or germination of seeds from different seed colour categories. Further investigations are therefore required to identify the mechanisms responsible for the stimulation of germination in seeds harvested from ozone-treated plants. It may be possible that a similar mechanism is responsible for the observed increase in precocious germination within the pods.

The findings of the present study are significant because a stimulation of seed germination could have important consequences for seedling establishment and plant growth. Ross and Harper (1972), for example, demonstrated that the growth of the earliest germinating seeds in monocultures of *Dactylis glomerata* was unaffected by inter-plant competition, whereas those germinating ten days later showed a negligible increase in weight after 35 days of growth. Providing that seedling vigour is not depressed in *B. campestris*, a stimulation of seed germination could provide a competitive advantage relative to seed from plants which have not experienced elevated concentrations of ozone or are resistant to ozone. If such an effect on germination was to occur in native species, it could have important consequences for both intraspecific and interspecific competition.

6.3. INFLUENCE OF THE REPRODUCTIVE GROWTH HABIT

The original hypothesis of this thesis was that the reproductive growth habit adopted by individual species may influence the impact of ozone on their reproductive biology (cf. Section 1.3.5). It was postulated that the reproductive development of *P. major* would be more severely affected by exposure to ozone than that of *B. campestris*, because the more determinate reproductive growth habit of the former would limit its ability to compensate for any ozone-induced losses of reproductive sites. Similarly, the reproductive development of *B. campestris* was expected to be more severely affected than that of the more indeterminate species *B. napus* (Bosac, 1992), because the shorter life cycle of *B. campestris* would limit its capacity to compensate for damage.

The results of this study have clearly shown that the reproductive structures of *B. campestris* and *P. major* are sensitive to ozone since seed abortion was significantly increased in both species following exposure to ozone. It was also shown that both species have the capacity to compensate for such seed losses by increasing the number of seeds which develop to maturity in other sites where they may be naturally aborted.

This suggests that the ability of plants to compensate for ozone-induced damage to the reproductive organs is not determined solely by the degree of reproductive determinacy.

However, the type of compensatory mechanisms available to plants is to some extent influenced by the degree of reproductive determinacy. For example, since the reproductive phase in *B. campestris* is restricted to a period of approximately 14 days, this may well preclude compensation for seed losses through the production of additional floral sites on the terminal or axillary racemes. In contrast, *B. napus* (cv. Tapidor) has a longer life cycle and produces a larger indeterminate inflorescence (c. 600 fertile sites), features which may allow this species to compensate for ozone-induced increases in bud abortion by increasing the length and number of raceme branches produced (Bosac *et al.*, 1994). In *P. major*, the determinate growth habit of individual spikes would prevent compensation for seed losses through an increase in the number of flowers on spikes which had formed prior to exposure.

The ability of plants to compensate for seed losses may also be influenced by aspects of the reproductive growth habit other than the degree of determinacy, including the morphology of the reproductive structures and the duration of the reproductive phase of development. For example, *B. campestris* may have a limited capacity to compensate for severe seed losses induced by high concentrations of ozone or long periods of exposure, since this species produces a relatively small number of pods (c. 30) during its life cycle into which excess resources may be diverted to increase seed yield. In contrast, *P. major* is a more perennial species which produces many reproductive spikes over an extended period of time, and may therefore have a greater opportunity for long-term compensation resulting from increases in spike production and/or seed yield in later formed spikes.

Four different populations of *P. major* were examined and, although each possessed the same type of reproductive growth habit, the effects of ozone on seed yield varied considerably between populations, suggesting that factors other than the type of reproductive growth habit are important in determining plant responses to ozone. These factors may include genetic differences in ozone sensitivity at the site(s) of impact. For example, genetic differences in the ability of tissues to exclude or detoxify ozone may determine the extent of injury to different types of reproductive tissues e.g. petals, anthers or stigmata, reproductive sites e.g. buds, flowers or pods or the reproductive organs as a whole. Importantly, the morphology of the reproductive organs may influence the extent of damage by providing protection to vulnerable tissues. For example, in *P. major* the ovary is surrounded by the corolla during flowering, perhaps protecting the ovary from direct contact with ozone. Similarly, the

development of a fleshy pod in *B. campestris* may protect the developing seeds from direct exposure to ozone.

The observed responses to ozone, however, depend not only upon the type and extent of damage to reproductive tissues but also upon the ability of the plants to alter physiological processes such as photosynthesis or carbon partitioning to repair and/or compensate for ozone damage. In certain circumstances, the photosynthetic capacity of the reproductive structures may influence this ability to repair and/or compensate for damage. For example, if the vegetative organs are damaged by exposure to ozone, the seed yields of species such as *P. major* may be more severely affected than those of species such as *B. campestris*, since reproductive development relies primarily upon assimilates from the vegetative organs in *P. major*, but is more closely related to assimilate production within the developing pods in *B. campestris*.

The responses of both vegetative and reproductive development to ozone may be made more complex and unpredictable because the ozone sensitivities of different tissues and/or the ability of the vegetative or reproductive structures to compensate for damage may vary with their age or stage of development. Thus the effects of ozone on seed yield may vary depending upon the timing of exposure to ozone, providing a possible explanation for the differing responses of *B. campestris* and *P. major*, population High Low, following exposure of their inflorescences at different stages of reproductive development.

The present study has not established the precise extent to which reproductive growth habit may influence responses to ozone due to the potential interacting factors mentioned above. Importantly, however, it has been demonstrated that the reproductive structures of plants may be damaged by direct exposure to ozone, and that potential compensation mechanisms for seed losses may also exist in both *B. campestris* and *P. major*.

6.4. IMPLICATIONS FOR FIELD GROWN PLANTS

The complex pattern of effects on vegetative and reproductive processes and the associated compensatory mechanisms observed in this study highlight the difficulties involved in defining species or varietal sensitivities to ozone. This problem is particularly important because ozone sensitivity is used as a basis for setting critical levels of ozone (AOT40) which are required to develop pollution control policies to reduce ozone exposures (CLAG, 1996).

To date, with the exception of crop yields, the effects of ozone on reproductive processes have been largely overlooked when setting AOT40 values. In general, AOT40 values for trees and native species have been established based upon studies which have examined the effects of ozone on visible injury or plant growth and used these to determine ozone sensitivity (Ashmore *et al.*, 1988, Reiling and Davison, 1992a, 1992d; CLAG, 1996). In addition, there is relatively little information available regarding the sensitivity of native species to ozone (Ashmore and Davison, 1996), thus it is not known whether current AOT40 values are sufficient to protect both the vegetative and reproductive development of wild plants. Based upon limited data, the current AOT40 for native species has been set at 3000 ppb.h, the same value as that set to protect arable crop species from a mean annual yield loss of 5 % (cf. Section 1.2.6.; CLAG, 1997). The results obtained in the present study suggest, however, that this value exceeds the threshold for damage to the physiology, growth and reproduction of both *P. major* and *B. campestris*. For example, total seed weight in *B. campestris* was reduced by 28 % following direct exposure of the inflorescence to an AOT40 of 1440 ppp.h, while an AOT40 of 1470 ppb.h was sufficient to reduce the seed yield of exposed spikes by 16 % in *P. major*. In the field, seed yield may be reduced still further due to additional effects of ozone on assimilate production and distribution from the vegetative tissues.

Clearly, it is exceedingly important to consider the potential effects of ozone on reproductive biology because, for many species, this may prove to be more sensitive than vegetative growth. In native plants, changes in seed yield or quality may have consequences for the survival or competitiveness of individual species and therefore the composition of semi-natural communities. It is apparent that further work is required to establish the effects of ozone on reproductive biology in a larger number of crop, tree and native species. In addition, since the present study has demonstrated that potential compensation mechanisms may exist, it is important that future studies consider the long-term effects of ozone on reproductive output in order to provide a more reliable indication of ozone sensitivity. This information can then be used to define more realistic AOT40 thresholds and therefore pollution control strategies which provide adequate protection for both agricultural and natural ecosystems.

While the present study has shown that the reproductive structures of *B. campestris* and *P. major* are sensitive to ozone and that both species possess potential compensation mechanisms, to be useful in the development of AOT40 values, the work needs to verified in the field to take account of other biotic and abiotic factors which are known to influence both the functioning of reproductive processes and plant responses

to ozone. Importantly, such interactions may serve to change the threshold level for responses to ozone and/or the effectiveness of compensation mechanisms.

In the field, biotic factors including pests and disease are known to modify plant responses to ozone, although these plant-ozone-insect interactions are complex. Evidence suggests that exposure to ozone may weaken plants, predisposing them to attack by insect herbivores (Stark *et al.*, 1968; Alstad *et al.*, 1982) and also that attack by pests may alter plant responses to ozone. Rosen and Runeckles (1976), for example, reported that a 6 d exposure to 20 ppb ozone for 6 h d^{-1} and infestation with greenhouse whitefly (*Trialeurodes vaporarium*) acted synergystically to accelerate chlorosis and senescence of the leaves of *Phaseolus vulgaris*. Competition for available resources is another biotic factor which may influence plant responses to ozone. Although limited data are available, studies of artificial grassland communities have shown that exposure to ozone may alter species composition (Ashmore and Ainsworth, 1995). This is thought to occur because decreases in growth of ozone-sensitive species allow more ozone-tolerant species to compete more effectively for available resources. The results of such studies suggest that it may not be possible to predict changes to plant communities from the responses of individual species under laboratory conditions.

Environmental factors including light, temperature, air humidity, windspeed, and soil moisture content are also known to influence responses to ozone by affecting ozone uptake (Guderian *et al.*, 1985; Mortensen, 1989). Soil water stress, for example, has been shown to reduce the impact of ozone by inducing stomatal closure and thereby decreasing the flux of ozone into the foliar tissues (Chevone *et al.*, 1990). The influence of differences in stomatal conductance on the extent of visible damage induced was clearly demonstrated in the present study of *B. campestris* (cf. 3.3.1.1). Soil nutrient deficiencies or competition for available resources may also affect plant health and therefore their ability to tolerate repair and compensate for ozone damage. These abiotic factors may also influence the rate of reproductive development which, in turn, may alter the duration of exposure at particularly vulnerable stages of development. Within plant communities, both abiotic and biotic factors may vary on a micro-scale, with the result that responses to ozone may vary between individual plants even though, due to the nature of ozone formation and distribution, neighbouring plants are likely to experience similar concentrations of ozone.

In addition, since ozone concentrations vary both spatially and temporally, the responses of plants to various concentrations of ozone and durations of exposure at different times during their life cycle need to be investigated. For example, repeated

exposures to ozone on separate or consecutive days may be more damaging to plant growth and development than a single exposure, although the concentration to which plants are exposed is also important since, for a given mean dose, short-term exposures to high concentrations of ozone are generally considered to be more damaging than long-term exposures to low concentrations (Finnan et al., 1996). Since the ozone sensitivity of leaf tissues is known to vary with age (Evans and Ting, 1974; Heath 1994), the timing of exposure to ozone will also be important in determining plant responses. The present study also suggested that the ozone sensitivity of inflorescences may change during development, since the seed yield of both B. campestris and P. major was more severely affected by exposure to ozone during the early rather than the later stages of reproductive development.

Another consideration when establishing ozone sensitivity is that plants are exposed simultaneously to several pollutants in the field, including sulphur dioxide, carbon dioxide and oxides of nitrogen; these may interact with ozone, resulting in effects which may be additive, synergistic or antagonistic (e.g. Heggestad et al., 1986; Deveau et al., 1987; Adaros et al., 1991a, b; Taylor and Bell, 1992). Elevated concentrations of CO_2 for example, have been shown to protect against ozone-induced damage to photosynthesis in wheat (Triticum aestivum cv. Minaret; Mulholland et al., 1997b); however, the maintenance of photosynthetic activity did not prevent significant yield losses (Mulholland et al., 1998a), suggesting that elevated CO_2 may not protect the reproductive structures from direct ozone damage. While research has considered the impact of pollutant mixtures on the vegetative growth and yield of crop species, little is known about the nature of their impact on reproductive processes such as flowering, pollination and seed set.

Clearly, the work reported here has identified the need for further research to investigate the effects of ozone and other pollutants on reproductive development in both crops and native species. Such research would be invaluable for the development of appropriate critical levels and more effective pollution control strategies. The screening of a large number of crop and native species would be required to establish whether current AOT40 values appropriate to protect crops from significant yield losses and natural or semi-natural plant communities from a significant loss of biodiversity or changes in species composition. Further research is also required to establish the precise biochemical and physiological mechanisms responsible for the ozone-induced damage and compensation identified in B. campestris and P. major, for example, are the observed effects of ozone on seed abortion due to changes in assimilate availability or a consequence of more direct effects on seed development? Such information regarding the responses of crop species to ozone stress would be of value to breeders

and geneticists working to develop more tolerant crop varieties for use in areas subject to chronic or acute ozone pollution.

BIBLIOGRAPHY

AARNES, H., BAUMANN, C., ERIKSEN, A.S., SOLAS, A., SUNDBYE, A. and SOLVERNES, K.A. (1993). Photosynthesis in ozone-exposed duckweed (*Lemna gibba*). *Physiologia Plantarum* **87**, 246-262.

ABDULLAH, W.D., POWELL, A.A. and MATTHEWS, S. (1991). Association of differences in seed vigour in long bean (*Vigna sesquipedalis*) with testa colour and imbibition damage. *Journal of Agricultural Science* **116**, 259-264.

ABEN, J.M.M., JANSSEN-JURKOVICOVA, M. and ADEMA, E.H. (1990). Effects of low-level ozone exposure under ambient conditions on photosynthesis and stomatal control of *Vicia faba* L. *Plant, Cell and Environment* **13**, 463-469.

ADAMS, M.B., KELLY, J.M. and EDWARDS, N.T. (1988). Growth of *Pinus taeda* L. seedlings varies with family and ozone exposure level. *Water, Air and Soil Pollution* **38**, 137-150.

ADAMS, R.M., GLYER, J.D. and McCARL, B.A. (1988). The NCLAN economic assessment: approach, findings and implications. In: Heck, W.W., Taylor, O.C. and Tingey, D.T. (Eds.). *Assessment of Crop Loss from Air Pollutants*. Elsevier Applied Science, London. pp. 473-552.

ADAROS, G., WEIGEL, H.J. and JÄGER, H.J. (1991a). Single and interactive effects of low levels of O_3, SO_2 and NO_2 on the growth and yield of spring rape. *Environmental Pollution* **72**, 269-286.

ADAROS, G., WEIGEL, H.J. and JÄGER, H.J. (1991b). Concurrent exposure to SO_2 and/or NO_2 alters growth and yield responses of wheat and barley to low concentrations of O_3. *New Phytologist* **118**, 581-591.

AHMED, S.U. and ZUBERI, M.I. (1971). Inheritance of seed coat color in *Brassica campestris* L. variety Toria. *Crop Science* **11**, 309-310.

AHUJA, Y.R. (1955). Environment and rate of growth of embryo in *Brassica campestris* var. Toria. *Current Science* **24**, 205-206.

ALSTAD, D.N., EDMUNDS, G.F.Jr., and WEINSTEIN, L.H. (1982). Effects of air pollutants on insect populations. *Annual Review of Entomology* **27**, 369-384.

298

ALTSHULLER, A.P. (1987). Estimation of the natural background of ozone present at surface rural locations. *Journal of the Air Pollution Control Association* **37**, 1409-1417.

AMUNDSON, R.G., KOHUT, R.J., SCHOETTLE, A.W., RABA, R.M. and REICH, P.B. (1987). Correlative reductions in whole-plant photosynthesis and yield of winter wheat caused by ozone. *Phytopathology* **77**, 75-79.

AMUNDSON, R.G., RABA, R.M., SCHOETTE, A.W and REICH, P.B. (1986). Response of soybean to low concentrations of ozone: II. Effects on growth, biomass allocation and flowering. *Journal of Environmental Quality* **15**, 161-167.

ANFOSSI, D., SANDRONI, S. and VIARENGO, S. (1991). Tropospheric ozone in the nineteenth century: The Moncalieri series. *Journal of Geophysical Research* **96D**, 17349-17352.

AREY, J., CROWLEY, D.E., CROWLEY, M., RESKETO, M. and LESTER, J. (1995). Hydrocarbon emissions from natural vegetation in the California South-coast-air-basin. *Atmospheric Environment* **29**, 2977-2988.

ASHENDEN, T.W. and McLEOD, A.R. (1993). Systems for exposing crops to gases. In: Jager, H.J., Unsworth, M., De Temmerman, L. and Mathy, P. (1993). *Effects of Air Pollution on Agricultural Crops in Europe. Proceedings of the Final Symposium of the European Open-Top Chamber Project.* Tervuren, Belgium. pp. 163-174.

ASHMORE, M.R. and AINSWORTH, N. (1995). The effects of ozone and cutting on the species composition of artificial grassland communities. *Functional Ecology* **9**, 708-712.

