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Ozone sensitivity and population genetics of Anthoxanthum odoratum L.

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Ozone Sensitivity and Population Genetics of *Anthoxanthum odoratum* L.

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by

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Abstract

The effects of ozone pollution on the perennial grass species *Anthoxanthum odoratum* L. were investigated in terms of ozone sensitivity to contrasting ozone pollution scenarios and intraspecific variation in relation to population genetic diversity. Four populations from North Wales, UK, were exposed to three ozone exposure profiles representing acute exposure and simulated current and future scenarios for both rural and upland areas. Population genetic diversity and differentiation were estimated for the nuclear genome using Amplified Fragment Length Polymorphism (AFLP) and microsatellite loci developed in this study using a streptavidin-biotin enrichment technique. Genetic variation within the chloroplast genome was assessed using Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) and chloroplast microsatellite loci.

A. odoratum was sensitive to ozone exposure in terms of premature senescence exhibiting a relatively fast response under all ozone profiles investigated. Exposure to chronic rural and upland ozone profiles demonstrated that elevated background concentrations may result in a similar or greater senescence response to increased ambient peak episodes. The development of premature senescence was only accompanied by significant reductions in above ground biomass under acute exposure. However, a trend for greater reduction in root biomass was also observed indicating a shift in resource allocation. The maintenance of shoot growth observed under chronic exposure may have also resulted from a reduction in resource allocation to root growth. Intraspecific variation in response was observed with the four populations differing in response to elevated ozone dependant on the assessment criteria applied and the timing of exposure in relation to the growing season and phenological stage.

AFLP and microsatellite markers revealed high levels of intrapopulation genetic diversity with low levels of population genetic differentiation maintained by high levels of pollen-mediated gene flow. Chloroplast markers also revealed high levels of intrapopulation genetic diversity although population differentiation was substantially greater indicating greater restrictions to seed-mediated gene flow between populations.

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Chapter 1: General Introduction

Terrestrial plant species experience a wide range of environmental stresses including those induced by air pollution. One such air pollutant is tropospheric ozone, which exerts strong oxidative stress on organisms within the biosphere. Although ozone and oxidative stress occur naturally, human activities have led to an increase in ozone concentrations and ozone-induced oxidative stresses. The phytotoxic effects of ozone have been recognised for 50 years (Richards *et al* 1958) and plant species have been shown to be highly variable in response to elevated ozone at both the intra- and inter-specific level (Davison and Barnes 1998). Tropospheric ozone concentrations are predicted to continue rising over the next century with potentially adverse effects on individual species, communities and ecosystems. In this thesis, the effects of ozone pollution on a common temperate grassland species, *Anthoxanthum odoratum* L., have been studied in relation to the population genetics of this species.

1.1 Tropospheric ozone

Ozone, a three atom allotrope of oxygen, is a natural component of the atmosphere produced through photochemical reactions. In relation to the biosphere, ozone has two contradicting roles being both beneficial and harmful dependant on its position within the atmosphere. The total column of atmospheric ozone acts to reduce the penetration of harmful UV-radiation to the surface through the absorption of UV-radiation in the photochemical reactions which both produce and destroy ozone (Runneckles and Krupa 1994). Tropospheric ozone, although still an absorbent of UV-radiation, has a mainly harmful influence exerting strong oxidative stress on both organisms and materials within the biosphere and is considered an air pollutant (Fuhrer *et al* 1997). Ozone is termed a secondary, regional air pollutant as it is not directly released through human activities but concentrations are influenced by other anthropogenically released molecules while long-range transport of both ozone and its precursor molecules make it a regional phenomenon (Metcalfe *et al* 2002). The processes and factors governing ozone concentrations, current and predicted future ozone concentrations and the

phytotoxic effects of ozone exposure and vegetation response are considered in this section.

1.1.1 Mechanisms of production and destruction

The troposphere is comprised of the planetary boundary layer (PBL) up to an altitude of around 2 km and the free troposphere which extends from the PBL to the tropopause, the boundary with the stratosphere at an altitude of 8-18km (Jacob 2000). Ozone concentrations within the troposphere are governed by the three main processes of *in situ* photochemical production and destruction, transport from the stratosphere, and deposition at ground level (Figure 1.1) (Beck and Grennfelt 1994, Jacob 2000).

1.1.1.1 Photochemical production and destruction

Photochemical production and destruction *in situ* is the dominant process determining tropospheric ozone concentrations. A complex series of photochemical reactions involving precursor molecules generate and destroy ozone within the troposphere. These precursor molecules include nitrogen oxides (NO_x), volatile organic compounds (VOC) and carbon monoxide (CO). Jacob (2000) described the production of ozone as a HO_x-catalysed chain of oxidation of CO and hydrocarbons in the presence of NO_x (Figure 1.1). A simplified relationship between ozone and NO_x is described in equations 1 to 3:

$$NO_2 + h\nu \rightarrow NO + O \qquad (1)$$

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

$$NO + O_3 \rightarrow NO_2 + O_2 \qquad (3)$$

Equations 1 and 2 describe the production of ozone through the photolysis of NO_2 by light ($h\nu$) followed by reaction with oxygen molecules and the third equation describes the destruction of ozone by NO. These reactions form a photostationary state with the production and destruction of ozone in equilibrium. Net production of ozone occurs when this equilibrium is disrupted by increases in NO_2 (Kleinman 2000). This is a very

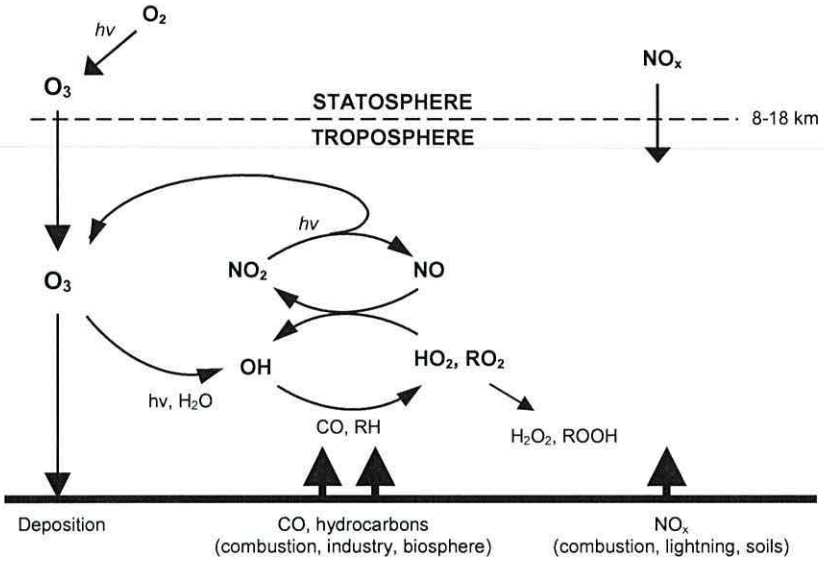

Figure 1.1 – A schematic diagram of tropospheric ozone chemistry involving hydroxides (HO_x), nitrogen oxides (NO_x), hydrocarbons (denoted by R) and light (*hv*). Adapted from Jacob (2000)

simplified view of ozone production and destruction which in reality involves the highly reactive hydroxyl (OH) and hydroperoxy (HO_2) radicals, hundreds of subsequent chemical reactions and thousands of different chemical species (Metcalfe *et al* 2002, NEGTAP 2001).

The precursor molecules to ozone are released from both natural and anthropogenic sources. Natural sources of ozone precursor molecules include biological emissions from vegetation and soil microbes, and the abiotic processes of lightning and biomass burning (Guenther *et al* 2000, Solomon *et al* 2000). In the absence of anthropogenic emissions, natural sources dominate the photochemical processes controlling tropospheric ozone levels, although the majority of these sources have now been influenced by human activities such as the application of nitrogen fertilisers to agricultural land. Anthropogenic sources of precursor molecules include vehicle emissions (Sawyer *et al* 2000), and industry and power plant emissions (Placet *et al* 2000). Human activities have increased the levels of precursor molecules through both influence on natural source emissions and the direct release of such molecules. This has altered the balance between the reactions producing and destroying ozone, resulting in a net increase of ozone within the troposphere from the background concentrations of 10 ppb in pre-industrial times to around 30 ppb in recent years (Volz and Kley 1988, Coyle *et al* 2002).

1.1.1.2 Transport from the stratosphere

Transport of ozone from the stratosphere occurs through stratospheric intrusions during tropopause folding events where ozone-rich stratospheric air mixes down into the troposphere resulting in the eventual transfer of ozone into the planetary boundary layer. Tropopause folding events occur in association with certain meteorological conditions including vigorous cold fronts, high pressure ridges and anticyclones. Although relatively uncommon and contributing a lesser amount to the total tropospheric ozone concentrations than *in situ* photochemical production, stratospheric intrusions can still have a significant effect on peak ozone concentrations especially in mountainous regions (Davies and Schuepbach 1994).

1.1.1.3 Deposition

The process of deposition is involved in the removal of ozone from the troposphere through the reaction of ozone with surfaces at ground level. Dry deposition rather than deposition through precipitation is the main pathway for ozone deposition due to the low solubility of ozone in water (Jacob 2000). Dry deposition is the process by which atmospheric chemicals are transported by air motions to the surface of the earth (Wesely and Hicks 2000). Once transported to ground level the deposition of ozone occurs rapidly on most surfaces due to the reactive nature of ozone (Coyle *et al* 2002). Deposition velocities at a few meters over vegetation can be moderately large at around 1cm s^{-1} (Wesely and Hicks 2000) and are enhanced by the absorption of ozone through plant stomata (Coyle *et al* 2002). It is the process of deposition through oxidative reactions which causes the harmful influence of tropospheric ozone on components of the biosphere.

1.1.2 UK spatial and temporal ozone distribution

The three processes of photochemical reaction, stratospheric transport and deposition govern the formation and removal of ozone within the troposphere. The interaction of these processes, both together and with factors such as meteorological conditions and

topography result in spatial and temporal variation in tropospheric ozone concentration (Hidy 2000). The meteorological conditions favourable for photochemical ozone production include high ambient temperatures and high levels of UV-radiation (Solomon *et al* 2000). Although ozone is largely formed in polluted urban and industrial areas where emissions of precursor molecules are highest, the effects do not remain local as persistence times of several days may result in long-range transport over distances of up to 1000 km (Metcalfe *et al* 2002). Wind speed, vertical mixing and horizontal transfer are important factors affecting the transport of ozone and its precursor molecules influencing local and regional distributions and the eventual surface deposition of ozone (Solomon *et al* 2000).

Tropospheric ozone concentrations are described as background concentrations, relating to the average annual mean ozone concentration, and as peak concentrations relating to maximum ozone concentrations. Photochemical ozone episodes refer to short time periods where peak ozone concentrations are reached. Ozone episodes occur when suitable meteorological conditions and an abundance of precursor molecules coincide resulting in high levels of photochemical ozone production (Solomon *et al* 2000), although stratospheric intrusions may have the same effect on rare occasions (Davies and Schuepbach 1994). The peak concentrations that constitute an ozone episode are dependent upon the critical level applied and the governmental policies to which the critical levels relate. In general, ozone episodes are considered to occur when peak concentrations exceed 50 parts per billion (ppb) as such levels are harmful to human health and vegetation (NEGTAP 2001). The duration of an ozone episode ranges from a few hours to several days dependant on the climatic conditions, precursor molecule concentrations and local topography.

The tropospheric ozone concentrations observed at any given location are the result of a complex interplay between a wide range of factors including *in situ* photochemical reactions, deposition rates, climatic conditions, local topography and the long-range transport of ozone and its precursor molecules. The shifting dominance of these factors in influencing the production and destruction of ozone result in concentrations which are highly variable between years and exhibit both seasonal and diurnal cycles.

1.1.2.1 Geographic variation

Mean annual tropospheric ozone concentrations in the UK follow a general trend for increased concentration with increasing altitude and a cline of increasing concentration from the North to the South in lowland areas (Figure 1.2 a). Background ozone concentrations vary with locality type with the lowest concentrations in urban and industrial areas at 10 ppb, rural areas at 25 ppb and with the highest concentrations of 35 ppb experienced in upland areas (NEGTAP 2001). The low concentrations in urban and industrial areas occur despite the highest precursor molecule emissions due to the accompanying high emission levels of Nitrogen oxide (NO) which facilitates the removal of ozone in these areas (Coyle *et al* 2002, Klumpp *et al* 2006). Rural areas experience higher concentrations due to the transport of ozone and precursor molecules from urban areas and lower levels of NO (Coyle *et al* 2002). The relationship between

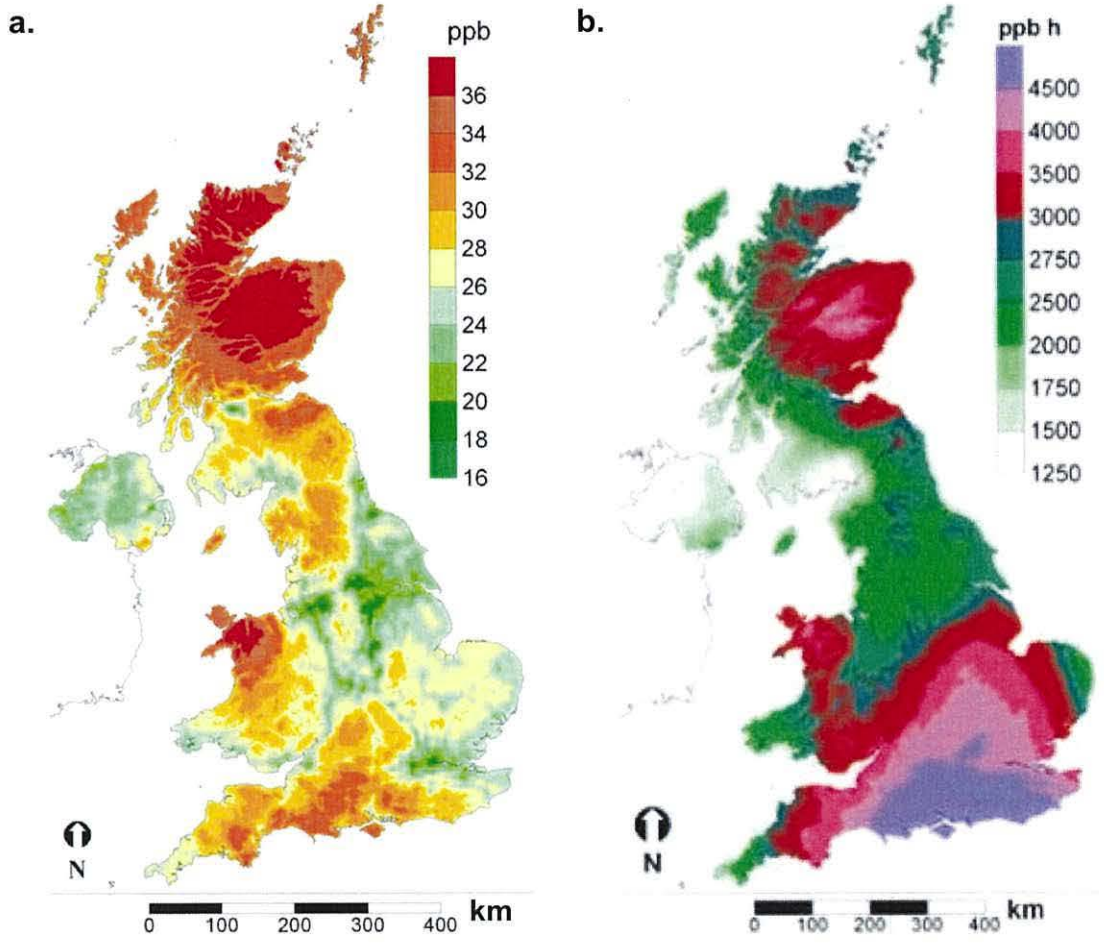

Figure 1.2 – Distribution of tropospheric ozone across the UK a) 2003 annual mean ozone concentration (ppb) and b) May to July AOT40 (ppb h) averaged over 1999 to 2003. (M. Coyle, pers comm.)

altitude and ozone concentration, with the highest concentrations occurring in upland areas, is due to the higher wind speeds in these areas. The more turbulent air conditions result in greater mixing down of ozone-rich air into the PBL replacing ozone removed through deposition and maintaining ozone concentrations (Coyle *et al* 2002, Ashmore *et al* 2002).

1.1.2.2 Seasonal variation

Ozone concentrations show seasonal variation with a general trend for maximum concentrations during the spring and summer months and minimum concentrations in autumn and winter (Coyle *et al* 2003a). Spring peaks in the Northern Hemisphere are thought to relate to photochemical ozone production involving precursor molecules accumulated over the polar region during the winter (Vingarzan 2004) while summer peaks are the result of favourable climatic conditions for the accumulation of precursor molecules and net photochemical ozone production (NEGTAP 2001, Coyle *et al* 2002). The majority of ozone episodes occur during the spring and summer months in the UK and coincide with the growing season for the majority of vegetation.

The seasonal trends also vary spatially with relatively unpolluted northern sites showing maximum concentrations in spring and summer maximums occurring at more southerly sites. This is illustrated by the prevalence of mean hourly ozone concentrations exceeding 40 ppb in Southern England in May to July (Figure 1.2 b). In addition to ozone and precursor emissions in the UK this pattern is also influenced by European precursor emissions (Beck and Grennfelt 1994, Metcalfe *et al* 2002) and hemispheric transport of the precursors of ozone pollution (Sitch *et al* 2007).

1.1.2.3 Diurnal cycles

Ozone concentrations also exhibit diurnal cycles typically reaching maximum levels in the afternoon and minimum levels between midnight and dawn. Diurnal cycles are governed by the levels of photochemical production and destruction, entrainment from the free troposphere, deposition, local topography and meteorological conditions. In the morning photochemical production of ozone begins, as does the turbulent mixing of the PBL through wind shear and thermal convection leading to the entrainment of ozone

from the free troposphere (Coyle *et al* 2002). Levels of entrainment decrease during the day as the well mixed PBL reaches its maximum depth while the photochemical production of ozone increases with the rising intensity of UV-radiation (Beck and Grennfelt 1994). Peak ozone concentrations are reached in the afternoon when UV-radiation levels are at a maximum and mixing of the PBL replaces ozone removed through deposition (Coyle *et al* 2002). During the night and early morning the atmosphere cools and the lower regions of the PBL become thermally stratified resulting in very reduced levels of ozone entrainment from the free troposphere. Concentrations decrease as ozone removed by deposition is not replaced by either photochemical production or entrainment (Coyle *et al* 2002).

The extent of variation in ozone concentration across the diurnal cycle varies both seasonally and spatially (Ashmore *et al* 2002). Diurnal cycles are most pronounced during the summer months when high levels of UV-radiation and more hours of daylight allow greater photochemical production of ozone than can occur during the winter months. Variation in the diurnal cycle at different localities is dependant on emission levels and the local topography and meteorology which affect the level of ozone depletion at night (Coyle *et al* 2002, Ashmore *et al* 2002, Solomon *et al* 2000). In urban and industrial areas peak concentrations are reached during the afternoon and night while minimum concentrations coincide with the morning and evening rush hours due to high levels of NO from vehicle emissions at these times resulting in the net depletion of ozone rather than net production (Figure 1.3) (Coyle *et al* 2002, Klumpp *et al* 2006).

In rural areas maximum concentrations occur in the afternoon and minimum concentrations between midnight and dawn (Coyle *et al* 2002). The maximum concentrations reached and timing of these maxima exhibits little variation among sites while the extent of night time depletion has a negative relationship with altitude resulting in more pronounced diurnal profiles at low altitude (Figure 1.4). In lowland rural areas there is a large decrease in ozone concentrations from the afternoon peak to the night time minimum due to the deposition of ozone without further replacement (Solomon *et al* 2000, Coyle *et al* 2002). In upland areas, although the lower layers of the atmosphere cool, thermal stratification does not occur to the same extent due to the thermally driven up- and down-slope air flows characteristic of valley-mountain

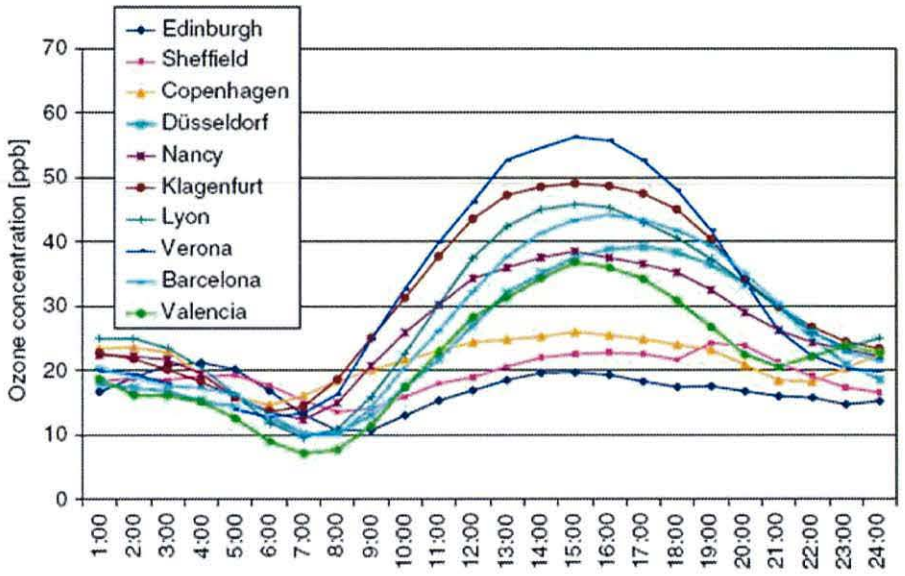

Figure 1.3 – Mean ozone concentrations across the diurnal cycle at European urban and suburban sites in April to September 2001 (Klumpp *et al* 2006).

interactions (Solomon *et al* 2000). These locally driven air flows result in the continued mixing of the PBL during the night and replacement of ozone removed through deposition by entrainment from the free troposphere. Coastal areas experience a similar ozone profile to upland areas. This is due to onshore winds which maintain PBL mixing and add relatively ozone-rich air that has accumulated over the sea due to the low ozone deposition velocities over water (Coyle *et al* 2002, Jacob 2000).

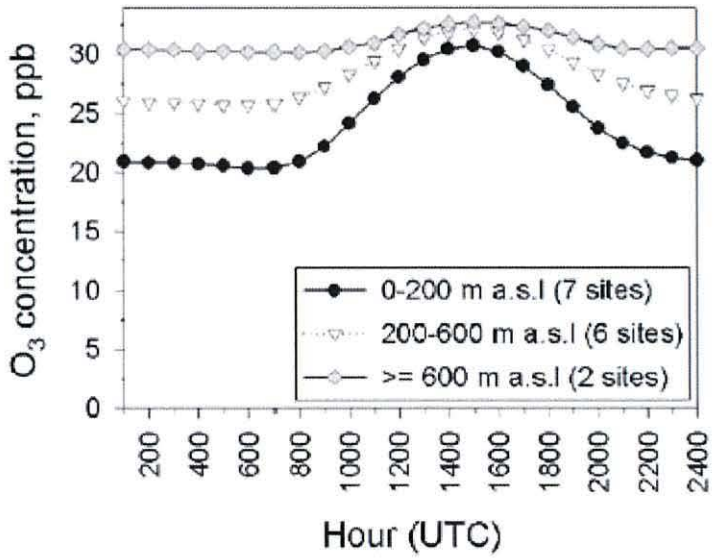

Figure 1.4 – Average diurnal cycles at rural monitoring sites for the years 1990-1996 averaged into groups by altitude (Coyle *et al* 2002).

1.1.3 Predicted trends for future ozone concentrations

Mean annual tropospheric ozone concentrations have more than doubled over the last century from pre-industrial levels of 10 ppb to 15 ppb to the current levels of 30 ppb to 35 ppb (Volz and Kley 1988, NEGTAP 2001). This is due to increasing anthropogenic precursor molecule emissions over this time increasing the pool of precursors available for photochemical ozone production (Volz and Kley 1988). Through the 1970's to 1990's ozone concentrations increased by 0.5 % to 2 % per year although this rate has slowed across Europe in recent years due to the implementation of air pollution policies reducing NO and CO emissions (Vingarzan 2004). In the UK, annual mean and maximum ozone concentrations from 1972 to 2000 have revealed a general trend for increasing background levels and decreasing maximum concentrations (NEGTAP 2001). The decrease in peak concentrations follows the reduction in NO and CO emissions.

At a global scale anthropogenic emissions are predicted to continue increasing over the next century causing background ozone concentrations to rise to 52 ppb by 2100 (NEGTAP 2001). This global trend is expected to be reflected in increases in the background ozone concentrations experienced in the UK while the magnitude of peak concentrations may decrease if local and regional emissions continue to fall leading to diurnal cycles in rural areas resembling those currently observed in upland areas (NEGTAP 2001). The spatial and seasonal variation in ozone concentrations are also predicted to change with increases in background concentrations expected to be greatest in winter and early spring and lowest in the summer months when local and regional precursor emissions dominate ozone levels. The increased background concentrations are expected to have the greatest impact in upland areas where ozone levels are less depleted over night (Ashmore *et al* 2002). Future UK ozone concentrations are expected to result a reduction in the magnitude of acute ozone exposure but an increase in the long term chronic exposure for vegetation (NEGTAP 2001). By the end of this century, mean ozone concentrations are predicted to exceed 75 ppb in most of Europe (Sitch et al 2007).

1.2 Phytotoxic effects of tropospheric ozone on vegetation

Ozone exposure exerts strong oxidative stress on vegetation and the potential for adverse effects from such exposure has been recognised for 50 years since the first description of ozone-induced visible injury by Richards *et al* (1958). The relationship between ozone exposure and plant response is governed by the dynamics of ozone uptake and plant energetics (Krupa *et al* 1995). Plants show both compensatory and defensive responses to ozone exposure including detoxification by chemical reactions, the alteration of metabolic pathways, repair of injured tissue and cell death (Alscher *et al* 1997, Pell *et al* 1997, Baier *et al* 2005). The induced responses are dependant upon the intensity of ozone exposure, environmental factors influencing photosynthesis and ozone deposition to the plant surfaces, and the inherent defence mechanisms (Musselmann and Massman 1999).

1.2.1 Ozone transport into plants

Ozone reacts with vegetation by deposition at the cuticular component of leaves and stomatal uptake. As the cuticle is virtually impermeable to ozone the main pathway for ozone into the plant is through the stomata (Rao *et al* 2000, Lyons *et al* 1999, Kerstiens and Lendzian 1989a, Kerstiens and Lendzian 1989b) and plant responses are more related to the ozone flux into the plant rather than the external ozone concentration (Pleijel *et al* 2000, Uddling *et al* 2004). The rate of ozone flux into the plant is dependant on the ozone concentration at the leaf surface and the conductance of ozone into the leaf tissue and is therefore regulated by stomatal opening and closure. Stomatal conductance is highest during the day but nocturnal conductance also occurs in a wide range of species, although at a much lower level, and may have a significant effect on total ozone flux into the plant (Musselmann and Minnick 2000). Species with higher levels of stomatal conductance may be at more risk due to greater ozone uptake and a greater occurrence of foliar injury has been associated with species with higher stomatal conductance although this relationship is not consistent (Bungener *et al* 1999b, Zhang *et al* 2001). Environmental factors which facilitate stomatal closure, such as drought, may provide plants with some protection by limiting ozone uptake and reduced foliar injury

has been associated with increased vapour pressure deficit (VPD) in some species (Bungener *et al* 1999b).

Reduced stomatal conductance under elevated ozone exposure has been reported in a wide variety of species (Balaguer *et al* 1995, Novak *et al* 2005, Pasqualini et al 2002, Power and Ashmore 2002, Ramo *et al* 2006b, Reiling and Davison 1994, 1995, Zhang *et al* 2001) suggesting an inhibitory effect on stomatal function. As the stomata are the pathway of ozone entry into the plant, the stomatal pores, subcellular cavities and guard cells may be the prime locations for ozone reaction (Robinson *et al* 1998). Torsethaugen *et al* (1999) found that ozone exposure in the broad bean, *Vicia faba*, appeared to have a direct effect upon the stomatal guard cells inhibiting the potassium channels that mediate the uptake of K^+ for the process of stomatal opening. Reduced stomatal opening due to the direct effects of ozone on the stomatal guard cells may help to protect the plant from further ozone induced oxidative stress by reducing ozone flux, but this may also affect the uptake of carbon dioxide for photosynthesis.

Intraspecific variation in stomatal response to ozone exposure has been related to differences in ozone sensitivity. Sensitive individuals of *Rudbeckia laciniata* were shown to exhibit a slower stomatal response to changes in light and VPD while insensitive individuals exhibited greater transpirational efficiency, maximum assimilation rates and carboxylation rates despite higher stomatal conductance and therefore ozone uptake (Grulke *et al* 2007). This suggests that a greater ability to control stomatal opening during ozone exposure may confer some level of ozone tolerance. Resistant populations of *Plantago major* also exhibited a smaller reduction in stomatal conductance than sensitive populations which exhibited a more transient response with a large initial reduction followed by a subsequent decrease in effect (Reiling and Davison 1995). A reduction in effect over time was observed in both the sensitive and resistant populations suggesting some level of acclimation to ozone exposure. The smaller initial reduction in stomatal conductance in the resistant populations may result from a faster and greater induction of detoxification mechanisms (Zheng *et al* 2000). The occurrence of intraspecific variation and the highly species specific nature of the relationship between adverse ozone effects and stomatal conductance are indicative of the variation in the detoxification and defence mechanisms among and within species.

1.2.2 Cellular level effects

Once ozone enters the intercellular leaf space it reacts with molecules in the aqueous phase of the cell wall (apoplast) to form the reactive oxygen species (ROS): hydroxyl radicals ($OH^{\bullet}$), superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). These molecules react with lipid membranes and biologically important macromolecules, including nucleic acids, inducing oxidative stress which activates a number of antioxidative stress-related defence mechanisms (Alscher *et al* 1997, Sharma and Davies 1997). Plants have inherent biochemical mechanisms to remove damaging ROS due to the natural production of ROS in normal aerobic metabolism (Sharma and Davis 1997, Pell *et al* 1997, Baier *et al* 2005), and the use of ROS as messengers in signal transduction cascades involved in many processes critical for plant development and defence including mitosis and cell death (Foyer and Noctor 2005).

One of the first lines of defence is apoplastic ascorbate which scavenges ROS and provides partial protection for the plasmalemma against oxidative damage (Zheng *et al* 2000, Ranieri *et al* 2003). A higher level of ascorbate within apoplastic washing fluid during ozone exposure has been related to ozone resistance in populations of *Plantago major* (Zheng *et al* 2000). Interestingly this relationship was not observed for plants under control conditions suggesting that resistant populations have a greater capacity for induction of antioxidants under ozone stress.

The presence of increased ROS levels within the cell as a result of ozone exposure can induce either programmed cell death (PCD) events similar to a hypersensitive response (HR) occurring during pathogen attack or the activation of defence genes (Rao and Davies 2001, Pellinen *et al* 2002). The formation of necrotic lesions typical of ozone-induced visible injury (Figure 1.5) are the result of such PCD events which are under partial genetic control (Miller *et al* 1999, Pasqualini *et al* 2003). The modulation of ozone-induced cell death is the result of a complex network of signalling pathways involving ethylene (ET), jasmonic acid (JA) and salicylic acid (SA) (Figure 1.6).

A greater induction of ET-inducible genes and higher ethylene production has been observed in sensitive compared to resistant *Arabidopsis* accessions (Tamaoki *et al*

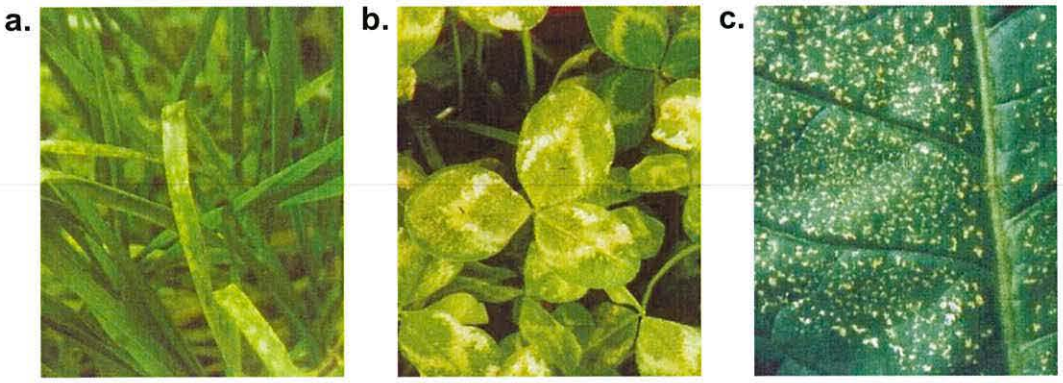

Figure 1.5 – Ozone induced visible injury as necrotic lesions on a) wheat, b) clover and c) tobacco (From Hayes *et al* 2007)

2003). An SA-inducible gene was also induced to a greater extent in the a sensitive *Arabidopsis* accession, which contrasts with results on hybrid poplar where a resistant clone exhibited a much greater induction of defence mechanisms including a pathogenesis-related gene (PR-1) which is indicative of an SA-mediated response (Koch *et al* 1998). Treatment with SA and methyl jasmonate (MeJA) also induced gene expression in the resistant but not the sensitive hybrid poplar clone indicating that sensitivity was due to a limited ability to perceive and then activate SA and JA mediated antioxidant defence response (Koch *et al* 2000). Diara *et al* (2005) also suggested that tolerance in the resistant hybrid poplar clone may result from an ability to maintain concentrations of these signalling molecules below a toxic threshold and therefore avoid excessive amplification of the defence response leading to cell death.