ASHMORE, M.R. and DAVISON, A.W. (1996). Towards a critical level of ozone for natural vegetation. In: Kärenlampi, L. and Skärby, L. (Eds.). *Critical Levels for Ozone in Europe: Testing and Finalising the Concepts. UN-ECE Workshop Report.* University of Kuopio, Department of Ecology and Environmental Science. pp. 58-71.

ASHMORE, M.R., BELL, J.N.B. and REILY, C.L. (1978). A survey of ozone levels in the British Isles using indicator plants. *Nature* **276**, 813-815.

ASHMORE, M.R., BELL, N. and RUTTER, J. (1985). The role of O₃ in forest damage in West Germany. *Ambio* **14**, 81-87.

ASHMORE, M.R., DALPRA, C. and TICKLE, A.K. (1988). Effects of ozone and calcium nutrition on native plant species. In: Mathy, P. (Ed.) *Air Pollution and Ecosystems*. Commission of the European Communities. Reidel Publishing Company, Dordrecht. pp. 647-652.

ASHMORE, M.R., BELL, J.N.B., DALPRA,C. and RUNECKLES, V.C. (1980). Visible injury to crop species by ozone in the United Kingdom. *Environmental Pollution (Series A)* **21**, 209-215.

ASHMORE, M.R., THWAITES, R.H., AINSWORTH, N., COUSINS, D.A., POWER, S.A. and MORTON, A.J. (1995). Effects of ozone on calcareous grassland communities. *Water, Air and Soil Pollution* **85**, 1527-1532.

ATKINS, D.H.F., COX, R.A. and EGGLETON, A.E.J. (1972). Photochemical ozone and sulphuric acid aerosol formation in the atmosphere over southern England. *Nature* **235**, 372-376.

BARNES, J.D. and DAVISON, A.W. (1988). The influence of ozone on winter hardiness of Norway spruce (*Picea abies* (L.) Karst). *New Phytologist* **108**, 159-166.

BARNES, J.D., DAVISON, A.W. and BOOTH, T.A. (1988). Ozone accelerates structural degradation of epicuticular wax on Norway spruce needles. *New Phytologist* **110**, 309-318.

BARNES, J.D., EAMUS, D. and BROWN, K.A. (1990). The influence of ozone, acid mist and soil nutrient status on Norway spruce: I. Plant-water relations. *New Phytologist* **114**, 713-720.

BARNES, R.L. (1972). Effects of chronic exposure to ozone on photosynthesis and respiration of pines. *Environmental Pollution* **3**, 133-138.

BELL, J.N.B. (1978). Ozone in the environment. *The Biologist* **25**, 279-287.

BELL, J.N.B. and COX, R.A. (1975). Atmospheric ozone and plant damage in the United Kingdom. *Environmental Pollution* **8**, 163-170.

BENNETT, J.H. and HILL, A.C. (1973). Absorption of gaseous air pollutants by a standardised plant canopy. *Journal of the Air Pollution Control Association* **23**, 203-206.

BENNETT, J.P. and OSHIMA, R.J. (1976). Carrot injury and yield responses to ozone. *Journal of the American Society of Horticultural Science* **101**, 638-639.

BENNETT, J.P. and RUNECKLES, V.C. (1977). Effects of low levels of ozone on plant competition. *Journal of Applied Ecology* **14**, 877-880.

BENNETT, J.P., OSHIMA, R.J. and LIPPERT, L.F. (1979). Effects of ozone on injury and dry matter partitioning in pepper plants. *Environmental and Experimetal Botany* **19**, 33-39.

BENOIT, L.F., SKELLY, J.M., MOORE, L.D. and DOCHINGER, L.S. (1983). The influence of ozone on *Pinus strobus* L. pollen germination. *Canadian Journal of Forest Research* **13**, 184-187.

BERGMANN, E., BENDER, J. and WEIGEL, H.J. (1995). Growth responses and foliar sensitivities of native herbaceous species to ozone exposures. *Water, Air and Soil Pollution* **85**, 1437-1442.

BERGMANN, E., BENDER, J. and WEIGEL, H.J. (1996). Effects of chronic ozone stress on growth and reproduction capacity of native herbaceous plants. In: *Exceedences of Critical Loads and Levels; Proceedings of International Workshop*, Vienna, 22-24 November 1995. pp. 177-185.

BERRANG, P., KARNOSKY, D.F. and BENNETT, J.P. (1989). Natural selection for ozone tolerance in *Populus tremuloides*: Field verification. *Canadian Journal of Forest Research* **19**, 519-522.

BLACK, J.N. (1957). The early vegetative growth of three strains of subterranean clover (*Trifolium subterraneum* L.) in relation to seed size. *Australian Journal of Agricultural Research* **8**, 1-14.

BLACK, V.J., COLLS, J.J., and BLACK, C.R. (1993). Ozone/sulphur dioxide interactions in temperate arable crops. In: Jackson, M.B. and Black, C.R. (Eds.). *Interacting Stresses on Plants in a Changing Climate*. NATO ISI series, Vol **16**, Springer-Verlag, Berlin. pp. 83-110.

BLACK, V.J., ORMROD, D.P. and UNSWORTH, M.H. (1982). Effects of low concentration of ozone, singly and in combination with sulphur dioxide on net photosynthesis rates of *Vricia faba* L. *Journal of Experimental Botany* **33**, 1302-1311.

BLUM, U. and HECK, W.W. (1980). Effects of acute ozone exposures on snap bean at various stages of its life cycle. *Environmental and Experimental Botany* **20**, 73-85.

BOSAC, C. (1992). *The Impact of Ozone and Sulphur Dioxide on Reproductive Development in Oilseed Rape*. PhD Thesis, University of Nottingham.

BOSAC, C., BLACK, V.J., BLACK, C.R., ROBERTS, J.A. and LOCKWOOD, F. (1993). Impact of O_3 and SO_2 on reproductive develpoment in oilseed rape (*Brassica napus* L.) I: Pollen germination and pollen-tube growth. *New Phytologist* **124**, 439-446.

BOSAC, C., BLACK, V.J., ROBERTS, J.A. and BLACK, C.R. (1998). Impact of ozone on seed yield and quality and seedling vigour in oilseed rape (*Brassica napus* L.). *Journal of Plant Physiology* (In Press).

BOSAC, C., ROBERTS, J.A., BLACK, V.J. and BLACK, C.R. (1994). Impact of O_3 and SO_2 on reproductive development in oilseed rape (*Brassica napus* L.) II. Reproductive site losses. *New Phytologist* **126**, 71-79.

BOTKIN, D.B., SMITH, W.H. and CARLSON, R.W. (1971). Ozone suppression of white pine net photosynthesis. *Journal of the Air Pollution Control Association* **21**, 778-780.

BOUTTIER, C. and MORGAN, D.G. (1992a). Ovule development and determination of seed number per pod in oilseed rape (*Brassica napus* L.). *Journal of Experimental Botany* **43**, 709-714.

BOUTTIER, C. and MORGAN, D.G. (1992b). Development of oilseed rape buds, flowers and pods *in vitro*. *Journal of Experimental Botany* **43**, 1089-1096.

BREWBAKER, J.L. and KWACK, B.H. (1963). The essential role of the calcium ion in pollen germination and pollen tube growth. *American Journal of Botany* **50**, 859-865.

BROWN, A.P. and DYER, A.F. (1991). Effects of low temperature storage on pollen of *Brassica campestris*, *B. oleracea* and *B. napus*. *Euphytica* **51**, 215-218.

BROWN, K.A. ROBERTS, T.M. and BLANK, L.W. (1987). Interaction between ozone and cold sensitivity in Norway spruce: A factor contributing to the forest decline in central Europe? *New Phytologist* **105**, 149-155.

BULL, K.R. (1991). The critical loads/ levels approach to gaseous pollutant emission control. *Environmental Pollution* **69**, 105-123.

CELA RENZONI, G., VEIGI, L., STEFANI, A. and ONNIS, A. (1990). Different *in vitro* germination responses in *Pinus pinea* pollen from two localities with different levels of pollution. *Annales Botanici Fennici* **27**, 85-90.

CHAMEIDES, W.L. and LODGE, J.P. (1992). Tropospheric ozone: Formation and fate. In: Lefohn, A.S. (Ed). *Surface Level Ozone Exposures and Their Effects on Vegetation*. Lewis Publishers, Chelsea, USA. pp. 5-30.

CHANG, C.W. and HEGGESTAD, H.E. (1974). Effects of ozone on Photosystem II in *Spinacia oleracea* chloroplasts. *Phytochemistry* **13**, 871-873.

CHEN, B.Y. and HENEEN, W.K. (1992). Inheritance of seed colour in *Brassica campestris* L. and breeding for yellow-seeds in *B. napus* L. *Euphytica* **59**, 157-163.

CHEVONE, B.I. and YANG, Y.S. (1985). CO_2 exchange rates and stomatal diffusive resistance in soybean exposed to O_3 and SO_2. *Canadian Jounal of Plant Science* **65**, 267-274.

CHEVONE, B.I., SEILER, J.R., MELKONIAN, J. and AMUNDSON, R.G. (1990). Ozone-water stress interactions. In: Alscher, R.G. and Cumming, J.R. (Eds.). *Stress Responses in Plants: Adaptation and Acclimation Mechanisms*. Plant Biology Volume 12. Wiley-Liss. Inc., New York. pp. 311-328.

CHIANG, M.S. (1974). Cabbage pollen germination and longevity. *Euphytica* **23**, 579-584.

CLAG (1996). *Critical Levels of Air Pollutants for the United Kingdom. Critical Loads Advisory Group, Sub-Group Report on Critical Levels of Air Pollutants.* Institute of Terrestrial Ecology, Penicuik, UK.

CLAPHAM, A.R., TUTIN, T.G. and WARBURG, E.F. (1989). *Excursion Flora of the British Isles.* Cambridge University Press, Third Edition.

CLARKE, B., HENNINGER, M. and BRENNAN, E. (1978). The effect of two antioxidants on foliar injury and tuber production in 'Norchip' potato plants exposed to ambient oxidants. *Plant Disease Reporter* **62**, 715-717.

CLARKE, J.M. and DePAUW, R.M. (1989). Water imbibition rate of wheat kernals as affected by kernal color, weather damage, and method of threshing. *Canadian Journal of Plant Science* **69**, 1-7.

COLBECK, I. and HARRISON, R.M. (1984). Tropospheric ozone. *Environmental Chemistry* **3**, 1-48.

COOLEY, D.R. and MANNING, W.J. (1987). The impact of ozone on assimilate partitioning in plants: A review. *Environmental Pollution* **47**, 95-113.

COOLEY, D.R. and MANNING, W.J. (1988). Ozone effects on growth and assimilate partitioning in alfalfa, *Medicago sativa* L. *Environmental Pollution* **49**, 19-36.

CORBET, S.A. and PLUMRIDGE, J.R. (1985). Hydrodynamics and the germination of oil-seed rape pollen. *Journal of Agricultural Science, Cambridge* **104**, 445-451.

COX, C.B. and MOORE, P.D. (1995). Patterns of distribution. In: Cox, C.B. and Moore, P.D. (Eds.). *Biogeography: An Ecological and Evolutionary Approach.* Blackwell Scientific, Oxford, UK. pp. 27-73.

COX, R.M. (1983). Sensitivity of forest plant reproduction to long range transported air pollutants: *In vitro* sensitivity of pollen to simulated acid rain. *New Phytologist* **95**, 269-276.

CRAKER, L.E. (1971). Ethylene production from ozone injured plants. *Environmental Pollution* **1**, 299-304.

CRAKER, L.E., WALDRON, P.F. and WERTHEIM, F.S. (1988). Acid rain, pollen germination and yield reductions in corn. *Environmental Pollution* **53**, 419-421.

CRAWLEY, M.J. and NACHAPONG, M. (1985). The establishment of seedlings from primary and regrowth seeds of ragwort (*Senecio jacobaea*). *Journal of Ecology* **73**, 255-261.

CRISOSTO, C.H., RETZLAFF, W.A., WILLIAMS, L.E., De JONG, T.M. and ZOFFOLI, J.P. (1993). Postharvest performance evaluation of plum (*Prunus salicina* Lindel., 'Casselman') fruit grown under three ozone concentrations. *Journal of the American Society of Horticultural Science* **118**, 497-502.

CRUTZEN, P.J. and GOLITZYN, G.S. (1992). Linkages between global warming, ozone depletion, acid deposition and other aspects of global environmental change. In: Mintzer, I.M. (Ed.). *Confronting Climate Change: Risks, Implications and Responses*. Cambridge University Press, UK. pp. 17-32.

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

DARRALL, N.M. (1989). The effects of air pollution in physiological processes in plants. *Plant, Cell and Environment* **12**, 1-30.

DAVISON, A.W. and REILING, K. (1995). A rapid change in ozone resistance of *Plantago major* after summers with high ozone concentrations. *New Phytologist* **131**, 337-344.

De BOUILLE, P., SOTTA, B., MIGINIAC, E. and MERRIEN, A. (1989). Hormones and pod development in oilseed rape (*Brassica napus*). *Plant Physiology* **90**, 876-880.

DEMCHIK, S.M. and DAY, T.A. (1996). Effect of enhanced UV-B radiation on pollen quantity, quality, and seed yield in *Brassica rapa* (Brassicaceae). *American Journal of Botany* **83**, 573-579.

DEVEAU, J.L., ORMROD, D.P., ALLEN, O.B. and BECKERSON, D.W. (1987). Growth and foliar injury responses of maize, soybean and tomato seedlings exposed to mixtures of ozone and sulphur dioxide. *Agriculture, Ecosystems and Environment* **19**, 223-240.

DIJAK, M. and ORMROD, D.P. (1982). Some physiological and anatomical characteristics associated with differential ozone sensitivity among pea cultivars. *Environmental and Experimental Botany* **22**, 395-402.

DOMINY, P.J. and HEATH, R.L. (1985). Inhibition of the K^+ stimulated ATPase of the plasmalemma of pinto bean leaves by ozone. *Plant Physiology* **77**, 43-45.

DUBAY, D.T. (1988). The direct effects of simulated acid rain on the sexual reproduction of selected agricultural crops. *Environmental Pollution* **53**, 422-423.

DUBAY, D.T. and MURDY, W.H. (1983). The impact of sulphur dioxide on plant sexual reproduction: *In vitro* and *In vivo* effects compared. *Journal of Environmental Quality* **12**, 147-149.

DUGGER, W.M. and TING, I.P. (1970). Air pollution oxidants - Their effects on metabolic processes in plants. *Annual Review of Plant Physiology* **21**, 215-234.

DUGGER, W.M., TAYLOR, O.C., THOMPSON, C.R. and CARDIFF, E. (1963). The effect of light on predisposing plants to ozone and PAN damage. *Journal of the Air Pollution Control Association* **13**, 423-428.

DUNGAN, G.H. (1924). Some factors affecting the water absorption and germination of seed corn. *Journal of the American Society of Agronomy* **16**, 473-481.

EAMUS, D., BARNES, J.D., MORTENSEN, L., RO-POULSON, H. and DAVISON, A.W. (1990). Persistent stimulation of CO_2 assimilation and stomatal conductance by summer ozone fumigation in Norway spruce. *Environmental Pollution* **63**, 365-379.

ENDRESS, A.G. and GRUNWALD, C. (1985). Impact of chronic ozone on soybean growth and biomass partitioning. *Agriculture, Ecosystems and Environment* **13**, 9-23.

ESCARRE, J., LEPART, J. and SENTUC, J.J. (1996). Effects of simulated herbivory in 3 old field compositae with different inflorescence architectures. *Oecologia* **105**, 501-508.

EVANS, L.S. and TING, I.P. (1974). Ozone sensitivity of leaves: Relationship to leaf water content, gas transfer resistance and anatomical characteristics. *American Journal of Botany* **61**, 592-597.

EVANS, L.S., ALBURY, K. and JENNINGS, N. (1996). Relationships between anatomical characteristics and ozone sensitivity of leaves of several herbaceous dicotyledonous plant species at Great Smokey Mountains National Park. *Environmental and Experimental Botany* **36**, 413-420.

EVANS, L.Y., BINGHAM, J., JACKSON, P. and SUTHERLAND, J. (1972). Effects of awns and drought on the supply of photosynthate and its distribution within wheat ears. *Annals of Applied Biology* **70**, 67-76.

FACTEAU, T.J. and ROWE, R.E. (1977). Effect of hydrogen fluoride and hydrogen chloride on pollen tube growth and sodium fluoride on pollen germination in Tilton apricot. *Journal of the American Society of Horticultural Science* **102**, 95.

FARAGE, P.K., LONG, S.P., LECHNER, E.G. and BAKER, N.R. (1991). The sequence of change within the photosynthetic apparatus of wheat following short-term exposure to ozone. *Plant Physiology* **95**, 529-535.

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

FEDER, W.A. (1970). Plant response to chronic exposure of low levels of oxidant type air pollution. *Environmental Pollution* **1**, 73-79.