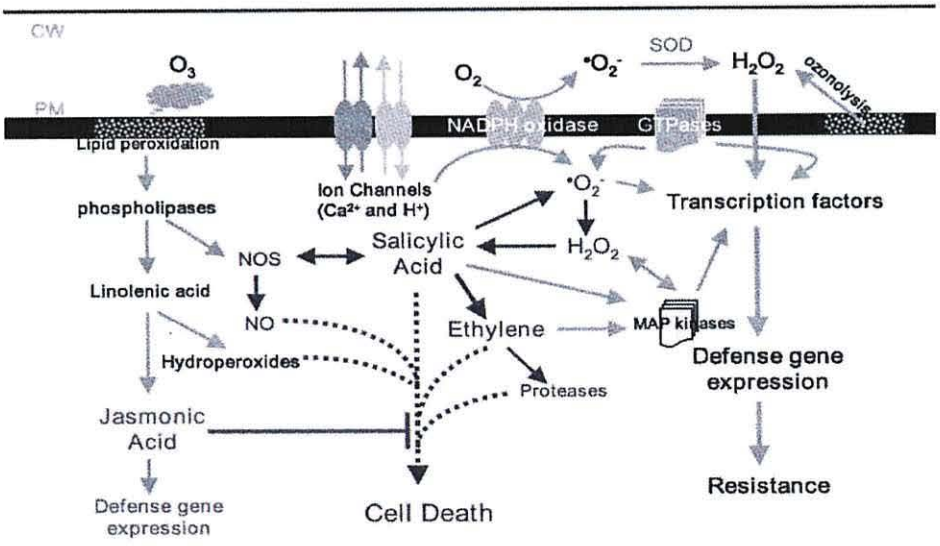

Figure 1.6 – A hypothetical model illustrating the complexity of the roles of salicylic acid-, ethylene- and jasmonic acid-signalling pathways in modulating O_3-induced cell death. CW, cell wall; PM, plasma membrane (Rao and Davies (2001)).

ROS are also produced in response to other stressors including wounding, pathogen attack, drought, high and low temperatures and high UV-B (Orvar *et al* 1997, Tamaoki *et al* 2003). Pre-treatment by wounding or jasmonate application has been shown to reduce the effects of subsequent ozone exposure in tobacco suggesting that prior activation of defence mechanisms involving similar signalling pathways provides some protection against ozone exposure (Orvar *et al* 1997). However, several studies have suggested that different defence pathways may be activated in response to different stressors. In birch, while a similar accumulation of H_2O_2 was observed in response to ozone, wounding and pathogen attack, the wounding induced gene expression differed from that induced by ozone and pathogen attack (Pellinen *et al* 2002). Comparison of gene expression patterns in *Arabidopsis* for 205 genes, identified as being up- or down-regulated by more than three-fold under ozone exposure, showed the greatest similarity with those induced by UV-B stress and less so with other stressors including drought and high and low temperature (Tamaoki *et al* 2004).

The majority of gene expression studies have involved responses to acute ozone exposure and these patterns may differ under chronic exposure. However, up-regulation of genes involved in signal transduction, defence and cell death, including various senescence associated genes (SAGs), and down-regulation of many photosynthesis involved genes were observed in trembling aspen under chronic ozone exposure (Gupta *et al* 2005) which corresponds with the up-regulation of SAGs and down-regulation of photosynthetic genes in *Arabidopsis* under acute exposure (Miller *et al* 1999). This indicates the activation of similar pathways under both forms of exposure, although the timing and magnitude of response are likely to differ. Responses to induced increases in ROS differ among stressors and both among and within species in relation to ozone exposure and although similar defence mechanisms and signalling pathways are involved, genetic variation underlying the extent of activation and expression of defence genes may play a significant role in the occurrence of differential ozone sensitivity both within and between species.

1.2.3 Vegetation response

Vegetation responses to ozone exposure have been most widely studied in crops (e.g. Fiscus *et al* 2005, Soja *et al* 2000, Ewert and Pleijel 1999, Ewert *et al* 1999, Bender *et al* 1999, Krupa *et al* 1995, Vandermeiren *et al* 2005) and forest trees (e.g. Bortier *et al* 2001, Braun *et al* 2007, Coleman *et al* 1995, Gerosa *et al* 2003, Inclan *et al* 1999, Matyssek *et al* 1995, Pell *et al* 1999, Uddling *et al* 2004, Zhang *et al* 2001). Since Davison and Barnes (1998) highlighted the need for greater determination of ozone sensitivity in semi-natural vegetation, as many species are expected to exhibit greater sensitivity than crop species, the implications of increased ozone concentrations for semi-natural species have received greater attention (e.g. Bassin *et al* 2007, Bungener *et al* 1999a, 1999b, Franzaring *et al* 2000, Hayes *et al* 2006, Power and Ashmore 2002, Ramo *et al* 2006a, Volk *et al* 2006).

The responses of vegetation to ozone exposure are highly variable and attempts to generalise among taxa by family, morphological and ecological characteristics and phenological traits such as growth strategy have been largely unsuccessful (Timonen *et al* 2004, Hayes *et al* 2007, Pleijel and Danielsson 1997). However, recent studies have suggested that Ellenberg Indicator values for light and salinity may provide the best prediction of plant responses (Hayes *et al* 2007, Jones *et al* 2007). Ozone responses in terms of foliar injury, photosynthesis, respiration, growth, resource allocation, reproductive output and intraspecific variation are discussed.

1.2.3.1 Foliar injury

Ozone induced foliar injury may occur as 'ozone-specific' chlorotic or necrotic stipple, flecking or mottling (Figure 1.5), while non-specific symptoms include chlorosis, necrosis and red pigmentation which may all be forms of premature senescence (Bergmann *et al* 1999, Bungener *et al* 1999b, Nebel and Fuhrer 1994, Reiling and Davison 1992a). Ozone-specific foliar injury is thought to result from programmed cell death events (Rao *et al* 2000, Koch *et al* 2000) while non-specific symptoms may result from the induction of various senescence associated genes and the acceleration of natural leaf senescence (Gupta *et al* 2005, Miller *et al* 1999).

The occurrence of visible foliar injury is a common response to ozone exposure and has been widely used to assess species ozone sensitivity and resistance (Bergmann *et al* 1999, Bermejo *et al* 2003, Hayes *et al* 2006, Nebel and Fuhrer 1994). However, the biological and ecological consequences of foliar injury, especially premature senescence, are uncertain as it does not necessarily confer reductions in growth or yield and some species exhibit reduced growth without the development of visible foliar injury (Davison and Barnes 1998, Power and Ashmore 2002, Inclan *et al* 1999, Pleijel and Danielsson 1997). One general consideration is that foliar injury reduces the green leaf area, which may inhibit photosynthesis (Vandermeiren *et al* 2005), and as plant growth is dependant on photosynthesis any inhibition should be translated into adverse effects on growth and yield (Runneckles and Krupa 1994).

1.2.3.2 Photosynthesis

Reductions in photosynthetic rate under elevated ozone exposure has been observed in a wide range of species including crops (Balaguer *et al* 1995, Fiscus *et al* 2005, Pasqualini *et al* 2002, Vandermeiren *et al* 2002, 2005), woody plants including trees (Dizengremel 2001, Novak *et al* 2005, Zeuthen *et al* 1997) and semi-natural vegetation species (Reiling and Davison 1994, van de Staaij *et al* 1997, Zheng *et al* 2000). Reductions in Rubsico quantity and quality have also been observed (Riikonen *et al* 2005, Wiese and Pell 1997, Yamaji *et al* 2003) and a loss of Rubisco was found to be the primary factor responsible for decreased CO_2 assimilation in *Plantago major* (Zheng et al 2002). Although such reductions are considered common responses they are not necessarily consistent among or within species. Pell *et al* (1999) found reductions in photosynthetic rate and Rubisco concentration in Black cherry and hybrid poplar but not in Sugar maple or Northern red oak while sensitive populations of *Plantago major* exhibited greater reductions in net CO_2 uptake than resistant populations (Reiling and Davison 1995).

The timing and magnitude of ozone induced reductions in photosynthesis were found to correspond to the onset of foliar injury in woody plant species (Novak *et al* 2005) and premature senescence in potato (Vandermeiren *et al* 2005) while a lack of response was also observed in asymptomatic leaves in tobacco (Wiese and Pell 1997). Photosynthetic responses also vary with leaf age, leaf position and developmental stage. While newly

expanded leaves of soybean were not found to exhibit any significant reduction in photosynthesis (Morgan *et al* 2004, Bernacchi *et al* 2006), a decline in photosynthetic capacity under ozone exposure occurred with increasing leaf age and leaf formation during flowering (Morgan *et al* 2004). A lack of effect on newly expanded leaves may relate to a compensatory photosynthesis response as observed in hybrid poplar where elevated photosynthetic rates occurred in young leaves, although this effect was only observed under low nutrient conditions when levels of premature senescence were highest (Bielenberg *et al* 2001). This variation highlights the importance of considering leaf age in the assessment of photosynthesis response to ozone exposure, especially where senescence is enhanced primarily in older leaves, and the interaction of such responses with other environmental conditions such as nutrient availability.

1.2.3.3 Plant growth

Plant growth responses to elevated ozone vary greatly among species (e.g. Bungener *et al* 1999a, Franzaring *et al* 2000, Hayes *et al* 2006, Inclan *et al* 1999, Reiling and Davison 1992a, Rudorff *et al* 1996). Bungener *et al* (1999a) found above ground biomass reduction, stimulation and no response in grassland species and similar variation in response has been observed in wetland species (Franzaring *et al* 2000), fen and fen-meadow species (Power and Ashmore 2002), and upland grassland species (Hayes *et al* 2006). Reductions in growth have been associated with the development of foliar injury in some species although this relationship is not consistent (Hayes *et al* 2006, Reiling and Davison 1992a, Power and Ashmore 2002). In some species, above ground biomass is maintained despite foliar injury as observed in Hayes *et al* (2006) where only half of the species exhibiting some form of foliar injury showed accompanying biomass reductions. Growth reductions without any development of foliar injury have also been reported in species including *Armeria maritime* (Hayes *et al* 2006) and most notably *Plantago major* (Reiling and Davison 1992a) which would be considered ozone resistant based on foliar injury alone (Nebel and Fuhrer 1994). However, enhanced senescence has been observed in some populations of *P. major* (Pearson *et al* 1996, Reiling and Davison 1992b). This highlights that the relationship between foliar injury and growth response is inconsistent even within species, and that some variation may result from the definition of foliar injury.

Various attempts have been made to explain the observed interspecific variation in above ground biomass responses. Differences in inherent growth rate and species growth strategy under Grime's C-S-R model were suggested and although stress tolerators exhibited lower biomass and growth rates, no significant trend in ozone response dependant on growth strategy was found (Bungener *et al* 1999a, Pleijel and Danielsson 1997). Timonen *et al* (2004) suggested a general trend for greater sensitivity in forbs than grass species although there were no clear trends among plant families. Legumes are often thought to be among the most sensitive species whether exposed in monocultures (Warwick and Taylor 1995, Nebel and Fuhrer 1994) or communities (Volk *et al* 2006). However, Ramo *et al* (2006a) found greater sensitivity in non-leguminous herbs and grasses compared to legumes grown in a seven species community although this difference may be attributable to the different legume species exposed, in particular *Trifolium medium*, which may be one of the less sensitive clover species (Ramo *et al* 2006a). Hayes *et al* (2007b), in a meta-analysis of 83 semi-natural vegetation species, found approximately 43% of species exhibited a change in above ground biomass of more than 10%, with reduction responses most common (33%) and stimulation responses less so (10%), and identified Legumes (*Fabaceae*) and plants with a therophyte lifeform as the most sensitive. However, high levels of within family variation in the *Fabaceae* and an under-representation of species in many families, Raunkiaer lifeforms and habitat types were highlighted as limitations to such generalisations.

A major limitation to the attempted generalisations, and assessment of growth response, is that the majority of studies have concentrated on ozone response of above ground biomass components. This is largely due to the inherent difficulties associated with determination of root biomass especially in long-term exposure studies (Grantz *et al* 2006). Reductions in root growth under elevated ozone have been reported in crops (Balaguer *et al* 1995, Ollerenshaw *et al* 1999), trees (Karlsson *et al* 2003, Woo and Hinkley 2005) and a variety of semi-natural vegetation species (Franzaring *et al* 2000, Reiling and Davison 1992a, 1992b, Power and Ashmore 2002). Several studies have suggested that root reductions are often greater than shoot reductions (Balaguer *et al* 1995, Power and Ashmore 2002, Ollerenshaw *et al* 1999) although instances of root growth maintenance and stimulation have been reported (Reiling and Davison 1992a, Yamaji *et al* 2003). Hayes *et al* (2006) found reduced above ground biomass after

overwintering in three species which had not exhibited any growth response during ozone exposure and suggested this was due to reduced resource allocation to root growth.

Grantz *et al* (2006), in a meta-analysis on combined data for 60 species exposed to both acute and chronic ozone exposures, found significant reductions in the root:shoot allocation coefficient (*k*) in 50 % of species analysed. Shifts in resource allocation from root to shoot growth may explain the maintenance or stimulation of shoot growth observed in some species (e.g. Balaguer *et al* 1995) and may result from greater investment in repair and defence mechanisms in the shoots. Yamaji *et al* (2003) found stimulation of root and shoot growth in birch clones accompanied by variation in the root to shoot biomass ratio (RSR) response among clones with reductions in RSR related to greater investment in high-cost antioxidants. Other species such as *Molinia caerlea* have been found to show stimulated growth of both biomass components and even exhibit a greater resource allocation to root growth (Franzaring *et al* 2000). Reiling and Davison (1992a) found some of the biggest shifts in the allometric root to shoot coefficient (*k*) exhibited by grass species with increases observed in *Arrhenatherum elatius*, *Brachypodium pinnatum*, *Bromus erectus,* and *Lolium perenne*, equating to four of the five species with significant increases in *k*, while decreases occurred in *Desmazeria rigida*, *Koeleria macrantha*, *Poa annua*, three of the nine species with significant decreases in *k*. Interestingly these shifts in resource allocation were only associated with significant changes in total growth rate in *Koeleria macrantha* and *Poa annua* which had significant reductions under ozone exposure, and highlights that shifts in resource allocation may occur without substantial effects on total biomass production.

Plant growth responses vary greatly among species with regards to root and shoot biomass production and resource allocation to these biomass components and this variation most likely results from differences in plant physiology and the inherent biochemical defence mechanisms. Shifts in resource allocation may be incredibly important in relation to plant longevity and competitive ability and the long-term effects of ozone exposure within communities and ecosystems. Additional factors determining growth responses include plant age and development stage in relation to the timing of ozone exposure. In *Plantago major*, ozone exposure has been shown to have the

greatest effects on growth rate during the seedling stage (Lyons and Barnes 1998, Reiling and Davison 1992b), while effects on *k* were transient especially when allocation to inflorescence production was considered (Reiling and Davison 1992b). It is therefore important to consider not only the effects of ozone on vegetative growth but also on reproduction, resource allocation to reproductive structures and the responses observed in vegetative growth in relation to phenological stage.

1.2.3.4 Reproduction

Ozone exposure has been found to decrease yield in crop species including wheat, corn (Rudorff *et al* 1996), oilseed rape (Ollerenshaw *et al* 1999), soybean (Morgan *et al* 2006) and potato (Vandermeiren *et al* 2005). In crops, reductions in seed or tuber quality may be of more concern than total yield as this determines the nutritive and economic value of many crops. Ollerenshaw *et al* (1999) found a reduction in flowering branches in oilseed rape was partially compensated by an increase in seeds per pod however seed quality and total yield were reduced. The effect of ozone exposure on tuber quality in potato was complex, with reduced tuber size accompanied by a beneficial decrease in the reducing sugar content but a detrimental increase in nitrogen concentration thought to be due reduced carbohydrate allocation (Vandermeiren *et al* 2005).

In semi-natural vegetation species ozone effects on reproduction include delayed flowering (Taylor *et al* 2002, Ramo *et al* 2007) and reductions in the number of flowers or flower spikes (Reiling and Davison 1992b, Taylor *et al* 2002, van de Staaij *et al* 1997), flower weight (Franzaring *et al* 2000, van de Staaij *et al* 1997) and seed or fruit production (Ramo *et al* 2007). As with other aspects of plant response to ozone exposure, effects on reproduction may depend on the timing of ozone exposure. Reproductive development was found to be particularly sensitive during the early stages of flowering in *Plantago major* (Lyons and Barnes 1998) while the development of foliar injury was greatest during the reproductive stage in *Centaurea jacea* (Bassin *et al* 2004). Assessment of reproductive responses are further complicated by intraspecific variation, as demonstrated in *P. major* where two populations of exhibited increases in reproductive effort at the expense of root growth while one population reduced allocation to seed production with a lesser effect on root growth (Pearson *et al* 1996).

It is still unclear whether impacts on reproduction are primarily due to direct effects of ozone on reproductive organs, although there is some evidence that ozone may suppress pollen germination (Roshchina and Mel'nikova 2001), or indirect effects involving vegetative growth and shifts in resource allocation (Black *et al* 2000). Furthermore, the effects of ozone on seed or fruit quality in semi-natural species may be more difficult to determine than for crop species and the implications of reductions in weight and quality of seeds or fruits for seedling development and survival have rarely been addressed (Black *et al* 2000). Such aspects may be of critical importance in determining species persistence and competitive ability in natural ecosystems.

1.2.3.5 Intraspecific variation in ozone response

Sensitive and resistant genotypes have been identified in various species including tobacco (e.g. Wellburn and Wellburn 1996, Degl'Innocenti *et al* 2002), *Arabidopsis* (e.g. Tamaoki *et al* 2003), *Trifolim repens* (e.g. D'Haese *et al* 2005, Kollner and Krause 2002, Nebel and Fuhrer 1994) and hybrid poplar (e.g. Koch *et al* 1998, 2000). Although these provide useful model systems for studying the mechanisms conferring ozone sensitivity, assessment of variation in response among natural populations may provide a better insight into the wider ecological impacts of elevated ozone. The majority of studies on semi-natural vegetation have concentrated on interspecific variation in ozone response. However, the body of evidence regarding intraspecific variation is increasing and a summary of studies assessing variation in ozone response among natural populations is given in Table 1.1.

The type of exposure method varied among studies from elevated ozone exposures in controlled environment chambers (CEC) and open-top chambers (OTC) to common-garden experiments and field observations under ambient air (AA) conditions. The majority of studies summarised in Table 1.1 exposed plants in CEC, which allow greater control over the growth environment but are not representative of natural growth conditions. OTCs allow exposure under more natural climatic conditions with ozone added to either charcoal-filtered (CF), which removes the potential effects of interactions with other air pollutants, or non-filtered (NF) air which allows comparison with AA to determine chamber effects. The OTC environment tends to result in increased temperatures, reduced humidity, reductions in photosynthetically active

Table 1.1 – Summary of studies investigating intraspecific variation in response to ozone exposure in natural populations.

Species	Populations		Age	Ozone treatment	Ozone response		Ref.
	n	provenance			Parameter	Main effects	
Centaurea jacea	5	Europe: Hungary, Italy, Norway, Slovenia, Switzerland	S	AA plots; AOT40 19.3 ppm.h, 8-9 weeks, AOT40 8.4 ppm.h, 10-11 weeks,	visible injury	Slovenia, Switzerland and Italy, earliest development; Slovenia and Switzerland, greatest; Hungary and Norway, least. Related to phonological stage: Slovenia, earliest reproduction; Hungary and Norway, remained in rosette stage.	Bassin *et al* (2004)
					genetic variation	Switzerland, highest; Norway, lowest;	
	4	Finland: Korppoo, Sälinkää. Switzerland: Neuchâtel S and R.	S	AA plots; OTC; NF, NF+ 1.5*AA, 9 hd^{-1}, 3 months	visible injury	Finnish, less and later development of light brown flecks. Swiss, greater purple pigmentation.	Ramo *et al* (2006b)
					leaf number	decrease	
					leaf dwt	Sälinkää, reduction; Neuchâtel R, lowest in NF	
					total dwt	no effect; greater in OTC's than AA	
					g_s	decrease	
					SPAD	No effect; lower in Swiss	
					Flowering	Neuchâtel R, delayed onset; Neuchâtel S, earlier onset. Finnish flowered later than Swiss.	
Elymus glaucus	2	USA: Southern and Northern California	S	CEC; 1-25 ppb or 30-215 ppb, episodic increase to peak at ~ 14:00 and decrease in afternoon and evening, varied daily peak, 16 weeks	leaf area and root mass	slightly reduced, greater in Southern	Yoshida *et al* (2001)
					tiller number	slightly increased, greater in Southern	
					arbuscular mycorrhizal colonization	reduction, greater in Northern	
Fragaria vesca	2	Finland: Kainuu (Eastern Central), Etelä-Häme (Southern)	S	AA plots; OTC; NF, NF+ 30 ppb (June) and 20 ppb (July) and 10 ppb (August), 8 hd^{-1}, 5 dw^{-1}, 2 seasons	Biomass	Year 1: No effect on runner biomass; Kainuu, reduction in root and shoot; Etelä-Häme, increase in root and shoot.	Manninen *et al* (2003)
					Foliar characteristics	Kainuu and Etelä-Häme, increased stomatal density, no effect on hair density or structure of epicuticular waxes	
					Reproduction	Kainuu, increase; Etelä-Häme, no effect.	
					Visible injury	Kainuu, increase in red, yellow and brown leaves	

A_t, transplant from field; F, *in situ* observation of plants in field; S, seedling; CEC, controlled environment chamber; OTC, open-top chamber; AA, ambient air; CF, charcoal filtered air; NF, non-filtered air; A, net CO_2 uptake; g_s, stomatal conductance; F_R, chlorophyll fluorescence; K, allometric root/shoot growth coefficient; AG, above ground; BG, below ground; RGR, relative growth rate; AWF, apoplastic washing fluid; RLE, residual leaf extracts;

Table 1.1 – Continued.

Species	Populations		Age	Ozone treatment	Ozone responses		Ref.
	n	provenance			Parameter	Main effects	
Plantago major	3	UK: ISP 1985 and 1991, Totley 1985 and 1991, Scaftworth 1988 & 1991	S	CEC; CF, CF+ 70 ppb, 7 hd^{-1}, 2 weeks CEC; CF, CF+ 70 ppb, 7 hd^{-1}, 5 d	RGR	Reduction; ISP and Totley, ozone resistance higher in 1991 compared to 1985 collections.	Davison & Reiling (1995)
					g_s	ISP and Scaftworth, reduction under ozone, reductions greater in Scaftworth, reductions greater in 1991 collections; Totley, reduction with change in effect over time in 1985 collection, constant reduction response in 1991 collection.	
					Dark respiration	1985 & 1988 collections, slight reduction; 1991 collections, significant increase.	
	22	Europe: Austria, Belgium, Finland, Germany, Greece, Italy, Spain, Switzerland, UK. America: USA	S	CEC; CF, CF+ 70 ppb, 7 hd^{-1}, 14 d	RGR	10 populations, significant reductions; Spanish, most sensitive with 13 % reduction; Greek, most resistant with no effect; Population ozone resistance related to recent ozone climate history.	Lyons *et al* (1997)
					K	No significant differences in response among populations; 3 populations, significant increase.	
	3	UK: Lullington, Great Dunn Fell (GDF), Bush	S	CEC; CF, O_{35} (35 ppb, 24 hd^{-1}), O_{70} (70ppb, 7 hd^{-1}), $O_{CF/70}$ (70 ppb, 7 hd^{-1} for 3 dw^{-1}), $O_{35/70}$ (35 ppb with 70 ppb 7 hd^{-1} for 3 dw^{-1}), 10 weeks	senescence leaf area	GDF, significant increase; Lullington and Bush, no effect Lullington, reduction in O_{70} and $O_{35/70}$; GDF and Bush, reduction in all treatments, greatest in O_{70}	Pearson *et al* (1996)
					shoot, root and total dwt	Lullington, largest reduction in $O_{35/70}$, effects greatest on root growth; GDF and Bush, reductions in O_{35}, O_{70} and $O_{35/70}$, increased root growth in $O_{CF/70}$, growth reductions not primarily due to root reductions	
					reproduction	Lullington, increased reproduction; GDF, increased reproductive effort in 35 ppb treatments, reductions in O_{70} and $O_{CF/70}$, greatest reduction in $O_{CF/70}$; Bush, significant reductions in all treatments, greatest reduction in $O_{CF/70}$	
					g_s	reduced in all treatments; Lullington and GDF, greatest reductions in O_{70} and $O_{35/70}$; Bush, smallest reductions, greatest effects in $O_{35/70}$.	

A_t, transplant from field; F, *in situ* observation of plants in field; S, seedling; CEC, controlled environment chamber; OTC, open-top chamber; AA, ambient air; CF, charcoal filtered air; NF, non-filtered air; *A*, net CO_2 uptake; g_s, stomatal conductance; F_R, chlorophyll fluorescence; *K*, allometric root/shoot growth coefficient; AG, above ground; BG, below ground; RGR, relative growth rate; AWF, apoplastic washing fluid; RLE, residual leaf extracts;

Table 1.1 – Continued.

Species	Populations		Age	Ozone treatment	Ozone responses		Ref.
	n	provenance			Parameter	Main effects	
Plantago major	2	Derbyshire, UK Athens, Greece	S	CEC; CF, CF+ 70 ppb, 7 hd^{-1}, 2 weeks	Visible injury	None.	Reiling & Davison (1992a)
					RGR	UK, decrease; Athens, no change.	
					K	UK, decrease; Athens, no change.	
				CEC; CF, CF+ 70 ppb, 7 hd^{-1}, 5 d	F_R	UK, initial increase then decrease; Athens, increase.	
	28	UK: England, Scotland, Wales	S	CEC; CF, CF+ 70 ppb, 7 hd^{-1}, 2 weeks	RGR	22 populations, significant reduction. 2 populations, no change. Scotland and N England populations more sensitive; S England populations more resistant. O_3 resistance correlated with ambient ozone climate	Reiling & Davison (1992c)
					K	4 populations, reduction.	
	3	UK: Lullington, ISP 1985, ISP 1991,	S	CEC; AA; AA+ rise 2.5 h, max 120 ppb for 3h, dec 2.5 h, 8 hd^{-1}; 2 d	Ethene	ISP 1985, increase; ISP 1991, decrease; Lullington, no change.	Wellburn & Wellburn (1995)
					Other antoxidants	Response varied between populations for different antioxidants.	
	9	Europe: Belgium, Germany, Greece, Spain, Switzerland, UK, America: USA	S	CEC; CF, CF+ 15 ppb to max 75 ppb between 12:00-16:00, 14 d	Ascorbate	Magnitude of effect varied among populations: 28% increase to 69% decrease in AWF, 54% increase to 77% decrease in RLE. Significant relationship between ascorbate in O_3 treated plants and population ozone resistance, not for CF plants.	Zheng *et al* (2000)

A_t, transplant from field; F, *in situ* observation of plants in field; S, seedling; CEC, controlled environment chamber; OTC, open-top chamber; AA, ambient air; CF, charcoal filtered air; NF, non-filtered air; *A*, net CO_2 uptake; g_s, stomatal conductance; F_R, chlorophyll fluorescence; *K*, allometric root/shoot growth coefficient; AG, above ground; BG, below ground; RGR, relative growth rate; AWF, apoplastic washing fluid; RLE, residual leaf extracts;

Table 1.1 – Continued.

Species	Populations		Age	Ozone treatment	Ozone responses		Ref.
	n	provenance			Parameter	Main effects	
Plantago major	2 (6)	UK: Lullington, Lullington S and R; Bush, Bush S and R.	S	CEC; CF,CF+ 70 ppb, 7 hd^{-1}, 14 d. artificial selection over four generations	RGR	Lullington, small reduction; Lullington R, no effect or change; Lullington S, significant reductions for generations 2 to 4. Bush, significant reduction; Bush R, significant reduction in generations 1 and 2 but not 3 and 4, rapid increase in resistance; Bush S, no effect or change. Artificial selection for sensitivity in a resistant population and resistance in a sensitive population.	Whitfield *et al* (1997)
				CEC; CF,CF+ 70 ppb, 7 hd^{-1}, 12 weeks	*A* (6 weeks)	Lullington and Lullington R, no effect; Lullington S, slight reduction. Bush and Bush R, no effect; Bush S, 13 % reduction.	
					g_s (6 weeks)	Lullington and Lullington R, no effect; Lullington S, slight reduction. Bush populations, reduction.	
					chlorophyll content (8 weeks)	Lullington S, increased chlorophyll; Lullington S and all Bush, increased carotenoids.	
					biomass (12 weeks)	Lullington and Lullington R, no effect; Lullington S, 30% reduction, equal root and shoot reductions. Bush populations, reduction; Bush S, greatest reduction of 42%.	
					Reproduction (12 weeks)	Lullington populations, no effect on total seed production; Lullington R, increased scape number, no change in seed number. Bush and Bush R, no effect; Bush S, 33% reduction in seed number, less seeds per scape.	

A_t, transplant from field; F, *in situ* observation of plants in field; S, seedling; CEC, controlled environment chamber; OTC, open-top chamber; AA, ambient air; CF, charcoal filtered air; NF, non-filtered air; *A*, net CO_2 uptake; g_s, stomatal conductance; F_R, chlorophyll fluorescence; *K*, allometric root/shoot growth coefficient; AG, above ground; BG, below ground; RGR, relative growth rate; AWF, apoplastic washing fluid; RLE, residual leaf extracts;

Table 1.1 – Continued.

Species	Populations		Age	Ozone treatment	Ozone responses		Ref.
	n	provenance			Parameter	Main effects	
Plantago major	2 (4)	UK (artificially selected): Lullington S and R, Bush S and R,	S	CEC; CF,CF+ 70 ppb, 7 hd^{-1}, 10-11 weeks Low (LN) and high (HN) nutrient treatments	biomass and root:shoot ratio (4 weeks)	Lullington R, no effect; Lullington S, biomass reduction with LN. Bush R, no effect in HN, reduction in R:S in LN; Bush S, biomass reduction in LN and HN.	Whitfield *et al* (1998)
					biomass (10-11 weeks)	Lullington R, no effect; Lullington S, reduced root growth in HN, reduced root and shoot growth in LN. Bush R, reduction in shoot growth in HN, reduction in root growth in LN and HN; Bush S, reduction in shoot and root growth in LN and HN.	
					Reproduction	Lullington S and R, no effect. Bush R, slight increase in reproductive effort; Bush S, slight decrease in reproductive effort, reduction in seeds per plant in LN.	
					A	Lullington S and R, no effect. Bush S and R, reduced in LN.	
					g_s	Lullington R, reduction in LN; Lullington S, reduced in LN and HN. Bush S and R, reduced in LN.	
					Ethylene	Lullinton S and R, no effect. Bush S, no effect; Bush R, reduction in LN.	
					Nitrogen content	Lullington R, reduction in HN, slight increase in LN; Lullinton S, no effect. Bush R, no effect; Bush S, reduction in HN.	
					Phosphorous content	Lullington S and R, no effect. Bush R, increase in LN; Bush S, reduction in HN.	
	5	UK: ISP 1985, 1994; Totley 1985, 1994; Aston Hill 1991, 1992; Lullington 1990, 1991, 1992; Scaftworth 1988, 1994.	S	Ozone resistance sourced from Reiling & Davison (1992c), Davison & Reiling (1995) and Lyons *et al* (1997): CEC; CF, CF+ 70 ppb, 7 hd^{-1}, 14 d	Genetic variation and ozone resistance (RGR)	ISP and Totley, significant increase in ozone resistance over time. ISP, Scaftworth and Aston Hill, genotype in later collections represent subset of earlier collections. Suggest change in ozone resistance due to selection of genotypes already present in populations.	Wolff *et al* (2000)

A_t, transplant from field; F, *in situ* observation of plants in field; S, seedling; CEC, controlled environment chamber; OTC, open-top chamber; AA, ambient air; CF, charcoal filtered air; NF, non-filtered air; *A*, net CO_2 uptake; g_s, stomatal conductance; F_R, chlorophyll fluorescence; *K*, allometric root/shoot growth coefficient; AG, above ground; BG, below ground; RGR, relative growth rate; AWF, apoplastic washing fluid; RLE, residual leaf extracts;

Table 1.1 – Continued.

Species	Populations		Age	Ozone treatment	Ozone responses		Ref.
	n	provenance			Parameter	Main effects	
Quercus coccifera	2	Spain: rock and garrigue sites	S	OTC; CF, NF+ 40 ppb, 9 hd^{-1}, 5 dw^{-1}, 2 seasons	Plant height, RGR_{height}, AG, BG & total biomass	No significant differences between treatments or populations.	Elvira *et al* (2004)
					A	Rock, no change; Garrigue, reduction with ozone	
					g_s	Rock, no change; Garrigue, reduction with ozone	
Rudbeckia laciniata	3	USA: Great Smoky Mountain National Park (GSMP)	F	Ambient	visible injury	Increase in injury with site elevation and higher ambient ozone exposure.	Chappelka *et al* (2003)
	2	USA: GSMP, Clingmans Dome, Purchase Knob,	F	Ambient	visible injury and genetic diversity	Clingmans Dome, different amounts of injury despite low genetic variation; Purchase Knob, greater genetic variation.	Davison *et al* (2003)
Spartina alterniflora	3	USA: New Jersey, South Carolina, Georgia	A_t	OTC; CF, CF+ 80 ppb, 8 hd^{-1}, 10 weeks	visible injury	increase in chlorotic and necrotic leaves	Taylor *et al* (2002)
					Shoot and leaf number	New Jersey and Georgia, reduction; South Carolina, no effect.	
					leaf biomass	no effect	
					Root and rhizome biomass	New Jersey, reduction in root growth; Georgia, reduction in root and rhizome growth; South Carolina, no effect.	
					Photosynthetic rate	New Jersey and Georgia, reduction; South Carolina, no effect.	
					Flowering	Only New Jersey, delayed onset and reduction in number of flower spikes	
Trifolium repens	6	Switzerland	A_t	CEC; 0 ppb overnight with max 30 ppb or 100 ppb at mid-day, 6 d	Visible injury	Low altitude population from frequently cut pasture least sensitive; Intermediate altitude population from wet Trisetion grassland most sensitive. Trend for lower sensitivity in plants from dry sites.	Nebel & Fuhrer (1994)

A_t, transplant from field; F, *in situ* observation of plants in field; S, seedling; CEC, controlled environment chamber; OTC, open-top chamber; AA, ambient air; CF, charcoal filtered air; NF, non-filtered air; A, net CO_2 uptake; g_s, stomatal conductance; F_R, chlorophyll fluorescence; K, allometric root/shoot growth coefficient; AG, above ground; BG, below ground; RGR, relative growth rate; AWF, apoplastic washing fluid; RLE, residual leaf extracts;

Table 1.1 – Continued.