FEDER, W.A. (1981). Bioassaying for ozone with pollen systems. *Environmental Health Perspectives* **37**, 117-123.

FEDER, W.A. and CAMPBELL, F.J. (1968). Influence of low levels of ozone on flowering of carnations. *Phytopathology* **58**, 1038-1039.

FEDER, W.A. and SHRIER, R. (1990). Combination of UV-B and ozone reduces pollen tube growth more than either stress alone. *Environmental and Experimental Botany* **30**, 451-454.

FEDER, W.A. and SULLIVAN, F. (1969a). Differential susceptibility of pollen grains to ozone injury. *Phytopathology* **59**, 399.

FEDER, W.A. and SULLIVAN, F. (1969b). Ozone: Depression of frond multiplication and floral production in duckweed. *Science* **165**, 1373-1374.

FELDHEIM, K. and CONNER, J.K. (1996). The effects of increased UV-B radiation on growth, pollination success, and lifetime fitness in two *Brassica* species. *Oecologia* **106**, 284-297.

FERNANDEZ-BAYON, J.M., BARNES, J.D., OLLERENSHAW, J.H. and DAVISON, A.W. (1993). Physiological effects of ozone on cultivars of watermelon (*Citrullus lanatus*) and muskmelon (*Cucumis melo*) widely grown in Spain. *Environmental Pollution* **81**, 199-206.

FINNAN, J.M., JONES, M.B. and BURKE, J.I. (1996). A time-concentration study on the effects of ozone on spring wheat (*Triticum aestivum* L.). 1. Effects on yield. *Agriculture, Ecosystems and Environment* **57**, 159-167.

FLINN, A.M. and PATE, J.S. (1970). A quantitative study of carbon transfer from pod and subtending leaf to the ripening seeds of the field pea. *Journal of Experimental Botany* **21**, 71-82.

FOOT, J.P., CAPORN, S.J.M., LEE, J.A. and ASHENDEN, T.W. (1997). Evidence that ozone exposure increases the susceptibility of plants to natural frosting episodes. *New Phytologist* **135**, 369-374.

FOSTER, P. (1997). Air pollution 'exceeds limits every five days'. *The Times* 26th September. p. 4.

FUHRER, J., PERLER, R. and SHARIAT-MADARI, H. (1993). Growth and gas exchange characteristics of two clones of white clover *Trifolium repens* L. differing in ozone sensitivity. *Angewandte Botanik* **67**, 163-167.

FUHRER, J., EGGER, A., LEHNHERR, B., GRANDJEAN, A., and TSCHANNEN, W. (1989). Effects of ozone on the yield of spring wheat (*Triticum aestivum* L. cv. Albis) grown in open-top field chambers. *Environmental Pollution* **60**, 273-289.

FUHRER, J., LEHNHERR, B., MOERI, P.B., TSCHANNEN, W. and SHARIAT-MADARI, H. (1990). Effects of ozone on the grain composition of spring wheat grown in open top field chambers. *Environmental Pollution* **65**, 181-192.

GAGNON, Z.E. and KARNOSKY, D.F. (1992). Physiological response of three species of *Sphagnum* to ozone exposure. *Journal of Bryology* **17**, 81-91.

GIFFORD, R.M. and EVANS, L.T. (1981). Photosynthesis, carbon partitioning, and yield. *Annual Review of Plant Physiology* **32**, 485-509.

GIMENO, B.S., PENUELA, J., PORCUNA, J.L. and REINERT, R.A. (1995). Biomonitoring ozone phytotoxicity in Eastern Spain. *Water, Air and Soil Pollution* **85**, 1521-1526.

GOMEZ, K.A. and GOMEZ, A.A. (1984). *Statistical Procedures for Agricultural Research* (2nd Ed). Wiley Interscience, Singapore.

GRIME, J.P., HODGSON, J.G. and HUNT, R. (1989). *Comparative Plant Ecology: A Functional Approach to Common British Species*. Unwin Hyman, London.

GROOT, J. and BOSCHHUIZEN, R. (1970). A preliminary investigation into the genecology of *Plantago major* L. *Journal of Experimental Botany* **21**, 835-841.

GRULKE, N.E., MILLER, P.R. and SCIOLI, D. (1996). Response of giant sequoia canopy foliage to elevated concentrations of atmospheric ozone. *Tree Physiology* **16**, 575-581.

GRUNWALD, C. and ENDRESS, A.G. (1988). Oil, fatty acid and protein content of seeds harvested from soybeans exposed to O_3 and/or SO_2. *Botanical Gazette* **149**, 283-288.

GUDERIAN, R., TINGEY, D.T. and RABE, R. (1985). Effect of photochemical oxidants on plants. In: Guderian, R. (Ed.). *Air Pollution by Photochemical Oxidants*. Springer-Verlag, Berlin. pp. 129-335.

GUICHERIT, R. and VAN DOP, H. (1977). Photochemical production of ozone in western Europe (1971-75) and its relation to meteorology. *Atmospheric Environment* **11**, 145-155.

GUZY, M.R. and HEATH, R.L. (1993). Response to ozone in common bean varieties. *New Phytologist* **124**, 617-625.

HAFNER, R. (1990). Fast plants - rapid-cycling *Brassicas*. *American Biology Teacher* **52**, 40-46.

HALEVY, A.H. (1984). Light and autonomous induction. In: Vince-Prue, D., Thomas, B. and Cockshull, K.E. (Eds.). *Light and the Flowering Process*. Academic Press, London. pp. 65-73.

HALEVY, A.H. (1987). Assimilate allocation and flower development. In: Atherton, J.G. (Ed.). *Manipulation of Flowering*. Butterworths. pp. 363-378.

HANSON, G.P., THORNE, L. and ADDIS, D.H. (1975). The ozone sensitivity of *Petunia hybrida* Vilm. as related to physiological age. *Journal of the American Society of Horticultural Science* **100**, 188-190.

HARRISON, B.H. and FEDER, W.A. (1974). Ultrastructural changes in pollen exposed to ozone. *Phytopathology* **64**, 257-258.

HASSAN, I.A., ASHMORE, M.R. and BELL, J.N.B. (1994). Effect of O_3 on the stomatal behaviour of Egyptian varieties of radish (*Raphanus sativus* L. cv. Baladey) and turnip (*Brassica rapa* L. cv. Sultani). *New Phytologist* **128**, 243-249.

HAWK, J.A. and CROWDER, L.V. (1978). *Brassica campestris* L., a higher plant with the potential for teaching genetics. *Journal of Heredity* **69**, 121-124.

HAWTHORN, W.R. (1974). The biology of Canadian weeds. 4. *Plantago major* and *P. rugelii*. *Canadian Journal of Plant Science* **54**, 383-396.

HEAGLE, A.S. (1989). Ozone and crop yield. *Annual Review of Phytopathology* **27**, 397-423.

HEAGLE, A.S., MCLAUGHLIN, M.R., MILLER, J.E., JOYNER, R.L. and SPRUILL, S.E. (1991). Adaptation of a white clover population to ozone stress. *New Phytologist* **120**, 29-37.

HEATH, R.L. (1975). Ozone. In: Mudd, J.B. and Kozlowski, T.T. (Eds.). *Responses of Plants to Air Pollution*. Academic Press, New York. pp. 23-28.

HEATH, R.L. (1980). Initial events in injury to plants by air pollutants. *Annual Review of Plant Physiology* **31**, 395-431.

HEATH, R.L. (1987). The biochemistry of ozone attack on the plasma membrane of plant cells. *Recent Advances in Phytochemistry* **21**, 29-54.

HEATH, R.L. (1994). Possible mechanisms for the inhibition of photosynthesis by ozone. *Photosynthesis Research* **39**, 439-451.

HECK, W.W. (1968). Factors influencing expression of oxidant damage to plants. *Annual Review of Phytopathology* **6**, 165-188.

HECK, W.W., TAYLOR, O.C. and TINGEY, D.T. (1988). *Assessment of Crop Loss from Air Pollutants*. Elsevier Applied Science, New York.

HEGGESTAD, H.E., BENNETT, J.H., LEE, E.H. and DOUGLASS, L.W. (1986). Effects of increasing doses of sulphur dioxide and ambient ozone on tomatoes: Plant growth, leaf injury, elemental composition, fruit yields, and quality. *Phytopathology* **76**, 1338-1344.

HELD, A.A., MOONEY, H.A. and GORHAM, J.N. (1991). Acclimation to ozone stress in radish: Leaf demography and photosynthesis. *New Phytologist* **118**, 417-423.

HESLOP-HARRISON, J.S. (1992). Cytological techniques to assess pollen quality. In: Cresti, M. and Tiezzi. A. (Eds). *Sexual Plant Reproduction*. Springer-Verlag, Berlin, Germany. pp. 41-48.

HEWITT, C.N., LUCAS, P., WELLBURN, A.R. and FALL, R. (1990). Chemistry of ozone damage to plants. *Chemistry and Industry* **15**, 478-481.

HILL, A.C. and LITTLEFIELD, N. (1969). Ozone effects on apparent photosynthesis, rate of transpiration and stomatal closure in plants. *Environmental Science and Technology* **3**, 52-56.

HILL, A.C., HEGGESTAD, H.E. and LINZON, S.N. (1970). Ozone. In: Jacobsen, J.S. and Hill, A.C (Eds). *Recognition of Air Pollution Injury to Vegetation; A Pictorial Atlas*. Journal of the Air Pollution Control Association, Pittsburg. B1-B6.

HOLOPAINEN, J.K., KAINULAINEN, P. and OKSANEN, J. (1997). Growth and reproduction of aphids and levels of free amino acids in Scots pine and Norway spruce in an open-air fumigation with ozone. *Global Change Biology* **3**, 139-147.

HORMAZA, J.I., PINNEY, K. and POLITO, V.S. (1996). Correlation in the tolerance to ozone between sporophytes and male gametophytes of several fruit and tree nut species (Rosaceae). *Sexual Plant Reproduction* **9**, 44-48.

HOUGH, A.M. and DERWENT, R.G. (1990). Changes in the global concentration of tropospheric ozone due to human activities. *Nature* **344**, 645-648.

HOUSTON, D.B and DOCHINGER, L.S. (1977). Effects of ambient air pollution on cone, seed and pollen characteristics in eastern white and red pines. *Environmental Pollution* **12**, 1-5.

HOV, O. (1984). Ozone in the troposphere: High level pollution. *Ambio* **13**, 73-79.

HOWE, H.F. and WESTLEY, L. (1991). Ecology of pollination and seed dispersal. In: Crawley, M.J. (Ed.). *Plant Ecology*. Blackwell Scientific. pp. 185-216.

HUNT, R. (1990). *Basic Growth Analysis*. Unwin Hyman, London.

IPCC (1995). *Climate Change 1995. The Science of Climate Change*. Contribution of Working Group 1 to the Second Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press.

IQBAL, M., ABDIN, M.Z., MAHMOODUZZAFAR, YANUS, M. and AGRAWAL, M. (1996). Resistance mechanisms in plants against air pollution. In: Yanus, M. and Iqbal, M. (Eds.). *Plant Responses to Air Pollution*. John Wiley and Sons Ltd. pp. 195-240.

JACOBSON, J.S. (1982). Ozone and the growth and productivity of agricultural crops. In: Unsworth, M.H. and Ormrod, D.P. (Eds.). *Effects of Gaseous Air Pollution in Agicuture and Horticulture*. Butterworth Scientific. pp. 293-304.

JÄGER, H.J., UNSWORTH, M., De TEMMERMAN, L. and MATHY, P. (1993). *Effects of Air Pollution on Agricultural Crops in Europe. Proceedings of the Final Symposium of the European Open-Top Chamber Project*. Tervuren, Belgium.

JEUFFROY, M-H and WAREMBOURG, F.R. (1991). Carbon transfer and partitioning between vegetative and reproductive organs in *Pisum sativum* L. *Plant Physiology* **97**, 440-448.

JOHNSON-FLANAGAN, A.M. and SPENCER, M.S. (1994). Ethylene production during development of mustard (*Brassica juncea*) and canola (*Brassica napus*) seed. *Plant Physiology* **106**, 601-606.

JOHNSON-FLANAGAN, A.M. and SPENCER, M.S. (1996). Chlorophyllase and peroxidase activity during degreening of maturing canola (*Brassica napus*) and mustard (*Brassica juncea*) seed. *Physiologia Plantarum* **97**, 353-359.

JONSSON, R. (1977). Breeding for improved oil and meal quality in rape (*Brassica napus* L.) and turnip rape (*Brassica campestris* L.). *Hereditas* **87**, 205-218.

KANGASJARVI, J., TALVINEN, J., UTRIAINEN, M. and KARJALAINEN, R. (1994). Plant defence systems induced by ozone. *Plant, Cell and Environment* **17**, 783-794.

KARNOSKY, D.F. and STAIRS, G.R. (1974). The effects of SO_2 on *in vitro* forest tree pollen germination and tube growth. *Journal of Environmental Quality* **3**, 406-409.

KARSSEN, C.M. (1970). The light-promoted germination of *Chenopodium album* L. VI. Pfr requirements during different stages of the germination process. *Acta Botanica Neerlandica* **19**, 297-312.

KASANA, M.S. (1991). Sensitivity of three leguminous crops to O_3 as influenced by different stages of growth and development. *Environmental Pollution* **69**, 131-149.

KHANNA-CHOPRA, R. and SINHA, S.K. (1976). Importance of fruit wall in seed yield of pea (*Pisum sativum* L.) & mustard (*Brassica campestris* L.). *Indian Journal of Experimental Biology* **14**, 159-162.

KICKERT, R.N. and KRUPA, S.V. (1990). Forest responses to tropospheric ozone and global climate change: An analysis. *Environmental Pollution* **68**, 29-65.

KNUDSON BUTLER, L. and TIBBITTS, T.W. (1979a). Variation in ozone sensitivity and symptom expression among cultivars of *Phaseolus vulgaris* L. *Journal of the American Society of Horticultural Science* **104**, 208-210.

KNUDSON BUTLER, L. and TIBBITTS, T.W. (1979b). Stomatal mechanisms determining genetic resistance to ozone in *Phaseolus vulgaris* L. *Journal of the American Society of Horticultural Science* **104**, 213-216.

KNUDSON BUTLER, L., TIBBITTS, T.W. and BLISS, F.A. (1979). Inheritance of resistance to ozone in *Phaseolus vulgaris* L. *Journal of the American Society of Horticultural Science* **104**, 211-213.

KOBAYASHI, K., OKADA, M. and NOUCHI, I. (1995). Effects of ozone on dry matter partitioning and yield of Japanese cultivars of rice (*Oryza sativa* L.). *Agriculture, Ecosystems and Environment* **53**, 109-122.

KOPPEL, A. and SILD, E. (1995). Bioindication of ozone in Estonia by using the tobacco variety Bel W3. *Water, Air and Soil Pollution* **85**, 1515-1519.

KRAUSE, G.H.M., RILEY, W.D. and FEDER, W.A. (1975). Effects of ozone on Petunia and tomato pollen tube elongation *in vivo*. *Proceedings of the American Phytopathological Society* **2**, 100.

KRESS, L.W. and MILLER, J.E. (1983). Impact of ozone on soybean yield. *Journal of Environmental Quality* **12**, 276-281.

KRESS, L.W. and SKELLY, J.M. (1982). Responses of several eastern forest tree species to chronic doses of ozone and nitrogen dioxide. *Plant Disease* **66**, 1149-1152.

KRUIPER, D. (1983). Genetic differentiation in *Plantago major*: Growth and root respiration and their role in phenotypic adaptation. *Physiologia Plantarum* **57**, 222-230.

KRUPA, S.V. and MANNING, W.J. (1988). Atmospheric ozone: Formation and effects on vegetation. *Environmental Pollution* **50**, 101-137.

KUIPER, P.J.C. and BOS, M. (1992) (Eds.). *Plantago: A Multidisciplinary Study*. Ecological Studies, Vol. **89**. Springer-Verlag, Berlin.

LACEY, E.P. and MARSHALL, C. (1992). Carbon integration in two *Plantago* species. *American Journal of Botany* **79**, 1108-1112.

LAISK, A., KULL, O. and MOLDAU, H. (1989). Ozone concentration in leaf intercellular air spaces is close to zero. *Plant Physiology* **90**, 1163-1167.

LANTING, R.W. (1984). Material damage by photochemical oxidants. In: Grennfelt, P. (Ed.). *The Evaluation and Assessment of the Effects of Photochemical Oxidants On Human Health, Agricultural Crops, Forestry, Materials and Visibility*. Swedish Environmental Research Institute, Göteborg. pp. 44-59.

LARDON, A. and TRIBOIBLONDEL, A.M. (1995). Cold and freeze stress at flowering - Effects on seed yields in winter rapeseed. *Field Crops Research* **44**, 95-101.

LAW, R. (1979). The cost of reproduction in annual meadow grass. *American Naturalist* **113**, 3-16.

LEHNHERR, B., MACHLER, F., GRANDJEAN, A. and FUHRER, J. (1987). The effect of ozone in ambient air on ribulose bisphosphate carboxylase/oxygenase activity decreases photosynthesis and grain yield in wheat. *Journal of Plant Physiology* **130**, 189-200.

LEHTILA, K. and SYRJANEN, K. (1995). Compensatory responses of 2 *Melampyrum* species after damage. *Functional Ecology* **9**, 511-517.

LENTINI, Z., MUSSELL, H., MUTSCHELER, M.A. and EARLE, E.D. (1988). Ethylene generation and reversal of ethylene effects during development *in vitro* of rapid cycling *Brassica campestris* L. *Plant Sciences* **54**, 75-81.

LESSER, V.M., RAWLINGS, J.O., SPRUILL, S.E. and SOMERVILLE, M.C. (1990). Ozone effects on agricultural crops: Statistical methodologies and estimated dose-response relationships. *Crop Science* **30**, 148-155.

LEUNG, J., FENTON, T.W., MUELLER, M.R. and CLANDININ, D.R. (1979). Condensed tannins of rapeseed meal. *Journal of Food Science* **44**, 1313-1316.

LEUNING, R., NEUMANN, M.H. and THURTHELL, G.W. (1979). Ozone uptake by corn: a general approach. *Agricultural Meterology* **20**, 115-135.

LOGAN, J.A. (1985). Tropospheric ozone: Seasonal behavior, trends and anthropogenic influence. *Journal of Geophysical Research* **90**, 10,463- 10,482.

LOTZ, L.A.P. (1990). The relation between the age and size at first flowering of *Plantago major* in various habitats. *Journal of Ecology* **78**, 757-771.

LOTZ, L.A.P. and BLOM, C.W.P.M. (1986). Plasticity in life-history traits of *Plantago major* ssp. *pleiosperma* Pilger. *Oecologia* **69**, 25-30.

LOTZ, L.A.P. and SPOORMAKERS, L.D.H. (1988). Differentiation in reproductive characteristics within a population of *Plantago major* L. ssp. *pleiosperma* Pilger. *Acta Oecologia* **9**, 11-18.

LOWENBERG, G.J. (1994). Effects of floral herbivory on maternal reproduction in *Sanicula arctopoides* (Apiaceae). *Ecology* **75**, 359-369.

MacDOWALL, F.D.H. (1965). Predisposition of tobacco to ozone damage. *Canadian Journal of Plant Science* **45**, 1-12.

MANSFIELD, T.A. and PEARSON, M. (1993). The physiological basis of stress imposed by ozone pollution. In: Fowden, L., Mansfield, T. and Stoddart, J. (Eds.). *Plant Adaptation to Environmental Stress*. Chapman & Hall, London. pp. 155-170.

MARTENS, M.J.M. (1992). Pollen for monitoring environmental pollution. In: Cresti, M. and Tiezzi. A. (Eds.). *Sexual Plant Reproduction*. Springer-Verlag, Berlin. pp. 219-224.

MASARU, N., SYOZO, F. and SABURO, K. (1976). Effects of exposure to various injurious gases on germination of lily pollen. *Environmental Pollution* **11**, 181-187.

McCOOL, P.M. and MENGE, J.A. (1983). Effect of O_3 on carbon partitioning in tomato: Potential role of carbon flow in the regulation of mycorrhizal symbiosis under conditions of stress. *New Phytologist* **94**, 241-247.

McGREGOR, D.I. (1981). Pattern of flower and pod development in rapeseed. *Canadian Journal of Plant Science* **61**, 275-282.

McLAUGHLIN, S.B. and TAYLOR, G.E. (1981) Relative humidity: Important modifier of pollutant uptake by plants. *Science* **211**, 167-168.

MEHLHORN, H. and WELLBURN, A.R. (1987). Stress ethylene formation determines plant sensitivity to ozone. *Nature* **327**, 417-418.

MEHLHORN, H., O'SHEA, J.M. and WELLBURN, A.R. (1991). Atmospheric ozone interacts with stress ethylene formation by plants to cause visible plant injury. *Journal of Experimental Botany* **42**, 17-24.

MENDHAM, N.J., SHIPWAY, P.A. and SCOTT, R.K. (1981). The effects of delayed sowing and weather on growth, development and yield of winter oilseed rape (*Brassica napus* L.). *Journal of Agricultural Science, Cambridge* **96**, 389-416.

MEREDITH, F.I., THOMAS, C.A. and HEGGESTAD, H.E. (1986). Effect of the pollutant ozone in ambient air on Lima beans. *Journal of Agricultural and Food Chemistry* **34**, 179-85.

MILLER, J.E. (1987). Effects of ozone and sulphur dioxide stress on growth and carbon allocation in plants. *Recent Advances in Phytochemistry* **21**, 55-100.

MILLER, P.R. (1983). Ozone effects in the San Bernadino National Forest. In: David, D.D., Miller, A.A. and Dochinger, L. (Eds.). *Air Pollution and the Productivity of the Forest*. Isaac Walton League of America, Arlington, Virginia. pp. 166-198.

MOLDAU, H. (1993). CO_2 exchange in plants under conditions of ozone stress. *Russian Plant Physiology* **40**, 467-472.

MOLDAU, H., SOBER, J. and SOBER, A. (1990). Differential sensitivity of stomata and mesophyll to sudden exposure of bean shoots to ozone. *Photosynthetica* **24**, 446-458.

MORGAN, D.G. (1974). Physiological determinants of yield in oilseed rape. *Journal of the Science of Food and Agriculture* **25**, 1057.

MORTENSEN, L.M. (1989). Review: Effects of ozone on plants in relation to other environmental conditions. *Meded. Norsk. Inst. Skogforst* **42**, 57-66.

MORTENSEN, L.M. (1992). Effects of ozone on growth of seven grass and one clover species. *Acta Agriculturae Scandinavica Section B, Soil and Plant Science* **42**, 235 - 239.

MORTENSEN, L.M. (1997). Effects of carbon dioxide concentrations on three grass species grown in mixture in two soil types at different ozone concentrations or temperatures. *Acta Agriculturae Scandinavica Section B, Soil and Plant Science* **47**, 14-19.

MORTENSEN, L.M. and NILSEN, J. (1992). Effects of ozone and temperature on growth of several wild plant species. *Norwegian Journal of Agricultural Sciences* **6**, 195-204.

MUDD, J.B. (1984). Effects of oxidants on metabolic function. In: Koziol, M.J. and Whatley, F.R. (Eds.). *Gaseous Air Pollutants and Plant Metabolism.* Butterworths, London.

MULCHI, C.L., LEE, E., TUTHILL, K. and OLINICK, E.V. (1988). Influence of ozone stress on growth processes, yields and grain quality characteristics among soybean cultivars. *Environmental Pollution* **53**, 151-169.

MULCHI, C.L., SAMMONS, D.J. and BAENZIGER, P.S. (1986). Yield and grain quality responses of soft red winter wheat exposed to ozone during anthesis. *Agronomy Journal* **78**, 593-600.

MULHOLLAND, B.J., CRAIGON, J., BLACK, C.R., COLLS, J.J., ATHERTON, J. and LANDON, G. (1997a). Effects of elevated carbon dioxide and ozone on the growth and yield of spring wheat (*Triticum aestivum* L.). *Journal of Experimental Botany* **48**, 113-122.

MULHOLLAND, B.J., CRAIGON, J., BLACK, C.R., COLLS, J.J., ATHERTON, J. and LANDON, G. (1997b). Impact of elevated atmospheric CO_2 and O_3 on gas exchange and chlorophyll content in spring wheat (*Triticum aestivum* L.). *Journal of Experimental Botany* **48**, 1853-1863.

MULHOLLAND, B.J., CRAIGON, J., BLACK, C.R., COLLS, J.J., ATHERTON, J. and LANDON, G. (1998a). Growth, light interception and yield response of spring wheat (*Triticum aestivum* L.) to elevated CO_2 and O_3 in open-top chambers. *Global Change Biology* **4**, 121-130.

MULHOLLAND, B.J., CRAIGON, J., BLACK, C.R., COLLS, J.J., ATHERTON, J. and LANDON, G. (1998b). Impact of elevated CO_2 and O_3 on grain growth and harvest index in spring wheat (*Triticum aestivum* L.). *Global Change Biology* (in press).

MUMFORD, R.A., LIPKE, H., LAUFER, D.A. and FEDER, W.A. (1972). Ozone induced changes in corn pollen. *Environmental Science and Technology* **6**, 427-430.

MUSSELMANN, R.C., McCOOL, P.M. and LEFOHN, A.S. (1994). Ozone descriptors for an air-quality standard to protect vegetation. *Journal of the Air and Waste Management Association* **44**, 1383-1390.

MYHRE, A., FORBERG, E., AARNES, H. and NILSEN, S. (1988). Reduction of net photosynthesis in oats after treatment with low concentrations of ozone. *Environmental Pollution* **53**, 265-271.

NEBEL, B. and FUHRER J. (1994). Interspecific and intraspecific differences in ozone sensitivity in seminatural plant communities. *Angewandte Botanik* **68**, 116-121.

NEIL, L.J., ORMROD, D.P. and HOFSTRA, G. (1973). Ozone stimulation of tomato stem elongation. *HortScience* **8**, 488-489.

NEY, B., DUTHION, C. and FONTAINE, E. (1993). Timing of reproductive abortions in relation to cell division, water content, and growth of pea seeds. *Crop Science* **33**, 267-270.

NEY, B., DUTHION, C. and TURC, O. (1994). Phenological response of pea to water stress during reproductive development. *Crop Science* **34**, 141-146.

O'CONNER, C.J., ASHFORD, K.P. and SPEDDING, D.J. (1987). Germination and metabolism of *Pinus radiata* pollen in the presence of sulphur dioxide. *Journal of Plant Physiology* **126**, 373-378.

OBESO, J.R. and GRUBB, P.J. (1994). Interactive effects of extent and timing of defoliation and nutrient supply on reproduction in a chemically protected annual *Senecio vulgaris*. *Oikos* **71**, 506-514.

OLIVEIRA, M de A., MATTHEWS, S. and POWELL, A.A. (1984). The role of split seed coats in determining seed vigour in commercial seed lots of soybean as measured by the electrical conductivity test. *Seed Science and Technology* **12**, 659-668.

OLSZYK, D.M. and TIBBITTS, T.W. (1981a). Stomatal response and leaf injury of *Pisum sativum* L. with SO_2 and O_3 exposures. 1 Influence of pollutant level and leaf maturity. *Plant Physiology* **67**, 539-544.

OLSZYK, D.M. and TIBBITTS, T.W. (1981b). Stomatal response and leaf injury of *Pisum sativum* L. with SO_2 and O_3 exposures. 2 Influence of moisture stress and time of exposure. *Plant Physiology* **67**, 545-549.

ONG, C.K., COLVILL, K.E. and MARSHALL, C. (1978). Assimilation of $^{14}CO_2$ by the inflorescence of *Poa annua* L. and *Lolium perenne* L. *Annals of Botany* **42**, 855-862.

ORMROD, D.P. (1996). Air pollution and seed growth and development. In: Yanus, M. and Iqbal, M. (Eds.). *Plant Responses to Air Pollution*. John Wiley and Sons Ltd. pp. 425-435.

OSHIMA, R.J., BENNETT, J.P. and BRAEGELMANN, P.K. (1978). Effect of ozone on growth and assimilate partitioning in parsley. *Journal of the American Society of Horticultural Science* **103**, 348-350.

OSHIMA, R.J., BRAEGELMANN, P.K., BALDWIN, D.W., VAN WAY, V. and TAYLOR, O.C. (1977). Reduction of tomato fruit size and yield by ozone. *Journal of the American Society of Horticultural Science* **102**, 289-293.

OSHIMA, R.J., BRAEGELMANN, P.K., FLAGLER, R.B. and TESO, R.R. (1979). The effects of ozone on the growth, yield, and partitioning of dry matter in cotton. *Journal of Environmental Quality* **8**, 474-479.

OSHIMA, R.J., TAYLOR, O.C., BRAEGELMANN, P.K. and BALDWIN, D.W. (1975). Effects of ozone on the yield and plant biomass of a commercial variety of tomato. *Journal of Environmental Quality* **4**, 463-464.

PÄÄKKÖNEN, E., PAASISALO, S., HOLOPAINEN, T. and KARENLAMPI, L. (1993). Growth and stomatal response of birch (*Betula pendula* Roth.) clones to ozone in open-air and chamber fumigation. *New Phytologist* **125**, 615-623.

PÄÄKKÖNEN, E., METSARINNE, S., HOLOPAINEN, T. and KARENLAMPI, L. (1995). The ozone sensitivity of birch (*Betula pendula*) in relation to the developmental stage of leaves. *New Phytologist* **132**, 145-154.

PALMBLAD, I.G. (1968). Competition in experimental populations of weeds with emphasis on the regulation of population size. *Ecology* **49**, 26-34.

PAOLETTI, E. and RADDI, P. (1995). Effects of acidity on *in vitro* germination and tube growth of pollen from declining *Fagus sylvatica*. *Journal of Applied Ecology* **32**, 836-840.

PARKER, R.E. (1979). *Introductory Statistics for Biology*. Arnold.

PAUSCH, R.C., MULCHI, C.L., LEE, E.H., FORSETH, I.N. AND SLAUGHTER, L.H. (1996). Use of ^{13}C and ^{15}N isotopes to investigate O_3 effects on C and N metabolism in soybeans. Part 1. C fixation and translocation. *Agriculture, Ecosystems and Environment* **59**, 69-80.

PEARSON, M. (1995). Effects of ozone on growth and gas exchange of *Eucalyptus globulus* seedlings. *Tree Physiology* **15**, 207-210.

PEARSON, S., DAVISON, A.W., REILING, K., ASHENDEN, T. and OLLERENSHAW, J.H. (1996). The effects of different ozone exposures on three contrasting populations of *Plantago major*. *New Phytologist* **132**, 493-502.

PECHAN, P.M., BASHFORD, J.A. and MORGAN, D.G. (1980). The use of radiography in the studies of pod growth in oilseed rape (*Brassica napus* L.). *Journal of Agricultural Science, Cambridge* **95**, 25-27.

PELL, E.J. and BRENNAN, E. (1973). Changes in respiration, photosynthesis, adenosine 5'-triphosphate and total adenylate content of ozonated pinto bean foliage as they relate to symptom expression. *Plant Physiology* **70**, 677-685.

PELL, E.J. and PEARSON, N.S. (1983). Ozone-induced reduction in quantity of ribulose-1,5-bisphosphate carboxylase in alfalfa foliage. *Plant Physiology* **73**, 185-187.

PELL, E.J. and WEISSBERGER, W.C. (1976). Histopathological characterisation of ozone injury to soybean foliage. *Phytopathology* **66**, 1711-1716.

PELL, E.J., ECKARDT, N. and ENYEDI, A.J. (1992). Timing of ozone stress and resulting status of ribulose bisphosphate carboxylase/oxygenase and associated net photosynthesis. *New Phytologist* **120**, 397-405.

PETRUZZELLI, L., HARREN, F. and REUSS, J. (1994). Patterns of C_2H_4 production during germination and seedling growth of pea and wheat as indicated by a laser-driven photoacoustic system. *Environmental and Experimental Botany* **34**, 55-61.

PLEIJEL, H., OJANPERA, K. and MORTENSEN, L. (1997). Effects of tropospheric ozone on the yield and grain protein content of spring wheat (*Triticum aestivum* L.) in the Nordic countries. *Acta Agriculturae Scandinavica Section B, Soil and Plant Science* **47**, 20-25.

PLEIJEL, H., SKÄRBY, L., WALLIN, G. and SELLENDEN, G. (1989). Yield and grain quality of spring wheat exposed to ozone in open-top chambers. In: Bonte, J. and Mathy, P. (Eds.). *The European Communities Research Project on Open-Top Chambers - Results on Agricultural Crops 1987-1988.* Air Pollution Research Report 19, CEC, Brussels. pp. 138-146.

POORTER, H. and LAMBERS, H. (1986). Gowth and competitive ability of a highly plastic and a marginally plastic genotype of *Plantago major* in a fluctuating environment. *Physiologia Plantarum* **67**, 217-222.

POTTER, L., FOOT, J.P., CAPORN, S.J.M. and LEE, J.A. (1996). The effects of long-term elevated ozone concentrations on the growth and photosynthesis of *Sphagnum recurvum* and *Polytrichum commune. New Phytologist* **134**, 649-656.

POWELL, A.A. and MATTHEWS, S. (1979). The influence of testa condition on the imbibition and vigour of pea seeds. *Journal of Experimental Botany* **30**, 193-197.