Species	Populations		Age	Ozone treatment	Ozone responses		Ref.
	n	provenance			Parameter	Main effects	
Trifolium pratense	6	Switzerland	A_t	CEC; 0 ppb overnight with max 30 ppb or 100 ppb at mid-day, 6 d	Visible injury	low altitude population from frequently cut pasture least sensitive; intermediate altitude population from wet Trisetion grassland most sensitive. Trend for lower sensitivity in plants from dry sites. Trend for decrease in sensitivity with increasing altitude.	Nebel & Fuhrer (1994)

A_t, transplant from field; F, *in situ* observation of plants in field; S, seedling; CEC, controlled environment chamber; OTC, open-top chamber; AA, ambient air; CF, charcoal filtered air; NF, non-filtered air; A, net CO_2 uptake; g_s, stomatal conductance; F_R, chlorophyll fluorescence; K, allometric root/shoot growth coefficient; AG, above ground; BG, below ground; RGR, relative growth rate; AWF, apoplastic washing fluid; RLE, residual leaf extracts;

radiation (PAR) and more constant air flow than under ambient conditions (Pleijel and Danielsson 1997, Nussbaum and Fuhrer 2000). Chamber effects have been recorded in a number of species (e.g. Pleijel and Danielsson 1997, Danielsson *et al* 1999) and were assessed in two studies of inter-population response (Table 1.1). Lower shoot and root biomass, runner number and runner biomass, and increased numbers of senesced leaves were observed in OTC compared to AA in *Fragaria vesca*, although differential population response was unclear (Manninen *et al* 2003). Ramo *et al* (2006b) found that OTCs had a greater stimulation effect on total biomass in Finnish compared to Swiss *Centaurea jacea* populations. Alterations in microclimate during OTC fumigation may affect plant ozone responses but it is unclear whether relative response among populations would be affected.

Alternatives to chamber experiments include exposure using free-air fumigation systems, although few studies have employed such methods (Kolliker *et al* 2008, Volk *et al* 2006) and none have considered inter-population ozone responses, or the comparison of population responses to ambient ozone conditions. *In situ* observations of variable levels of foliar injury among populations of *Rudbeckia laciniata* were related to differences in ambient ozone exposures and site elevation (Chappelka *et al* 2003). However, differing environmental conditions among sites, especially light levels, were considered more important than ozone climate in determining this variation (Davison *et al* 2003). Bassin *et al* (2004) compared foliar injury among European populations of *Centaurea jacea* under AA in a common-garden experiment. Although the populations exhibited differential responses to the same ozone exposure, levels of foliar injury were related to phenological stage which was in turn related to geographic origin. Differences in climatic conditions such as day length between the experimental site and site of origin may explain the variation in plant development and therefore ozone response. These studies highlight the difficulty in comparing responses in the field and among populations from different ambient climates. However, population comparison by whichever method of ozone exposure necessitates some standardisation of other environmental conditions, but comparisons among populations from broadly similar ambient climates is preferable.

The majority of studies have considered plant responses to chronic ozone exposure with ozone concentrations of less than 80 ppb while the duration of exposure ranging from

two days to two seasons (Table 1.1). Where concentrations exceeded 80 ppb, exposure duration was usually for less than seven days and involved the determination of foliar injury (Nebel and Fuhrer 1994) or antioxidant responses (Wellburn and Wellburn 1996). An exception was for *Elymus glaucus* where exposure was for 16 weeks, although daily peaks varied throughout the exposure period from 30 ppb a maximum of 215 ppb (Yoshida *et al* 2001). The majority of inter-population studies have also been performed on plants germinated from seeds collected in the field with exposure during the seedling stage. Growth stage during exposure may be an important factor influencing plant response (Bassin *et al* 2004, Lyons and Barnes 1998, Reiling and Davison 1992b) and results from *Plantago major* have suggested that although ozone resistance determined during short-term exposures may relate to responses under longer term exposure, this relationship varies among populations (Whitfield *et al* 1997).

Studies varied greatly in the criteria used to assess differential sensitivity including foliar injury, growth, reproductive output, stomatal conductance and antioxidant production. The most widely studied species is *Plantago major*, in which intraspecific variation was considered in half of the studies summarised in Table 1.1, and which has been studied in relation to the largest number of populations and range of assessment criteria. The potential for sensitivity and intraspecific variation in this species was highlighted by Reiling and Davison (1992a) and confirmed in numerous subsequent studies (e.g. Reiling and Davison 1992c, Lyons *et al* 1997, Pearson *et al* 1996). Differential population ozone resistance in this species has been related to the ozone climate of source populations in relation to geographic variation (Reiling and Davison 1992c, Lyons *et al* 1997) and temporal variation (Davison and Reiling 1995) and to levels of ascorbate in ozone treated plants (Zheng *et al* 2000).

In other species intraspecific responses have most often been studied in terms of foliar injury (Table 1.1), those in which growth has been assessed include two grasses, *Elymus glaucus* (Yoshida *et al* 2001) and *Spartina alterniflora* (Taylor *et al* 2002), two herbaceous species *Centaurea jacea* (Ramo *et al* 2006b) and *Fragaria vesca* (Manninen *et al* 2003), and one tree species *Quercus coccifera* (Elvira *et al* 2004). Growth responses in these species have been determined based on longer term exposures than the majority of studies in *Plantago major*, with a minimum of 10 weeks duration. Differential population growth responses were observed in the herbaceous and grass

species but not in *Q. coccifera*, however some represented subtle differences in population response (Manninen *et al* 2003, Yoshida *et al* 2001). The majority of studies considering intraspecific variation among natural populations demonstrate the complexity of responses to ozone exposure, especially with regard to differential sensitivity among assessment criteria, and highlight the difficulties associated with determining ozone sensitivity not only at the species level but also for interspecific comparison.

1.2.4 Potential for evolution of ozone resistance

Ozone and oxidative stress are natural components of life within the biosphere and as such current terrestrial vegetation has developed mechanisms to combat oxidative stress over evolutionary time (Runneckles and Krupa 1994). The wide variation in plant responses to ozone exposure both among and within species, including variation in biochemical defence mechanisms and gene expression (e.g. Tamaoki *et al* 2003, Koch *et al* 1998, 2000), suggests the potential for the evolution of ozone resistance as tropospheric ozone levels rise (Davison and Barnes 1998). Whether the evolution of resistance ozone resistance occurs will depend largely on the strength of the selective pressure imposed, the traits affected by exposure and the heritability of such traits (Bradshaw and McNeilly 1991).

Evolution of resistance has been demonstrated in relation to other pollutants, especially metal contamination (Bradshaw and McNeilly 1991). Reciprocal transplant experiments have large found differences in metal tolerance among adjacent populations at mine sites (e.g. Antonovics 1972) and more recent studies have demonstrated greatly reduced genetic diversity and genetic isolation of metal tolerant populations (e.g. Mengoni *et al* 2001). Demonstration of the evolution of resistance to air pollutants is more difficult as unlike metal contamination, air pollutants rarely show sharp boundaries in pollution levels. Early studies on resistance to sulphur dioxide (SO_2) pollution were reviewed by Bell *et al* (1991). Differential resistance in *Lolium perenne* was related to different levels of SO_2 pollution with less variation in response in populations from polluted sites suggesting a reduction in genetic variation and selection for resistance. Temporal

variation in SO_2 resistance was also found in *L. perenne*, *Poa pratensis* and *Phleum pratense* in relation to point source pollution levels where resistance increased rapidly over five years and then decreased and this variation corresponded to the temporal changes in SO_2 pollution. Resistance to acute injury, such as visible foliar injury, was also found to occur more rapidly and in more species than resistance to chronic injury, such as growth effects.

In relation to the evolution of ozone resistance, although various studies have demonstrated variation in response among populations (see Table 1.1), the majority have not provided strong evidence for evolution (Bell *et al* 1991, Barnes *et al* 1999). Bell *et al* (1991) highlighted two methods of study by which evolution for resistance may be assessed, firstly by the comparison of populations from different geographic origins, and secondly by comparison of samples collected at different points in time with corresponding changes in ambient pollution levels. The majority of inter-population response studies have involved the first method, which may often be confounded by variation in other environmental conditions among source populations. Additional methods include the demonstration of heritability in traits linked to ozone resistance and the direct assessment of genetic variation in relation to ozone resistance.

The majority of evidence regarding the evolution of ozone resistance has been based on studies of *Plantago major* (Table 1.1) and variation in ozone resistance has been studied using all of the methods described above. Variation among populations from different geographical origins has been demonstrated in numerous studies (e.g. Reiling and Davison 1992a, Lyons *et al* 1997, Pearson *et al* 1996) and ozone resistance based on changes in relative growth rate was related to variation in ambient ozone climate in Reiling and Davison (1992c). Ozone resistance was also found to increase over a short time period of six years in several populations corresponding to increases in ambient ozone levels (Davison and Reiling 1995). Further evidence for the evolution in ozone resistance in this species was provided by artificial selection experiments. Whitfield *et al* (1997) demonstrated the heritability of ozone resistance in two populations differing in sensitivity by selection for both sensitivity and resistance in both populations. Increased sensitivity in a resistant population and increased resistance in a sensitive population were produced, although it was not possible to increase either resistance or sensitivity in the respective populations. Finally, temporal shifts in ozone resistance in

several populations were related to shifts in genetic composition (Wolff *et al* 2000). The more resistant populations sampled at a later date were found to represent a subset of the original population suggesting that the temporal shifts in resistance were the result of *in situ* selection on genotypes present within the populations rather than gene flow from another more resistant population (Wolff *et al* 2000). These studies demonstrate the potential for the evolution of resistance over relatively short time periods, however the predominantly selfing nature of this species may promote such events and similar responses may require greater selection pressure and longer time periods in outcrossing species.

The existence of biochemical mechanisms to combat oxidative stress and the natural variation in both genotype and ozone responses within species provides the basis for the evolution of ozone resistance to occur as tropospheric ozone levels continue to rise. The strength of the selection pressure will determine whether ozone resistance evolves within populations and species or whether extinction events will occur. It is likely that resistance will evolve rather than species going extinct but this will also depend on the interaction with other factors, such as competition, which may lead to exclusion of species which are less ozone tolerant (Davison and Barnes 1998).

1.2.5 Ozone Critical Levels for Vegetation

Exposure indices are used to determine the relationship between ozone concentrations and observed response for implementation in air pollution polices (Fuhrer *et al* 1997). The current critical levels employed in Europe are based on a cumulative concentration approach using the AOT (accumulated over a threshold) index. The threshold for the AOT-based critical levels for crops, trees and semi-natural vegetation has been set at 40 ppb (i.e. AOT40) based on empirical data from open top chamber experiments for impacts of growth or yield (Fuhrer *et al* 1997). AOT40 is defined as the sum of the differences between the hourly mean ozone concentration (in ppb) and 40 ppb when the concentration exceeds 40 ppb during daylight hours, accumulated over a stated time period (LRTAP Convention, 2007). This exposure index integrates exposure over time and places more weight on higher concentrations but it does not consider the dominance

of either the frequency or intensity of levels exceeding 40 ppb at a certain site (Tuovinen 2000), and can be very sensitive to fluctuations in ozone concentrations or the threshold level used (Sofiev and Tuovinen 2001). Another disadvantage to this exposure index is that ozone concentrations above the canopy rarely reflect the actual ozone flux into plants and therefore AOT40 does not provide a very accurate exposure-response relationship. A cumulative stomatal flux approach is required for the accurate estimation of ozone impacts in the field (Fuhrer *et al* 1997) and flux-based methods have been included in the LRTAP Convention Mapping Manual since 2004. These take into account the effect of temperature, humidity, light, soil water content, phenology and ozone concentration on stomatal aperture and ozone flux (LRTAP Convention, 2007). A further improvement would be the inclusion of detoxification mechanisms into flux-based models although quantification of such mechanisms can be difficult (Musselman *et al* 2006). The majority of flux-based models have been developed for agricultural crops and forest trees, and the AOT index with a threshold value of 40 ppb is still recommended for semi-natural and natural vegetation (LRTAP Convention, 2007).

1.3 Genetic diversity

Studies of genetic diversity can provide insights into the ecological and evolutionary processes that influence the genetic composition of species and populations underlying variation in phenotypic traits. Plant species provide an interesting model for the study of genetic variation and phenotypic variation in relation to environmental heterogeneity due to the sedentary nature of adult individuals and the differential modes of genome transmission and dispersal. The development of molecular markers has enabled the direct estimation of genetic diversity and differentiation among populations and species. Plant genomes and the application of several molecular marker systems for the assessment of genetic diversity are discussed in this section.

1.3.1 Plant genomes

Plants contain three genomes, the nuclear and mitochondrial genomes which are ubiquitous among eukaryotic organisms and the plastid (chloroplast) genome which is specific to plants. These genomes exhibit different modes of inheritance and levels of sequence conservation among species while rates of mutation and levels of variation differ not only between genomes but among regions and genes within each genome.

The nuclear genome is biparentally inherited through both pollen and seeds receiving one copy of each chromosome from each parent and undergoes recombination during sexual reproduction (Ouborg *et al* 1999). Recombination plays a significant role in the generation and maintenance of nucleotide variation in the nuclear genome. Rates of recombination vary among loci depending upon chromosomal position, lower rates occur near centromeres and telomeres, and the structural features of the sequence (Zhang and Hewitt 2003). The nuclear genome is the fastest evolving of the three plant genomes exhibiting the highest rates of nucleotide substitution (Wolfe *et al* 1987, Gaut 1998, Muse 2000). Nucleotide substitution rates vary among genomic regions depending on function. Protein-coding regions are associated with lower substitution rates as mutations occurring at the first and second codon positions often lead to amino acid replacement and altered protein function. Non-coding regions, including introns and intergenic spacers, are not constrained by coding function and usually exhibit higher nucleotide substitution rates (Zhang and Hewitt 2003). The biparental mode of inheritance, recombination and high substitution rates result in the nuclear genome being the most variable of the three plant genomes.

The two plant organelle genomes are usually uniparentally inherited and are homoplastic with all copies within an individual identical (Ouborg *et al* 1999). The mitochondrial genome is usually maternally inherited through seeds while chloroplast genome inheritance varies with maternal inheritance in angiosperm species and paternal inheritance in gymnosperm species (Ouborg *et al* 1999). Although uniparental inheritance is the norm, partial bi-parental inheritance or paternal leakage of chloroplast DNA have been observed (Chat *et al* 2003, McCauley *et al* 2007, Hansen *et al* 2007).

The plant mitochondrial genome is characterised by a multipartite organisation consisting of master circles and subgenomic circles and extensive levels of intramolecular recombination (Sugiyama *et al* 2005). The structure, organisation and size of the mitochondrial genome varies greatly among plant lineages due to varying amounts of gene transfer and genome reorganisation throughout evolutionary history (Mackenzie and McIntosh 1999, Adams *et al* 2002, Sugiyama *et al* 2005). Despite the high level of structural variation the mitochondrial genome exhibits the lowest rates of nucleotide substitution and is considered the least variable of the three genomes (Wolfe *et al* 1987, Gaut 1998, Muse 2000). However, rates of nucleotide substitution vary among genomic regions with higher rates of nucleotide substation in non-coding compared to coding-regions and some mitochondrial genes have been found to exhibit rates of evolution comparable to those of chloroplast genes (Larouche *et al* 1997, Muse *et al* 2000).

The chloroplast genome, in comparison to the mitochondrial genome, is relatively conserved in size, structure and organisation among species (Provan *et al* 2001) although gene transfer and reorganisations have occurred (Stegemann *et al* 2003, Katayama and Ogihara 1996, Michelangeli *et al* 2003).The chloroplast genome usually consists of two inverted repeats (IR) separated by one large single-copy region (LSC) and one small single-copy region (SSC) (Shaw *et al* 2007). Rates of nucleotide substitution are higher in the single-copy regions than the inverted repeats regions and in non-coding intron and intergenic spacer regions compared to protein coding-regions (Wolfe *et al* 1987, Gaut 1998). In addition, within the single-copy regions intergenic spacers exhibit more variation than introns (Shaw *et al* 2007). Despite the conserved nature of the chloroplast genome it has the highest rates of evolution of the two organelles at approximately half that of the nuclear genome (Wolfe *et al* 1987, Gaut 1998, Muse 2000) and exhibits extensive intraspecific and interspecific variation (Provan *et al* 2001, Petit *et al* 2003).

1.3.2 Polyploidy

Polyploidy can be defined as the presence of three or more chromosome sets in the nuclear genome of an organism (Soltis *et al* 2003) such as triploidy (3x), tetraploidy (4x) and hexaploidy (6x). Polyploidization is the process of genome duplication resulting from genomic doubling, gametic non-reduction or polyspermy (Otto and Whitton 2000). Polyploids are typically separated into two main categories: autopolyploids which originate from intraspecific genome duplications and have multiple copies of the same genome, and allopolyploids which originate from genome duplication events during interspecific hybridization and contain two or more distinct genomes (Lawton-Rauh 2003, Soltis *et al* 2003). Amounts of polyploidization are thought to relate to lineage age (Meyers and Levin 2006), mating system and life history (Otto and Whitton 2000). Extensive and rapid intra- and inter-genomic reorganisation can occur following polyploidization and this may lead to 'diploidization' where the genome is no longer structured as a polyploid (Soltis and Soltis 1999). Diploidization is thought to have occurred following ancient polyploid events with the re-establishment of disomic segregation and differentiation or loss obscuring gene copies (Otto and Whitton 2000). Current theories suggest that polyploidization is a recurrent phenomenon and that most organisms have polyploidy in their evolutionary history even if they now function as diploids (Soltis *et al* 2003).

Polyploidy is widespread throughout the plant kingdom with 70 % of angiosperms and 95 % of ferns thought to be polyploids (Otto and Whitton 2000, Soltis *et al* 2003). Although polyploidy has occurred in animal species including fish, reptiles, amphibians and insects it is much rarer and this may relate to lower levels of interspecific hybridization among animal species than plant species (Otto and Whitton 2000). Polyploidy is a special type of mutation and is thought to play an important role in plant speciation (Soltis *et al* 2003, Meyers and Levin 2006) with an estimated 2 % to 4 % of angiosperm speciation events associated with polyploidization (Otto and Whitton 2000). Many polyploid species exhibit broader ecological tolerances and more generalist life histories than their diploid progenitors and polyploidy is often thought to confer increased heterozygosity and a greater potential for adaptive evolution (Otto and Whitton 2000).

1.3.3 Molecular markers

Molecular marker systems commonly applied to assess genetic variation include allozymes, Amplified Fragment Length Polymorphisms (AFLP), Random Amplified Polymorphic DNA (RAPD), Intersimple Sequence Repeats (ISSR), Restriction Fragment Length Polymorphisms (RFLP) and microsatellites (Parker *et al* 1998). The utility of different marker systems depends upon the specific nature of the marker, technical issues such as development time and cost, the genomes and organisms of interest and the ecological and evolutionary processes under consideration. The molecular markers and techniques detailed in this section are those which were employed in this study.

1.3.3.1 Polymerase Chain Reaction (PCR)

The development of the Polymerase Chain Reaction (PCR) (Saiki *et al* 1985, Mullis and Faloona 1987, Saiki *et al* 1988) revolutionised the field of molecular biology allowing rapid *in vitro* DNA amplification from small quantities of template DNA. The PCR technique involves heat denaturation of double-stranded DNA, annealing of primers to sites flanking the DNA region to be amplified, and primer extension where DNA polymerase mediates synthesis of DNA strands complementary to the region between the primer sites. These three steps are cycled repeatedly to synthesize multiple copies of the target sequence for subsequent analysis (Avise 1994). This technique is fundamental to the majority of modern molecular marker systems used to assess genetic variation.

1.3.3.2 DNA sequencing

Sequencing involves the direct determination of DNA nucleotide composition. Polymorphism results from variation in the nucleotide base present at a specific site and types of polymorphism detected include nucleotide substitutions and insertion or deletion events. DNA sequencing provides the most complete information regarding genetic variation and is a powerful tool for the reconstruction of phylogenetic relationships, usually based on organelle sequences (Catalan *et al* 1997, Gugerli *et al* 2001, Mathews *et al* 2000, Yamane and Kawahara 2005), and for species identification

(Greenstone *et al* 2005, Jarman *et al* 2004). Sequence data has also provided insights into nucleotide substitution rates (Cho *et al* 2004, Laroche *et al* 1997, Shaw *et al* 2007), pseudogenes (Bailey *et al* 2003), genome reorganisations (Sugiyama *et al* 2005), polyploidization (Valcarcel *et al* 2003) and genetic diversity and structuring (Ishikawa *et al* 2001). In addition to the use of DNA sequencing to directly assess genetic variation sequencing is also employed in the development and characterisation of other molecular marker systems such as microsatellites (Zane *et al* 2002, Cai *et al* 2003, Cordeiro *et al* 2000) and PCR-RFLP (Horning *et al* 2006) for the subsequent indirect assessment of genetic variation.

Traditional Sanger sequencing techniques involve PCR amplification of short stretches of DNA using the dideoxy chain termination method (Avise 1994) and although advances in automated sequencing continue to reduce costs, Sanger sequencing still remains relatively costly (Hutchinson 2007). New 'massively parallel' sequencing (MPS) techniques such as 'pyrosequencing', which is based on real-time detection of DNA synthesis monitored by bioluminescence (Ahmadian *et al* 2006), have dramatically reduced the time and costs associated with sequence generation. However, sequence length and accuracy are currently limiting factors in the application of such techniques (Rogers and Venter 2005, Hutchinson 2007). Single-molecule approaches such as 'nanopore sequencing' and 'fluorescence-based enzymatic sequencing' may further reduce costs and may provide the best chance of obtaining the 'thousand dollar genome' (Bayley 2006). Continual improvements in MPS and single-molecule sequencing techniques are likely to result in the replacement of Sanger sequencing in the near future. Direct sequencing may eventually replace the current methods for marker development and even the use of other molecular markers although parallel improvements in bioinformatics are also required (Pop and Salzberg 2008). In relation to this study sequencing was employed for the development of microsatellites and PCR-RFLP markers using Sanger sequencing (see Chapters 5 and 6).

1.3.3.3 AFLP fingerprinting

Amplified Fragment Length Polymorphism (AFLP) is a restriction-based universal fingerprinting technique for the assessment of genetic diversity across the nuclear genome of any organism (Vos *et al* 1995). This technique involves the fragmentation of

genomic DNA by restriction digest with two restriction endonucleases, one rare-cutter with a 6 bp recognition sequence and a frequent-cutter with a 4 bp recognition sequence, usually *Eco*RI and *Mse*I respectively. Enzyme specific adapter molecules, consisting of a core sequence and an enzyme specific sequence, are ligated to the DNA fragments to provide universal primer sites for PCR amplification. A subset of the DNA fragments are PCR amplified using primers complementary to the adapter molecules, amplifying fragments cut by both restriction endonucleases. The addition of selective nucleotides to the primers, usually 3 nucleotides per primer, further reduces the subset of DNA fragments amplified based on the nucleotide composition of the fragmented DNA. The number of selective nucleotides required to produce a readable AFLP profile depends on the nuclear DNA content and genome size of the organism under study (Vos *et al* 1995, Fay *et al* 2005). One primer is radioactively or fluorescently end-labelled to allow the detection of fragments through electrophoretic separation on acrylamide gels. Polymorphism results from variation in the nucleotide sequence of the fragmented DNA in relation to the primer annealing sites for the selective nucleotides and is detected by the presence or absence of amplified fragments of the same size. A detailed AFLP protocol is described in chapter 6.

AFLP has emerged as a rapid cost effective alternative to many other molecular marker systems such as microsatellites and RFLP (Mueller and Wolfenbanger 1999) and has become one of the primary marker systems applied in plant species as it requires no prior sequence knowledge and produces a large number of reproducible loci providing a genome wide scan of genetic variation (Vos *et al* 1995, Bensch and Akesson 2005, Jones *et al* 1997). AFLP markers have been widely applied for the study of species hybridization (Beismann *et al* 1997, Fady *et al* 2003, O'Hanlon *et al* 1999, Salmon *et al* 2005), parentage analysis (Gerber *et al* 2000), cultivar identification (Loh *et al* 2000), population differentiation (Cardoso *et al* 2000, Kreivi *et al* 2005), spatial genetic structure (Jump *et al* 2007, Wu and Campbell 2005), QTL mapping (Guo *et al* 2003, Price *et al* 2000) and interspecific and intraspecific phylogenetics (Despres *et al* 2002, Despres *et al* 2003, Kebede *et al* 2007, Lambertini *et al* 2006, Martinez-Ortega *et al* 2004, Pimentel *et al* 2007).

Despite the universal nature and wide range of applications there are several limitations to the AFLP technique. AFLPs are dominant markers, being scored as either present or

absent, and as such provide no information regarding heterozygosity. This can be a limiting factor in studies where the estimation of allele frequencies is required for the inference of population genetic parameters (Mueller and Wolfenbanger 1999). However, the low per-locus information content may be compensated by the high number of loci produced and wide genome coverage, while advances in statistical analysis have enabled the estimation of allele frequencies from dominant data under the assumption of Hardy-Weinberg equilibrium (Bonin *et al* 2007). AFLPs are assumed to provide a genome wide scan of genetic variation however some studies have suggested that loci may be clustered within genomic regions. A mapping study in rye demonstrated clustering of *Eco*RI/*Mse*I AFLP loci around the centromere on several chromosomes (Saal and Wricke 2002). The use of different restriction endonuclease combinations may overcome this problem, although other combinations may exhibit similar clustering depending on the genomic distribution of the recognition sites. Some AFLP loci have also been shown to include repetitive DNA sequences. The presence of few strongly amplifying fragments is thought to relate to high copy numbers of repetitive DNA sequences especially in organisms with large genomes (Fay *et al* 2005) and may represent retrotransposons in some cases (Reamon-Buttner *et al* 1999). AFLP markers, as with any fragment length based marker system, require the assumption of homology of co-migrating fragments. The occurrence of size homoplasy, where co-migrating fragments of the same size are non-homologous representing different genome regions, violates this assumption and can result in incorrect inferences. However, size homoplasy at AFLP loci is most prevalent in higher taxonomic level comparisons while it is limited among closely related taxa (O'Hanlon and Peakall 2000) and the assumption of homology should be applicable for comparisons at the congeneric or conspecific level (Bonin *et al* 2007).

In the case of polyploid species, AFLP markers have been widely applied for the study of genetic diversity, hybridization, polyploidization events and phylogeography in polyploid species and ploidy-variable complexes (Anamthawat-Jonsson *et al* 1999, Lambertini *et al* 2006, Paun *et al* 2006, Perez-Collazos and Catalan 2006, Salmon *et al* 2005, Pimentel *et al* 2007). The inability of dominant AFLP markers to detect heterozygosity is less of a disadvantage than for diploids as even co-dominant markers such as microsatellites may not provide full genotypic data in polyploids (Esselink *et al* 2004). As such both co-dominant and dominant markers may provide a similar level of

information in polyploids. In addition, ploidy level does not appear to affect the quality of AFLP profiles although additional selective nucleotides may be required to generate readable profiles (Fay *et al* 2005).

The analysis of polyploid AFLP data may be more restricted than for data from diploid species although few studies have actually addressed this issue. Traditional band-based analysis methods concerned with the similarity of AFLP banding patterns are applicable regardless of ploidy level, while the more recently developed allele frequency-based methods may not be appropriate. In diploids, AFLP data scored as fragment presence (1) or absence (0) can represent four allelic configurations at a locus: homozygote for absence (00), homozygote for presence (11) and heterozygote for presence (01 or 10). Allele frequency-based analysis methods utilise the known frequency of the absence homozygote to estimate allele frequencies under the assumption of Hardy-Weinberg equilibrium (HWE):

$$p^2 + 2pq + q^2 = 1$$

where p is the presence allele and q is the absence allele in a diploid organism. In tetraploids, the estimation of allele frequencies becomes more complex as 16 allele configurations are possible: homozygote for absence (0000) and homozygote for presence (1111) and 14 possible heterozygotes with multiple allele configurations for each of one presence allele (1000, 0100, 0010,0001), two presence alleles (1100, 1010, 0101, 0110, 1001, 0011) and three presence alleles (1110, 1101, 1011, 0111). The HWE equation has been expanded for a tetraploid model:

$$p^4 + 4p^3q + 6p^2q^2 + 4pq^3 + q^4 = 1$$

where p is the presence allele and q is the absence allele in a tetraploid organism. However, currently this has not been incorporated into analysis methods to allow the estimation of allele frequencies from polyploid AFLP data. The available allele frequency-based AFLP analysis methods require the assumption of HWE, including diploidy, to estimate allele frequencies from dominant AFLP data (Bonin *et al* 2007) and traditional band-based methods remain the most suitable for analysis of polyploid data.

1.3.3.4 PCR-RFLP

Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) is a restriction-based single locus technique combining PCR amplification and restriction digestion. This technique involves PCR amplification of a specific genomic region and subsequent fragmentation of the amplification product with restriction endonucleases. Polymorphism results from presence or absence of enzyme specific recognition sequences due to variation in the nucleotide sequence producing variable fragment numbers and length variants. Polymorphism is detected by electrophoretic separation on agarose gels or on acylamide gels with radioactive or fluorescent end-labelling of one primer.

PCR-RFLP markers can provide a rapid and cost effective method for assessing interspecific and intraspecific genetic variation especially when specific or universal primers are available for PCR amplification (Dumolin-Lapegue *et al* 1997, Horning *et al* 2006, Provan *et al* 2001). Development of PCR-RFLP can be achieved by screening amplified regions for variation with multiple restriction endonucleases or by using prior sequence knowledge to assess variable nucleotide sites and determine suitable restriction endonucleases for segregation at these sites. Although the level of polymorphism detected by PCR-RFLP is lower than can be obtained by sequence analysis of amplified fragments, PCR-RFLP is relatively inexpensive in comparison allowing the screening of more individuals (Horning *et al* 2006).

In plant species this technique has been primarily applied for assessing genetic variation in the chloroplast genome due its relatively conserved nature, the presence of intraspecific variation and availability of universal primers (Dumolin-Lapegue *et al* 1997, Weising and Gardner 1999, Saltonstall 2001, Shaw *et al* 2007). Chloroplast PCR-RFLP markers have been employed in the study of invasive species (Baumel *et al* 2001), species identification (Brunner *et al* 2001), population genetic diversity and differentiation (McCauley 1998, Dutech *et al* 2000, Yannic *et al* 2004), glacial refugia (Petit *et al* 2003) phylogeography (Fineshi *et al* 2005, Ge *et al* 2005, Palme *et al* 2003) and chloroplast inheritance modes (McCauley *et al* 2007).

1.3.3.5 Microsatellites

Microsatellites, also known as simple sequence repeats (SSRs), short tandem repeats (STRs) and variable number tandem repeats (VNTRs) are PCR-based single locus markers consisting of short DNA sequences containing tandemly repeated units of 1 to 6 nucleotide base pairs. Microsatellite profiling involves PCR amplification of individual loci using locus specific primers with one primer radioactively or fluorescently end-labelled to allow the detection of fragments through electrophoretic separation on acrylamide gels. Polymorphism results from variation in the repeat unit number generating different length variants at the locus and is detected by the presence of amplified fragments of different size.

Microsatellites are abundant throughout nuclear eukaryotic genomes, are codominant markers inherited in a Mendelian fashion and are characterised by relatively high mutation rates and high levels of per-locus polymorphism (Li *et al* 2002, Zane *et al* 2002, Selkoe and Toonen 2006). These properties have led to microsatellites becoming one of the most popular molecular markers for studies of intraspecific nuclear genetic variation especially in animal species and have been employed in studies including parentage assignment (Gerber *et al* 2000, Saltonstall 2003), dispersal and population structuring (Heuertz *et al* 2003, Lian *et al* 2003), genome mapping and quantitative trait analysis (Andaya and Mackill 2003, Shen *et al* 2003).

The majority of microsatellite loci are thought to evolve neutrally (Schlotterer 2000). However, the assumption of neutrality is questionable as these loci occur in both non-coding and coding regions where they are thought to play a role in coding and regulatory processes and may contribute to quantitative trait variation (Kashi and Soller 1999, Zhang and Hewitt 2003). Rates of mutation at nuclear microsatellite loci are high, ranging from 10^{-6} to 10^{-2} mutations per locus per meiosis, and vary both among species and among loci within the same genome (Zhang and Hewitt 2003). Polymorphism occurs by mutation resulting in the addition or deletion of repeat units. The two mutational mechanisms responsible generating this polymorphism are DNA replication slippage and recombination. DNA replication slippage occurs due to slip-strand mispairing errors during DNA replication and is thought to be the primary mechanism generating microsatellite length variation (Schlotterer 2000, Li *et al* 2002). Several

mutation models for DNA replication slippage have been proposed including the Stepwise Mutation Model (SMM) where mutation occurs by the loss or gain of single repeat units (Kimura and Ohta 1978), and the Infinite Allele Model (IAM) where mutation can result in the loss or gain of multiple repeat units (Kimura and Crow 1964). These models are simplifications of the complex mutation processes involved in microsatellite evolution which are influenced by factors such as repeat number, repeat type, flanking sequence composition and efficiency of the mismatch-repair system (Schlotterer 2000). An additional factor is the occurrence of size homoplasy at microsatellite loci where fragments of identical size are not identical by descent. This can occur when fragments exhibiting the same repeat number are produced by convergent mutation (Estoup *et al* 2002) and when variation in the flanking sequence leads to fragments of identical size differing in repeat number (Grimaldi and CrouauRoy 1997).

A major limiting factor to the application of microsatellite markers is the often costly, time-consuming and inefficient process of marker development (Squirrell *et al* 2003, Zane *et al* 2002). Although cross-species amplification of homologous loci is possible (Fitzsimmons *et al* 1995, Rico *et al* 1996, Jones *et al* 2001), in plant species this is largely restricted to closely related taxa (Peakall *et al* 1998, Yamamoto *et al* 2002, Kolliker *et al* 2001, Jump *et al* 2002). A lack of previously developed markers in congeneric species often necessitates the isolation of microsatellites in new study species (Zane *et al* 2002). Numerous microsatellite isolation methods have been reported including traditional genomic library screening and various enrichment methods (reviewed in Zane *et al* 2002) and a selective hybridization enrichment technique is detailed in chapter 5. Enrichment methods are considered most appropriate for species with large genomes or relatively low microsatellite abundance such as plant species. Although such methods are more efficient, microsatellite isolation remains relatively costly and time-consuming (Squirrell *et al* 2003, Zane *et al* 2002) and may explain the slower rate of application of microsatellites in plant species compared to animals.