POWELL, A.A., OLIVEIRA, M DE A. and MATTHEWS, S. (1986a). Seed vigour in cultivars of dwarf French bean (*Phaseolus vulgaris*) in relation to the colour of the testa. *Journal of Agricultural Science, Cambridge* **106**, 419-425.

POWELL, A.A., OLIVEIRA, M DE A. and MATTHEWS, S. (1986b). The role of imbibition damage in determining the vigour of white and coloured seed lots of dwarf French beans (*Phaseolus vulgaris*). *Journal of Experimental Botany* **37**, 716-722.

PRICE, H. and TRESHOW, M. (1972). Effects of ozone on the growth and reproduction of grasses. In: *Proceedings of the International Clean Air Conference.* Melbourne, Clean Air Society of Australia and New Zealand. pp. 275-280.

PRIMACK, R. B. (1978). Regulation of seed yield in *Plantago*. *Journal of Ecology* **66**, 835-847.

PYE, J.M. (1988). Impact of ozone on the growth and yield of trees: A review. *Journal of Environmental Quality* **17**, 347-360.

RAJPUT, C.B.S. and ORMROD, D.P. (1986). Stimulation of plant growth in pumpkin by ozone. *HortScience* **21**, 498-499.

RAO, G.U., JAIN, A. and SHIVANA, K.R. (1992). Effects of high temperature stress on *Brassica* pollen: Viability, germination and ability to set fruits and seeds. *Annals of Botany* **68**, 193-198.

RATHORE, R.K.S. and SINGH, R.P. (1968). Embryological studies in *Brassica campestris* L. var. Yellow Sarson Prain. *Journal of the Indian Botanical Society* **47**, 341-349.

REICH, P.B. (1983). Effects of low concentrations of ozone on net photosynthesis, dark respiration and chlorophyll contents in ageing poplar leaves. *Plant Physiology* **73**, 291-296.

REICH, P.B. (1987). Quantifying plant response to ozone: A unifying theory. *Tree Physiology* **3**, 63-91.

REICH, P.B. and AMUNDSON, R.C. (1985). Ambient levels of ozone reduce net photosynthesis in tree and crop species. *Science* **230**, 566-570.

REICH, P.B. and LASSOIE, J.P. (1984). Effects of low level O_3 exposure on leaf diffusive conductance and water-use efficiency in hybrid poplar. *Plant, Cell and Environment* **7**, 661-668.

REICH, P.B. and LASSOIE, J.P. (1985). Influence of low concentrations of ozone on growth, biomass partitioning and leaf senescence in young hybrid poplar plants. *Environmental Pollution* **39**, 39-51.

REILING, K. (1990). *The Effects of O_3 on British Native Plants*. PhD Thesis. Newcastle Upon Tyne.

REILING, K. and DAVISON, A.W. (1992a). The response of native, herbaceous species to ozone: Growth and fluorescence screening. *New Phytologist* **120**, 29-37.

REILING, K. and DAVISON, A.W. (1992b). Effects of a short ozone exposure given at different stages in the development of *Plantago major* L. *New Phytologist* **121**, 643-647.

REILING, K. and DAVISON, A.W. (1992c). Ozone sensitivity. In: Hendry, G.A.F. and Grime, J.P. (Eds.). *Methods in Comparative Plant Ecology - a Laboratory Manual.* Chapman and Hall, London. pp. 63-65.

REILING, K. and DAVISON, A.W. (1992d). Spatial variation in ozone resistance of British populations of *Plantago major* L. *New Phytologist* **122**, 699-708.

REILING, K. and DAVISON, A.W. (1994). Effects of exposure to ozone at different stages in the development of *Plantago major* L. on chlorophyll fluorescence and gas exchange. *New Phytologist* **128**, 509-514.

REILING, K. and DAVISON, A.W. (1995). Effects of ozone on stomatal conductance and photosynthesis in populations of *Plantago major* L. *New Phytologist* **129**, 587-594.

REINERT, R.A., TINGEY, D.T. and HERSHELL, C.B. (1972). Ozone induced foliar injury in lettuce and radish cultivars. *Journal of the American Society of Horticultural Science* **97**, 711-714.

RETZLAFF, W.A., WILLIAMS, L.E. and DeJONG, T.M. (1997). Growth and yield response of commercial bearing-age 'Casselman' plum trees to various ozone partial pressures. *Journal of Environmental Quality* **26**, 858-865.

RICH, S., WAGGONER, P.E. and TOMLINSON, H. (1970). Ozone uptake by bean leaves. *Science* **169**, 79-80.

RICHARDS, G.A., MULCHI, C.L. and HALL, J.R. (1980). Influence of plant maturity on the sensitivity of turfgrass species to ozone. *Journal of Environmental Quality* **9**, 49-53.

RIGNEY, L.P. (1995). Postfertilisation causes of differential success of pollen donors in *Erythronium grandiflorum* (Liliaceae) - Nonrandom ovule abortion. *American Journal of Botany* **82**, 578-584.

RILEY, W.D. and FEDER, W.A. (1974). Pollen tube susceptibility to ozone as a function of tube extension. *Proceedings of the American Phytopathological Society* **1**, 143.

ROBERTS, T.M. (1984). Effects of air pollutants on agriculture and forestry. *Atmospheric Environment* **18**, 629-652.

ROSEN, P.M. and RUNECKLES, V.C. (1976). Interactions of ozone and greenhouse whitefly in plant injury. *Environmental Conservation* **3**, 70-71.

ROSS, M.A. and HARPER, J.L. (1972). Occupation of biological space during seedling establishment. *Journal of Ecology* **60**, 77-78.

RUDORFF, B.F.T., MULCHI, C.L., FENNY, P., LEE, E.H. and ROWLAND, R. (1996). Wheat-grain quality under enhanced tropospheric CO_2 and O_3 concentrations. *Journal of Environmental Quality* **25**, 1384-1388.

RUNECKLES, V.C. (1992). Uptake of ozone by vegetation. In: Lefohn, A.S. (Ed). *Surface Level Ozone Exposures and Their Effects on Vegetation.* Lewis Publishers, Chelsea, USA. pp. 157-188.

RUNECKLES, V.C. and CHEVONE, B.I. (1992). Crop responses to ozone. In: Lefohn, A.S. (Ed). *Surface Level Ozone Exposures and Their Effects on Vegetation.* Lewis Publishers, Chelsea, USA. pp. 189-270.

S.A.P.S. (1990a). *Rapid-cycling Brassicas.* Science and Plants for Schools, Cambridge.

S.A.P.S. (1990b). *Growing Instructions for Rapid-cycling Brassica campestris (fast plants).* Science and Plants for Schools, Cambridge.

S.A.P.S. (1990c). *Investigations With Rapid-cycling Brassicas.* Science and Plants for Schools, Cambridge.

SAGAR, G.R. and HARPER, J.L. (1960). Factors affecting the germination and early establishment of plantains (*Plantago lanceolata, P. media* and *P. major*). In: Harper, J.L. (Ed.). *The Biology of Weeds. First Symposium of the British Ecological Society.* Blackwell Scientific, Oxford. pp. 236-245.

SAGAR, G.R. and HARPER, J.L. (1964). Biological Flora of the British Isles; *Plantago major* L., *P. media* L. and *P. lanceolata* L. *Journal of Ecology* **52**, 189-222.

SALONEN, V. and SUHONEN, J. (1995). Effects of seed weight on growth, reproduction and competitive ability of *Linum usitatissimum* seedlings. *Annales Botanici Fennici* **32**, 101-106.

SALTER, L. and HEWITT, C.N. (1992). Ozone-hydrocarbon interactions in plants. *Phytochemistry* **31**, 4045-4050.

SANDELIUS, A.S., NASLUND, K., CARLSSON, A.S., PLEIJEL, H. and SELLDEN, G. (1995). Exposure of spring wheat (*Triticum aestivum*) to ozone in open-top chambers. Effects on acyl lipid composition and chlorophyll content of flag leaves. *New Phytologist* **131**, 231-239.

SANDERS, G.E., COLLS, J.J. and CLARK, A.G. (1992). Physiological changes in *Phaseolus vulgaris* in response to long-term ozone exposure. *Annals of Botany* **69**, 123-133.

SAXE, H. (1990). *Air Pollution, Primary Plant Physiological Responses and Diagnostic Tools.* Thesis, Royal Veterinary and Agricultural University, Thorvaldsensvig, Denmark.

SCHMIEDEN, U. and WILD, A. (1995). The contribution of ozone to forest decline. *Physiologia Plantarum* **94**, 371-378.

SCHREIBER, U., VIDAVER, W., RUNECKLES, V.C. and ROSEA, P. (1978). Chlorophyll fluorescence assay for ozone injury to intact plants. *Plant Physiology* **61**, 80-84.

SCOTTI, I.A., SILVA, S. and BOTTESCHI, G. (1994). Effects of ozone on grain quality of wheat grown in open-top chambers: three years of experimentation. *Environmental Pollution* **86**, 31-35.

SEARCY, K.B. and MULCAHY, D.L. (1985). The parallel expression of metal tolerance in pollen and sporophytes of *Silene dioica* (L.) Clairv., *S. alba* (Mill.) Krause and *Mimulus guttatus* DC. *Theoretical and Applied Genetics* **69**, 579-602.

SETTER, T.L. (1990). Transport/harvest index: photosynthate partitioning in stressed plants. In: Alscher, R.G. and Cumming, J.R. (Eds.). *Stress Responses in Plants: Adaptation and Acclimation Mechanisms*. Plant Biology Volume 12. Wiley-Liss. Inc., New York. pp. 17-36.

SHANNON, J.G. and MULCHI, C.L. (1974). Ozone damage to wheat varieties at anthesis. *Crop Science* **14**, 335-337.

SHARMA, P. and GHILDIYAL, M.C. (1992). Contribution of leaf and pod photosynthesis to seed yield in mustard. *Photosynthetica* **26**, 91-94.

SHEORAN, I.S., SAWHNEY, V., BABBAR, S. and SINGH, R. (1991). *In vivo* fixation of CO_2 by attached pods of *Brassica campestris* L. *Annals of Botany* **67**, 425-428.

SINGAL, H.R., SHEORAN, I.S. and SINGH, R. (1987). Photosynthetic carbon fixation characteristics of fruiting structures of *Brassica campestris* L. *Plant Physiology* **83**, 1043-1047.

SKÄRBY, L., TROENG, E. and BOSTROM C. (1987). Ozone uptake and effects on the transpiration, net photosynthesis, and dark respiration in Scots pine. *Forest Science* **33**, 801-808.

SLAUGHTER, L.H., MULCHI, C.L. and LEE, E.H. (1993). Wheat-kernel growth characteristics during exposure to chronic ozone pollution. *Environmental Pollution* **81**, 73-79.

SPENCE, R.D., RYKIEL, E.J.Jr. and SHARPE. P.J.H. (1990). Ozone alters carbon allocation in loblolly pine: assessment with carbon-11 labelling. *Environmental Pollution* **64**, 93-106.

SQUIRE, G.R. (1993). *The Physiology of Tropical Crop Production*. CAB International, Wallingford, UK.

STANLEY, R.G. (1971). Pollen chemistry and tube growth. In: Heslop-Harrison, J.S. (Ed.). *Pollen: Development and Physiology*. Butterworth Scientific. pp. 131-155.

STARK, R.W., MILLER, P.R., COBB, F.W.Jr., WOOD, D.L. and PARMETER, J.R.Jr. (1968). Incidence of bark beetle infestation in injured trees. *Hilgardia* **39**, 121-126.

STIRLING, C.M. and BLACK, C.R. (1991). Stages of reproductive development in groundnut (*Arachis hypogaea*) most susceptible to environmental stresses. *Tropical Agriculture* **68**, 296-300.

SULZBACH, C.W. and PACK, M.R. (1972). Effects of fluoride on pollen germination, pollen tube growth and fruit development in tomato and cucumber. *Phytopathology* **62**, 1247.

SUTTON, R. and TING, I.P. (1977). Evidence for the repair of ozone-induced membrane injury. *American Journal of Botany* **64**, 404-411.

TAKEMOTO, B.K., BYTNEROWICZ, A. and OLSZYK, D.M. (1988). Depression of photosynthesis, growth and yield in field-grown green pepper (*Capsicum annuum* L.) exposed to acidic fog and ambient ozone. *Plant Physiology* **88**, 477-482.

TAKEMOTO, B.K., BYTNEROWICZ, A. and OLSZYK, D.M. (1989). Physiological responses of field-grown strawberry (*Fragaria* x *ananassa* Duch.) exposed to acidic fog and ambient ozone. *Environmental and Experimental Botany* **29**, 379-386.

TAYLOR, G.E.Jr., TINGEY, D.T. and RATSCH, H.C. (1982). Ozone flux in *Glycine max* (L.) Merr.: sites of regulation and relationship to leaf injury. *Oecologia* **53**, 179-186.

TAYLOR, H.J. and BELL, J.N.B. (1992). Tolerance to SO_2, NO_2 and their mixture in *Plantago major* L. populations. *Environmental Pollution* **76**, 19-24.

TAYO, T.O. and MORGAN, D.G. (1975). Quantitative analysis of the growth, development and distribution of flowers and pods in oilseed rape (*Brassica napus* L.). *Journal of Agricultural Science, Cambridge* **85**, 103-110.

TEMPLE, P.J. (1986). Stomatal conductance and transpirational responses of field-grown cotton to ozone. *Plant, Cell and Environment* **9**, 315-321.

TENGA, A.Z. and ORMROD, D.P. (1990). Diminished greenness of tomato leaves exposed to ozone and post-exposure recovery of greenness. *Environmental Pollution* **64**, 29-41.

THEANDER, O., AMAN, P., MIKSCHE, E. and YASUDA, S. (1977). Carbohydrates, polyphenols and lignin in seed hulls of different colors from turnip rapeseed. *Journal of Agriculture and Food Chemistry* **25**, 270-273.

THURLING, N. (1974a). Morphophysiological determinants of yield in rapeseed (*Brassica campestris* and *Brassica napus*). I. Growth and morphological characters. *Australian Journal of Agricultural Research* **25**, 697-710.

THURLING, N. (1974b). Morphophysiological determinants of yield in rapeseed (*Brassica campestris* and *Brassica napus*). II. Yield components. *Australian Journal of Agricultural Research* **25**, 711-721.

TIEDEMANN, A.V. and FEHRMANN, H. (1988). Secondary effects of photochemical oxidants on cereals: Alterations in susceptibility to fungal leaf diseases. In: Mathy, P. (Ed.) *Air Pollution and Ecosystems*. Commission of the European Communities. Reidel Publishing Company, Dordrecht. pp. 653-658.

TING, I.P. and MUHERJI, S.K. (1971). Leaf anatomy as a factor in susceptibility to ozone: Amino acid and carbohydrate changes during expansion. *American Journal of Botany* **58**, 497-504.

TINGEY, D.T., FITES, R.C. and WICKLIFF, C. (1973). Foliar sensitivity of soybean to ozone as related to several leaf parameters. *Environmental Pollution* **4**, 193-192.

TINGEY, D.T., HECK, W.W. and REINERT, R.A. (1971). Effect of low concentrations of ozone and sulphur dioxide on foliage, growth and yield of radish. *Journal of the American Society of Horticultural Science* **96**, 369-371.

TODD, G.W. (1956). The effects of gaseous ozone, hexene and their reaction products upon the respiration of lemon fruit. *Physiologia Plantarum* **9**, 421-428.

TOMPKINS, S.P. and WILLIAMS, P.H. (1990). Fast plants for finer science - an introduction to the biology of rapid-cycling *Brassica campestris (rapa)* L. *Journal of Biological Education* **24**, 239-250.

TRIVEDI, S. and TRIPATHI, R.S. (1982). The effects of soil texture and moisture on reproductive strategies of *Spergula arvensis* L. and *Plantago major* L. *Weed Research* **22**, 41-49.

UKPORG (1987). *Ozone in the United Kingdom. United Kingdom Photochemical Oxidants Review Group Interim Report.* Department of the Environment, London.

UKPORG (1993). *Ozone in the United Kingdom. Third Report of the United Kingdom Photochemical Oxidants Review Group.* Department of the Environment, London.

UKPORG (1997). *Ozone in the United Kingdom. Fourth Report of the Photochemical Oxidants Review Group.* Department of the Environment, Transport and the Regions, London.

UNSWORTH, M.H. and GEISSLER, P. (1993). Results and achievements of the European open-top chamber network. In: Jäger, H.J., Unsworth, M., De Temmerman, L. and Mathy, P. (1993). *Effects of Air Pollution on Agricultural Crops in Europe. Proceedings of the Final Symposium of the European Open-Top Chamber Project.* Tervuren, Belgium. pp. 5-22.

UNSWORTH, M.H. and ORMROD, D.P. (1982). (Eds). *Effects of Gaseous Air Pollution in Agriculture and Horticulture.* Butterworth Scientific.

VAN RYN, D.M., JACOBSON, J.S. and LASSOIE, J.P. (1986). Effects of acidity on *in vitro* pollen germination and tube elongation in four hardwood species. *Canadian Journal of Forest Research* **76**, 379-400.

VANDERMEIREN, K., DETEMMERMAN, L. and HOOKHAM, N. (1995). Ozone sensitivity of *Phaseolus vulgaris* in cultivar differences, growth stage and growing conditions. *Water, Air and Soil Pollution* **85**, 1455-1460.

VARSHNEY, S.R.K. and VARSHNEY, C.K. (1981). Effect of sulphur dioxide on pollen germination and pollen tube growth. *Environmental Pollution* **24**, 87-92.

VAUGHAN, J.G. (1970). *The Structure and Utilisation of Oilseeds*. Chapman and Hall Ltd., London.

VAUGHAN, J.G. (1977). A multidisciplinary study of the taxonomy and origin of *Brassica* crops. *Bioscience* **27**, 35-40.

VOZZO, S.F., MILLER, J.E., HEAGLE, A.S. and PURSLEL, W.A. (1988). Effects of ozone and water stress on net photosynthetic rate of field grown soybean leaves. *Environmental Pollution* **53**, 471-473.

WALMSLEY, L. ASHMORE, M.R. and BELL, J.N.B. (1980). Adaptation of radish *Raphanus sativus* L. in response to continuous exposure to ozone. *Environmental Pollution*, series A **23**, 165-177.

WARD, K., SCARTH, R., DAUN, J.K. and VESSEY, J.K. (1995). Chlorophyll degradation in summer oilseed rape and summer turnip rape during seed ripening. *Canadian Journal of Plant Science* **75**, 413-420.

WARWICK, K.R. and TAYLOR, G. (1995). Contrasting effects of tropospheric ozone on 5 native herb which coexist in calcareous grassland. *Global Change Biology* **1**, 143-151.

WATKINSON, A.R. (1991). Plant population dynamics. In: Crawley, M.J. (Ed.). *Plant Ecology*. Blackwell Scientific. pp. 137-184.

WAYNE, R. P. (1987). The photochemistry of ozone. *Atmospheric Environment* **21**, 1683-1694.

WEBER, J.A., SCOTT CLARK, C. and HOGSETT, W.E. (1993). Analysis of the relationships among O_3 uptake, conductance, and photosynthesis in needles of *Pinus ponderosa*. *Tree Physiology* **13**, 157-172.

WELLBURN, F.A.M. and WELLBURN, A.R. (1996). Variable patterns of antioxidant protection but similar ethene emission differences in several ozone-sensitive and ozone-tolerant plant selections. *Plant, Cell and Environment* **19**, 754-760.

WILLIAMS, P.H. and HILL, C.B. (1986). Rapid cycling populations of *Brassica*. *Science* **232**, 1385-1389.

WILLSON, M.F. and BURLEY, N. (1983). *Mate Choice in Plants: Tactics, Mechanisms and Consequences*. Princeton University Press, New Jersey.

WINFIELD, A.L. (1962). Effect of early bud removal on subsequent development of some *Brassica* seed plants. *Plant Pathology* **11**, 17-23.

WOLTERS, J.H.B. and MARTENS, M.J.M. (1987). Effects of air pollutants on pollen. *The Botanical Review* **53**, 372-414.

YANG, Y.S., SKELLY, J.M., CHEVONE, B.I. and BIRCH, J.B. (1983). Effects of long-term ozone exposure on photosynthesis and dark respiration of eastern white pine. *Environmental Science and Technology* **17**, 371-373.

ZELITCH, I. (1982). The close relationship between net photosynthesis and crop yield. *Bioscience* **10**, 796-802.

APPENDIX ONE: SUMMARY OF SEED YIELD FOR *BRASSICA CAMPESTRIS* IN A REPEAT EXPOSURE TO 100 PPB OZONE FOR 6 H DAY^{-1} ON FOUR CONSECUTIVE DAYS BETWEEN 17-20 DAS (SEE CHAPTER FOUR FOR RELEVANT TEXT).
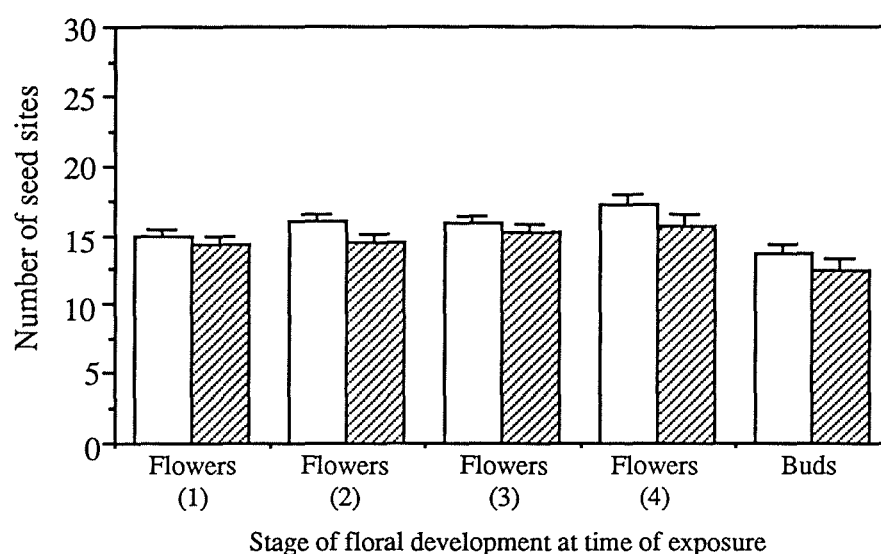
Figure 1.1. Mean numbers of a) aborted, b) germinated and c) mature seeds per pod in *Brassica campestris* for floral sites exposed on four consecutive days between 17-20 DAS to filtered air (control) or 100 ppb ozone for 6 h day^{-1} as open flowers (open on day 1, 2, 3 or 4 of exposure as indicated) or buds. □, control; ▨ ozone-treated. Single standard errors are shown. * and ** indicate significance at P<0.05 and P<0.01.

335
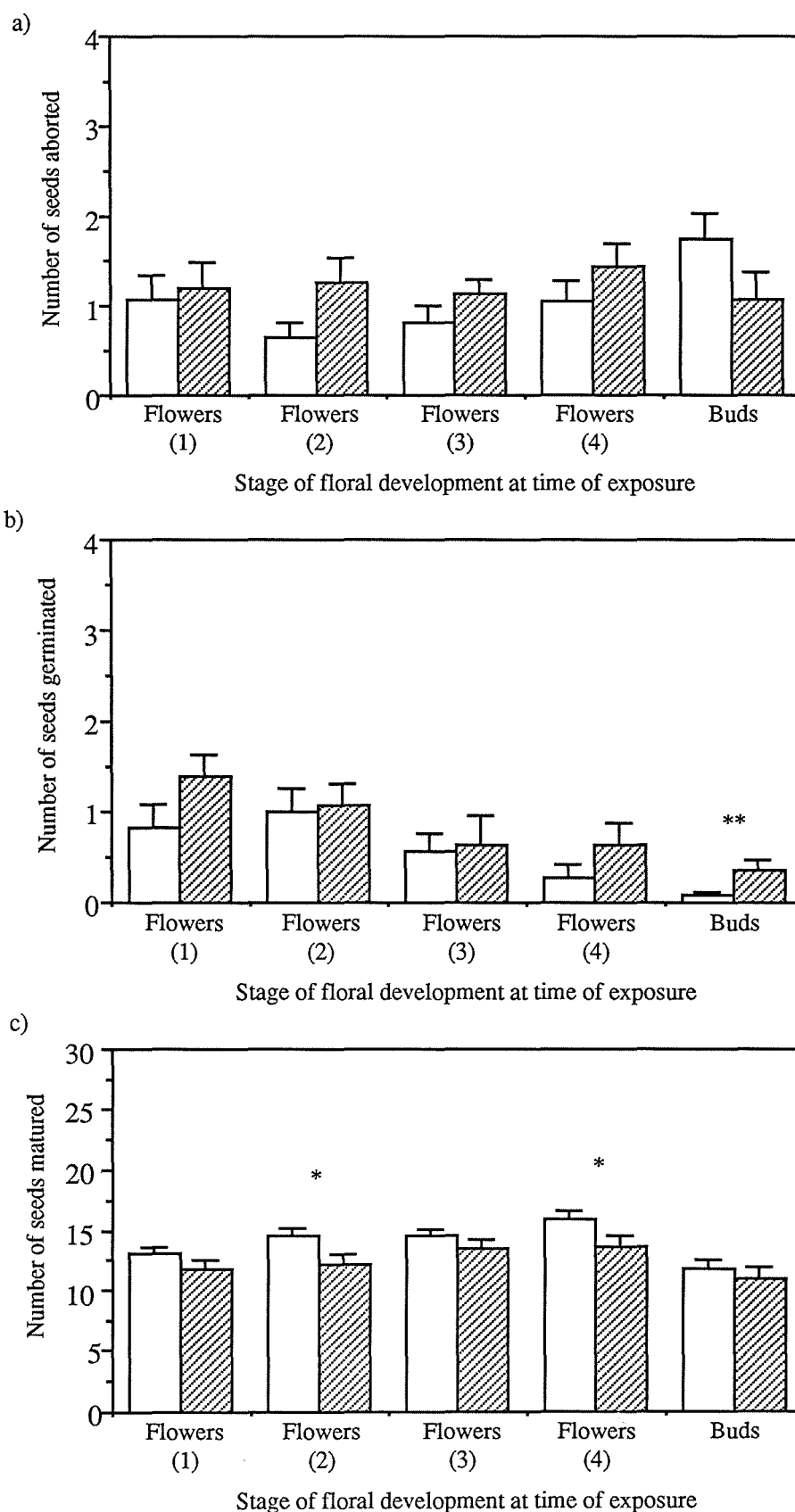
Figure 1.2. Mean total number of seed sites per pod for floral sites of *Brassica campestris* exposed on four consecutive days between 17-20 DAS to filtered air (control) or 100 ppb ozone for 6 h day $^{-1}$ as open flowers (open on day 1, 2, 3 or 4 of exposure as indicated) or buds. □, control; ▨, ozone-treated. Single standard errors are shown. There was no significant difference between the means of treated and control plants for any of the stages of floral development.

Stage of floral development at time of exposure

Table 1.1. Mean percentage of aborted, germinated and mature seeds per pod in *Brassica campestris* for floral sites which were exposed on four consecutive days between 17-20 DAS to filtered air (control) or 100 ppb ozone for 6 h day $^{-1}$ as open flowers (open on day 1, 2, 3 or 4 of exposure as indicated) or buds.

Stage of floral development at time of exposure	Treatment	Aborted seeds	Germinated seeds	Mature seeds
Flowers (1)	Control	7.1	5.5	87.4
	Ozone	8.3	9.6	82.1
Flowers (2)	Control	4.0	6.2	89.8
	Ozone	8.6	7.3	84.1
Flowers (3)	Control	5.0	3.6	91.4
	Ozone	7.3	4.1	88.6
Flowers (4)	Control	6.0	1.6	92.4
	Ozone	9.1	3.9	87.0
Buds	Control	12.6	0.6	86.8
	Ozone	8.5	2.9	88.6

Figure 1.3. Timecourse of germination for fresh seeds of *Brassica campestris* collected from floral sites exposed on four consecutive days between 17-20 DAS to filtered air (control) or 100 ppb for 6 h day $^{-1}$ as open flowers (open on day 1, 2, 3 or 4 of exposure as indicated) or buds. Solid circles, controls; open circles, ozone-treated. Double standard errors are shown. *, ** and *** indicate significance at P<0.05, P<0.01 and P<0.001; n=4.
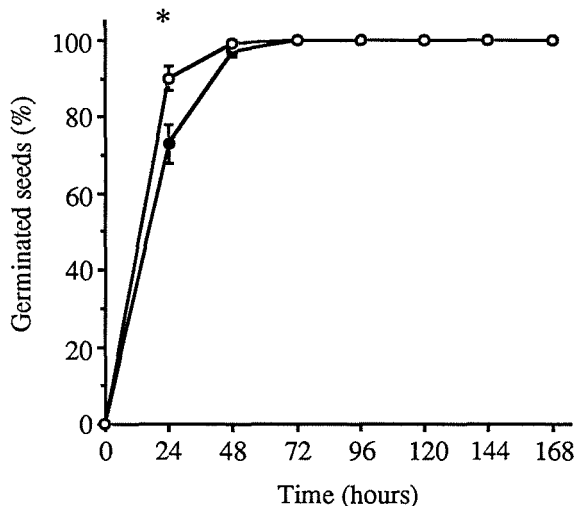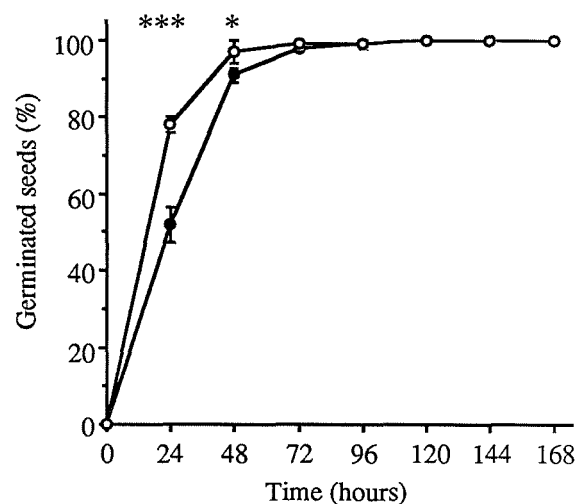

a) Open flowers (1)

b) Open flowers (2)

c) Open flowers (3)

d) Open flowers (4)