An additional factor limiting the application of microsatellites in plants is the prevalence of polyploid species. A major advantage of microsatellite markers compared to markers such as AFLP is co-dominance, which enables the determination of

heterozygotes and the precise estimation of allele frequencies for the inference of population genetic parameters (Mueller and Wolfenbanger 1999). In polyploids co-dominance may not provide such an advantage as although heterozygotes can be distinguished, full microsatellite genotypes may not be obtained unless copy number can be determined through either prior knowledge of segregation ratios or variation in band intensity (Obbard *et al* 2006, Esselink *et al* 2004, Becher *et al* 2000). In such cases both co-dominant and dominant markers may provide a similar level of information and universal fingerprinting techniques such as AFLP may be preferable as they do not require prior marker isolation. Recent developments for the determination of allele copy number such as the Microsatellite DNA Allele Counting – Peak Ratios (MAC-PR) method (Esselink *et al* 2004), and in the statistical analysis of partial genotype data such as the computer programs AUTOTET (Thrall and Young 2000) and TETRASAT (Markwith *et al* 2006), have increased the applicability of microsatellite markers for polyploid species although the use of such techniques is still limited (Landergott *et al* 2006, Ng *et al* 2004, Hochu *et al* 2006, Truong *et al* 2007, Markwith and Scanlon 2007, Markwith and Parker 2007).

In addition to the nuclear genome, microsatellite loci are also present in the haploid organelle genomes of plant species. Mononucleotide microsatellite repeats have been found in many regions of the chloroplast genome in all species for which comprehensive sequence data is available (Provan *et al* 2001) and some studies have also reported the presence of mononucleotide repeats in the mitochondrial genome (Nishizawa *et al* 2000, Mitton *et al* 2000, Sperisen *et al* 2001). Chloroplast microsatellites have been more widely studied due to the relatively conserved nature of the chloroplast genome among species compared with the mitochondrial genome which exhibits lower nucleotide substitution rates and extensive levels of intramolecular recombination (Provan *et al* 2001). Chloroplast microsatellites are typically short in length, usually consisting of fewer than 15 repeats (Provan *et al* 2001), with mutation rates of 10^{-5} which are lower than many nuclear microsatellites but higher than nucleotide substitution rates elsewhere in the chloroplast genome (Provan *et al* 1999). The relatively conserved nature of chloroplast sequences has facilitated the development of universal primers for chloroplast microsatellites (Weising and Gardner 1999, Provan *et al* 2004) and extensive intraspecific variation has been observed in many species (Provan *et al* 2001). The conservation of loci across species allowing cross-species

amplification and the presence of intraspecific variation have led to the wide application of these markers for the study of genetic diversity (Bryan *et al* 1999), population structuring (Heuertz *et al* 2001), dispersal (Lian *et al* 2003), metapopulation dynamics (Navascues *et al* 2006), chloroplast genome modes of inheritance (Cheng *et al* 2003, Morand-Prieur *et al* 2002), and inter- and intra-specific phylogeny (Gugerli *et al* 2001, Ishii and McCouch 2000) and phylogeography (Heuertz *et al* 2004).

1.4 Study species and sample sites

1.4.1 *Anthoxanthum odoratum* L.

Anthoxanthum odoratum is a morphologically highly variable, tufted, perennial grass species and the most widespread of the four perennial species within the *Anthoxanthum* L. *sensu lato* polyploid complex in Europe (Pimentel *et al* 2007). This tetraploid (2n = 4*x* = 20) species is a short-lived perennial, rarely exceeding 5 years (Antonovics 1972), wind pollinated predominantly outcrossing species (Wu and Jain 1980, Silvertown *et al* 2002) and reproducing primarily through seed production with vegetative propogation of minor importance (Antonovics 1972). The origin of this polyploid species has been suggested as autotetraploid (Felber 1987), however, allotetraploidy was also considered highly probable based on ecology, morphology and cytology (Hedberg 1990) and supported by phylogenetics (Pimental *et al* 2007). Previous studies have suggested local adaptation of this species within relatively short time scales over small geographic distances in response to fertilizer and liming treatments (Snaydon 1970, Snaydon and Davies 1972, Snaydon and Davies 1976, Davies and Snaydon 1976, Silvertown *et al* 2005) and zinc pollution (Antonovics 2006). In relation to ozone sensitivity *A. odoratum* has previously been classified as an intermediate species for ozone sensitivity (Nebel and Fuhrer 1994) and exhibits a fast response in terms of premature senescence (Hayes *et al* 2006). In addition, screening studies identified high levels of intraspecific variation in response to ozone exposure suggesting the potential for evolution of ozone resistance in this species (G. Mills, pers comm.).

1.4.2 Sample sites and collection

1.4.2.1 Sample sites

Four populations of *Anthoxanthum odoratum* were sampled from four sample sites across North Wales (Figure 1.7 a). Permission to sample was obtained for the sampling sites from the relevant authorities. Sample sites are illustrated in Figure 1.8 and details of each site are given in Table 1.2. The four study sites were chosen based on suitable semi-natural vegetation habitat unlikely to have undergone extensive management and to encompass a range of altitudes and topographies which should influence the natural ozone profiles received at the sites. Background ozone concentrations at these sites are predicted to range from 25 ppb to 40 ppb during the growing season (Coyle, pers. comm., Figure 1.7 b) with the highest concentrations at the highest altitude site, Cwm Idwal.

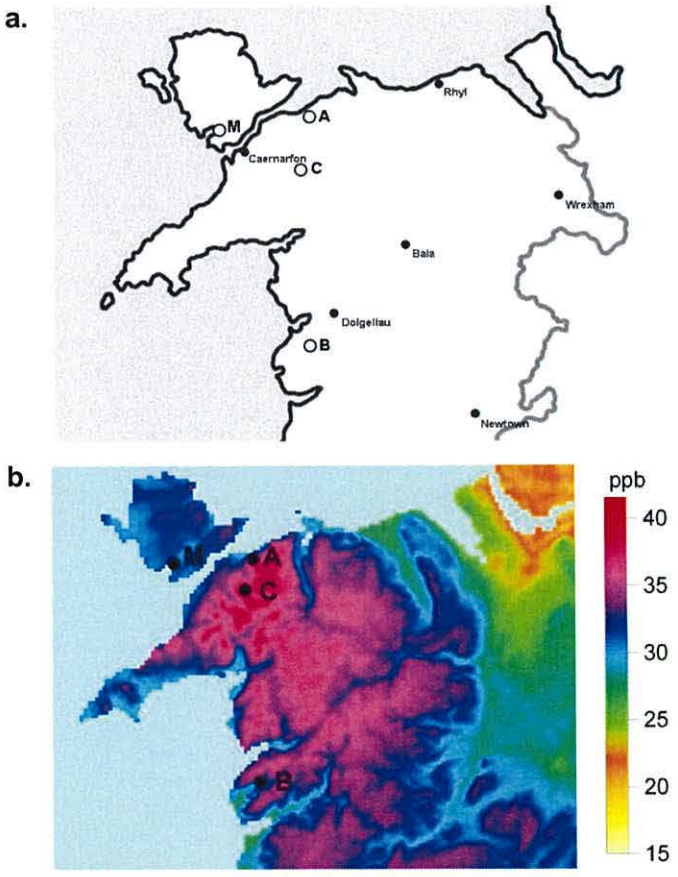

Figure 1.7 –Map of North Wales detailing a) sample sites and b) mean ozone concentrations from April to September 2003 (M. Coyle, pers comm.). Sample sites labelled by prefix: Aber (A), Birds Rock (B), Cwm Idwal (C) and Maltraeth (M).

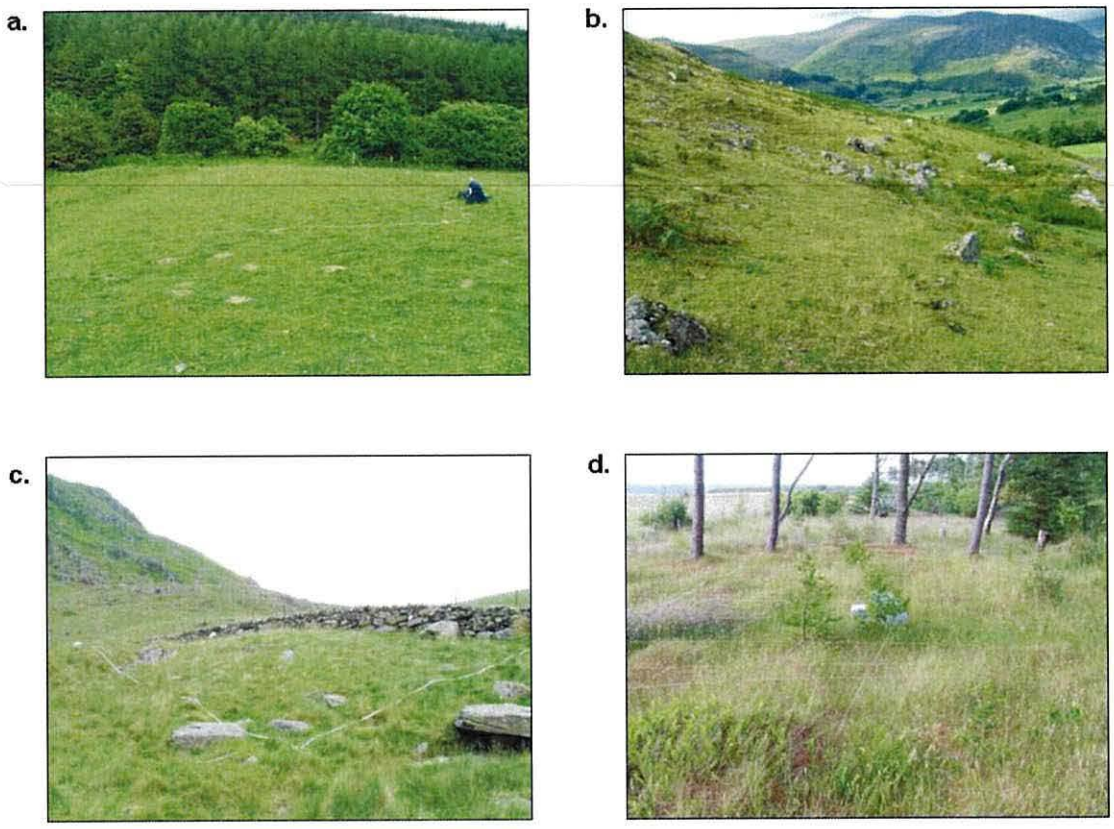

Figure 1.8 – Sample sites of the four North Wales *A.odoratum* populations a) Aber Valley, b) Cwm Idwal, c) Bird's Rock and d) Maltraeth.

Table 1.2 – Details of sample sites including grid reference, altitude, aspect, climatic conditions and habitat type. Authority from which sample permission was granted and date of sample collection are also provided.

Sample site	Grid reference	Altitude (m)	Aspect	Temperature range[†§]	Annual precipitation[‡§]	Habitat type	Grazing	Collection date
Aber Valley[a]	SH 666711	150	NW	0.1 – 17.9 (17.8)	1143	Old pasture, acid grassland	Sheep: Medium density	8 June 2004
Cwm Idwal[a]	SH 643599	380	SE	-1.4 – 16.6 (18.0)	1416	Open upland, acid grassland	none	9 June 2004
Maltraeth[b]	SH 411671	5	NW	2.2 – 18.5 (16.3)	915	Marginal woodland (Corsican & Scots pine), acid, partial shading	none	30 June 2004
Bird's Rock[c]	SH 649070	130	NE	1.0 – 18.1 (17.1)	1152	Open upland, acid grassland	Sheep: Medium density	17 July 2004

Sampling permission: [a], Countryside Council for Wales; [b]. Forestry Commission; [c], Snowdonia National Park Authority.
Climatic conditions: [†], annual temperature minimum and maximum (range) °C; [‡], precipitation in mm; [§], Data averaged for 1950-2000 obtained from WORLDCLIM version 1.4 and BIOCLIM at 1km^2 resolution in DIVA-GIS version 5.2.0.2.

1.4.2.1 Sample collection

Thirty individuals from each population were randomly sampled from within 1 m^2 quadrats on a 20 m by 10 m grid. Quadrats on the grid were numbered from 1 to 200 and chosen using randomly generated numbers. Samples were taken from as close to the centre of each quadrat as possible, labelled from 1 to 30 and stored in plastic collection bags. The position of each quadrat on the 20m by 10m grid from which each sample was taken was recorded. Samples were subsequently potted up into 11.5cm square pots in an ericaceous/sand mix compost consisting of 40 litres ericaceous compost and 25kg horticultural sharp sand. One tiller of *A. odoratum* from each sample was isolated to ensure only one individual was present, vegetatively propagated and maintained in a glasshouse at the Pen-y-Ffridd Research Station, Bangor. Stock plants were watered regularly, received half strength nutrient application on a monthly to bimonthly cycle ('phostrogen': PBI Home and Garden, UK) and were cut back to 7 cm each spring. Plants were treated with the commercial fungicides and pesticides Bavistan (Active Ingredient (AI): carbendazim, BASF), Rovral (AI: iprodione, BASF), Radar (AI: propiconazole, INTEG®), Dynamec® (AI: abamectin, MSD AGVET), Nemolt (AI: teflubenzuron, BASF), Provado® Ultimate Bug Killer Concentrate (AI: imidichloprid, Bayer Garden) and Nicotine shreds (AI: nicotine, Dow Agrosciences Limited) as required to prevent rust and mildew infection and thrip and aphid infestation.

1.5 Aims

The potential for adverse effects of tropospheric ozone on semi-natural vegetation species is of concern in the UK where concentrations in many areas, especially rural and upland areas, exceed 40 ppb on a regular basis. The predicted increase in tropospheric ozone concentrations over the next century has potential impacts not only on individual species but at the community and ecosystem level. An important factor governing the potential long-term effects on semi-natural vegetation habitats is the potential for species to adapt and the evolution of ozone resistance. The widespread nature of *A. odoratum* presents an ideal opportunity to study ozone sensitivity in populations which experience different ambient ozone profiles and may exhibit different responses due to

local adaptation. The highly variable nature of this species also suggests that genetic diversity may be high and may increase the potential for adaptation to environmental stresses. In addition, any adverse effects of tropospheric ozone exposure on this species have implications for a wide range of semi-natural communities due to the occurrence of *A. odoratum* in a wide range of grassland habitats.

The aims of this study were to:

1) Assess the ozone sensitivity of *A. odoratum* populations in relation to intraspecific variation and different scenarios for predicted future increases in ozone concentration.

2) Develop molecular marker systems in *A. odoratum* for the assessment of genetic variation.

3) Assess the genetic diversity and differentiation among *A. odoratum* populations underlying differential response to ozone exposure

Chapter 2: Intraspecific ozone sensitivity of *Anthoxanthum odoratum* in response to acute ozone exposure

2.1 Introduction

The effects of ozone exposure on semi-natural vegetation have received greater attention in recent years although only a small proportion of species have so far been assessed (Bassin *et al* 2007, Hayes *et al* 2007b). Species from semi-natural grassland habitats are of particular interest as such habitats constitute approximately 50 % of the land area in Europe and are important in terms of conservation and agricultural use (Bassin *et al* 2007, Ashmore *et al* 2007). Various attempts have been made to generalise responses among taxa for the prediction of sensitive species and communities (Timonen *et al* 2004, Hayes *et al* 2007b, Pleijel and Danielsson 1997). Several studies have suggested a general trend for greater ozone sensitivity in legumes compared to other forbs and grasses (Warwick and Taylor 1995, Volk *et al* 2006, Hayes *et al* 2007b, Timonen *et al* 2004). However, such generalisations are often based on a limited number of species and even the 'less sensitive' families or functional groups contain sensitive species (Hayes *et al* 2007b). Therefore, determination of ozone response of individual species is still necessary to increase the knowledge base for such generalisations.

The majority of studies on semi-natural vegetation have concentrated on interspecific comparisons of ozone sensitivity (Hayes *et al* 2006, Bergmann *et al* 1999, Bungenner *et al* 1999a, Bungenner *et al* 1999b, Franzaring *et al* 2000) and disregarded the potential differential response among individuals and populations of the same species which may result from adaptation to local environmental conditions, differences in detoxification mechanisms and genetic diversity (Davison and Barnes 1998). Intraspecific variation in response among or within populations has been observed in several species including *Plantago major* (Pearson *et al* 1996, Lyons *et al* 1997), *Trifolium repens* (Nebel and Fuhrer 1994), *Rudbeckia laciniata* (Davison *et al* 2003) and *Fragaria vesca* (Manninen *et al* 2003). The occurrence of intraspecific variation in ozone sensitivity highlights the

potential for the evolution of ozone resistance which may mitigate the potential adverse effects of elevated ozone levels for both individual species and communities (Davison and Barnes 1998).

Anthoxanthum odoratum L. is a perennial, tufted grass species widespread throughout the UK and Europe (Hubbard 1992, Pimentel *et al* 2007). This species has previously been classified as an intermediate species for ozone sensitivity (Nebel and Fuhrer 1994) and exhibits a fast response in terms of premature senescence (Hayes *et al* 2006). A preliminary screening study suggested that this species was highly variable in response to ozone exposure and a suitable candidate species for assessment of intraspecific variation (G. Mills, pers comm.). The aim of this study was to assess intraspecific variation in response to acute ozone exposure in terms of visible injury and plant growth among *A. odoratum* populations from the relatively small geographic area of North Wales, UK, which are thought to differ in relation to ambient ozone climate.

2.2 Methods

2.2.1 Plant material

Seven rametes per individual were vegetatively propagated in July 2006 from stock plants collected from four populations in North Wales in June-July 2004 (see Chapter 1, section 1.4 for details of sample collection). Plants were grown in an ericaceous-sand mix compost (ratio 3:2, litres:kg) at the Pen-y-Ffridd Research Station, Bangor. Rametes were established from single tillers of similar size in a heated glasshouse (day 18 °C and night 16 °C) with supplementary lighting. After three weeks rametes were transplanted into 1 litre 10 cm square pots, lined with perforated black plastic to contain root growth within the pot, and transferred to a sheltered outside position under ambient conditions. At four weeks ramete size was determined by tiller number. Seventy six individuals, comprising 16 to 20 per population, were selected on the basis of plant health and ramete size uniformity. Three rametes per individual were selected and one

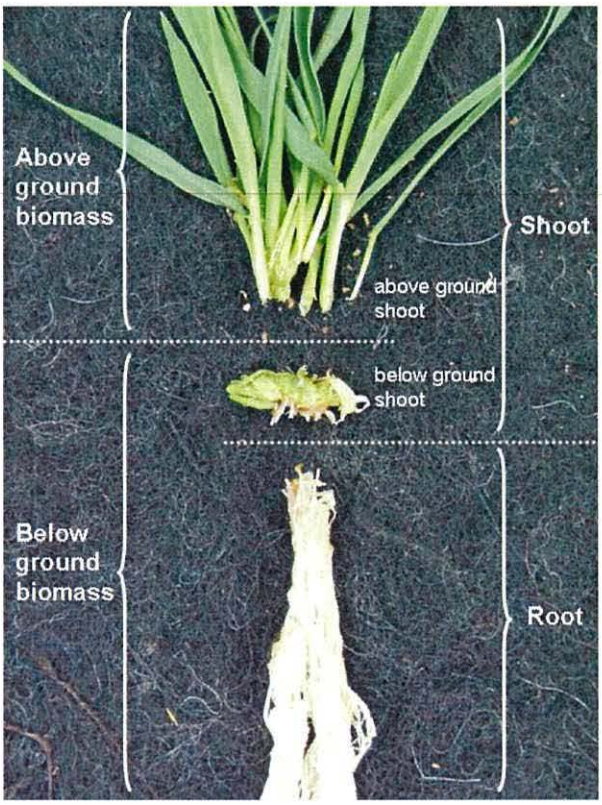

Figure 2.1 –Separation of biomass components into above or below ground and root or shoot during destructive harvest.

ramete destructively harvested to determine pre-exposure above and below ground biomass. Above ground biomass was removed to soil level and plant material then dried at 65 °C for at least four days for dry weight measurement. Below ground biomass was cleaned of soil to a consistent standard, dried at 65 °C for at least seven days and dry weight determined for separated root and below ground shoot biomass components (Figure 2.1). At five weeks the remaining two rametes per individual were transferred to the CEH ozone exposure facility, Abergwyngregyn.

2.2.2 Ozone exposure

2.2.2.1 Ozone fumigation system

Plants were exposed to ozone in eight replicate ventilated hemispherical glasshouses (solardomes, Figure 2.2) 2 metres high and 3 metres diameter receiving approximately two complete air changes per minute. Ozone was generated by a G11 ozone generator

Figure 2.2 – CEH Bangor solardome ozone exposure facility at Abergwyngregyn.

from oxygen supplied by a Workhorse 8 oxygen generator (both Dryden Aqua, UK Ltd) and delivered to the solardomes by a computer controlled (Labview, version 7) mass-flow controller system. Ozone was added to charcoal filtered air and concentrations in one solardome were continuously monitored on a Model 49C ozone analyser (Thermo Electron) with ozone supply to all solardomes adjusted accordingly via a feedback control loop. Ozone concentrations in all solardomes were monitored on a 30 minute cycle by two API400 ozone analysers (Envirotech) of matched calibration. Hourly mean ozone concentrations (ppb) were calculated from half hourly measurements and used to calculate the accumulated ozone over a threshold of X ppb (AOTX ppm.h) for 12 daylight hours (7 am to 7 pm) and 24 hours per day over the experimental period. Weekly AOTXs were calculated from the start of ozone exposure to 9 am on the corresponding assessment day.

2.2.2.2 Ozone treatments and experimental design

Two ozone treatments were applied in a weekly episodic regime with a background ozone concentration of 20 ppb maintained throughout in the control treatment ($O_3(20)$). A five-day ozone episode at a continuous 150 ppb was applied in the elevated ozone treatment ($O_3(150)$) with the background concentration of 20 ppb maintained at all other times (Figure 2.3).

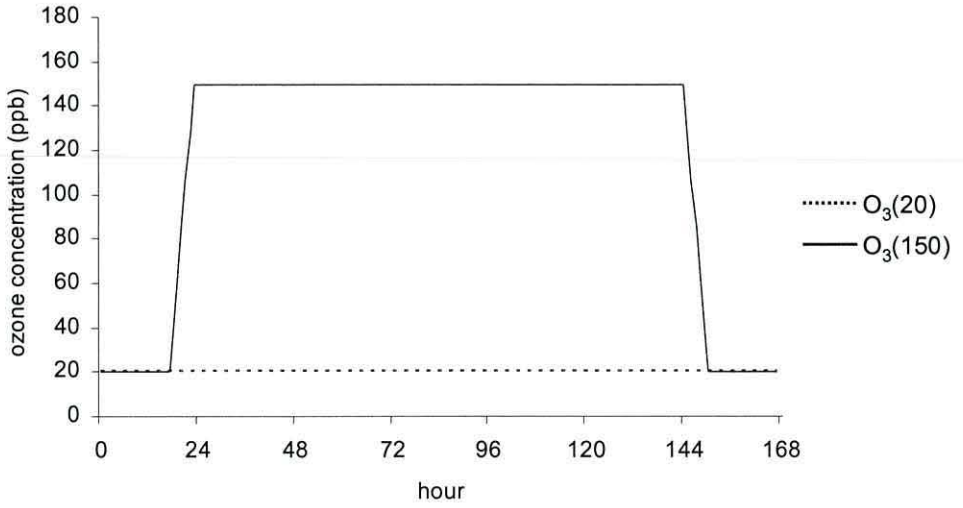

Figure 2.3 – Weekly ozone profiles for control ($O_3(20)$) and elevated ozone ($O_3(150)$) treatments.

Solardomes were split into four randomised blocks of two treatments with four to five individuals per population per replicate block. Plants were randomly assigned to solardomes within each replicate with one ramete per individual in each solardome. Plants were arranged on trays (0.5 m x 0.4 m) with drainage holes 10 mm above the base and lined with capillary matting. Tray positions were duplicated among solardomes and plants were randomly assigned to 19 positions across two trays within each solardome. Plant pots were spaced approximately 5 cm apart on each tray and trays were rotated weekly within each solardome. Plants were transferred to the solardomes five weeks after propagation and allowed to acclimatise in $O_3(20)$ for five days prior to ozone exposure. Plants were exposed to ozone treatments for four weeks from 23rd August to 19th September 2006 receiving four ozone episodes. Plants were irrigated daily by hose system into the trays, ensuring the capillary matting remained moist, and received overhead watering by hand once a week.

2.2.2.3 Visual assessment and chlorophyll content.

All plants were assessed weekly for non-specific foliar injury as senescence and chlorophyll content prior to and throughout the exposure period. Whole plant senescence was visually assessed as the percentage of leaves with $\geq$ 25 % necrotic senescence (Figure 2.4).

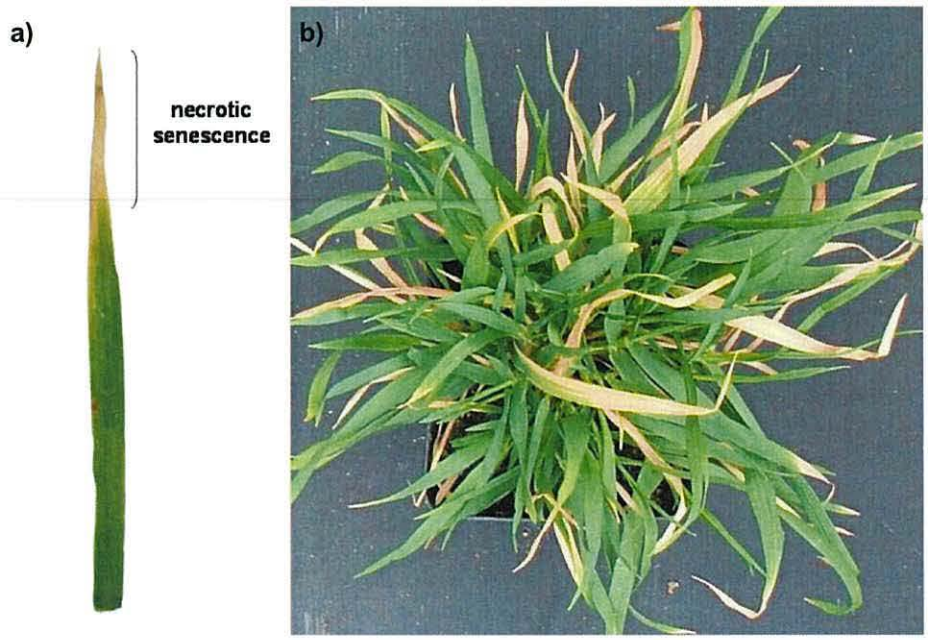

Figure 2.4 – a) Individual leaf necrotic senescence progressing from the distal to proximal end of the lamina and b) whole plant necrotic senescence.

Chlorophyll meter readings (SPAD values) were made on the mid lamina of the second fully expanded leaf down from the flag leaf for three tillers per plant using the SPAD-502 Chlorophyll meter (Minolta).

2.2.2.4 Plant growth assessment

Plants were destructively harvested after four ozone episodes to determine post-exposure biomass. Above ground biomass was removed to soil level, fresh weight determined and plant material then dried at 65 °C for at least four days for dry weight measurement. Below ground biomass was cleaned of soil to a consistent standard and dried at 65 °C for at least seven days. Dry weight was determined for total below ground biomass and separated root and shoot biomass components. Root to shoot (R:S) and below ground to above ground (BG:AG) ratios were calculated for post-exposure dry weight biomass. Relative growth rate (RGR) was calculated for all dry weight biomass variables using pre- and post-exposure measurements as:

$$RGR = (\ln w_2 - \ln w_1)/(t_2 - t_1)$$

where w is weight (g), t is time (week) and subscript denotes harvest date.

2.2.2.5 Relative ozone sensitivity

A relative ozone sensitivity index was determined for comparison of ozone effects between different assessment criteria and populations. Foliar injury assessed as the percentage of whole plant senescence was converted to the percentage of healthy leaves for this purpose as:

$$\% \text{ healthy leaves} = 100 - \% \text{ whole plant senescence}$$

Relative sensitivity (RS) of plant growth and individual week foliar injury variables were calculated using treatment means as the proportion under elevated ozone ($O_3(150)$) relative to the control ($O_3(20)$) where:

$$RS = 1 + ((O_{3\ elevated} - O_{3\ control}) / O_{3\ control})$$

RS values of <1 or >1 are obtained with a reduction or stimulation respectively for plant growth, % healthy leaves or chlorophyll content (SPAD value) under elevated ozone.

For the rate of development of foliar injury, assessed as $wpns_{coeff}$ and $SPAD_{coeff}$, the relative sensitivity was calculated using treatment means as the rate under control conditions relative to the rate under elevated ozone where:

$$RS = O_{3\ control} / O_{3\ elevated}$$

RS values of <1 and >1 are obtained for an increase or decrease respectively in the rate of development under elevated ozone.

The relative ozone sensitivity index describes the effects of elevated ozone in relation to the control with a relative sensitivity of 1 indicating no response, <1 indicating sensitivity or reduction and >1 indicating resistance or stimulation.

2.2.3 Statistical analysis

Arcsine transformation was performed on individual week senescence data and post-exposure fresh and dry weight biomass data were log transformed. Values for the four to five plants per population within each dome were averaged to provide four replicates per treatment per population and the population dome averages for each variable used for all subsequent analysis. Time-course data for whole plant necrotic senescence (wpns) and chlorophyll content (SPAD value) were analysed by regression in Minitab version 14 and the resulting regression coefficients saved for subsequent analysis as variables $wpns_{coeff}$ and $SPAD_{coeff}$ respectively. Variables were analysed for main and interaction effects of ozone treatment and population using an analysis of variance (ANOVA) model with a randomized block design in Genstat version 8. Populations exhibited some inequality of variance which may have violated the assumptions of the analysis model and were reanalysed separately for ozone treatment as a main effect. ANOVA model for overall and population level analysis given in Table 2.1. Ozone dose response at the species and population level was analysed by quadratic regression of relative whole plant necrotic senescence against relative AOTX, both as the difference from the control, using treatment means from all assessment weeks throughout the exposure period in Minitab version 14.

Table 2.1. – ANOVA model for overall and individual population analysis.

Overall		Individual populations	
Source of variation	**DF**	**Source of variation**	**DF**
Block	3	**Block**	3
Treatment‡	1	**Treatment**‡	1
residual	*3*	*residual*	*3*
Population‡	3	**Total**	7
Treatment x Population‡	3		
residual	*18*		
Total	31		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

2.3 Results

2.3.1 Received ozone exposure

Average total and weekly ozone concentrations received by the control ($O_3(20)$) and elevated ($O_3(150)$) ozone treatments were within 10 ppb of the target concentration, with the elevated ozone treatment receiving an average 125 ppb increase above the control treatment (Table 2.2). Average peak ozone concentrations met the target concentration for the $O_3(20)$ treatment and were between 5 ppb and 10 ppb below for the $O_3(150)$ treatment. The difference between the expected 20 ppb and actual average background ozone concentration was greater in the $O_3(20)$ treatment than the $O_3(150)$ treatment. This can be ascribed to low total system ozone demand when running at 20 ppb, differences in capacity and calibration of the mass-flow controllers required for each treatment and the inclusion of periods when the ozone supply was suspended for access into the solardomes (receiving charcoal filtered air with < 10 ppb ozone) in the calculation of average background concentrations.

In the first week of exposure one replicate solardome for the $O_3(150)$ treatment received over 200 ppb above the target peak concentration during the ozone episode. The hourly average ozone concentration increased steadily over 15 hours, from 150 ppb at 7 pm on day three to 360 ppb at 10 am on day four, due to deterioration and failure of the mass-flow controller supplying ozone to the solardome. The mean hourly ozone concentration for this treatment at that time reached 200 ppb (Figure 2.5) although the average peak

Table 2.2 – Total and weekly average ozone concentrations (ppb) received for background and peak times during the weekly profile for control ($O_3(20)$) and elevated ozone ($O_3(150)$) treatments.

Ozone Treatment		Average Ozone Concentration (ppb)				
		week 1	week 2	week 3	week 4	Total
$O_3(20)$	background	12.6 ±1.5	13.7 ±0.9	12.5 ±0.4	12.8 ±0.9	12.9 ±0.4
	peak	19.1 ±0.2	20.0 ±0.1	19.7 ±0.2	19.8 ±0.2	19.6 ±0.1
$O_3(150)$	background	19.4 ±1.6	18.8 ±0.8	19.9 ±0.5	18.2 ±0.9	19.2 ±0.4
	peak	146.1 ±2.1	139.0 ±1.6	144.8 ±1.8	144.4 ±1.9	143.5 ±0.9

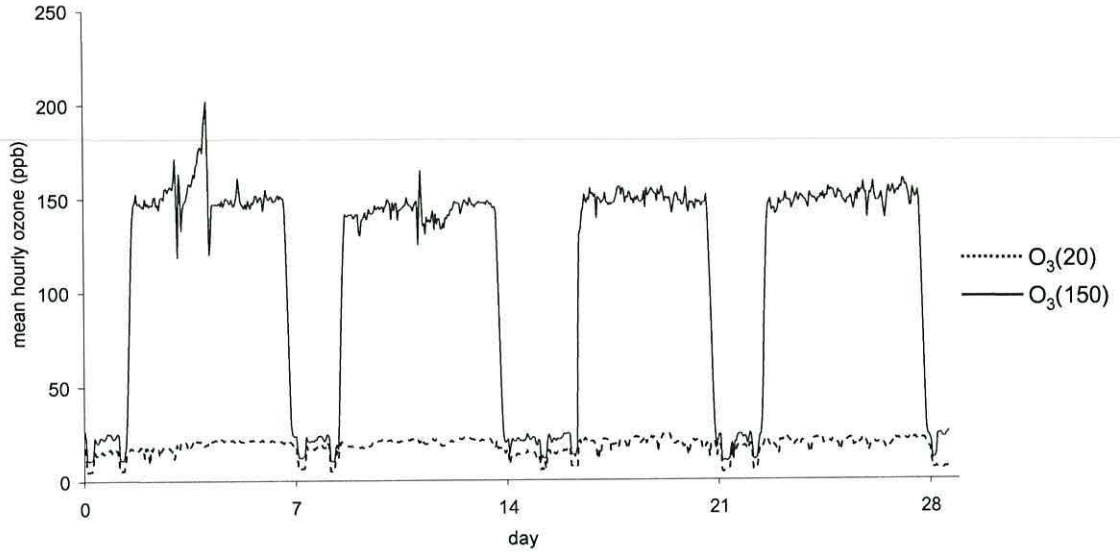

Figure 2.5 – Mean hourly ozone concentration (ppb) received over 28 days of exposure for control (O_3(20)) and elevated ozone (O_3(150)) treatments.

ozone concentration for week one remains comparable with the following weeks (Table 2.2). The hourly mean ozone concentrations remained stable for the rest of the exposure period. The intended weekly profile comprising a five-day ozone episode was applied throughout the exposure period with the exception of week three, which received a four-day episode, due to adverse weather conditions in week two preventing entry into the solardomes which postponed plant assessment and commencement of the peak episode.

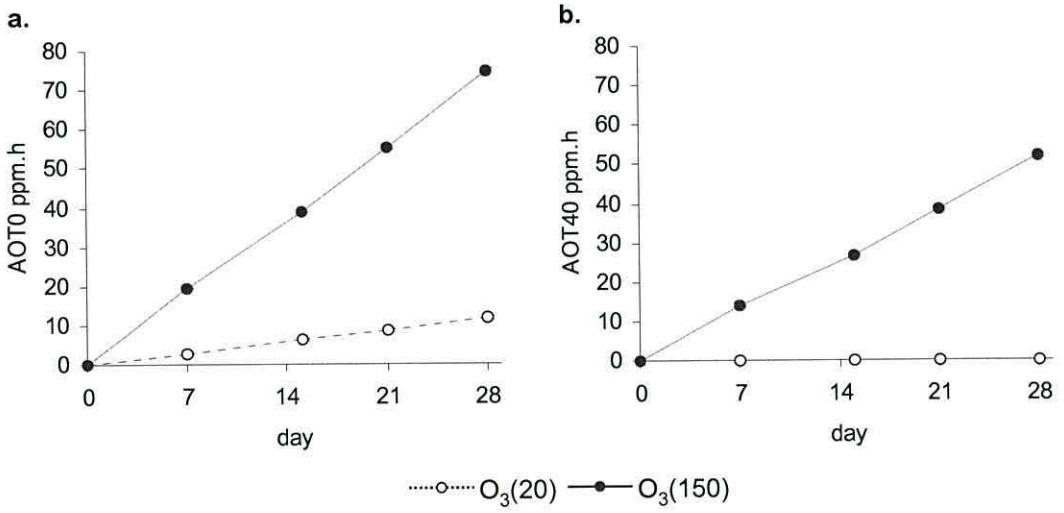

Figure 2.6 – Mean weekly accumulated ozone (ppm.h) over a 28 day exposure period for control (O_3(20)) and elevated ozone (O_3(150)) treatments. Ozone accumulated over a threshold of a) 0 ppb and b) 40 ppb for 24 hours per day. Bars represent standard errors of the mean.

After 28 days of exposure the received ozone dose, measured as accumulated ozone over a threshold of X ppb (AOTX ppm.h) for 24 hours per day, was 11.8 ±0.3 ppm.h and 0 ppm.h for the $O_3(20)$ treatment and 74.8 ±0.6 ppm.h and 51.9 ±0.4 ppm.h for the $O_3(150)$ treatment with threshold values of 0 ppb and 40 ppb respectively (Figure 2.6).

2.3.2 Visual assessment and chlorophyll content

Increased/premature senescence with elevated ozone was observed at the species and population level with whole plant senescence significantly higher ($P<0.05$) under elevated ozone for every assessment week throughout the exposure period (Figures 2.7 a and 2.8). Summary of ANOVA results are given in Table 2.4 and full details in Appendix I. The greatest increases in whole plant senescence under elevated ozone in relation to the control treatment were observed in the first two weeks. Average senescence at the species level was 3.7 and 4.2 times higher under elevated ozone after one and two ozone episodes respectively. Although this decreased with the natural development of senescence over time, senescence under elevated ozone remained at least double that observed in the control treatment. The difference in senescence between the $O_3(20)$ and $O_3(150)$ treatments increased from 10.2 % to 18.4 % after one

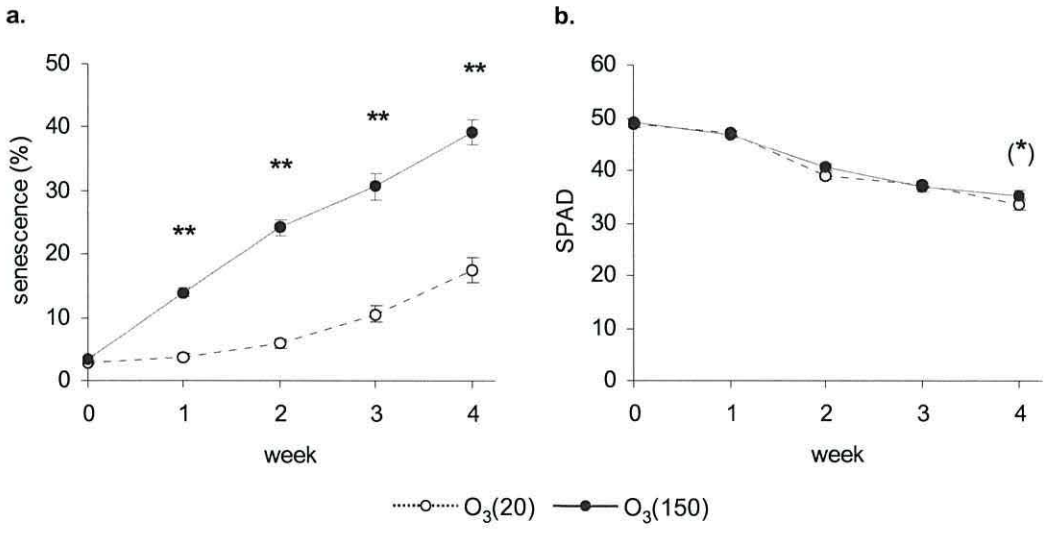

Figure 2.7 – Species level progression of a) whole plant senescence and b) chlorophyll content (SPAD value) over time for control ($O_3(20)$) and elevated ($O_3(150)$) ozone treatments. Significant differences are indicated by ** = $P<0.01$, * = $P<0.05$ and $P<0.1$ by (*). Bars represent standard errors of the mean.

and two episodes and continued to increase at a slower rate with a difference of 21.8 % observed after 4 weeks of exposure (Figure 2.9).

Populations exhibited increased/premature senescence under elevated ozone ($P<0.05$) with three populations showing the greatest increases under elevated ozone in relation to the control treatment after one and two episodes while this extended to week three for the Maltraeth population (Figure 2.8). The reduction in this increase observed after three episodes in the Birds Rock population, and to a lesser extent in the Cwm Idwal population, may have been due to plants receiving a four-day rather than five-day ozone episode in the third week of exposure. Populations differed in the amount of senescence during the final two weeks of exposure ($P<0.05$) with the highest levels of senescence in both the elevated ozone and control treatments observed for the Aber population.

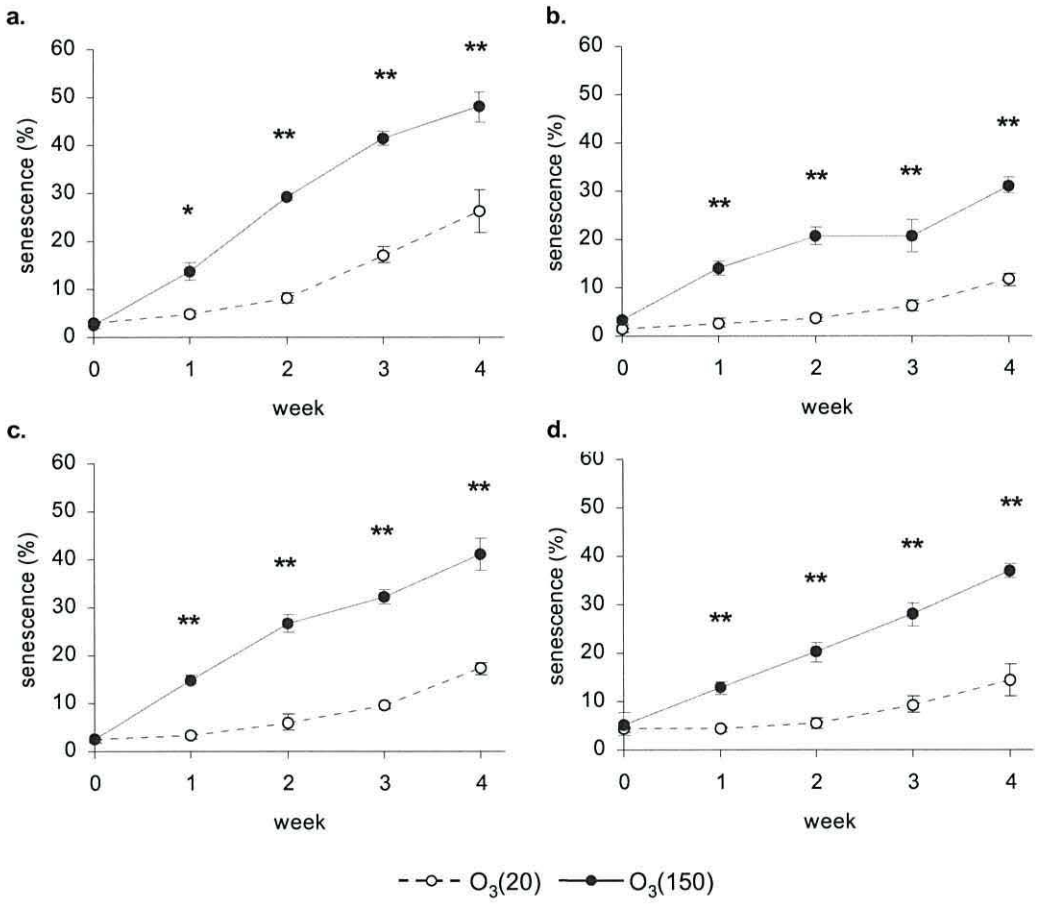

Figure 2.8 – The development of whole plant senescence over time for populations a) Aber, b) Birds Rock, c) Cwm Idwal and d) Maltraeth for control ($O_3(20)$) and elevated ($O_3(150)$) ozone treatments. Significant differences are indicated by ** = $P<0.01$ and * = $P<0.05$. Bars represent standard errors of the mean.

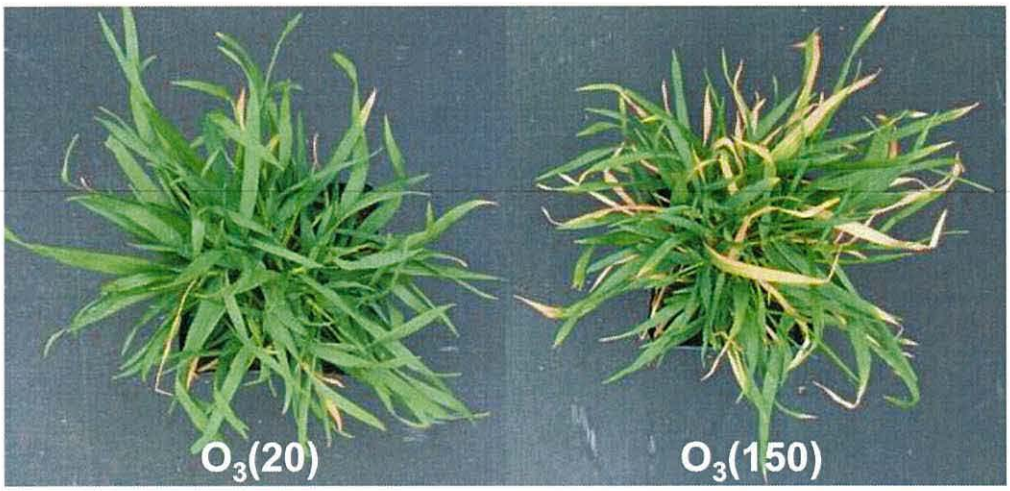

Figure 2.9 – Whole plant senescence on rametes of one individual from Cwm Idwal after 4 weeks exposure to control ($O_3(20)$) and elevated ozone ($O_3(150)$) treatments.

The rate of development of senescence, assessed by the coefficients of regression for whole plant senescence against days of exposure ($wpns_{coeff}$), increased under elevated ozone at the species and population level ($P<0.05$, Figure 2.10). Populations differed in the rate of senescence ($P<0.001$) with the Aber population exhibiting the highest rate and Birds Rock the lowest. Under elevated ozone, $wpns_{coeff}$ was double to triple that observed in the control treatment with Maltraeth exhibiting the highest increase and Aber the lowest. The Maltraeth population had the highest level of senescence prior to ozone exposure and exhibited very little increase under the control treatment until week three which may have resulted in a low rate in the development of natural senescence and the large increase under elevated ozone.

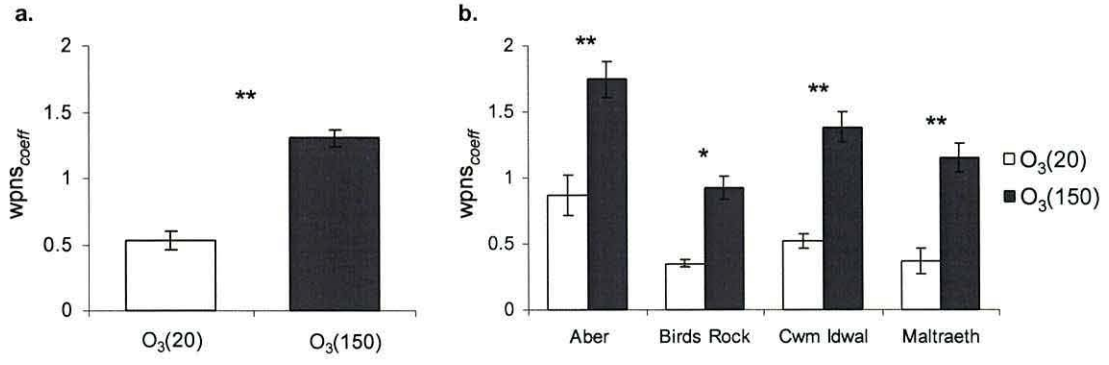

Figure 2.10 – Rate of development of senescence ($wpns_{coeff}$) over 28 days exposure to control ($O_3(20)$) and elevated ($O_3(150)$) ozone treatments at a) Species level and b) population level. Significant differences are indicated by ** = $P<0.01$, * = $P<0.05$ and $P<0.1$ by (*). Bars represent standard errors of the mean.

Leaf chlorophyll content (SPAD value) decreased through the exposure period regardless of ozone treatment. After four ozone episodes a slight non-significant 5 % increase in SPAD was observed under elevated ozone at the species level ($P<0.1$, Figure 2.7 b). Populations differed significantly in leaf chlorophyll content throughout the exposure period with the highest SPAD values observed in the Birds Rock population ($P<0.05$). Three populations exhibited a trend for increased SPAD throughout the exposure period while the Aber population exhibited a trend for decrease with an 8.3% reduction after three weeks ($P<0.1$). By the end of ozone exposure, three populations exhibited no significant response to ozone exposure whilst a significant 10 % increase occurred in the Maltraeth population ($P<0.05$).

The rate of change in leaf chlorophyll content, assessed by the coefficients of regression for SPAD against days of exposure ($SPAD_{coeff}$), exhibited a non-significant trend for a lower rate of chlorophyll content reduction under elevated ozone at the species level. The populations differed significantly regardless of ozone treatment with the highest rate observed in the Aber population and the lowest in the Birds Rock population ($P<0.01$). The Cwm Idwal population exhibited a 10.1% lower rate under elevated ozone significant at the $P<0.1$ level. The Birds Rock and Maltraeth populations exhibited larger but non-significant reductions of 12.0% and 17.2% respectively. The Aber population exhibited the opposite trend with a non-significant 8.1% higher rate of chlorophyll content reduction under elevated ozone.

2.3.3 Plant growth

The separation of dry weight biomass into either above ground and below ground or shoot and root components resulted in no significant treatment differences (data not presented). Plant growth results are reported for the above ground and below ground separation for comparison between fresh and dry weight biomass measurements and continuity between ozone exposure experiments. Summary of ANOVA results are given in Table 2.4 and full details in Appendix I.

Plant growth response to 28 days of exposure to elevated ozone varied between biomass components and fresh or dry weight with either no effect or decrease at the species level and increase, decrease or no effect for individual populations. No significant effects were found for the below ground to above ground dry weight (BG:AG) or dry weight to fresh weight (dwt:fwt) biomass ratios at either the species or individual population level (Figures 2.11 and 2.12). Although not significant there was a trend for reduction in both biomass ratios at the species level and for the majority of populations. The exceptions were the Birds Rock and Aber populations which exhibited no response for BG:AG and dwt:fwt respectively.

Above ground fresh weight showed no effect of ozone treatment at the species level (Figure 2.13 a). There were no significant interaction effects between ozone treatment and population, although the populations exhibited different trends for above ground fresh weight response (Figure 2.13 b). When analysed individually, two populations showed no difference with ozone treatment while a 10.5 % increase was observed in the Cwm Idwal population ($P<0.01$) and the Birds Rock population showed a non-significant 11.6 % decrease ($P=0.282$). Although there was a significant response in the Cwm Idwal population this result may be an artefact of the analysis model as the standard errors of the mean showed a large overlap (Figure 2.13 b). However, when the underlying variation at the replicate level was considered, the large standard errors were due to greater among replicate than within replicate variation and the response to elevated ozone was consistent (Figure 2.14, Table AII.2 in Appendix II). The analysis model applied accounts for variation among replicates and the significance of this

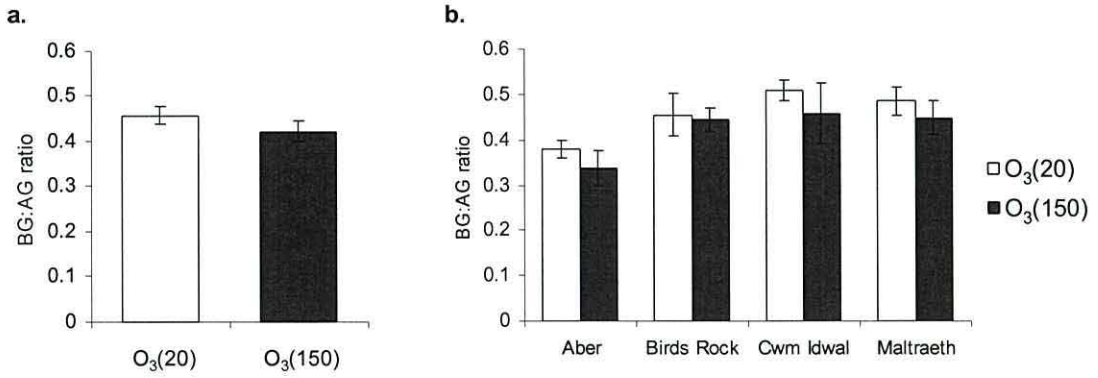

Figure 2.11 – Below ground to above ground dry weight biomass ratio at a) Species level and b) population level. No significant differences were observed. Bars represent standard errors of the mean.

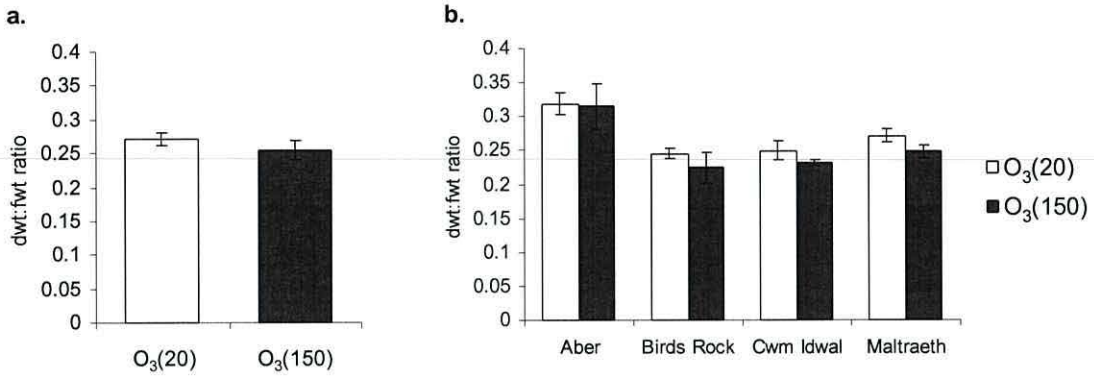

Figure 2.12 –Dry weight to fresh weight biomass ratio at a) Species level and b) population level. No significant differences were observed. Bars represent standard errors of the mean.

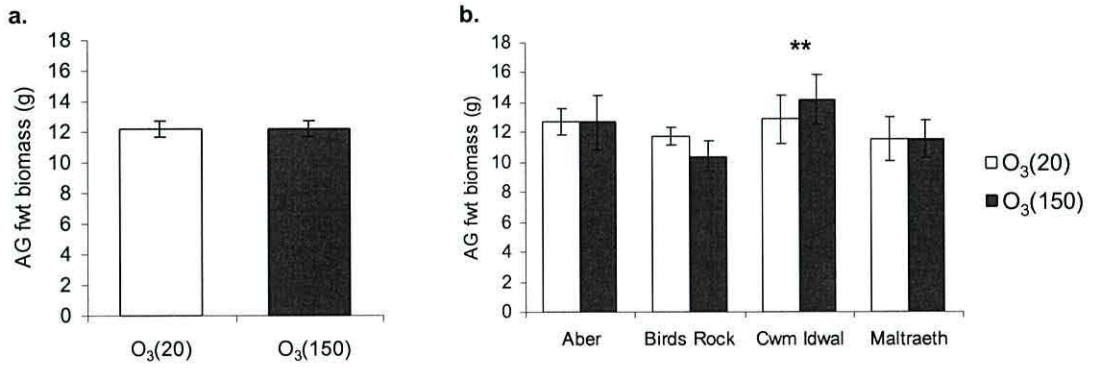

Figure 2.13 – Above ground fresh weight at a) Species level and b) population level. Significant differences are indicated by ** = $P<0.01$, * = $P<0.05$ and $P<0.1$ by (*). Bars represent standard errors of the mean.

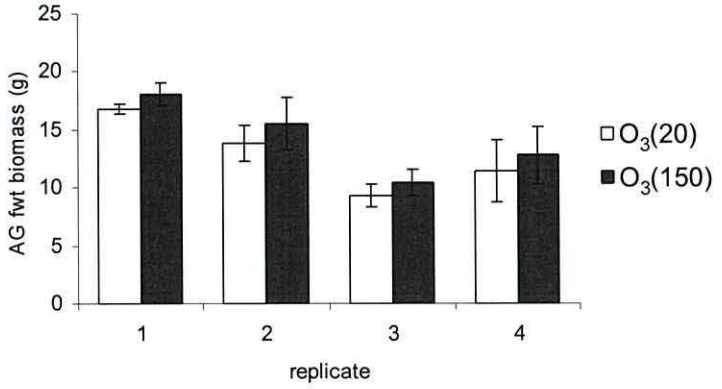

Figure 2.14 – Within and among replicate variation in above ground fresh weight biomass for the Cwm Idwal population. Bars represent standard errors of the mean.

response can therefore be accepted. The contrasting responses between the populations result in the lack of response for above ground fresh weight at the species level.

Dry weight biomass decreased by 10.5 % ($P<0.1$) under elevated ozone at the species level with a significant 8.3 % ($P<0.05$) reduction for the above ground component and non-significant 15.0 % reduction for the below ground component (Figure 2.15 b). The below ground biomass component constituted approximately one third of the total biomass and reductions in this component would need to be large to be reflected in the total biomass. Three populations followed the same trend for decreased dry weight biomass under elevated ozone with a significant reduction of 22.8 % ($P<0.05$) for Birds Rock and a non-significant trend of 9.2 % ($P=0.162$) and 10.6 % ($P=0.379$) reduction for Aber and Maltraeth respectively (Figure 2.16 c). The Cwm Idwal population exhibited no effect of ozone treatment on total dry weight biomass. The same pattern of response was observed for the above ground biomass component with a 22.5 % reduction ($P<0.01$) in shoot growth for Birds Rock (Figure 2.16 a). All populations exhibited a trend for reduction in below ground biomass under elevated ozone with a 13.3 % decrease significant at the $P<0.1$ level for the Aber population. Although the Birds Rock and Maltraeth populations showed a greater decrease than Aber, 22.4 % and 15.3 % respectively, these were non-significant. The Cwm Idwal population had the smallest decrease in below ground growth (Figure 2.16 b).

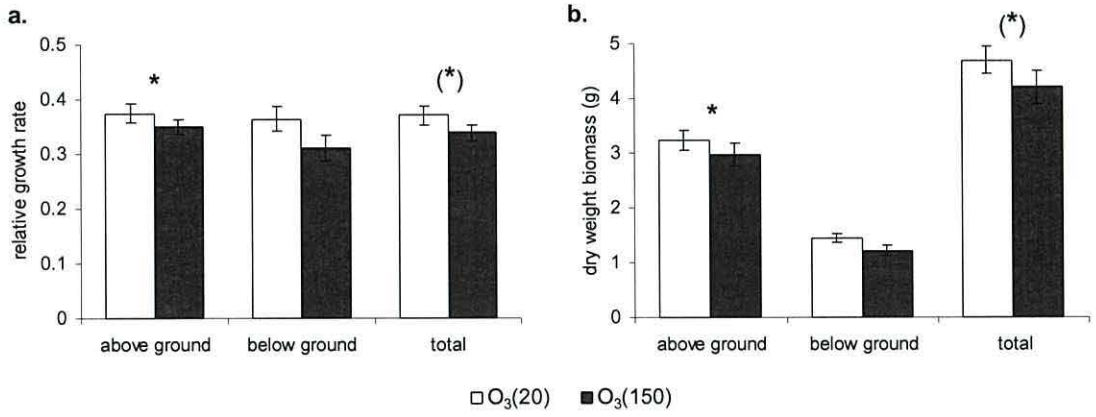

Figure 2.15 – Species level a) relative growth rate b) dry weight biomass for total, above ground and below ground biomass components. Significant differences are indicated by * = $P<0.05$ and (*) = $P<0.1$. Bars represent standard errors of the mean.

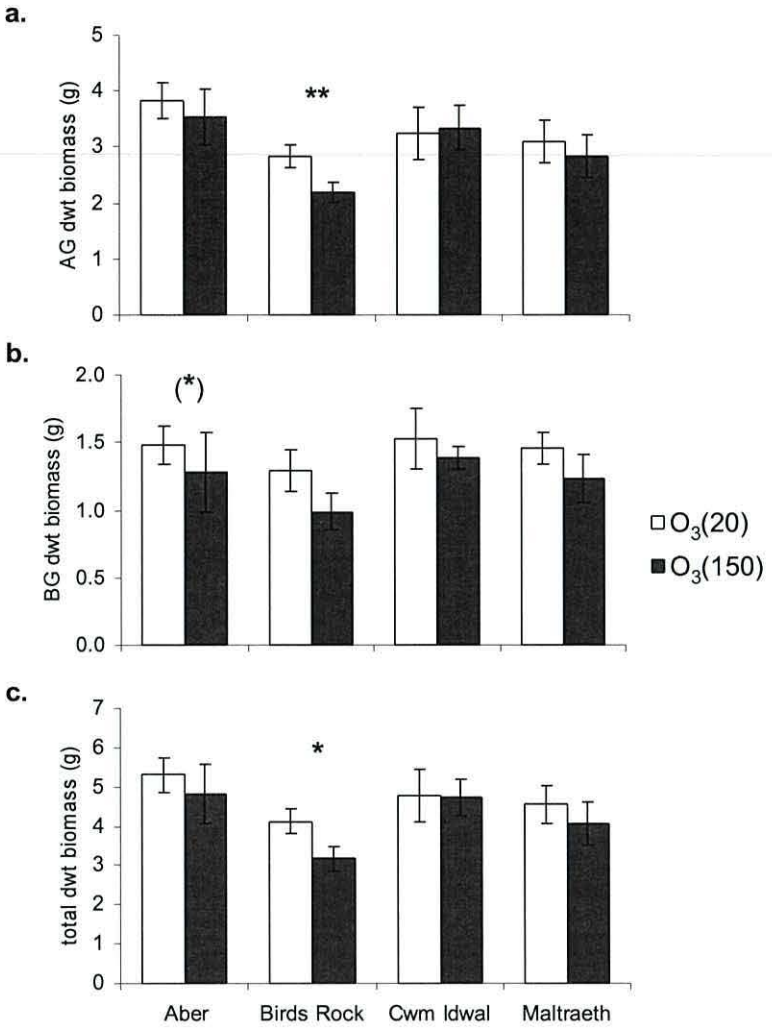

Figure 2.16 – Population level dry weight biomass response for a) above ground, b) below ground and c) total biomass components. Significant differences are indicated by ** = $P<0.01$, * = $P<0.05$ and $P<0.1$ by (*). Bars represent standard errors of the mean.

Relative growth rate (RGR) followed the same trend as dry weight biomass for reduction under elevated ozone with slightly lower percentage reductions of 8.4 % for total RGR ($P<0.1$), 6.4 % for above ground RGR ($P<0.05$) and a non-significant reduction of 14.4 % for below ground RGR at the species level (Figure 2.15 a). Populations differed significantly in total RGR ($P<0.05$) and below ground RGR ($P<0.01$) with Aber showing the lowest rate for both and Cwm Idwal the highest below ground RGR. Under control conditions, $O_3(20)$ treatment, within population RGR differed for the separate biomass components with below ground growth rate lower in Aber and higher in Cwm Idwal than the above ground component (Table 2.3). Birds Rock and Maltraeth showed a similar growth rate for above ground and below ground

Table 2.3 – Population level relative growth rate (RGR) for control ($O_3(20)$) and elevated ozone ($O_3(150)$) treatments and percentage change in RGR under elevated ozone (% Δ RGR) for above ground, below ground and total biomass components. Significant differences are indicated by ** = $P<0.01$, * = $P<0.05$ and $P<0.1$ by (*).

Relative Growth Rate	Population			
	Aber	Birds Rock	Cwm Idwal	Maltraeth
Above ground				
mean $O_3(20)$	0.333	0.408	0.378	0.377
mean $O_3(150)$	0.315	0.340	0.386	0.358
% Δ RGR	-5.5	-16.7 **	2.2	-5.0
Below ground				
mean $O_3(20)$	0.257	0.401	0.414	0.382
mean $O_3(150)$	0.191	0.329	0.386	0.338
% Δ RGR	-25.7 (*)	-18.0	-6.8	-11.5
Total				
mean $O_3(20)$	0.307	0.407	0.387	0.378
mean $O_3(150)$	0.281	0.337	0.386	0.352
% Δ RGR	-8.4 (*)	-17.1 *	-0.3	-6.9

biomass components. Population relative growth rates showed a similar response to elevated ozone exposure as dry weight biomass with a significant decrease in total biomass of 17.1 % in the Birds Rock population ($P<0.05$) and a reduction of 8.4 % significant at the $P<0.1$ level for Aber. All populations exhibited a trend for reduced growth rate in the below ground biomass component with the largest decrease of 25.7 % significant at the $P<0.1$ level for Aber and the smallest non-significant decrease of 6.8 % occurring in the Cwm Idwal population. Responses for above ground RGR varied with the Birds Rock population having a highly significant 16.7 % reduction ($P<0.01$), two populations exhibiting a trend for decrease under elevated ozone and Cwm Idwal showing a slight 2.2 % non-significant increase.

Table 2.4 – Summary of ANOVA analysis results.

			wpns week 4			$wpns_{coeff}$			Above ground dwt			Below ground dwt		
	Source of variation	**DF**	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***
Overall	**Block**	3	0.0402	0.0134	17.03	0.4266	0.1422	14.15	0.4734	0.1578	17.43	0.7210	0.2403	2.67
	Treatment‡	1	0.5679	0.5679	721.54**	4.7660	4.7660	474.18**	0.0989	0.0989	10.92*	0.4062	0.4062	4.52
	residual	*3*	0.0024	0.0008	0.18	0.0302	0.0101	0.30	*0.0272*	*0.0091*	*0.15*	*0.2697*	*0.0899*	*1.24*
	Population‡	3	0.1459	0.0486	11.32**	2.0529	0.6843	20.52**	0.5511	0.1837	3.02(*)	0.3815	0.1272	1.75
	Treatment x Population‡	3	0.0014	0.0005	0.11	0.1189	0.0396	1.19	0.0950	0.0317	0.52	0.0552	0.0184	0.25
	residual	*18*	0.0774	0.0043		0.6002	0.0334		*1.0957*	*0.0609*		*1.3048*	*0.0725*	
	Total	31	0.8351			7.9949			2.3410			3.1384		
Aber	**Block**	3	0.0428	0.0143	7.71	0.4398	0.1466	8.42	0.3912	0.1304	10.52	0.9597	0.3199	10.42
	Treatment‡	1	0.1265	0.1265	68.43**	1.5402	1.5402	88.52**	0.0296	0.0296	2.39	0.2034	0.2034	6.62(*)
	residual	*3*	*0.0056*	*0.0019*		*0.0522*	*0.0174*		*0.0372*	*0.0124*		*0.0921*	*0.0307*	
	Total	7	0.1749			2.0322			0.4579			1.2552		
Birds Rock	**Block**	3	0.0049	0.0016	1.30	0.0384	0.0128	0.59	0.1356	0.0452	13.91	0.3585	0.1195	3.15
	Treatment‡	1	0.1298	0.1298	104.68**	0.6517	0.6517	29.83*	0.1481	0.1481	45.58**	0.1673	0.1673	4.40
	residual	*3*	*0.0037*	*0.0012*		*0.0656*	*0.0219*		*0.0098*	*0.0033*		*0.1139*	*0.0380*	
	Total	7	0.1384			0.7556			0.2935			0.6397		
Cwm Idwal	**Block**	3	0.0088	0.0029	1.00	0.1385	0.0462	2.64	0.5257	0.1752	60.80	0.2547	0.0849	2.53
	Treatment‡	1	0.1550	0.1550	53.18**	1.4803	1.4803	84.65**	0.0022	0.0022	0.75	0.0251	0.0251	0.75
	residual	*3*	*0.0088*	*0.0029*		*0.0525*	*0.0175*		*0.0087*	*0.0029*		*0.1005*	*0.0335*	
	Total	7	0.1725			1.6713			0.5365			0.3804		
Maltraeth	**Block**	3	0.0338	0.0113	2.89	0.2213	0.0738	4.53	0.3876	0.1292	3.85	0.3560	0.1187	5.93
	Treatment‡	1	0.1580	0.1580	40.49**	1.2128	1.2128	74.43**	0.0140	0.0140	0.42	0.0657	0.0657	3.28
	residual	*3*	*0.0117*	*0.0039*		*0.0489*	*0.0163*		*0.1006*	*0.0335*		*0.0600*	*0.0200*	
	Total	7	0.2035			1.4830			0.5023			0.4817		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

Table 2.4 – Continued.