e) Buds

337
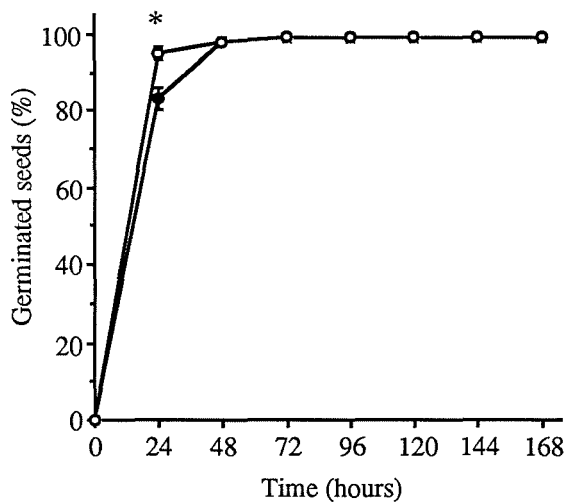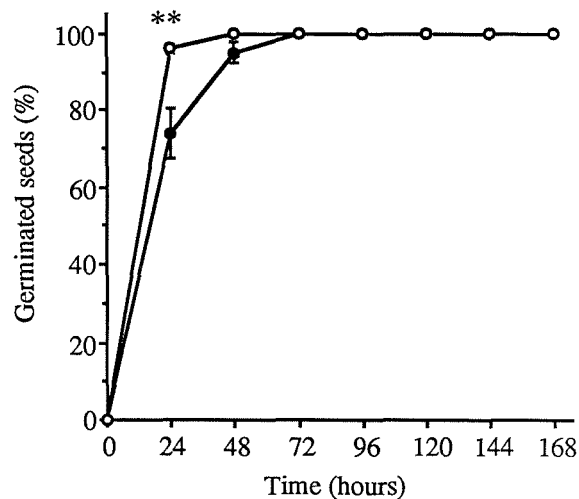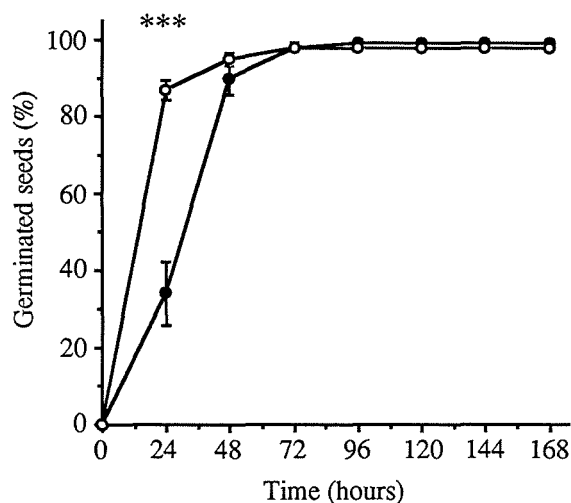
APPENDIX TWO : **TIMECOURSES OF SEED GERMINATION FOR** *PLANTAGO MAJOR* **POPULATIONS EXAMINED IN CHAPTER FIVE.**
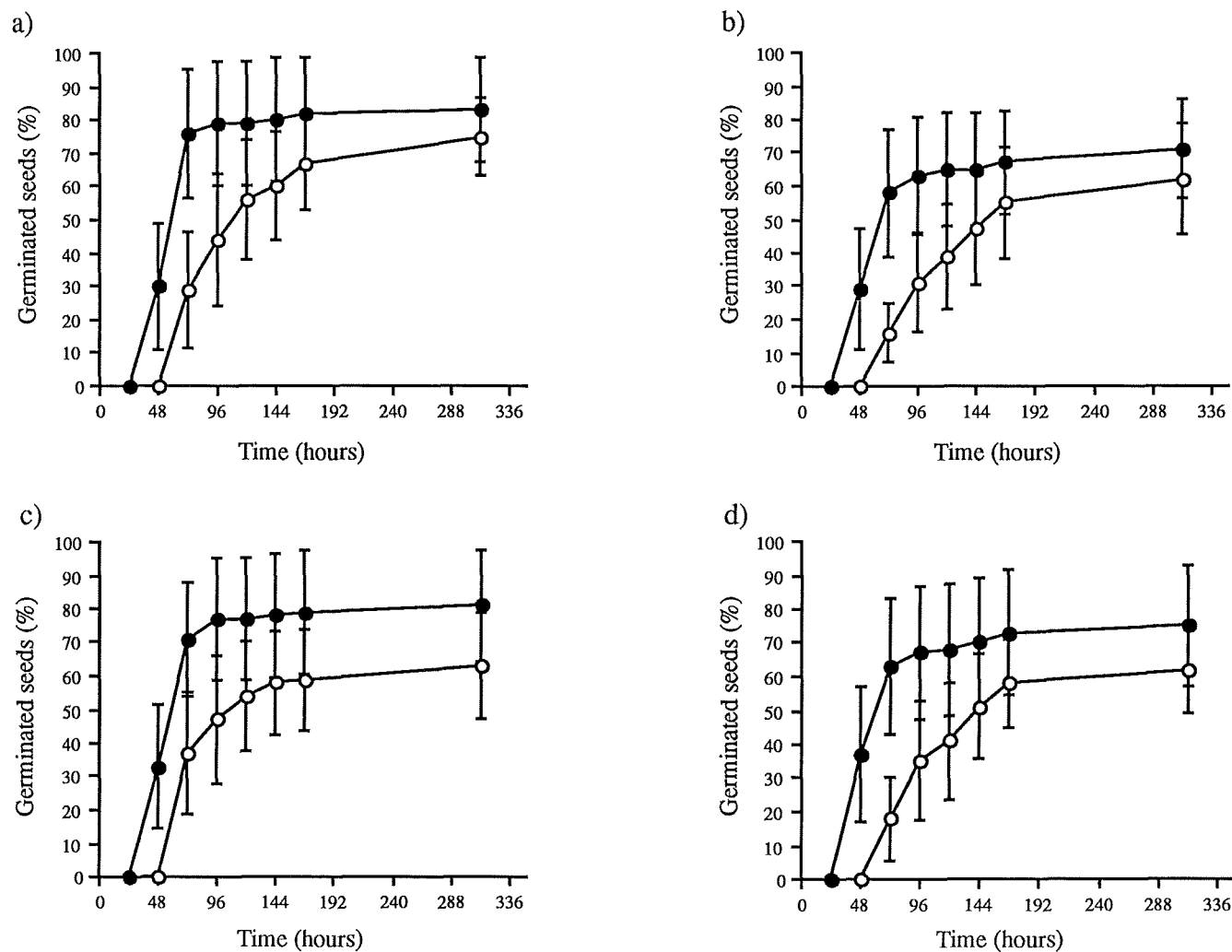
Figure 2.1. Timecourses of percentage germination of seeds from plants of *Plantago major* population Lullington in which the first spike was exposed to filtered air (control treatment) or to 120 ppb O_3 for 7 h d^{-1} for 9 d during flowering. Seeds were germinated from a) lower exposed spike, b) upper exposed spike, c) lower unexposed spike and d) upper unexposed spike. Solid circles, controls; open circles, ozone treated. Means and double standard errors are shown; n=5.
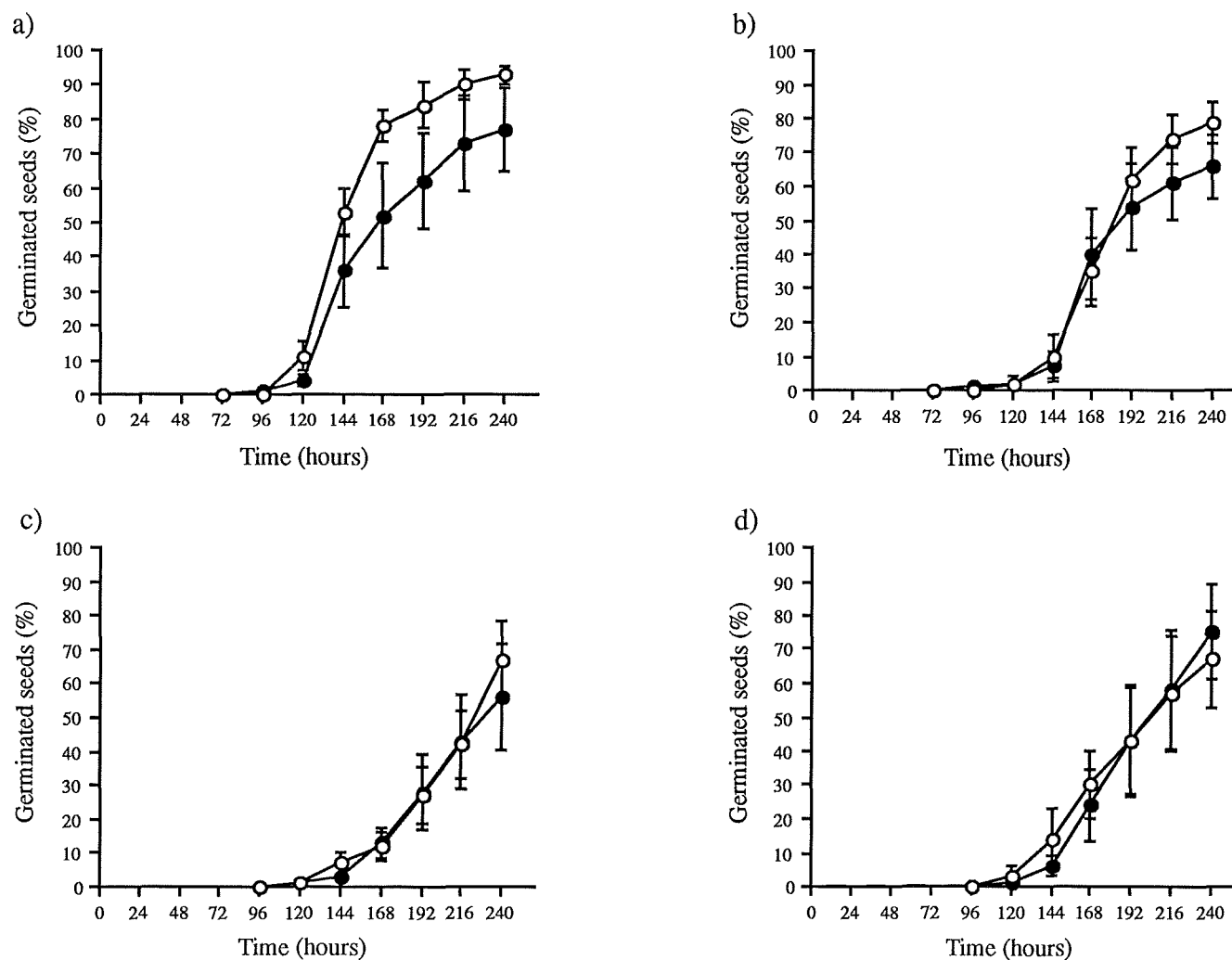
Figure 2.2. Timecourses of percentage germination of seeds from plants of *Plantago major* population Penicuik in which the first spike was exposed to filtered air (control treatment) or to 120 ppb O_3 for 7 h d^{-1} for 9 d during flowering. Seeds were germinated from a) lower exposed spike, b) upper exposed spike, c) lower unexposed spike and d) upper unexposed spike. Solid circles, controls; open circles, ozone treated. Means and double standard errors are shown; n=5.
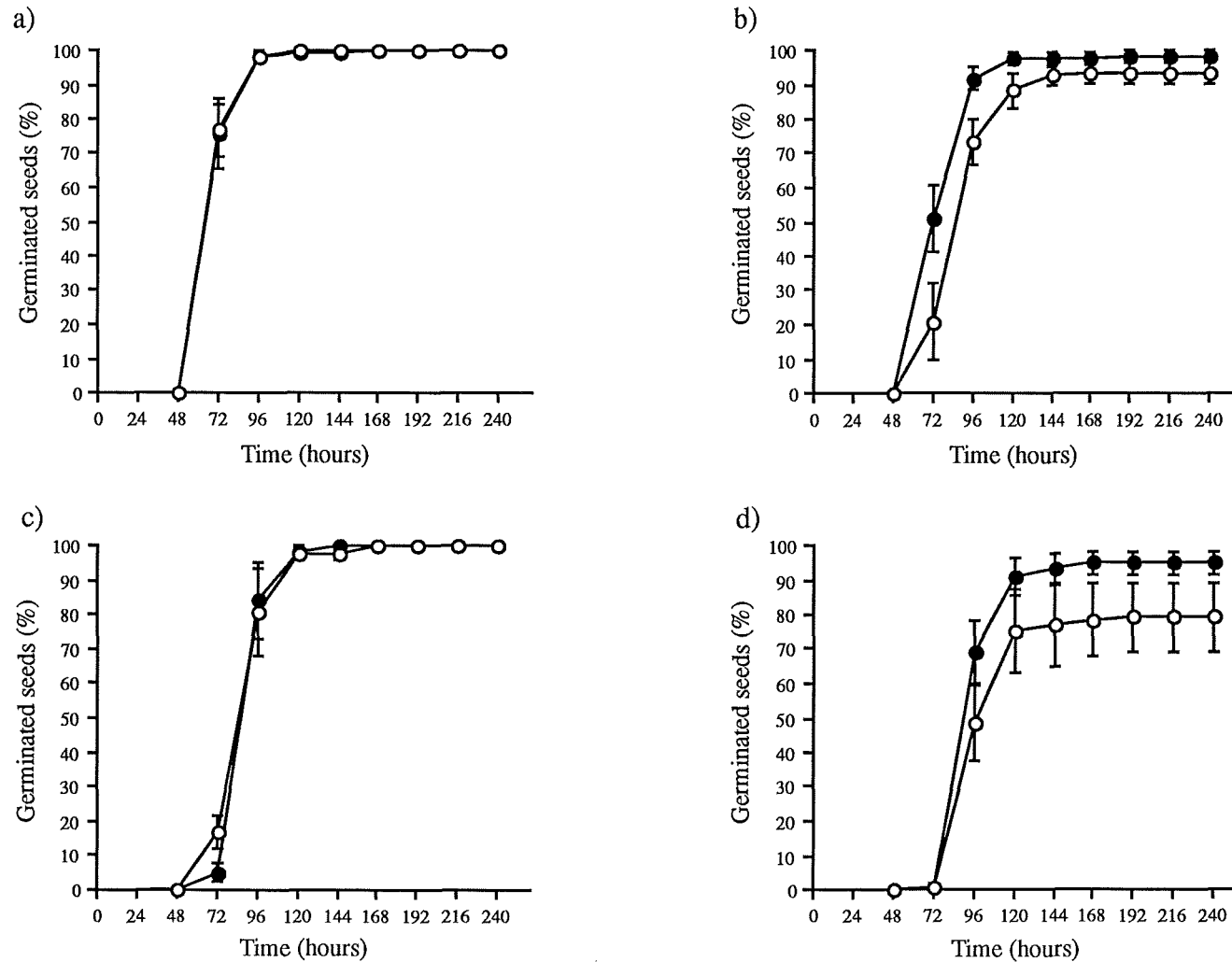
Figure 2.3. Timecourses of percentage germination of seeds from plants of *Plantago major* population Sibton in which the first spike was exposed to filtered air (control treatment) or to 120 ppb O_3 for 7 h d^{-1} for 9 d during flowering. Seeds were germinated from a) lower exposed spike, b) upper exposed spike, c) lower unexposed spike and d) upper unexposed spike. Solid circles, controls; open circles, ozone treated. Means and double standard errors are shown; n=6.
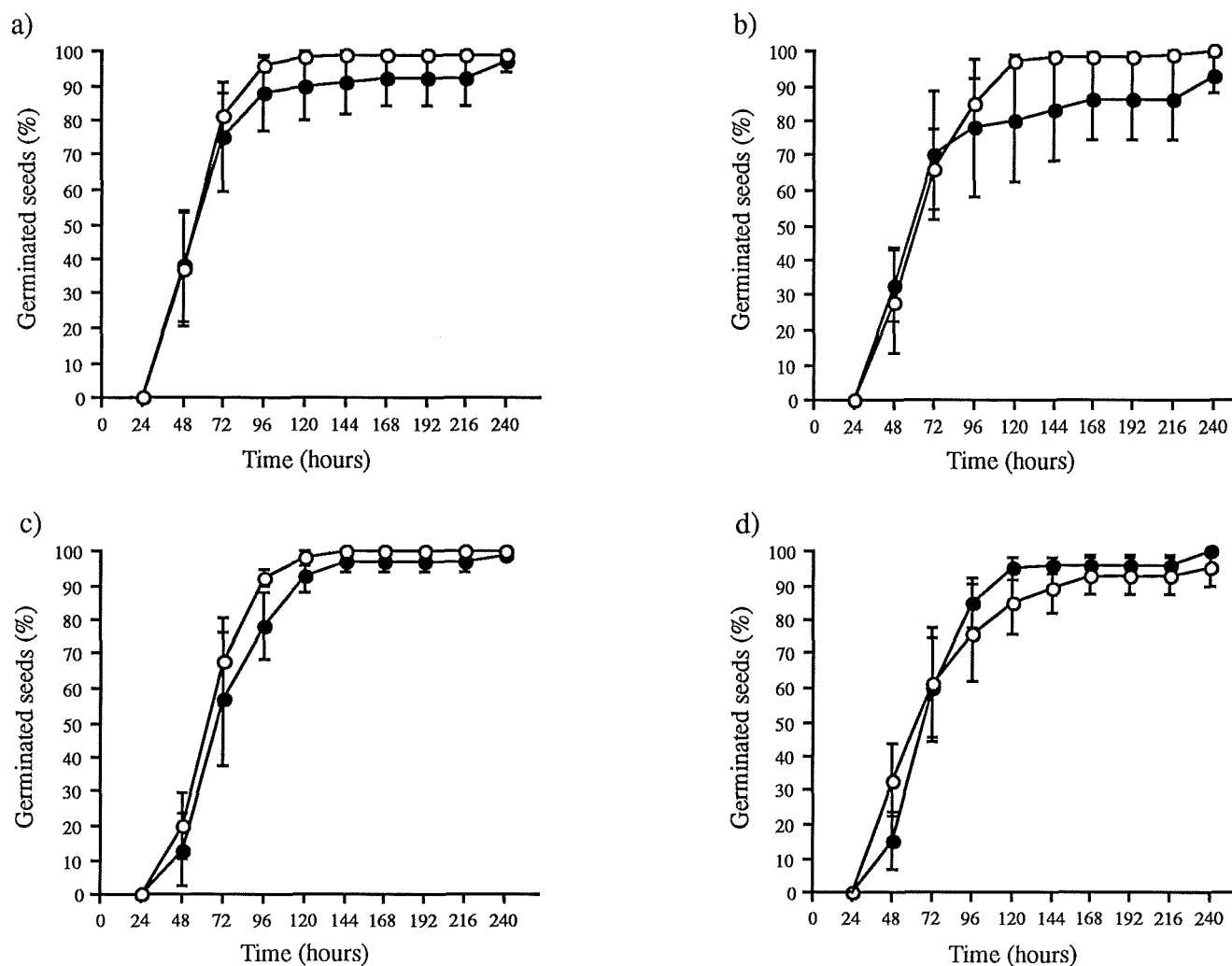
Figure 2.4. Timecourses of percentage germination of seeds from plants of *Plantago major* population High Low in which the first spike was exposed to filtered air (control treatment) or to 120 ppb O_3 for 7 h d^{-1} for 9 d during flowering from 37 DAS. Seeds were germinated from a) lower exposed spike, b) upper exposed spike, c) lower unexposed spike and d) upper unexposed spike. Solid circles, controls; open circles, ozone treated. Means and double standard errors are shown; n=7.
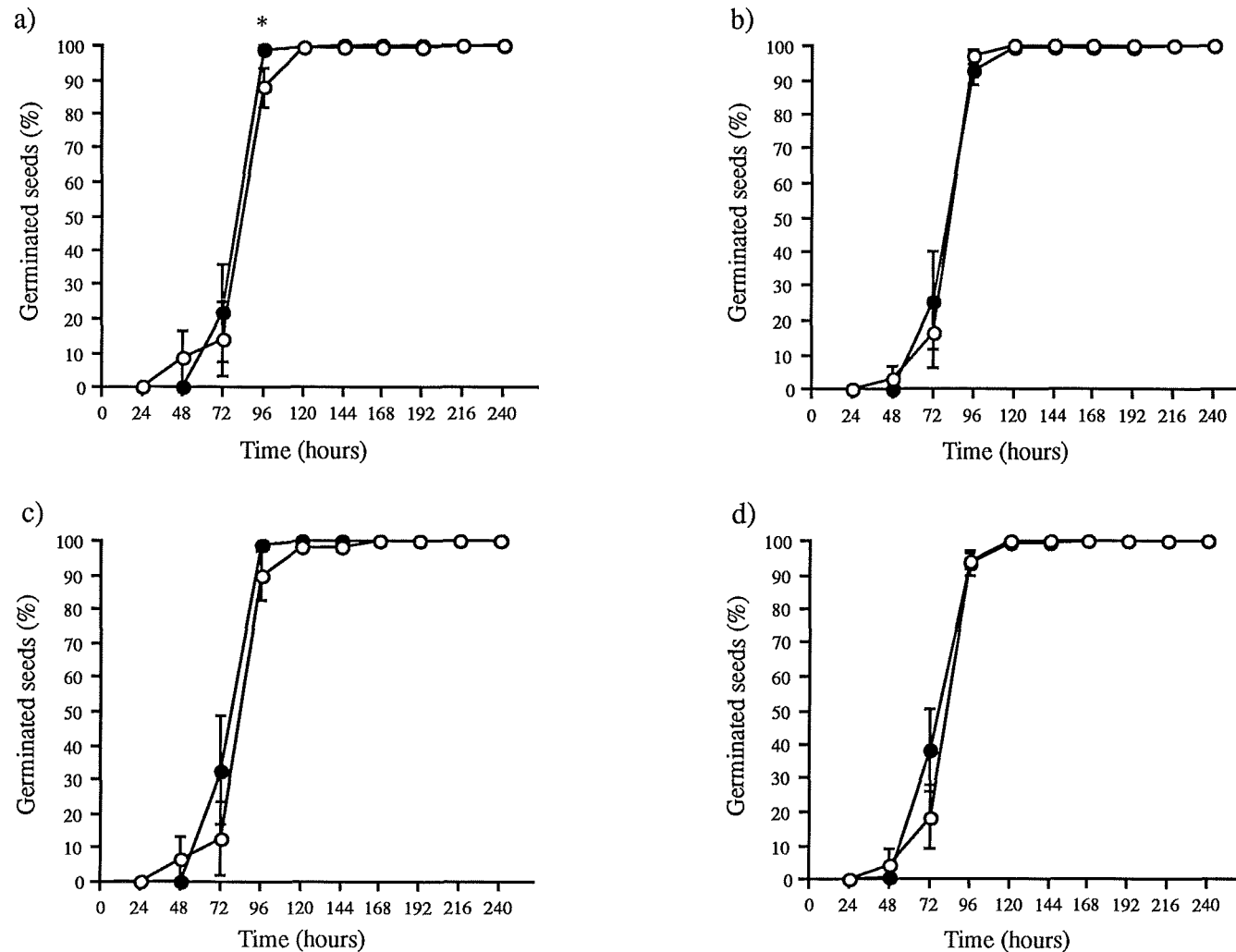
Figure 2.5. Timecourses of percentage germination of seeds from plants of *Plantago major* population High Low in which the first spike was exposed to filtered air (control treatment) or to 120 ppb O_3 for 7 h d^{-1} for 9 d during flowering from 51 DAS. Seeds were germinated from a) lower exposed spike, b) upper exposed spike, c) lower unexposed spike and d) upper unexposed spike. Solid circles, controls; open circles, ozone treated. Means and double standard errors are shown. * indicates significance of P<0.05; n=9.