			Above ground RGR			Below ground RGR			BG:AG ratio			dwt:fwt ratio		
	Source of variation	**DF**	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***
Overall	**Block**	3	0.0140	0.0047	10.87	0.0335	0.0112	2.47	0.0559	0.0187	1.41	0.0040	0.0013	1.43
	Treatment‡	1	0.0048	0.0048	11.12*	0.0220	0.0220	4.86	0.0095	0.0095	0.72	0.0021	0.0021	2.27
	residual	*3*	0.0013	0.0004	0.11	0.0136	0.0045	1.42	0.0398	0.0133	4.92	0.0028	0.0009	0.77
	Population‡	3	0.0163	0.0054	1.39	0.1448	0.0483	15.17**	0.0753	0.0251	9.32**	0.0339	0.0113	9.42**
	Treatment x Population‡	3	0.0061	0.0020	0.52	0.0025	0.0008	0.26	0.0017	0.0006	0.22	0.0005	0.0002	0.13
	residual	*18*	0.0702	0.0039		0.0573	0.0032		0.0485	0.0027		0.0216	0.0012	
	Total	31	0.1126			0.2736			0.2308			0.0649		
Aber	**Block**	3	0.0426	0.0142	106.47	0.0176	0.0059	6.65	0.0165	0.0055	3.16	0.0125	0.0042	3.05
	Treatment‡	1	0.0007	0.0007	5.12	0.0086	0.0086	9.69(*)	0.0033	0.0033	1.89	0.0000	0.0000	0.01
	residual	*3*	*0.0004*	*0.0001*		*0.0027*	*0.0009*		*0.0052*	*0.0017*		*0.0041*	*0.0014*	
	Total	7	0.0437			0.0288			0.0250			0.0166		
Birds Rock	**Block**	3	0.0005	0.0002	0.88	0.0017	0.0006	0.24	0.0190	0.0063	1.15	0.0028	0.0009	0.66
	Treatment‡	1	0.0093	0.0093	45.58**	0.0105	0.0105	4.40	0.0002	0.0002	0.04	0.0009	0.0009	0.61
	residual	*3*	*0.0006*	*0.0002*		*0.0071*	*0.0024*		*0.0165*	*0.0055*		*0.0043*	*0.0014*	
	Total	7	0.0104			0.0193			0.0357			0.0079		
Cwm Idwal	**Block**	3	0.0155	0.0052	28.63	0.0168	0.0056	2.67	0.0434	0.0145	3.08	0.0019	0.0006	2.92
	Treatment‡	1	0.0001	0.0001	0.75	0.0016	0.0016	0.75	0.0051	0.0051	1.09	0.0006	0.0006	2.89
	residual	*3*	*0.0005*	*0.0002*		*0.0063*	*0.0021*		*0.0141*	*0.0047*		*0.0006*	*0.0002*	
	Total	7	0.0161			0.0247			0.0626			0.0031		
Maltraeth	**Block**	3	0.0193	0.0064	3.16	0.0485	0.0162	13.20	0.0119	0.0040	0.67	0.0016	0.0005	2.35
	Treatment‡	1	0.0008	0.0008	0.37	0.0039	0.0039	3.18	0.0026	0.0026	0.44	0.0011	0.0011	4.91
	residual	*3*	*0.0061*	*0.0020*		*0.0037*	*0.0012*		*0.0177*	*0.0059*		*0.0007*	*0.0002*	
	Total	7	0.0261			0.0561			0.0322			0.0033		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

2.3.4 Ozone dose response

There was a significant relationship between species level relative whole plant necrotic senescence and the received ozone dose, measured as accumulated ozone over a threshold of X ppb (AOTX) relative to the control, across the exposure period (Table 2.5). A quadratic relationship provided the best fit as relative senescence increased to around 20 % over the first two weeks of exposure and then increased at a slower rate through the remaining two weeks (see section 2.3.2, Figure 2.7 a) despite the continued ozone dose accumulation. The reduced rate of increase in relative senescence is attributed to the natural development of senescence under control conditions. The quadratic relationship remained highly significant ($P<0.01$) regardless of whether a 0 ppb, 20 ppb or 40 ppb threshold value was applied and there was no difference for AOTXs calculated over a 24 hour or 12 hour per day period. Therefore, regression relationships for AOT0, AOT20 and a 12 hd^{-1} period are not considered further as AOT40 is more comparable with previous studies and a 24 hd^{-1} period accounts for ozone concentrations maintained overnight and potential nocturnal uptake.

Table 2.5 – Species level quadratic regression analysis of the relationship between relative whole plant senescence and relative accumulated ozone over a threshold (AOT) of X ppb calculated for 12 and 24 hd^{-1} periods throughout the exposure period.

AOT(X ppb)	Period	Quadratic regression			
		R^2	adj R^2	F value	*P* value
AOT0	**24 hd^{-1}**	0.995	0.989	182.55	0.005
	12 hd^{-1}	0.995	0.990	191.82	0.005
AOT20	**24 hd^{-1}**	0.994	0.989	178.65	0.006
	12 hd^{-1}	0.995	0.989	186.39	0.005
AOT40	**24 hd^{-1}**	0.994	0.988	169.46	0.006
	12 hd^{-1}	0.994	0.989	177.79	0.006

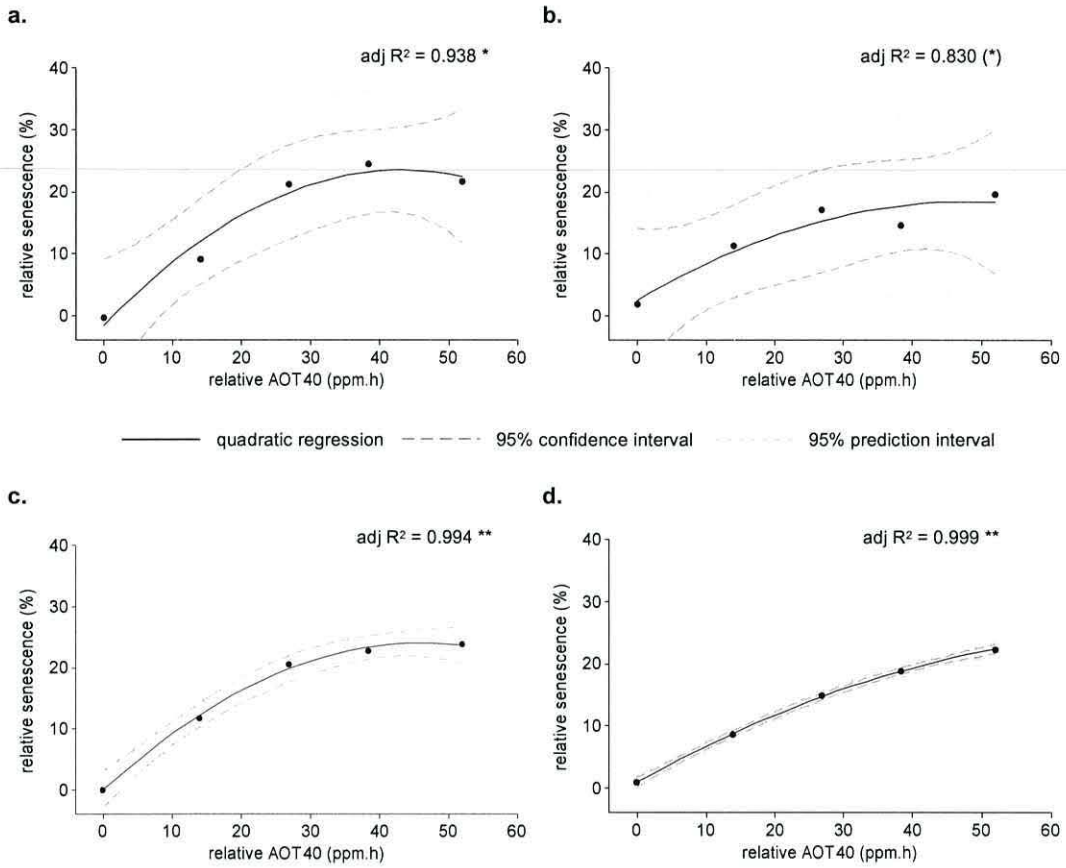

Figure 2.17 - Relationship between relative whole plant senescence and received ozone dose (relative AOT40 (24 hd^{-1})) throughout the exposure period with 95 % confidence and prediction intervals for populations a) Aber, b) Birds Rock, c) Cwm Idwal and d) Maltraeth. Significant adjusted R^2 for quadratic regression indicated by ** = $P<0.01$, * = $P<0.05$ and (*) = $P<0.1$.

Significant quadratic relationships between relative senescence and relative AOT40 (24 hd^{-1}) were obtained for the Cwm Idwal, Maltraeth (both $P<0.01$) and Aber ($P<0.05$) populations and at the $P<0.1$ level for Birds Rock (Figure 2.17). The highly significant relationships obtained for Aber, Maltraeth and Cwm Idwal suggest that the observed increase in senescence corresponded more to the received ozone dose than in the Birds Rock population. The almost linear nature of the relationship in Maltraeth is attributable to the slower rate of development of natural senescence in this population under control conditions.

2.3.5 Relative ozone sensitivity

A. odoratum exhibits the greatest relative ozone sensitivity for foliar injury with increased senescence resulting in a reduction in the percentage of healthy leaves under elevated ozone (Table 2.6). The populations all exhibit sensitivity to ozone for this assessment criterion with the greatest relative sensitivity observed in Aber and Cwm Idwal throughout the exposure period. However, Aber was the least sensitive in terms of the rate of development of senescence ($wpns_{coeff}$, Table 2.7).

Despite the reduction in healthy leaves, leaf chlorophyll content showed a trend for stimulation under elevated ozone at the species level although the relative sensitivity of the populations differed with both reduction and stimulation (Table 2.6). Aber was the only population to exhibit any sensitivity to ozone while Maltraeth was the most resistant with the greatest stimulation effect. The populations exhibit the same pattern of relative sensitivity when assessed for the rate of development of chlorophyll content ($SPAD_{coeff}$, Table 2.7).

The greatest relative sensitivity in plant growth was observed for the below ground biomass component although the most significant effects occurred in the above ground component (Table 2.6). Below ground growth was the most sensitive in all populations while the relative sensitivity of the above ground component differed between populations. The Birds Rock population was the most sensitive in terms of plant growth while the Cwm Idwal population was the most resistant.

The populations differed in relative sensitivity dependant upon the assessment criteria applied. All populations were considered sensitive for foliar injury as the percentage of healthy leaves, however, this did not always equate to a similar sensitivity in plant growth or leaf chlorophyll content.

Table 2.6 – Species and population level relative ozone sensitivity (RS) for foliar injury and plant growth assessment criteria. Ozone effect illustrated as a reduction (-), increase (+) or no response (0) under elevated ozone. *doe* denotes days of exposure. RS values of <1 and >1 represent sensitivity and resistance respectively. Significant differences from the control are indicated by **=$P<0.01$, * = $P<0.05$ and (*) = $P<0.1$.

Assessment criteria			Species		Aber		Birds Rock		Cwm Idwal		Maltraeth	
		doe	O_3 effect	RS	O_3 effect	RS	O_3 effect	RS	O_3 effect	RS	O_3 effect	RS
Foliar injury												
% healthy leaves		7	-	0.89 **	-	0.90 *	-	0.88 **	-	0.88 **	-	0.91 **
		15	-	0.80 **	-	0.77 **	-	0.82 **	-	0.78 **	-	0.84 **
		21	-	0.78 **	-	0.71 **	-	0.85 **	-	0.75 **	-	0.79 **
		28	-	0.74 **	-	0.71 **	-	0.78 **	-	0.71 **	-	0.74 **
Chlorophyll content (SPAD)		7	-	0.99	-	0.97	0	1.00	+	1.01	-	0.99
		15	+	1.04	+	1.01	+	1.02	+	1.03	+	1.09 (*)
		21	-	0.99	-	0.92 (*)	+	1.01	+	1.02	+	1.02
		28	+	1.05 (*)	-	0.98	+	1.03	+	1.08	+	1.11 **
Plant growth												
Fresh weight	*AG*	28	0	1.00	0	1.00	-	0.88	+	1.10 **	0	1.00
Dry weight	*AG*	28	-	0.92 *	-	0.92	-	0.77 **	+	1.03	-	0.92
	BG	28	-	0.85	-	0.87 (*)	-	0.77	-	0.91	-	0.85
	total	28	-	0.90 (*)	-	0.91	-	0.77 *	-	0.99	-	0.89
Relative growth rate	*AG*	28	-	0.93 *	-	0.94	-	0.83 **	+	1.02	-	0.95
	BG	28	-	0.86	-	0.75 (*)	-	0.82	-	0.93	-	0.88
	total	28	-	0.92 (*)	-	0.92 (*)	-	0.83 *	0	1.00	-	0.93
Biomass ratio	*BG:AG*	28	-	0.92	-	0.89	-	0.98	-	0.90	-	0.93
	dwt:fwt	28	-	0.94	-	0.99	-	0.92	-	0.93	-	0.91

Table 2.7 – Species and population level relative ozone sensitivity (RS) for the rate of development of foliar injury assessed as senescence ($wpns_{coeff}$) and leaf chlorophyll content ($SPAD_{coeff}$). Ozone effects illustrated as a reduction in rate (-) or an increase in rate (+) under elevated ozone. RS values of <1 and >1 represent sensitivity and resistance respectively. Significant differences from the control are indicated by **=P<0.01, * = P<0.05 and (*) = P<0.1.

	$wpns_{coeff}$				$SPAD_{coeff}$			
	O_3(20)	O_3(150)	O_3 effect	RS	O_3(20)	O_3(150)	O_3 effect	RS
Species	0.53	1.30	+	0.41 **	-0.59	-0.55	-	1.07
Population								
Aber	0.87	1.75	+	0.50 **	-0.68	-0.73	+	0.92
Birds Rock	0.35	0.92	+	0.38 **	-0.51	-0.45	-	1.12
Cwm Idwal	0.53	1.39	+	0.38 **	-0.57	-0.51	-	1.10 (*)
Maltraeth	0.37	1.15	+	0.32 **	-0.60	-0.50	-	1.17

2.4 Discussion

Plants exhibit varying chronic responses to oxidative stress imposed by exposure to high ozone concentrations. Ozone sensitivity can affect competitive fitness (Schraudner *et al* 1997) and has implications for species longevity and community and ecosystem composition. In addition to interspecific differences, many species exhibit intraspecific variation in response with sensitive and resistance clones, ecotypes and populations (Kollner and Krause 2002, Pasqualini *et al* 2002, Burkey *et al* 2005, Manninen *et al* 2003). This intraspecific variation is an important factor in determining the ozone sensitivity of a species in relation to the potential impacts of increasing tropospheric ozone concentration at both the species and community level. This study demonstrates the presence of intraspecific variation in chronic response to ozone exposure in a widespread grass species, *A. odoratum*, over a relatively small geographic range. The results are discussed first in relation to the experimental design and received ozone exposure, second in terms of the species level response and finally in terms of the evidence for intraspecific variation.

2.4.1 Experimental design and received ozone exposure

Solardomes allow ozone exposure under climatic conditions closer to ambient than indoor growth chambers with temperature fluctuations and day length following the ambient conditions. However, there is still a potential chamber effect. The solardome environment has been found to reduce irradiance in the photosynthetically active radiation (PAR) region of the spectrum by 18% despite the use of high quality UV-B transmitting glass (Rafarel *et al* 1995). An increase in temperature is also expected although this is not believed to exceed 2 °C above ambient due to constant air flow through the solardome. Chamber effects during ozone exposure in open-top chambers (OTC) have been reported for some species including wild strawberry, *Fragaria vesca* (Manninen *et al* 2003) and European beech, *Fagus sylvatica* (Zeuthen *et al* 1997). Nussbaum and Fuhrer (2000) found that chamber effects were species specific in six grassland species and suggested the OTC environment altered the microclimatic conditions and affected stomatal resistance and ozone uptake. It is likely that the solardome environment has a similar chamber effect to that in OTCs. The chamber effect was not investigated in this study and was assumed to be consistent between solardomes.

In addition to the chamber effect, plant responses may differ between pot- and ground-grown plants and those in a monoculture or community due to differences in root restriction, canopy shading and competition. In a comparative study of pot- and ground-grown soybean, *Glycine max*, Booker *et al* (2005) found higher stomatal conductance and above ground biomass in pot-grown plants while both had a similar response to ozone exposure, although the ground-grown plants exhibited reductions for more biomass components. In an earlier study, *Plantago major* exhibited restricted growth with small pot size (Whitfield *et al* 1996). However, these plants best resembled the growth form of those observed in the field and it was concluded that although pot-grown plants may experience restricted root growth this would also be expected under competition in the natural environment and that a realistic assessment of ozone effect should involve some degree of root restriction (Whitfield *et al* 1996). Plants in a pot-grown monoculture also experience greater edge effects and higher levels of ozone exposure than those in a community (Wilbourne *et al* 1995). The relatively small pot

size used in this study may have caused some restricted growth, although the effects on below ground growth are likely to be small as plants were harvested at a relatively young age of 10 weeks after propagation. In addition, the spacing of pots 5 cm apart to reduce canopy growth competition would have increased the edge effects experienced by each plant.

Chamber effects, pot related growth restrictions and edge effects all have a potential impact on the observed response to elevated ozone. However, these effects should be consistent between solardomes and the randomisation and rotation procedures applied should have minimised any confounding effects of within solardome position. The comparison between treatments should therefore provide an adequate assessment of relative ozone sensitivity.

Plants were exposed to acute ozone episodes of a constant 150 ppb for five days per week with an AOT40 calculated over a 24 hd^{-1} period (AOT40 (24 hd^{-1})) of 51.9 ppm.h after 28 days. A 24 hd^{-1} period was used as ozone concentrations were maintained overnight. An acute ozone exposure was applied to screen populations of *A. odoratum* for intraspecific variation. However, the responses may not be as ecologically significant as those obtained with a longer-term chronic ozone exposure (Franzaring *et al* 2000).

The system failure in the first week of exposure resulted in a high peak of ozone received overnight in one replicate solardome of the $O_3(150)$ treatment. Despite the high concentrations reached the potential effect on the observed responses may have been mitigated by low nocturnal ozone uptake. However, Musselman and Minnick (2000) illustrated that although stomatal conductance is generally lower at night a considerable ozone flux can still occur. A large effect of this peak would be expected to result in greater variation in the $O_3(150)$ treatment compared to the control. The results for foliar injury and plant growth do not indicate any such increased variation and it is therefore considered to have had a minimal impact on the observed responses.

2.4.2 *A. odoratum* response to elevated ozone

Increased/premature senescence was observed after seven days of ozone exposure in the O_3(150) treatment where the accumulated ozone dose was 19.4 ppm.h AOT0 (24 h) and 13.9 ppm.h AOT40 (24 h). This response was observed throughout the exposure period with the rate of development of senescence more than doubled under elevated ozone. Leaf senescence under elevated ozone developed in the same manner as under control conditions progressing from the distal end to the proximal end of the lamina. *A. odoratum* has previously been shown to exhibit premature senescence under chronic ozone exposure after four weeks (Hayes *et al* 2006) and was classified as an intermediate species for ozone sensitivity by Nebel and Fuhrer (1994) after nine days exposure to daytime peaks of 100 ppb to 150 ppb. Although increased/premature senescence is an unspecific form of ozone induced foliar injury it is considered a typical response in many wild plant species (Bergmann *et al* 1995, Bergmann *et al* 1999, Franzaring *et al* 2000) and was observed in 67% of the Poaceae species screened for ozone sensitivity by Bermejo *et al* (2003).

A variety of different methods have been used for the determination of increased/premature senescence in wild plant species including visual assessment, ratio of the number senescent leaves to total leaves, dry weight of senescent leaves and ratio of green biomass to senescent biomass and chlorophyll content (Hayes *et al* 2006, Bermejo *et al* 2003, Ommen *et al* 1999). Visual assessment was used in this study as it is a non-destructive assessment method where leaf counts are impractical. However, visual assessment of either ozone specific foliar injury or unspecific senescence is a subjective measurement and is prone to assessor bias (Bussotti *et al* 2003). To reduce this bias, assessments were made by one assessor following training and using a standardized methodology.

The accelerated rate of senescence under exposure to elevated ozone may be due to the induction of senescence-associated genes and promoted cell death (Miller *et al* 1999, Pell *et al* 1997). Increased senescence reduces the green leaf area and can result in reductions in photosynthesis and the resources available for growth (Vandermeinen *et al* 2005, Gay and Thomas 1995). However, ozone induced foliar injury is not always

associated with a reduction in plant growth and in some species growth responses occur without the development of visible injury (Franzaring *et al* 2000, Inclan *et al* 1999, Woo and Hinckley 2005, Hayes *et al* 2006, Bender *et al* 2006). This may be a result of lower resource allocation to the roots while maintaining shoot biomass under elevated ozone (Grantz *et al* 2006). The loss of root reserves may result in longer-term reductions in plant vitality and competitive ability especially in perennial species. This is supported by Hayes *et al* (2006) where *Galium sacatile*, *Nardus stricta* and *Saxifraga stellaris* exhibited no effect on shoot growth after exposure but a reduction in shoot regrowth occurred following overwintering, although root biomass was not assessed.

Leaf chlorophyll content (SPAD value) decreased during the exposure period although the response to ozone exposure varied between assessment weeks with a slight increase observed after two and four weeks and a trend for a lower reduction under elevated ozone. The relatively small change and inconsistency of response between weeks is consistent with previous studies on white clover, *Trifolium repens* (Kollner *et al* 2002) and five Egyptian semi-natural vegetation species (El-Khatib 2003). The trend for a lower rate of chlorophyll reduction under elevated ozone does not reflect the increased rate of senescence. Differences in leaf age, anatomy and the amount of senescence may have influenced the obtained SPAD values. The higher rate of senescence may have resulted in increased leaf turnover under elevated ozone with a younger relative leaf age at the second expanded leaf position and a higher chlorophyll content. Younger leaf age has been related to higher chlorophyll content in *Lolium temulentum* (Gay and Thomas 1995). Although measurements were made on the mid lamina which was generally not senescent, leaf morphology may have altered with the development of senescence at the distal end. Changes in leaf anatomy in response to elevated ozone have been demonstrated in *Betula pendula* (Paakkonen *et al* 1995, Oksanen *et al* 2005). Differences in leaf morphology and anatomy may have altered the measurement path length for determination of SPAD value and resulted in inaccurate assessment of chlorophyll content.

Total biomass dry weight and relative growth rate were decreased after 28 days exposure with reductions in both above ground and below ground growth. The decrease was greatest for root growth although only shoot growth reductions were significant. The non-significance of root growth reductions were attributed to greater variation in

biomass measurements for this component which may reflect some loss of fine root biomass and inconsistency during soil removal. The larger root growth reductions indicate a shift in resource allocation and this was reflected in the trend for a reduction in BG:AG ratio under acute ozone exposure. This result is supported by Grantz *et al* (2006) where a meta-analysis on combined data for 60 species exposed to both acute and chronic ozone exposures found significant reductions in the root:shoot allocation coefficient (*k*) in 50 % of species and in 9 of the 14 monocot species included.

Above ground fresh weight did not change under elevated ozone despite the reduction in dry weight of this component. This was reflected in the trend for reduction in dwt:fwt ratio and indicates a greater water content under elevated ozone. This may be related to changes in tiller morphology with resources allocated to the production of new tillers rather than the growth of existing tillers as foliar injury increased. A greater number of small tillers equated to an increased leaf to stem ratio in *Festuca arundinacea* (Sugiyama 1995) and reductions in tiller sheathing base size have been related to increased defoliation in *Nardus stricta* (Grant *et al* 1996). A greater leaf to sheath ratio may result in a lower dry matter density (DMD) and greater water content as DMD decreases from leaf base to leaf tip with cell elongation (Maurice *et al* 1997). However, few studies have considered tillering in response to ozone and results from spring wheat cv. Minaret suggest there is very little effect (Ewert *et al* 1999). Further investigation of tillering and tiller morphology in relation to ozone exposure is necessary as it may effect not only plant development, biomass production and resource allocation but physiology as tiller size has been related to within plant carbon availability (Sugiyama 1995).

A significant ozone dose response relationship between relative senescence and relative AOTX occurred through the exposure period. The significance of the relationship did not differ between the threshold values of 0 ppb, 20 ppb and 40 ppb as the ozone concentrations in the $O_3(150)$ treatment during peak episodes always exceeded these thresholds. The quadratic form of the dose response relationship was attributable to the effect of natural development of senescence under control conditions and the finite nature of measuring senescence as a percentage. This result is comparable to foliar injury dose response functions reported for *Tanacetum vulgare* and *Trifolium arvense* based on one chronic ozone exposure (Bergmann *et al* 1999).

2.4.3 Intraspecific variation in ozone sensitivity

The four *A. odoratum* populations studied differed significantly for the majority of assessment criteria including the amount and rate of senescence and chlorophyll content, relative growth rate and both biomass ratios. This was consistent with the polymorphic morphological nature of this species (Hubbard 1992). The observed population differences may have been influenced by vegetative propagation (Schwaegerle 2005). However, common treatment of plants should have reduced any bias associated with vegetative propagation and differences in response to ozone exposure should result from parental population/genotypic effects.

Whole plant senescence under control conditions ranged from 1.5 % - 5 % at the start of exposure to 11.8 % - 26.4 % in week four. The populations differed significantly in the amount of senescence from week two onwards and rank from lowest to highest as Birds Rock (*BR*) < Maltraeth (*M*) < Cwm Idwal (*CI*) < Aber (*A*). The rate of development of senescence ($wpns_{coeff}$) also differed between populations and can be ranked as *BR*, *M* < *CI* < *A*. All of the populations exhibited a significant increase in senescence and reduction in healthy leaves throughout the exposure period and an increased rate of senescence under elevated ozone. Aber exhibited the greatest effects in the shortest time with a relative sensitivity (RS) of 0.71 after three weeks. At the end of exposure the populations rank by RS as *BR* < *M* < *CI*, *A* suggesting that higher natural levels of senescence relate to a greater response to acute ozone exposure.

The response in terms of the rate of senescence RS did not follow the same pattern with populations ranked as *A* < *CI*, *BR* < *M*. This may relate to the use of linear rather than non-linear regression to assess the rate of senescence. Aber exhibited an equal extent of senescence in weeks three and four indicating a peak and then reduction in the development of senescence over time. Early response followed by a reduction in the extent of senescence under elevated ozone has previously been reported in this species (Hayes *et al* 2006) and is related to the continual production of new leaves so that the rate of increase reduces over time. These results suggest that where higher natural rates of senescence are observed, the response to elevated ozone may occur earlier in exposure.

Increased / premature senescence results in a decrease in photosynthetic capacity (Gay and Thomas 1995) and a greater response earlier in the season may have a larger impact on resource accretion, overwintering potential and long-term competitive ability than slower responses. In this respect, Aber would be considered the most sensitive population. *A. odoratum* tends to lose the majority of leaves over winter (Schippers *et al* 1999) and the observed ozone induced premature senescence suggests that this state would be reached at an earlier date. The natural development of senescence may follow a logistic curve through a growing season and ozone exposure may alter the shape of this curve reaching the logarithmic and stationary phases at earlier dates. Areas of study which may prove useful in determining relative sensitivity in a longer-term chronic exposure include the time to reach the logarithmic phase, the rate of increase during this phase and the time to reach the stationary phase.

The leaf chlorophyll content (SPAD) differed among populations under control conditions throughout the exposure period with populations ranked as *M*, *CI* < *A* < *BR*. However, the relationship between SPAD value and actual chlorophyll content was not determined and these differences may relate more to variation in lamina morphology. A trend for reduced destruction of chlorophyll was observed under elevated ozone in the Birds Rock, Cwm Idwal and Maltraeth populations while Aber exhibited the opposite trend. The obtained SPAD values did not show a consistent response between weeks nor with the observed level of senescence, as discussed above. The results indicate some intraspecific variation in response but assessment of chlorophyll content using a destructive method may provide a clearer picture.

The populations differed significantly under control conditions for total and below ground RGR, BG:AG ratio and dwt:fwt ratio. Aber was significantly different from all other populations with the lowest RGR and BG:AG ratio and the highest dwt:fwt ratio. The differences in above ground dry weight were almost significant with populations ranked as *BR* < *M* < *CI* < *A*. The greatest intraspecific variation in relative ozone sensitivity was observed for plant growth. This is consistent with results for wild strawberry, *Fragaria vesca* (Manninen *et al* 2003) and *Plantago major* (Lyons *et al* 1997) where populations differed in growth response while there was little difference in or no visible injury response respectively.

The relative sensitivity ranking of populations for growth response differed between the measurement criteria. In terms of total biomass dry weight and RGR, the Birds Rock population exhibited the greatest reductions while Cwm Idwal showed no response to elevated ozone and the populations rank by RS as *BR* < *A*, *M* < *CI*. The RS ranking did not alter for above ground growth measured as either dry weight or RGR and the Cwm Idwal population was the most resistant with a slight non-significant stimulation of above ground growth. The RS ranking of populations for below ground growth measured as dry weight was similar to that obtained for total and above ground growth. However, this differed when measured as RGR with the greatest reductions observed in Aber and a RS ranking of *A* < *BR* < *M* < *CI*. The results for above ground growth response in the Cwm Idwal population are consistent with those obtained for plants sourced from the same locality under chronic ozone exposure, where little change in growth occurred despite significant increases in senescence (Hayes *et al* 2006). In the current study, the small response in shoot growth in this population was concurrent with a trend for reduction in root growth. A greater response to elevated ozone in root growth compared to shoot growth was also observed in Aber and Maltraeth. This was reflected in the non-significant reductions in BG:AG ratio observed in these populations suggesting a shift in resource allocation to shoot growth under elevated ozone (Grantz *et al* 2006). However, in the most sensitive population, Birds Rock, no shift in resource allocation was observed as the above and below ground components were equally sensitive to ozone exposure.

The foliar injury ozone dose response relationship differed among populations. The Aber, Cwm Idwal and Maltraeth populations all exhibited significant response relationships suggesting that the development of premature senescence in these populations was more highly related to the received ozone dose than in the Birds Rock population. This is consistent with the Birds Rock population ranking as the least sensitive of in terms of foliar injury. The quadratic nature of the response relationship related to the development of natural senescence under control conditions with an almost linear relationship in the Maltraeth population corresponding to the slowest rate of natural senescence. The among population differences in dose response are comparable to variation in foliar injury dose response between semi-natural vegetation species (Bergmann *et al* 1999). Investigation of dose response in terms of plant growth would provide further elucidation of the intraspecific variation in *A. odoratum*.

However, this was not possible in the current study due to time and space limitations precluding the use of additional rametes for destructive harvest through the exposure period.

The classification of population ozone sensitivity was dependant upon the assessment criteria used with Aber exhibiting the greatest sensitivity for foliar injury while Birds Rock was the most sensitive and Cwm Idwal the least sensitive in terms of plant growth. As with previous studies comparing ozone sensitivity between semi-natural vegetation species, there was no clear relationship between the development of foliar injury and the observed plant growth response (Bergmann *et al* 1995, Warwick and Taylor 1995, Power and Ashmore 2002, Hayes *et al* 2006). The complexity of intraspecific response to ozone exposure among populations of *A. odoratum* as observed in the current study, is consistent with previous reports of intraspecific variation in *Trifolium repens* and *T. pratense* (Nebel and Fuhrer 1994), *Phleum pratense* (Danielsson *et al* 1999), *Fragaria vesca* (Manninen *et al* 2003) and most notably in *Plantago major*, the most extensively studied semi-natural vegetation species in terms of ozone sensitivity (Reiling and Davison 1992c, Reiling and Davison 1995, Pearson *et al* 1996, Lyons *et al* 1997, Whitfield *et al* 1997). These results highlight the difficulty in the classification of ozone sensitivity at both the species and population level, especially when focused on single assessment criteria such as foliar injury, and demonstrate the importance of considering intraspecific variation in the assessment of species specific ozone sensitivity and for interspecific comparison.

Chapter 3: Effects of simulated current and future rural ozone profiles on *Anthoxanthum odoratum*

3.1 Introduction

In the UK ozone concentrations are highest during the spring and summer months corresponding to the growing season for the majority of vegetation (Coyle *et al* 2003a) and critical levels for adverse effects have been developed for semi-natural vegetation based on a cumulative concentration approach using AOT40. In Northern Europe critical levels are set at an AOT40 of 3 ppm.h accumulated over three months from mid-April to mid-July for communities dominated by annual species and an AOT40 of 5 ppm.h over six months from mid-April to mid-October for communities dominated by perennial species (LRTAP Convention, 2007). Ozone concentrations currently exceed these critical levels across much of the UK (Coyle *et al* 2002).