Figure 2.6. Timecourses of percentage germination of seeds from plants of *Plantago major* population High Low in which the first spike was exposed to filtered air (control treatment) or to 120 ppb O_3 for 7 h d^{-1} for 9 d during flowering from 60 DAS. Seeds were germinated from a) lower exposed spike, b) upper exposed spike, c) lower unexposed spike and d) upper unexposed spike. Solid circles, controls; open circles, ozone treated. Means and double standard errors are shown; n=6.
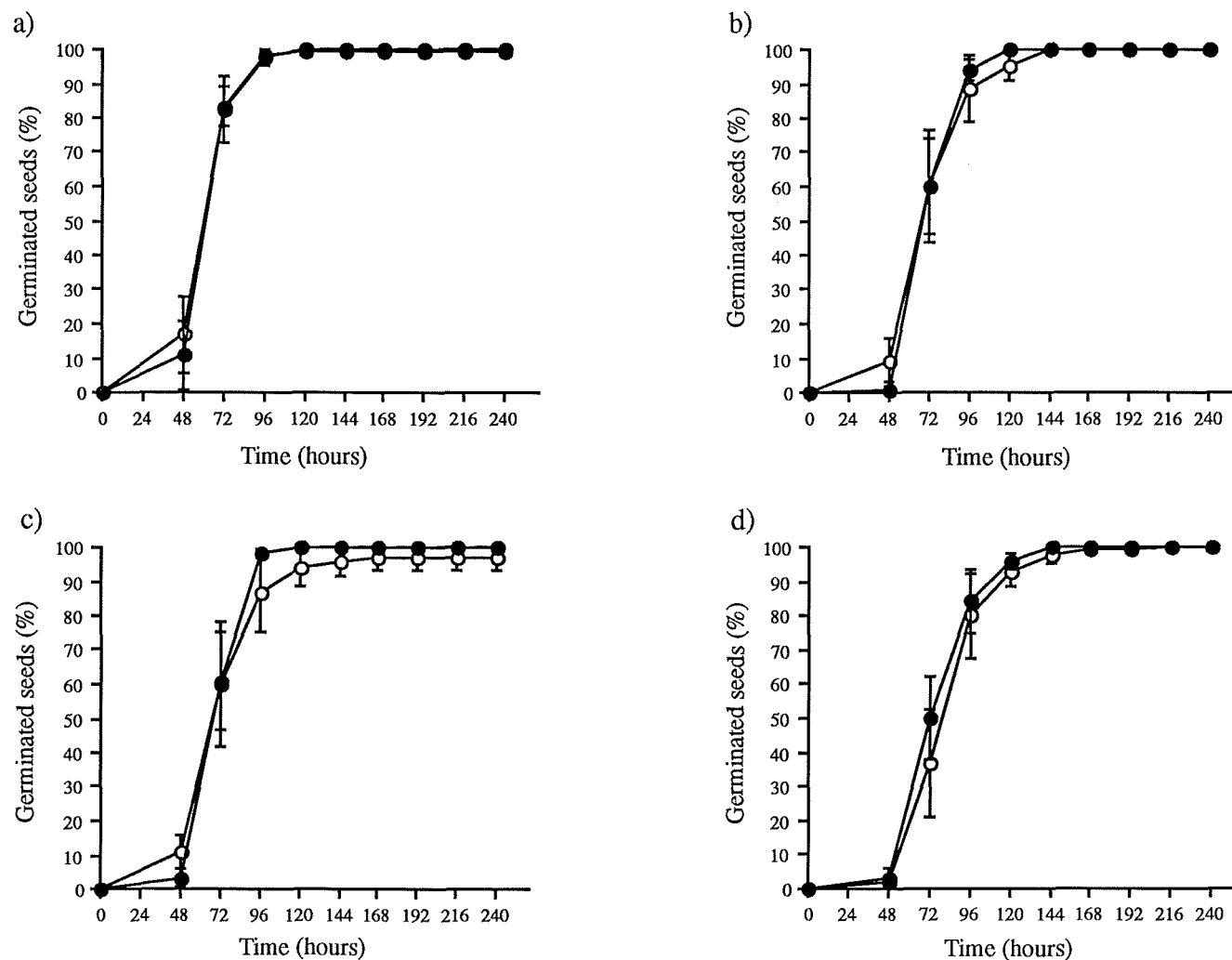
Figure 2.7. Timecourses of percentage germination of seeds from plants of *Plantago major* population High Low in which the first spike was exposed to filtered air (control treatment) or to 70 ppb O_3 for 7 h d^{-1} for 7 d during flowering. Seeds were germinated from a) lower exposed spike, b) upper exposed spike, c) lower unexposed spike and d) upper unexposed spike. Solid circles, controls; open circles, ozone treated. Means and double standard errors are shown. * and ** indicate significance of P<0.05 and P<0.01; n=5.
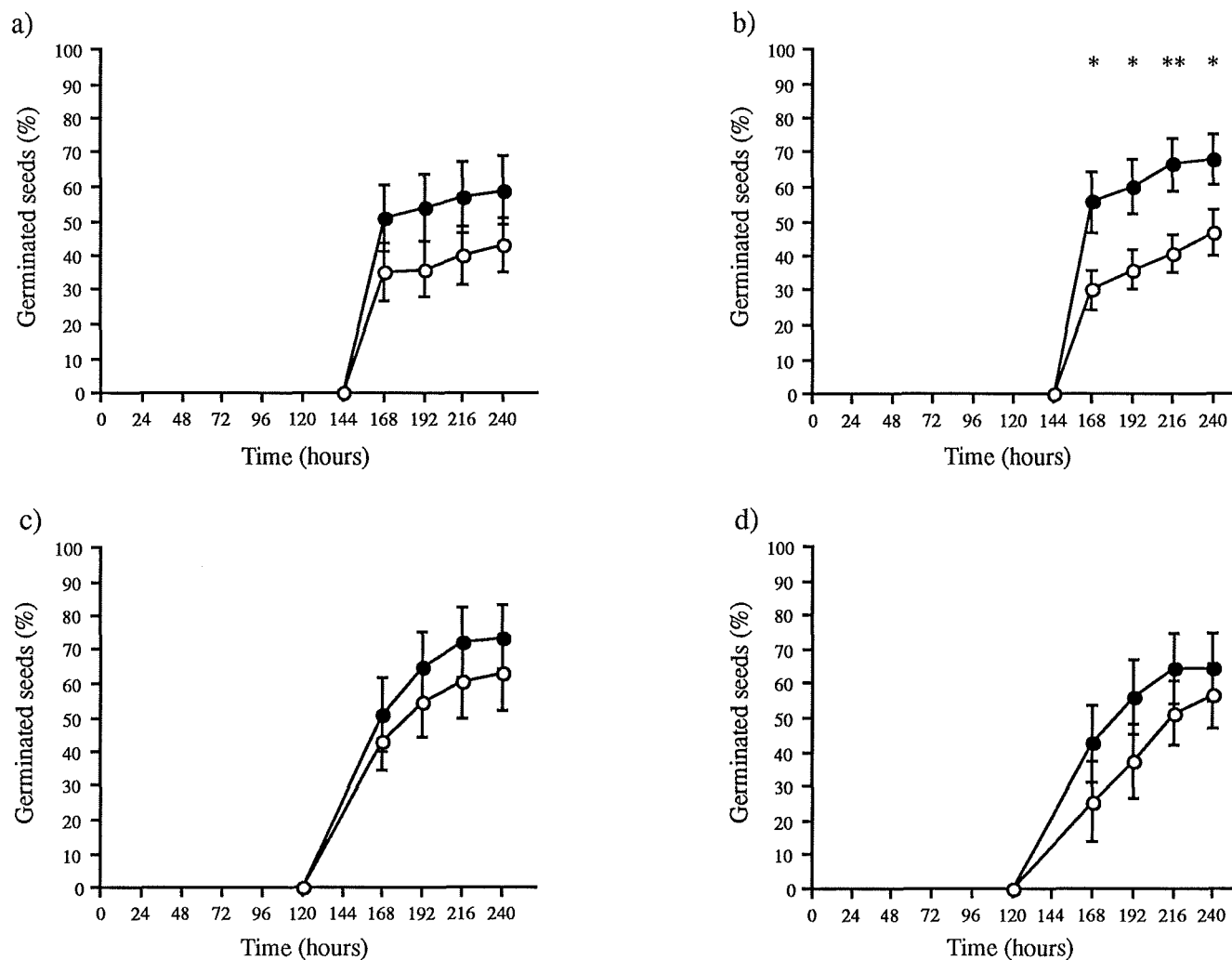

345

Figure 2.8. Timecourses of percentage germination of seeds from plants of *Plantago major* population High Low in which the first spike was exposed to filtered air (control treatment) or to 120 ppb O_3 for 7 h d^{-1} for 14 d during seed filling. Seeds were germinated from a) lower exposed spike, b) upper exposed spike, c) lower unexposed spike and d) upper unexposed spike. Solid circles, controls; open circles, ozone treated. Means and double standard errors are shown; n=5.
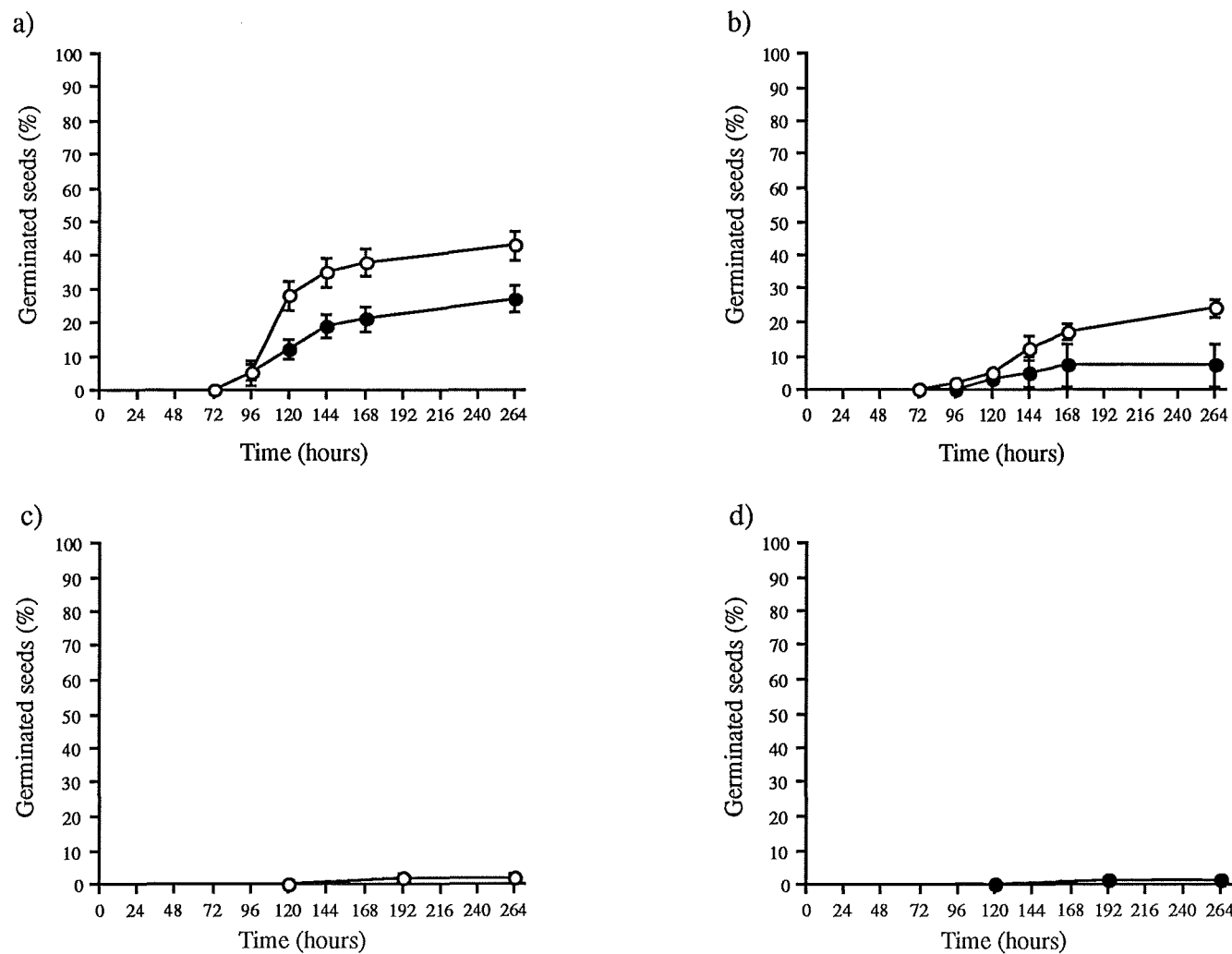
Figure 2.9. Timecourses of percentage germination of seeds from plants of*Plantago major* population High Low in which whole plants were exposed to filtered air (control treatment) or to 54 ppb O_3 for 7 h d^{-1} for 14 d during flowering. Seeds were germinated from a) lower first spike, b) upper first spike, c) lower second spike and d) upper second spike. Solid circles, controls; open circles, ozone treated. Means and double standard errors are shown. * indicates significance at P<0.05; n=6.