Ambient ozone conditions in rural areas of the UK are typified by background concentrations of around 25 ppb and peak concentrations during photochemical episodes of up to 150 ppb although concentrations of 70 ppb to 100 ppb are more common (NEGTAP 2001, Figure 3.1). Although the magnitude of peak concentrations tend to be similar across the UK, background concentrations increase with altitude to around 35 ppb in upland rural areas (NEGTAP 2001). This is due to the higher wind speeds in these areas and more turbulent air conditions resulting in greater mixing down of ozone-rich air into the Planetary Boundary Layer (PBL) replacing ozone removed through deposition (Coyle *et al* 2002, Ashmore *et al* 2002). In addition to spatial variation, ozone concentrations also exhibit diurnal cycles with maximum concentrations occurring in the afternoon and minimum concentrations between midnight and dawn in rural areas (Coyle *et al* 2002). The extent of variation across the diurnal cycle is dependant on the levels of photochemical ozone production and destruction, entrainment from the free troposphere, deposition, local topography and meteorological conditions. On average ozone concentrations are reduced by 2.6 ppb to 10.4 ppb overnight in upland and rural areas respectively (Coyle *et al* 2002). However,

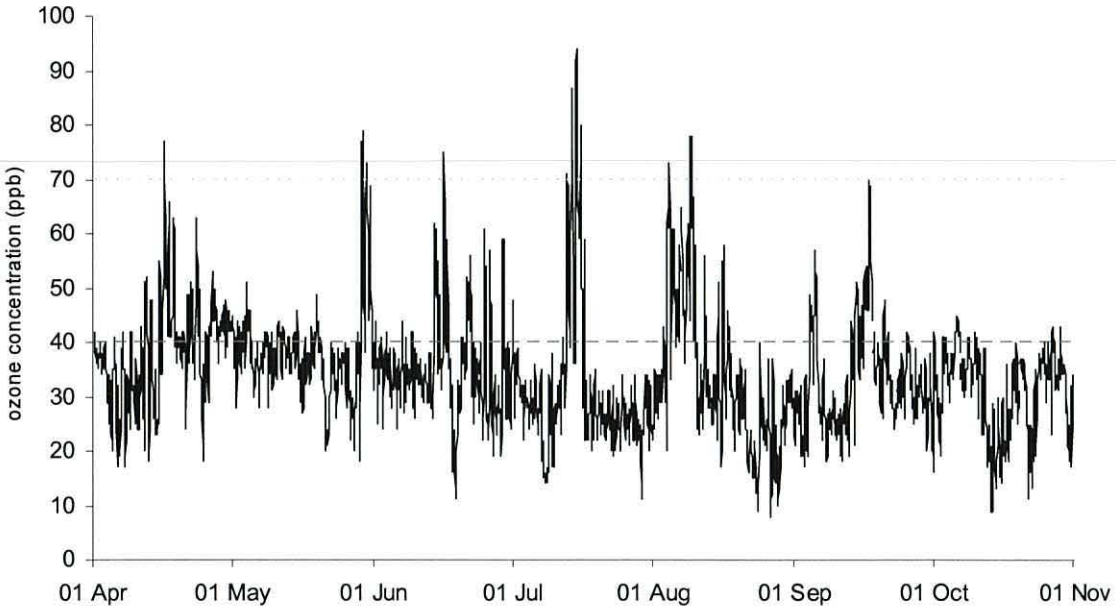

Figure 3.1 – Hourly ozone concentrations at the rural monitoring site in Aston Hill, UK during April to November 2003. Horizontal lines indicate concentrations of 40 ppb and 70 ppb. (Data obtained from www.airquality.co.uk).

diurnal cycles are most pronounced during the summer months when high levels of UV-radiation and more hours of daylight allow greater photochemical production of ozone (Ashmore *et al* 2002) and peak concentrations of greater than 40 ppb above the background are often observed during ozone episodes (Figure 3.1, Figure 3.2).

Tropospheric ozone concentrations have more than doubled over the last century (Volz and Kley 1988, NEGTAP 2001) and are predicted to continue rising though this century (NEGTAP 2001, Vingarzan 2004). Over the last 30 to 40 years annual mean and maximum UK ozone concentrations have revealed a general trend for a decline in peak concentrations and an increase in background concentrations (NEGTAP 2001). The reduction in peak concentrations is due to the implementation of air pollution polices across Europe reducing emissions of Nitrogen oxides and Carbon monoxide (Vingarzan 2004) while increasing background concentrations result from an increase in anthropogenic emissions on a global scale (NEGTAP 2001, Vingarzan 2004). Background ozone concentrations in the UK are predicted to reach 30 ppb to 40 ppb in rural areas resulting in a doubling of AOT40 values by 2030 and to continue increasing to 40 ppb to 50 ppb by 2100 (Coyle *et al* 2003b). Future ozone concentrations are

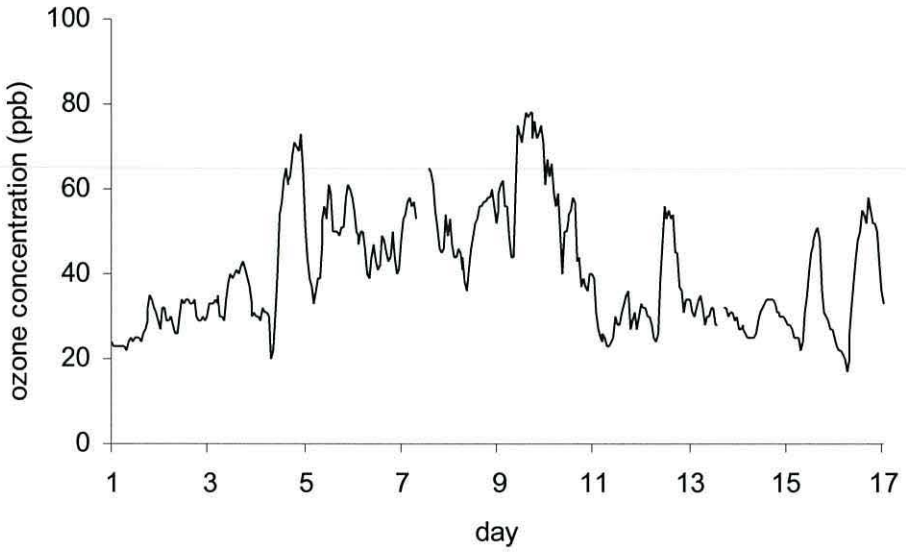

Figure 3.2 – Hourly ozone concentrations at the rural monitoring site in Aston Hill, UK during an ozone episode in the period 1st August to 17th August 2003. (Data obtained from www.airquality.co.uk).

expected to result in a reduction in the magnitude of acute ozone exposure but an increase in the long term chronic exposure for vegetation (NEGTAP 2001).

The response of semi-natural vegetation to elevated background concentrations requires further investigation as the majority of previous studies have included peak episodes rather than maintained background concentrations during ozone exposure (Ashmore *et al* 2002, Coyle *et al* 2003b). The aim of this study was to investigate the comparative effects of ambient peak episodes, elevated background concentrations and the combination of elevated background and peak episodes. The peak episodes applied were a stylised version of a peak episode recorded at the rural monitoring site Aston Hill, UK in August 2003 (Figure 3.2). The effects of these contrasting ozone scenarios were investigated in the perennial, tufted grass species *Anthoxanthum odoratum* L. in terms of visible injury and plant growth. Intraspecific variation in ozone response and stomatal conductance were compared among four populations from North Wales, UK previously investigated for response to acute ozone exposure (Chapter 2).

3.2 Materials and Methods

3.2.1 Plant material

Twenty rametes per individual were vegetatively propagated in May 2005 from stock plants collected from four populations in North Wales in June-July 2004 (see Chapter 1, section 1.4 for details of sample collection). Plants were grown in an ericaceous-sand mix compost (ratio 3:2, litres:kg) at the Pen-y-Ffridd Research Station, Bangor. Rametes were established from single tillers of similar size in a heated glasshouse (day 18 °C and night 16 °C) with supplementary lighting, transplanted after three weeks and transferred to a cool, unlit glasshouse. At five weeks rametes were transplanted into one litre 10 cm square pots and treated with fungicides Bavistan and Rovral (active ingredients (AI): carbendazim and iprodione, BASF) against rust and mildew infection, allowed to recover for one week and then transferred to a sheltered outside position under ambient conditions. At eight weeks ramete size was determined by tiller number. Seventy six individuals, comprising 18 to 20 per population, were selected on the basis of plant health and ramete size uniformity. Twelve rametes per individual were selected and at 10 weeks the eight rametes allocated for ozone exposure were transferred to the CEH ozone exposure facility, Abergwyngregyn. The remaining three rametes were maintained at the Pen-y-Ffridd Research Station under ambient conditions for determination of stomatal conductance.

3.2.2 Ozone exposure

3.2.2.1 Ozone exposure system

Plants were exposed to ozone in eight replicate ventilated hemispherical glasshouses (solardomes) two metres high and three metres diameter receiving approximately two complete air changes per minute. Ozone was generated by a G11 ozone generator from

oxygen supplied by a Workhorse 8 oxygen generator (both Dryden Aqua, UK Ltd) and delivered to the solardomes by a computer controlled (Labview, version 7) mass-flow controller system. Ozone was added to charcoal filtered air and concentrations within the solardomes monitored and recorded on a 30 minute cycle by two API400 ozone analysers (Envirotech) of matched calibration. Hourly mean ozone concentrations (ppb) were calculated from half hourly measurements and used to calculate the accumulated ozone over a threshold of X ppb (AOTX ppm.h) for 12 daylight hours (7 am to 7 pm) and 24 hours per day over the experimental period. Weekly AOTXs were calculated from the start of ozone exposure to 9 am on the corresponding assessment day.

3.2.2.2 Ozone treatment profiles

Four ozone treatments were applied as weekly profiles in a factorial design with two background levels combined with two peak episode levels. Levels are described as low (L) or high (H) with treatments designated by background level * peak level, for example low background * high peak (LH). Background concentrations were 20 ppb (L) or 45 ppb (H) increasing by 5 ppb during daylight hours (9 am to 8 pm) and peak concentrations were elevated by 0 ppb (L) or 50 ppb (H) above the background level. Weekly ozone profiles comprised six days of ozone exposure with background concentrations maintained throughout and an ozone episode with four consecutive days of peak concentrations during daylight hours (Figure 3.3), and a seventh day in which plants received charcoal filtered air to allow access to the solardomes for measurements.

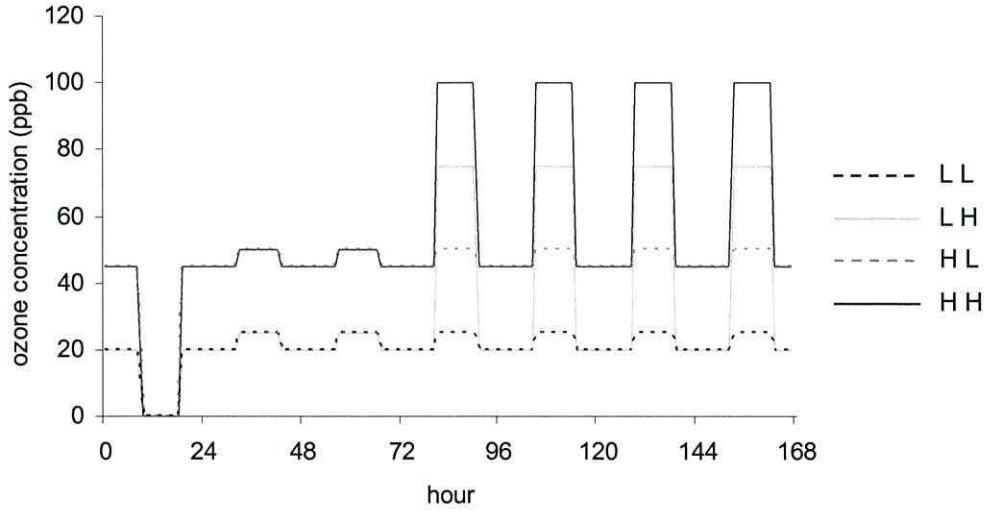

Figure 3.3 – Weekly ozone treatment profiles for low background * low peak (LL), low background * high peak (LH), high background * low peak (HL) and high background * high peak (HH).

3.2.2.3 Experimental design

Solardomes were split into two randomised blocks of four treatments. Plants were randomly assigned to solardomes with one ramete per individual in each solardome. Plants were arranged on trays (0.5m x 0.4m) lined with sand and with drainage holes 10 mm above the base. Tray positions were duplicated among solardomes. Within each solardome plants were randomly assigned to 76 positions across four trays. The 19 plants per tray were arranged in five rows and orientated with row one to five representing outer to inner of the solardome and relative distance from the air inlet tubing. Plants were transferred to the solardomes 10 weeks after propagation and allowed to acclimatise at 20 ppb for four days prior to ozone exposure. Plants were exposed to ozone treatments for 12 weeks from 8th July to 27th September 2005, receiving 12 simulated ozone episodes.

Plants were irrigated with a timed automatic misting system for 30 to 60 minutes every night. The duration was adjusted to account for changes in canopy size and climatic conditions over the exposure period with supplemental watering by hand as necessary. Plants were sprayed with the commercial pesticides Provado® Ultimate Bug Killer Concentrate (AI: imidichloprid, Bayer Garden) and No-Fid® (AI: nicotine, Certis) in weeks six and seven of exposure to control aphid infestation and Radar (AI: propiconazole, INTEG®) in week eight to control rust infection. Nutrients were applied at half strength after six ozone episodes ('phostrogen', PBI Home and Garden, UK).

3.2.2.4 Visual assessment

Plants were visually assessed for percentage of necrotic senescence throughout the exposure period. Whole plant senescence was assessed as the percentage of leaves with $\geq$ 25% necrotic senescence after three, five, eight and eleven ozone episodes. On each plant one central tiller with three or more fully expanded leaves was tagged with coloured thread prior to ozone exposure for assessment of individual leaf senescence. The second and third leaves down from the flag leaf were assessed biweekly for percentage of senescence, starting after two episodes for the second leaf and four episodes for the third leaf. Once this tiller had become fully senesced a new tiller was tagged following the same criteria and the change of tiller was recorded.

3.2.2.5 Plant growth assessment

Plants were destructively harvested to soil level after 12 ozone episodes and the above ground biomass dried at 65 °C for at least four days for dry weight biomass assessment. Post-exposure tiller number was determined from dried plant material. Relative tillering (R_{till}) was calculated for each plant using pre- and post-exposure tiller number as:

$$R_{till} = (\ln tn_2 - \ln tn_1)/(t_2 - t_1)$$

where tn is tiller number, t is time (wk) and subscript denotes measurement week or harvest date.

3.2.2.6 Relative ozone sensitivity

A relative ozone sensitivity index was determined for comparison of ozone effects between different assessment criteria and populations. Foliar injury assessed as the percentage of whole plant necrotic senescence was converted to the percentage of healthy leaves for this purpose as:

$$\% \text{ healthy leaves} = 100 - \% \text{ whole plant senescence}$$

Relative sensitivity (RS) of plant growth and individual week foliar injury variables were calculated using treatment means as the proportion under elevated ozone low background * high peak (LH), high background * low peak (HL) and high background * high peak (HH) treatments relative to the control (low background * low peak (LL)) where:

$$RS = 1 + ((O_{3\ elevated} - O_{3\ control}) / O_{3\ control})$$

The relative ozone sensitivity index describes the effects of elevated ozone in relation to the control with a relative sensitivity of 1 indicating no response, <1 indicating sensitivity or reduction and >1 indicating resistance or stimulation for plant growth and % healthy leaves.

3.2.3 Stomatal conductance

Three rametes per individual were maintained at the Pen-y-Ffridd Research Station in a sheltered outside position under ambient conditions during the 12 weeks of the ozone exposure experiment then transferred to a heated glasshouse (day 18 °C and night 16 °C) with supplementary lighting. Plants were completely randomised and allowed to acclimatise for two weeks. One central tiller with three or more fully expanded leaves was tagged with coloured thread on every plant. Stomatal conductance (g_s) was measured using a cycling porometer AP4 (Delta-T Devices Ltd, UK) on the mid lamina abaxial and adaxial surfaces of the second and third leaves down from the flag leaf on the tagged tiller. For each plant and leaf surface g_s (mmol m^{-2} s^{-1}), leaf temperature (°C), leaf irradiance (μmol m^{-2} s^{-1}) and time of measurement were recorded.

3.2.4 Statistical analysis

3.2.4.1 Ozone effects

Arcsine transformation was performed on individual week senescence data and natural log transformation on above ground dry weight biomass data. Values for the 18 to 20 plants per population within each dome were averaged to provide two replicates per treatment per population and the population dome averages for each variable used for all subsequent analysis. Variables were analysed for main and interaction effects of background ozone level, peak ozone level and population using a factorial analysis of variance (ANOVA) model with a randomized block design in Genstat version 8. ANOVA model for overall and population level analysis given in Table 3.1. Populations exhibited some inequality of variance which may have violated the assumptions of the analysis model and were reanalysed separately for main and interaction effects of background and peak ozone levels. Ozone dose response was analysed by regression of whole plant senescence against AOTX using treatment means for individual assessment weeks and across the entire exposure period using Minitab version 14.

Table 3.1. – ANOVA model for overall and individual population analysis.

Overall		Individual populations	
Source of variation	**DF**	**Source of variation**	**DF**
Block	1	**Block**	1
Background‡	1	**Background**‡	1
Peak‡	1	**Peak**‡	1
Background x Peak‡	1	**Background x Peak**‡	1
residual	*3*	*residual*	*3*
Population‡	3	**Total**	7
Background x Population‡	3		
Peak x Population‡	3		
Background x Peak x Population‡	3		
residual	*12*		
Total	31		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

3.2.4.2 Stomatal conductance

Values for the three replicate rametes per individual were averaged to give 18 to 20 individual means per population. Data from leaf temperature (°C), leaf irradiance (μmol m^{-2} s^{-1}) and time of measurement were investigated for inclusion as covariates by one-way ANOVA for among population differences. Natural log transformation was performed on stomatal conductance data. Species level and individual population stomatal conductance were analysed separately for effects of the four leaf surfaces (leaf two adaxial, leaf two abaxial, leaf three adaxial and leaf three abaxial) using one-way ANOVA with Tukey HSD test for post-hoc multiple comparisons. Stomatal conductance for each leaf surface was analysed for population differences using one-way ANOVA. All analyses were performed in Genstat version 8.

3.3 Results

3.3.1 Ozone exposure

3.3.1.1 Received ozone exposure

Average total ozone concentrations received by all treatments across the exposure period were lower than expected due to low concentrations during weeks one, seven and eight (Table 3.2). In the first week of exposure the average concentrations ranged between 10 ppb and 30 ppb due to stabilisation of the ozone supply system over the first five days of exposure. In weeks seven and eight there were interruptions in the ozone supply to the solardomes with plants receiving charcoal filtered air with <10 ppb ozone and ozone episodes of one and two day duration respectively (Figure 3.4). Excluding

Table 3.2 – Total and weekly average ozone concentrations (ppb ±standard error of the mean) received for background and peak times during weekly profile for low background * low peak (LL), low background * high peak (LH), high background * low peak (HL) and high background * high peak (HH) ozone treatments.

	Ozone Treatment							
	LL (O_3(20,25))		LH (O_3(20,75))		HL (O_3(45,50))		HH (O_3(45,100))	
Week	bgnd	peak	bgnd	peak	bgnd	peak	bgnd	peak
1	10.1 ±0.6	21.3 ±1.2	10.3 ±0.6	26.1 ±2.3	14.7 ±1.4	24.4 ±1.3	15.4 ±1.6	27.9 ±3.0
2	15.4 ±0.3	19.8 ±0.3	14.5 ±0.3	55.8 ±1.8	31.6 ±0.7	38.0 ±0.6	36.0 ±0.7	82.6 ±1.8
3	15.4 ±0.3	19.8 ±0.2	15.0 ±0.3	55.8 ±1.6	29.4 ±0.6	36.4 ±0.5	36.5 ±0.8	82.5 ±1.9
4	14.8 ±0.3	19.5 ±0.2	14.8 ±0.3	55.3 ±1.6	27.5 ±0.7	35.2 ±0.4	34.4 ±0.8	80.8 ±1.8
5	15.2 ±0.5	17.6 ±1.2	15.9 ±0.5	47.4 ±3.9	29.8 ±1.2	35.2 ±2.6	32.8 ±1.4	75.3 ±5.4
6	14.5 ±0.7	23.0 ±0.3	15.6 ±0.7	65.5 ±2.0	32.6 ±1.7	49.6 ±0.5	31.6 ±1.6	91.2 ±1.9
7	6.8 ±0.6	7.6 ±0.8	6.5 ±0.6	16.7 ±3.7	12.3 ±1.4	12.8 ±1.7	9.4 ±1.2	19.2 ±4.7
8	12.8 ±0.6	9.7 ±0.9	14.1 ±0.6	28.7 ±4.2	25.3 ±1.4	16.3 ±2.1	26.2 ±1.5	37.4 ±5.9
9	13.4 ±0.5	22.7 ±0.7	13.6 ±0.7	73.3 ±2.1	27.3 ±1.5	47.1 ±1.3	24.0 ±1.6	94.2 ±2.2
10	17.7 ±0.4	18.1 ±0.7	19.9 ±0.4	63.3 ±3.1	39.2 ±0.9	39.1 ±1.5	38.5 ±0.9	83.5 ±4.0
11	17.4 ±0.4	22.2 ±0.2	18.5 ±0.4	62.1 ±1.6	36.2 ±1.1	42.6 ±0.6	37.7 ±1.0	84.8 ±1.7
12	16.6 ±0.5	23.7 ±0.3	17.4 ±0.5	67.4 ±1.7	34.5 ±1.2	46.2 ±0.6	36.5 ±1.2	91.5 ±1.8
Total	14.3 ±0.2	18.9 ±0.3	14.8 ±0.2	52.3 ±1.0	28.9 ±0.4	35.7 ±0.6	30.5 ±0.4	72.1 ±1.4

these weeks, the low and high background concentrations were within 5 ppb and 15 ppb of the target concentration respectively and were consistent between treatments at each level. The concentrations reached during ozone episodes in the high peak treatments were on average 40 ppb to 45 ppb above the corresponding background level. Although the target concentrations were not met, the received ozone exposure remained stable and maintained the factorial treatment structure for the early season (weeks two through six) and late season (week nine onwards) of the 12 week exposure period. The intended weekly ozone treatment profile including a four-day ozone episode was applied for half the exposure period, with interruptions in weeks one, seven and eight and a five-day episode applied from week 10 onwards.

The received ozone dose, measured as accumulated ozone over a threshold of X ppb (AOTX) increased in a linear fashion for weeks two through six and showed a greater increase from week eight onwards reflecting the extra day of peak ozone episode received in weeks 10 to 12 (Figure 3.5). After 12 weeks of exposure the HH treatment had received 82.4 ppm.h or 23.4 ppm.h for AOT0 and AOT40 respectively calculated over a 24 hour per day (hd^{-1}) period. The received ozone dose, calculated with a threshold of 0 ppb, showed a progression from low background to high background treatments with a larger difference between treatments at the different background levels than between those with or without peak episodes at the same background level.

A larger difference in AOT0 was observed between treatments at the same background level than between the LH and HL treatments, which received a similar ozone dose of 49.7 ppm.h and 59.9 ppm.h respectively. The received ozone dose measured as AOT40 was greater in the high peak treatments with the amount doubling from the HL treatment (4.1 ppm.h) to the LH (10.6 ppm.h) and doubling again for the HH treatment (23.4 ppm.h). The threshold values of 0 ppb and 40 ppb attribute differing importance to high background and peak episodes in the calculation of ozone dose.

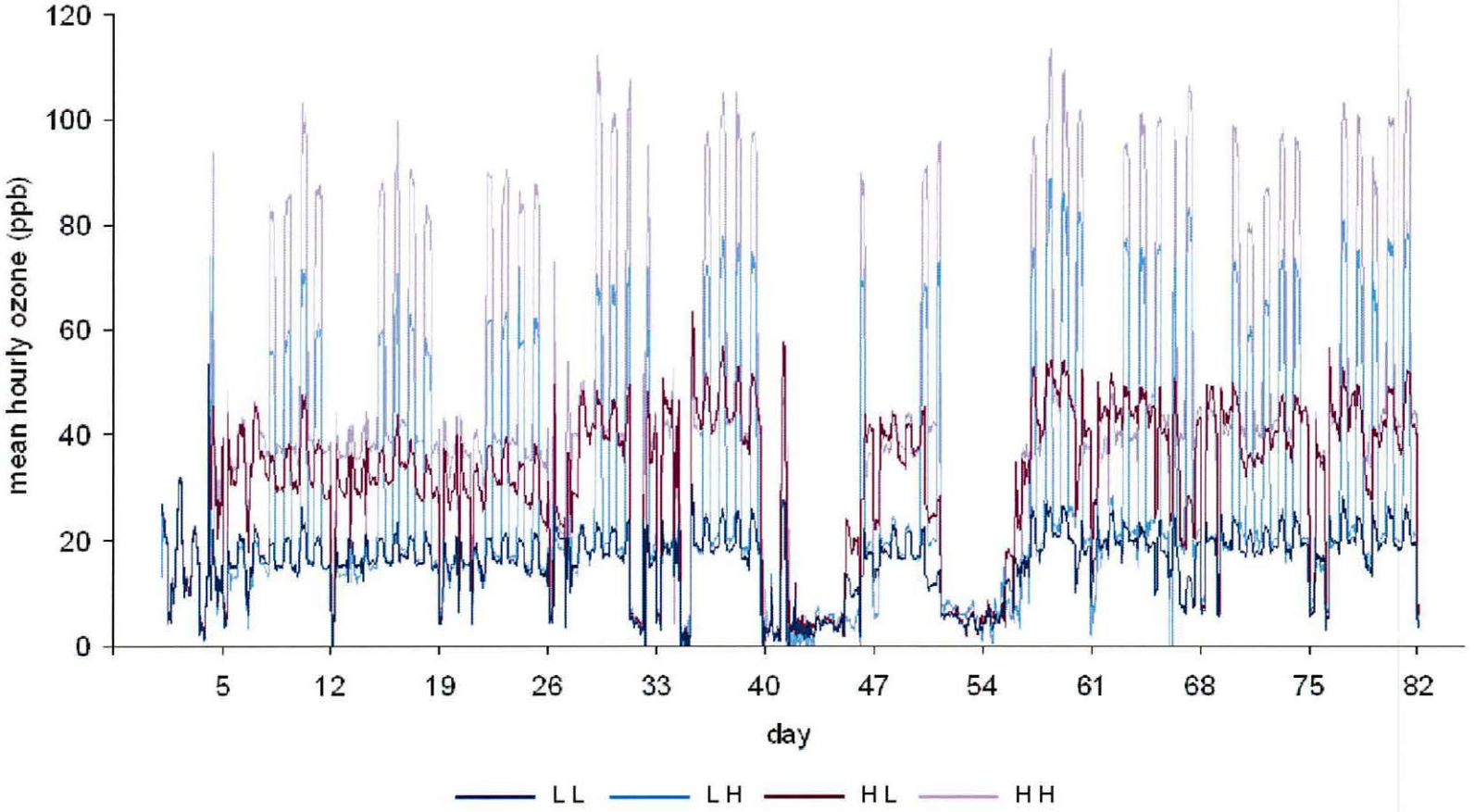

Figure 3.4 – Hourly mean ozone concentration (ppb) received over 12 weeks of exposure for low background * low peak (LL), low background * high peak (LH), high background * low peak (HL) and high background * high peak (HH) ozone treatments.

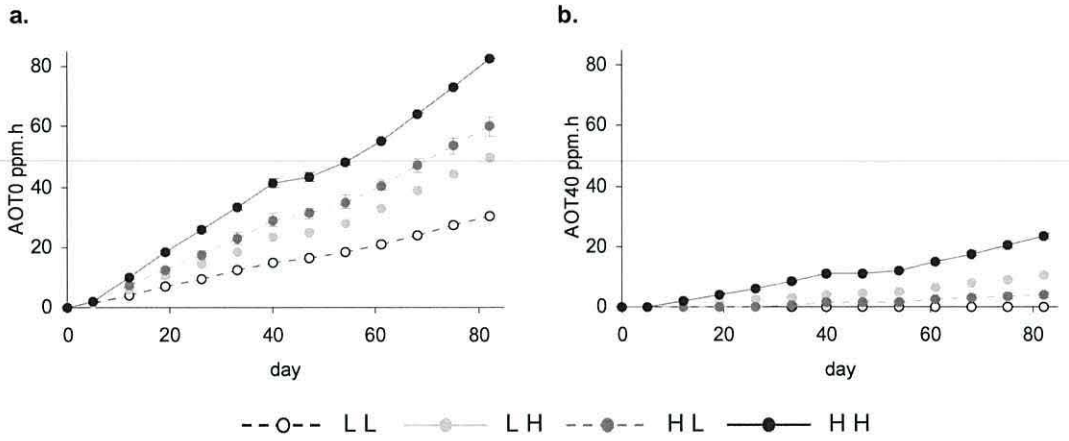

Figure 3.5 – Mean weekly accumulated ozone (ppm.h) over a 12 week exposure period for low background * low peak (LL), low background * high peak (LH), high background * low peak (HL) and high background * high peak (HH) ozone treatments. Ozone accumulated over a threshold of a) 0 ppb and b) 40 ppb for 24 hours per day. Bars represent the standard error of the mean.

3.3.1.2 Visual assessment

Increased/premature whole plant senescence was observed under elevated ozone at the species level. Summary of overall ANOVA results are given in Table 3.3 and full details in Appendix II. The most pronounced differences occurred between background ozone levels for early season senescence with increases of 10.2 % ($P<0.01$) and 10.1 % ($P<0.05$) under high background for weeks three and five respectively (Figure 3.6 a). Although the amount of senescence increased with the application of peak ozone episodes this was only significant to the $P<0.1$ level for a 4.9 % increase in week three (Figure 3.6 b). The increase in senescence with peak ozone episode at each background level was half that observed between background levels with the same peak treatment applied. The amount of early season senescence increased progressively between the LL, LH, HL and HH ozone treatments with the greatest increases above the control (LL treatment) of 15.1 % and 15.2 % in the HH treatment for weeks three and five respectively (Figure 3.6 c).

After eight weeks of exposure a significant 5.2 % ($P<0.05$) increase with background level was observed while there was no effect of peak ozone episodes. An interaction between background level and peak episode became significant at the $P<0.1$ level with

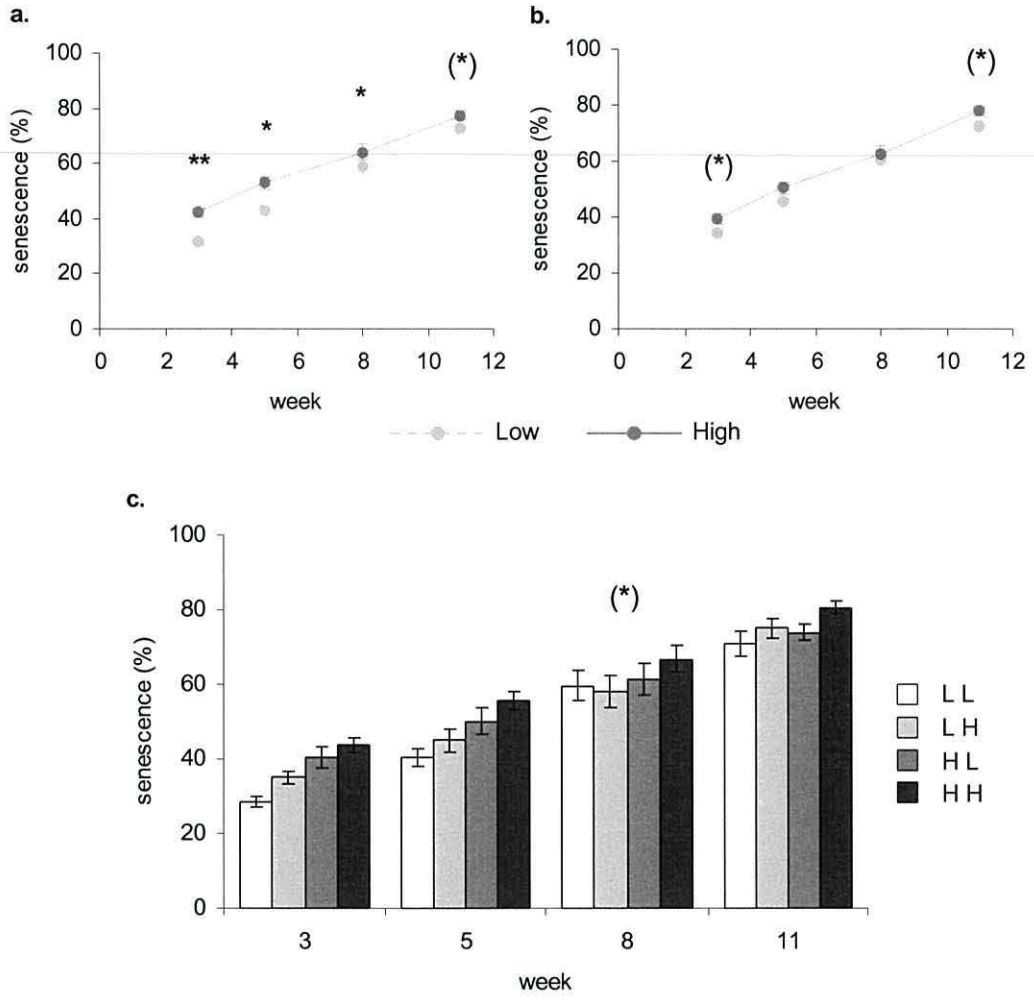

Figure 3.6 – Species level development of whole plant senescence measured after three, five, eight and eleven weeks of exposure to low background * low peak (LL), low background * high peak (LH), high background * low peak (HL) and high background * high peak (HH) ozone treatments. Significant differences are indicated as ** = $P<0.01$, * = $P<0.05$ and (*) = $P<0.1$ for effects of a) background ozone level, b) peak ozone level and c) interaction effects of background ozone level*peak ozone level treatments. Bars represent standard errors of the mean.

peak episodes resulting in a slight decrease at the low background level and a 5.5 % increase at the high background level (Figure 3.6 c). A number of factors from the preceding two weeks may have influenced this result including interruptions in the ozone supply, variation in the levels of aphid infestation and rust infection between solardomes and the application of nutrients and pesticides.

The late season whole plant senescence, assessed after 11 weeks, reached 70.8 % and 80.7 % in the LL and HH treatments respectively. Both background and peak episode treatment effects were only significant at the $P<0.1$ level with a slightly larger increase of 5.5 % for high peak episodes than the 4.4 % increase with high background (Figure

3.6). The larger increase with peak episodes may be due to the additional day of peak episode applied in weeks 10 and 11.

Populations differed throughout the exposure period in the amount of whole plant senescence regardless of ozone treatment ($P<0.01$) with the Aber and Maltraeth populations exhibiting higher levels of senescence than the Birds Rock and Cwm Idwal populations. Differences in the response of individual populations to elevated ozone exposure were observed in weeks five and eleven ($P<0.05$). In week five differences occurred in response to the interaction of background ozone level and peak episode, with the Aber and Cwm Idwal populations exhibiting an increase with peak episode at both background levels while Birds Rock and Maltraeth only exhibited an increase at high background and low background respectively. In week 11 the Birds Rock and Maltraeth populations differed from the other populations in response to background ozone treatment with no difference in senescence between low and high background levels.

Increase/premature early season senescence was exhibited by all populations with significant increases at the high background level in weeks three and five ($P<0.05$, Figure 3.7). Summary of population level ANOVA results are given in Table 3.4 and full details in Appendix II. The greatest effects of high background level were observed in the Cwm Idwal population with increases of 13.6 % ($P<0.01$) and 12.2 % ($P=0.05$) for weeks three and five respectively. Cwm Idwal exhibited a significant increase of 7.4 % with peak episodes in week three ($P<0.05$) while increases with peak episodes were significant at the $P<0.1$ level in the Birds Rock population for weeks three and five and Maltraeth for week three. The Maltraeth population also exhibited a significant interaction effect in week three ($P<0.05$) as the amount of senescence was similar for all three elevated ozone treatments with a 9.6 % increase with peak episode at low background but no difference at high background.

In week eight only two populations exhibited any significant increase under elevated ozone, with significant increases at the high background level of 6.6 % and 6.0 % in Aber and Cwm Idwal respectively ($P<0.05$, Figure 3.7). The Birds Rock and Maltraeth populations showed no significant effect of background level or peak episode with

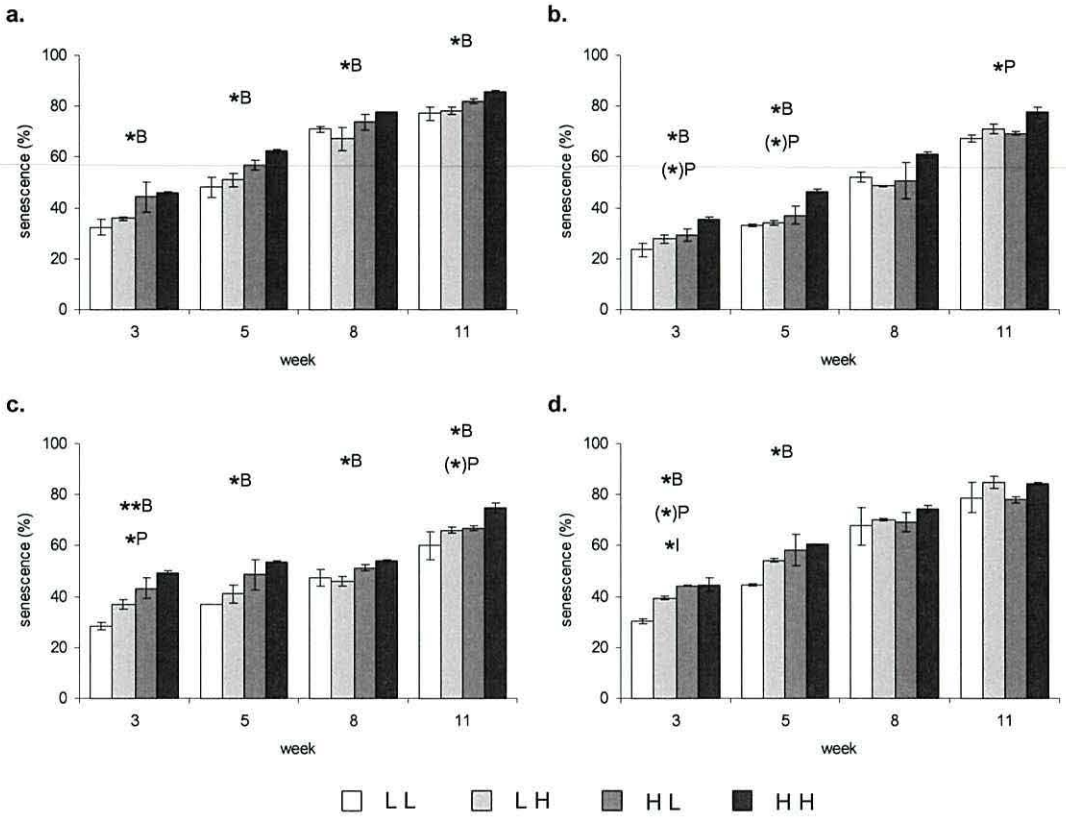

Figure 3.7 – The development of whole plant senescence for populations a) Aber, b) Birds Rock, c) Cwm Idwal and d) Maltraeth over 12 weeks exposure to low background * low peak (LL), low background * high peak (LH), high background * low peak (HL) and high background * high peak (HH) ozone treatments. Significant differences are indicated as ** = $P<0.01$, * = $P<0.05$ and (*) = $P<0.1$ for background, peak and interaction effects (suffix B, P or I respectively). Bars represent standard errors of the mean.

similar amounts of senescence in all treatments for Maltraeth and the amount of senescence in the LL treatment higher than that observed in both the LH and HL treatments for Birds Rock. The Aber, Birds Rock and Cwm Idwal populations exhibited higher senescence in the control than the LH treatment which may be due to damage from both aphid infestation and rust infection and the influence of ozone supply interruptions all of which occurred in the two weeks preceding assessment.

After 11 weeks of ozone exposure three populations exhibited significant increases in late season whole plant senescence with elevated ozone (Figure 3.7). Significant effects of background ozone level were observed with increases of 6.2 % and 7.6 % at high background for Aber and Cwm Idwal ($P<0.05$). The Birds Rock population exhibited a significant increase of 6.1 % with peak episodes ($P<0.05$) and Cwm Idwal an increase of 7.0 % significant at the $P<0.1$ level. Maltraeth exhibited a trend for increased

senescence with peak episodes and no increase with elevated background although there were no significant differences.

Individual leaf senescence, assessed on the second and third leaves down from the flag leaf, showed significant increases in early season and late season senescence under elevated ozone (Figure 3.8). Increased early season senescence was observed for leaf three with significant effects of both background level and peak episodes in week four ($P<0.05$). The greatest differences on this leaf were observed between the control and HH treatments with an increase of 11.6 % in week four ($P<0.05$) and a non-significant increase of 11.3 % in week six. A significant interaction occurred in week two for leaf two although it is likely that this was due to pre-exposure differences as the observed senescence levels are all below 10 % and there were no significant effects of either background level or peak episodes.

The amount of individual leaf senescence under elevated ozone increased through the exposure period in relation to that observed in the control treatment. Significant effects of peak episodes for leaf two ($P<0.05$) and both background level and peak episodes for leaf three ($P<0.05$) were observed for late season senescence after 10 weeks of exposure (Figure 3.8). The greatest increases of 17.4 % and 15.7 % occurred between the control and HH treatments for leaves two and three respectively. Both leaves exhibited a higher amount of senescence in the LH treatment compared to the HL treatment although the difference was relatively small with increases of 4.2 % and 4.6 % for leaves two and three respectively. The trend in late season individual leaf senescence is for a greater effect of peak episodes than background level which may be due to the additional day of peak episode applied during the tenth episode.

Populations differed significantly in the amount of individual leaf senescence regardless of ozone treatment ($P<0.01$) with the highest amounts of senescence observed for both leaves in the Aber and Maltraeth populations (Figure 3.9). In week four, populations differed significantly in the response of leaf two to the interaction of background level and peak episodes ($P<0.05$) although there were no significant treatment effects and senescence levels were all below 20 %. The Cwm Idwal population exhibited a significant interaction effect in this week ($P<0.05$) with an increase of 8.9 % with peak

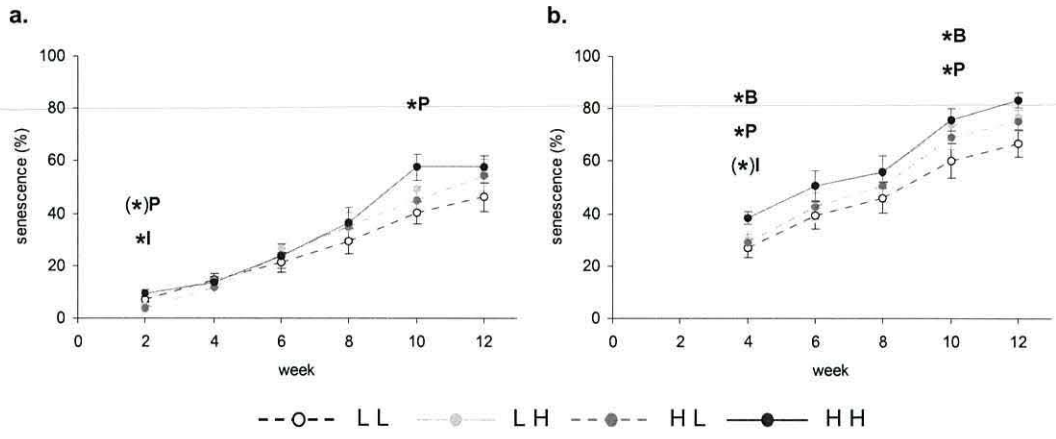

Figure 3.8 – Species level progression of senescence for individual leaves a) leaf 2 and b) leaf 3 over 12 weeks exposure to low background * low peak (LL), low background * high peak (LH), high background * low peak (HL) and high background * high peak (HH) ozone treatments. Significant differences are indicated as ** = $P<0.01$, * = $P<0.05$ and (*) = $P<0.1$ for background, peak and interaction effects (suffix B, P or I respectively). Bars represent standard errors of the mean.

episode at the low background level and a decrease of 2.6 % at the high background level and no significant treatment effects. The populations differed in the response of both leaves to background ozone levels in week 10 ($P<0.05$) due to higher amounts of senescence in the LL treatment than the HL treatment in the Birds Rock population. The differences were 5.3 % and 3.9 % for leaves two and three respectively although there were no significant treatment effects overall or for this population.

Elevated ozone had the greatest effect on leaf three senescence at the population level (Figure 3.9). The Cwm Idwal population exhibited increased leaf three early season senescence after four weeks of exposure with significant effects of both background level ($P<0.05$) and peak episodes ($P<0.05$). In week eight a significant peak episode effect was observed for leaf three in Aber ($P<0.05$). Three populations exhibited increased late season leaf three senescence under elevated ozone in week 10 with significant background effects observed in Aber and Cwm Idwal ($P<0.05$) and a significant effect of peak episodes in Maltraeth ($P<0.05$). The Aber population also exhibited increased leaf two senescence in week 10 with significant effects of both background level ($P<0.05$) and peak episodes ($P<0.05$). No significant increases in individual leaf senescence were observed in the Birds Rock population throughout the exposure period.

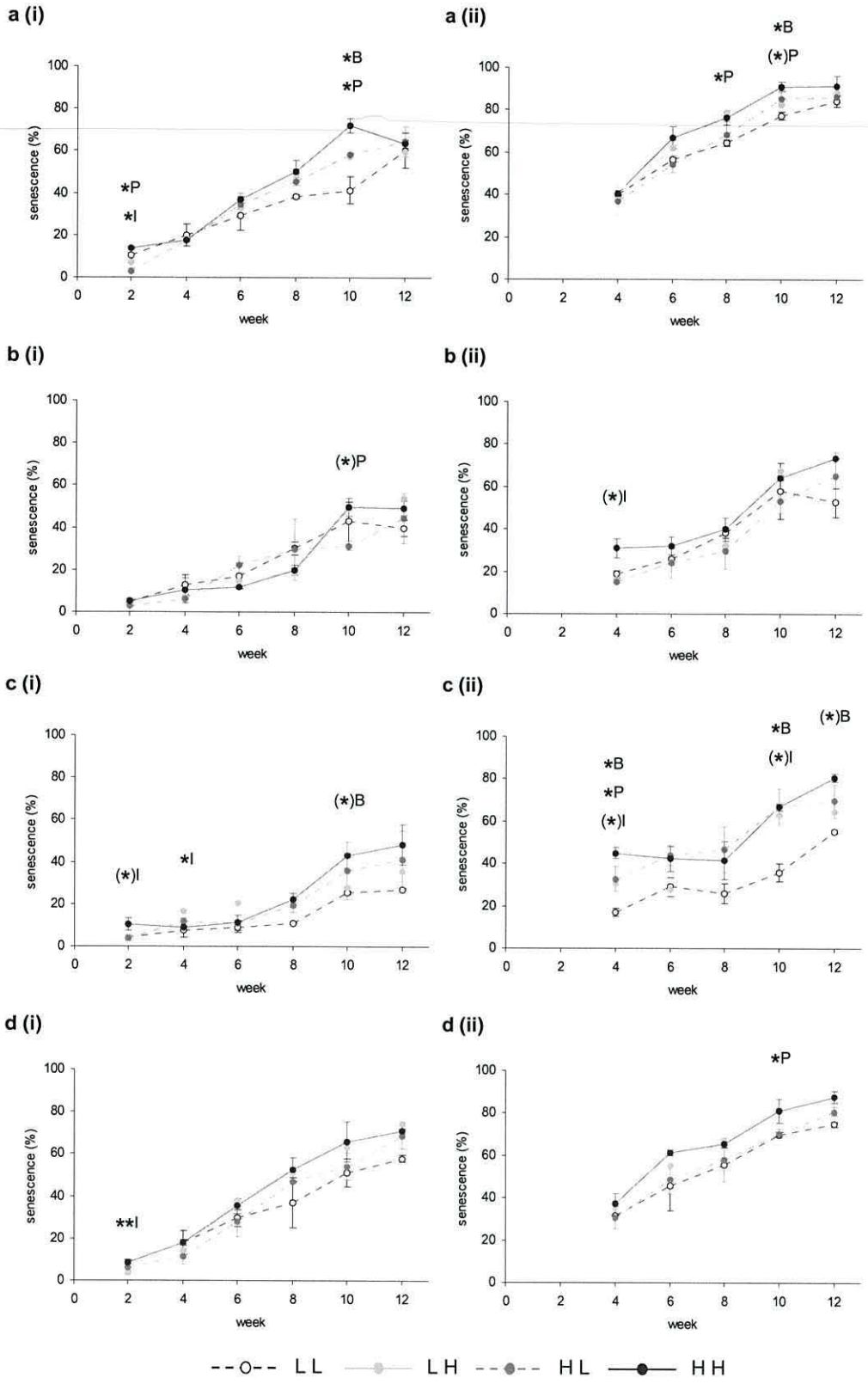

Figure 3.9 – Individual leaf senescence for populations a) Aber, b) Birds Rock, c) Cwm Idwal and d) Maltraeth. The development of (i) leaf two and (ii) leaf three senescence over 12 weeks exposure to low background * low peak (LL), low background * high peak (LH), high background * low peak (HL) and high background * high peak (HH) ozone treatments. Significant differences are indicated as ** = $P<0.01$, * = $P<0.05$ and (*) = $P<0.1$ for background, peak and interaction effects (suffix B, P or I respectively). Bars represent standard errors of the mean.

3.3.1.3 Plant growth

Plant growth assessed by above ground dry weight biomass (Figure 3.10) and relative tillering (Figure 3.11) exhibited no significant effects of either background ozone level or peak episodes at the species level. Above ground dry weight biomass exhibited a slight trend for increased growth in the LH and HL treatments with increases of 3.7 % and 4.3 % respectively, and decreased growth in the HH treatment with a 2.1% reduction although no significant interaction effects were observed. Tiller number after 12 weeks of exposure exhibited a significant 1.8 % increase under high background ozone levels ($P<0.01$, Figure 3.12). Although this was significant, the actual difference of 1.7 tillers was very small and the standard errors of the mean showed a large overlap, suggesting that this may be an artefact of the analysis model. In addition, this response was not observed for relative tillering. Summary of overall ANOVA results are given in Table 3.3 and full details in Appendix II.

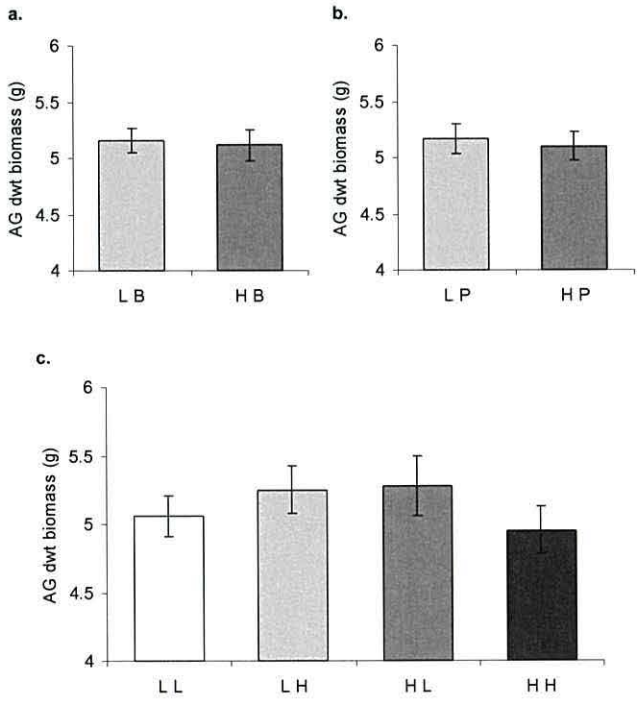

Figure 3.10 – Species level above ground dry weight biomass response to a) background ozone level: low (LB) and high (HB), b) peak episode level: low (LP) and high (HP), and c) background level* peak episode ozone treatments: low background * low peak (LL), low background * high peak (LH), high background * low peak (HL) and high background * high peak (HH). No significant differences were observed. Bars represent standard errors of the mean.

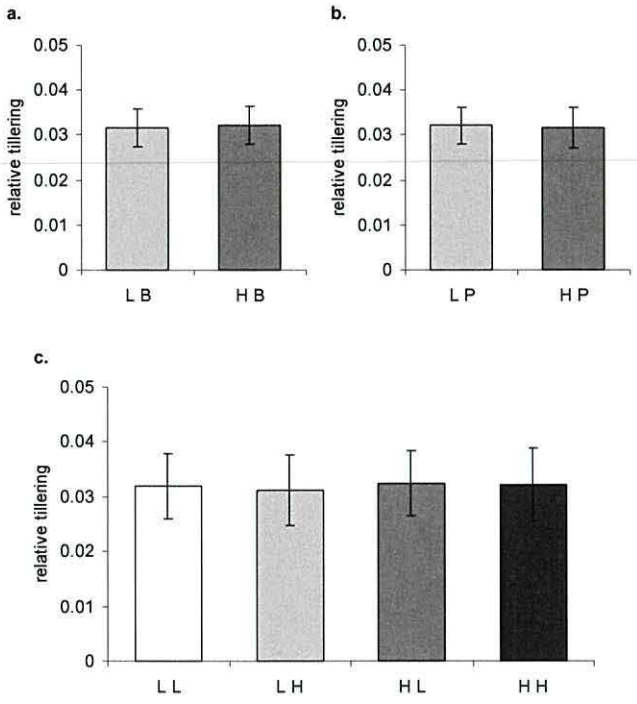

Figure 3.11 – Species level relative tillering response to a) background ozone level: low (LB) and high (HB), b) peak episode level: low (LP) and high (HP), and c) background level* peak episode ozone treatments: low background * low peak (LL), low background * high peak (LH), high background * low peak (HL) and high background * high peak (HH). No significant differences were observed. Bars represent standard errors of the mean.

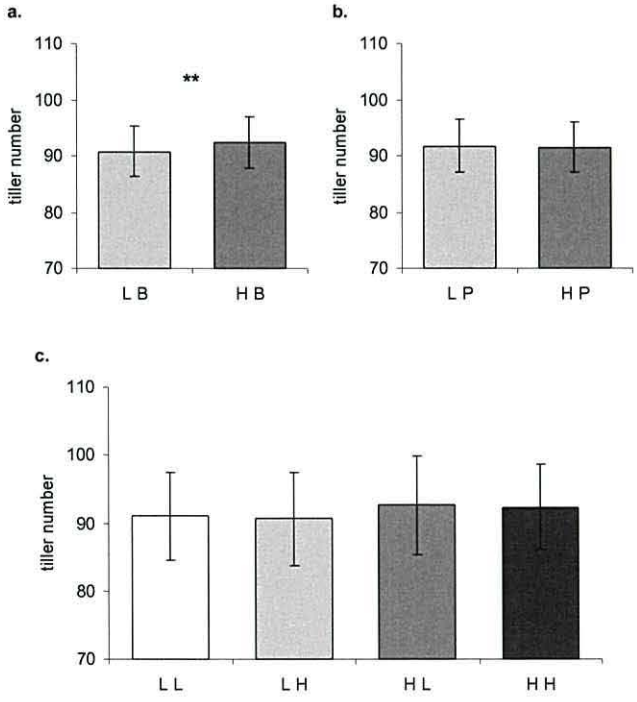

Figure 3.12 – Species level tiller number after 12 weeks of ozone exposure for a) background ozone level: low (LB) and high (HB), b) peak episode level: low (LP) and high (HP), and c) background level* peak episode ozone treatments: low background * low peak (LL), low background * high peak (LH), high background * low peak (HL) and high background * high peak (HH). Significant differences indicated as **=P<0.01. Bars represent standard errors of the mean.

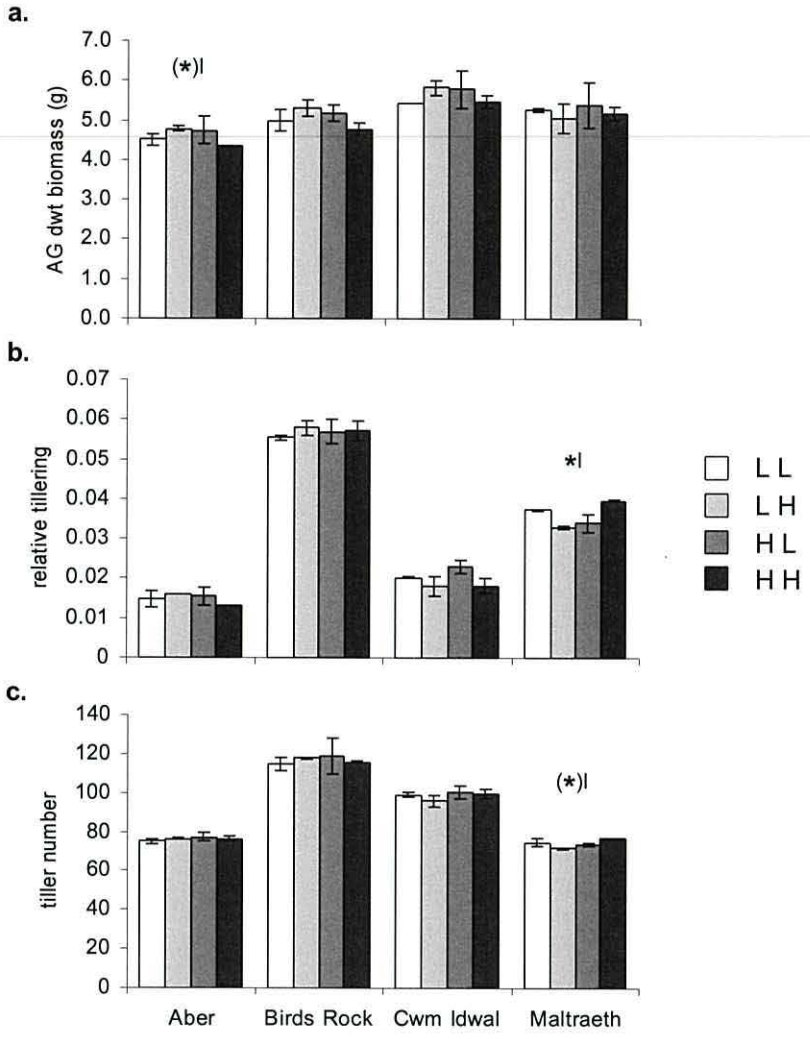

Figure 3.13 – Population level response for a) above ground dry weight biomass, b) relative tillering and c) tiller number after 12 weeks exposure to low background * low peak (LL), low background * high peak (LH), high background * low peak (HL) and high background * high peak (HH) ozone treatments. No significant background ozone level or peak episode effects were found. Significant background level * peak episode interaction effects are indicated as ^{*l} = P<0.05 and $^{(*)l}$ = P<0.1.

Populations differed significantly for all plant growth variables (P<0.01) with the highest values in Cwm Idwal for above ground dry weight biomass and in Birds Rock for relative tillering and tiller number (Figure 3.13). The lowest values were observed in the Aber population except for tiller number where there was no difference between Aber and Maltraeth.

Populations differed significantly in response to the interaction of background ozone level and peak episodes for above ground dry weight biomass and relative tillering (P<0.05) although there were no significant effects of background level or peak

episodes overall or for individual populations. Summary of population level ANOVA results are given in Table 3.4 and full details in Appendix II. Three populations exhibited a trend for increased above ground dry weight biomass with peak episodes at the low background level and a decrease with peak episodes at the high background level (Figure 3.13 a). This interaction was significant at the $P<0.1$ level for the Aber population. The Maltraeth population exhibited a different response with a non-significant trend for decreased above ground dry weight biomass with peak episodes at both background levels. Relative tillering responses differed for all populations (Figure 3.13 b). Cwm Idwal exhibited no response. Aber exhibited a similar trend to that found for above ground dry weight biomass. Birds Rock exhibited a decrease with the application of peak episodes at both background levels and an increase in the HL treatment. The Maltraeth population exhibited a significant interaction effect with a decrease in relative tillering at the low background level and an increase at the high background level ($P<0.05$). Populations did not differ significantly in the response of tiller number to elevated ozone although the Maltraeth population showed a similar trend to that observed for relative tillering with an interaction effect significant to the $P<0.1$ level (Figure 3.13 c).

The observed plant growth responses to elevated ozone at both the species and population level may have been affected by small pot size, with plants becoming pot bound during the exposure period causing restricted growth and leading to more uniform growth measurements after 12 weeks of exposure than may have been observed if harvested during early or mid season periods. The late season plant growth results may also have been influenced by the mid season aphid infestation and rust infection, nutrient and pesticide applications and interruptions to the ozone supply.

Table 3.3 – Summary of overall ANOVA analysis results.

		wpns week 3			wpns week 5			wpns week 8			wpns week 11		
Source of variation	**DF**	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***
Block	1	0.0078	0.0078	3.05	0.0032	0.0032	0.71	0.0169	0.0169	9.57	0.0072	0.0072	2.26
Background‡	1	0.0985	0.0985	38.39**	0.0897	0.0897	20.03*	0.0281	0.0281	15.89*	0.0193	0.0193	6.05(*)
Peak‡	1	0.0257	0.0257	10.00(*)	0.0229	0.0229	5.12	0.0034	0.0034	1.92	0.0309	0.0309	9.66(*)
Background x Peak‡	1	0.0022	0.0022	0.85	0.0002	0.0002	0.05	0.0105	0.0105	5.93(*)	0.0013	0.0013	0.40
residual	*3*	*0.0077*	*0.0026*	*4.53*	*0.0134*	*0.0045*	*6.09*	*0.0053*	*0.0018*	*1.03*	*0.0096*	*0.0032*	*4.42*
Population‡	3	0.0801	0.0267	47.15**	0.1996	0.0665	90.51**	0.4842	0.1614	93.78**	0.2310	0.0770	106.59**
Background x Population‡	3	0.0047	0.0016	2.75(*)	0.0016	0.0005	0.72	0.0033	0.0011	0.64	0.0126	0.0042	5.8*
Peak x Population‡	3	0.0024	0.0008	1.38	0.0005	0.0002	0.23	0.0045	0.0015	0.86	0.0030	0.0010	1.36
Background x Peak x Population‡	3	0.0032	0.0011	1.90	0.0102	0.0034	4.64*	0.0048	0.0016	0.93	0.0012	0.0004	0.53
residual	*12*	*0.0068*	*0.0006*		*0.0088*	*0.0007*		*0.0207*	*0.0017*		*0.0087*	*0.0007*	
Total	31	0.2391			0.3502			0.5816			0.3247		

		Above ground dwt			R_{till}			Tiller number		
Source of variation	**DF**	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***
Block	1	0.0004	0.0004	0.02	0.00000	0.00000	0.13	8.2100	8.2100	17.87
Background‡	1	0.0003	0.0003	0.02	0.00000	0.00000	0.34	22.190	22.190	48.34**
Peak‡	1	0.0005	0.0005	0.03	0.00000	0.00000	0.21	0.7300	0.7300	1.59
Background x Peak‡	1	0.0275	0.0275	1.62	0.00000	0.00000	0.03	0.0400	0.0400	0.09
residual	*3*	*0.0509*	*0.0170*	*10.06*	0.00003	0.00001	*1.87*	*1.3800*	*0.4600*	*0.02*
Population‡	3	0.2080	0.0693	41.14**	0.00859	0.00286	539.57**	9738.1	3246.0	151.91**
Background x Population‡	3	0.0049	0.0016	0.97	0.00001	0.00000	0.61	2.8500	0.9500	0.04
Peak x Population‡	3	0.0012	0.0004	0.24	0.00003	0.00001	1.88	6.9600	2.3200	0.11
Background x Peak x Population‡	3	0.0199	0.0066	3.94*	0.00006	0.00002	3.80*	48.390	16.130	0.75
residual	*12*	*0.0202*	*0.0017*		*0.00006*	*0.00001*		*256.42*	*21.370*	
Total	31	0.3338			0.00879			10085.3		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

Table 3.4 – Summary of population level ANOVA analysis results.

			wpns week 3			wpns week 11			Above ground dwt			Tiller number		
Population	**Source of variation**	**DF**	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***
Aber	**Block**	1	0.0068	0.0068	5.68	0.0015	0.0015	2.71	0.0009	0.0009	0.22	12.087	12.087	16.49
	Background‡	1	0.0296	0.0296	24.74*	0.0128	0.0128	23.32*	0.0011	0.0011	0.26	2.2874	2.2874	3.12
	Peak‡	1	0.0020	0.0020	1.66	0.0018	0.0018	3.30	0.0000	0.0000	0.00	0.2041	0.2041	0.28
	Background x Peak‡	1	0.0001	0.0001	0.11	0.0007	0.0007	1.25	0.0233	0.0233	5.85(*)	2.5313	2.5313	3.45
	residual	*3*	*0.0036*	*0.0012*		*0.0017*	*0.0006*		*0.0120*	*0.0040*		*2.1987*	*0.7329*	
	Total	7	0.0420			0.0185			0.0372			19.308		
Birds Rock	**Block**	1	0.0024	0.0024	3.25	0.0002	0.0002	0.27	0.0005	0.0005	0.12	63.850	63.850	1.58
	Background‡	1	0.0117	0.0117	15.82*	0.0043	0.0043	5.49	0.0013	0.0013	0.31	2.7600	2.7600	0.07
	Peak‡	1	0.0075	0.0075	10.15*	0.0087	0.0087	11.00*	0.0001	0.0001	0.03	0.0100	0.0100	0.00
	Background x Peak‡	1	0.0001	0.0001	0.12	0.0014	0.0014	1.80	0.0128	0.0128	3.03	19.220	19.220	0.48
	residual	*3*	*0.0022*	*0.0007*		*0.0024*	*0.0008*		*0.0126*	*0.0042*		*121.13*	*40.380*	
	Total	7	0.0239			0.0170			0.0273			206.96		
Cwm Idwal	**Block**	1	0.0025	0.0025	2.62	0.0048	0.0048	4.03	0.0002	0.0002	0.04	0.1000	0.1000	0.01
	Background‡	1	0.0404	0.0404	42.46**	0.0143	0.0143	12.12*	0.0002	0.0002	0.04	13.060	13.060	0.69
	Peak‡	1	0.0126	0.0126	13.25*	0.0103	0.0103	8.72(*)	0.0000	0.0000	0.00	7.1400	7.1400	0.38
	Background x Peak‡	1	0.0005	0.0005	0.56	0.0003	0.0003	0.22	0.0102	0.0102	2.13	2.0000	2.0000	0.11
	residual	*3*	*0.0029*	*0.0010*		*0.0036*	*0.0012*		*0.0143*	*0.0048*		*56.850*	*18.950*	
	Total	7	0.0589			0.0332			0.0249			79.150		
Maltraeth	**Block**	1	0.0000	0.0000	0.03	0.0023	0.0023	0.74	0.0004	0.0004	0.04	0.8130	0.8130	0.27
	Background‡	1	0.0215	0.0215	33.10*	0.0004	0.0004	0.14	0.0026	0.0026	0.25	6.9380	6.9380	2.32
	Peak‡	1	0.0059	0.0059	9.12(*)	0.0130	0.0130	4.25	0.0016	0.0016	0.16	0.3400	0.3400	0.11
	Background x Peak‡	1	0.0047	0.0047	7.17(*)	0.0001	0.0001	0.02	0.0012	0.0012	0.12	24.675	24.675	8.24(*)
	residual	*3*	*0.0020*	*0.0007*		*0.0092*	*0.0031*		*0.0306*	*0.0102*		*8.9810*	*2.9940*	
	Total	7	0.0341			0.0249			0.0364			41.747		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

3.3.1.4 Ozone dose response

The response of species level whole plant senescence to the received ozone dose, measured as accumulated ozone over a threshold of X ppb (AOTX) for a 24 hour per day period, differed with the applied threshold value for early and late season senescence. Early season senescence, assessed after three ozone episodes, exhibited a significant ozone dose response relationship with AOT0 (adj R^2=0.872, P=0.044) but not with AOT40 (adj R^2=0.185, P=0.324) (Figure 3.14 a). This difference relates to the greater importance ascribed to peak episodes in the calculation of AOT40 while AOT0 ascribes a higher ozone dose for a constant elevated background (HL treatment) than for a four day peak episode during daylight hours at low background concentrations (LH treatment). In comparison, the ozone dose response relationship for late season

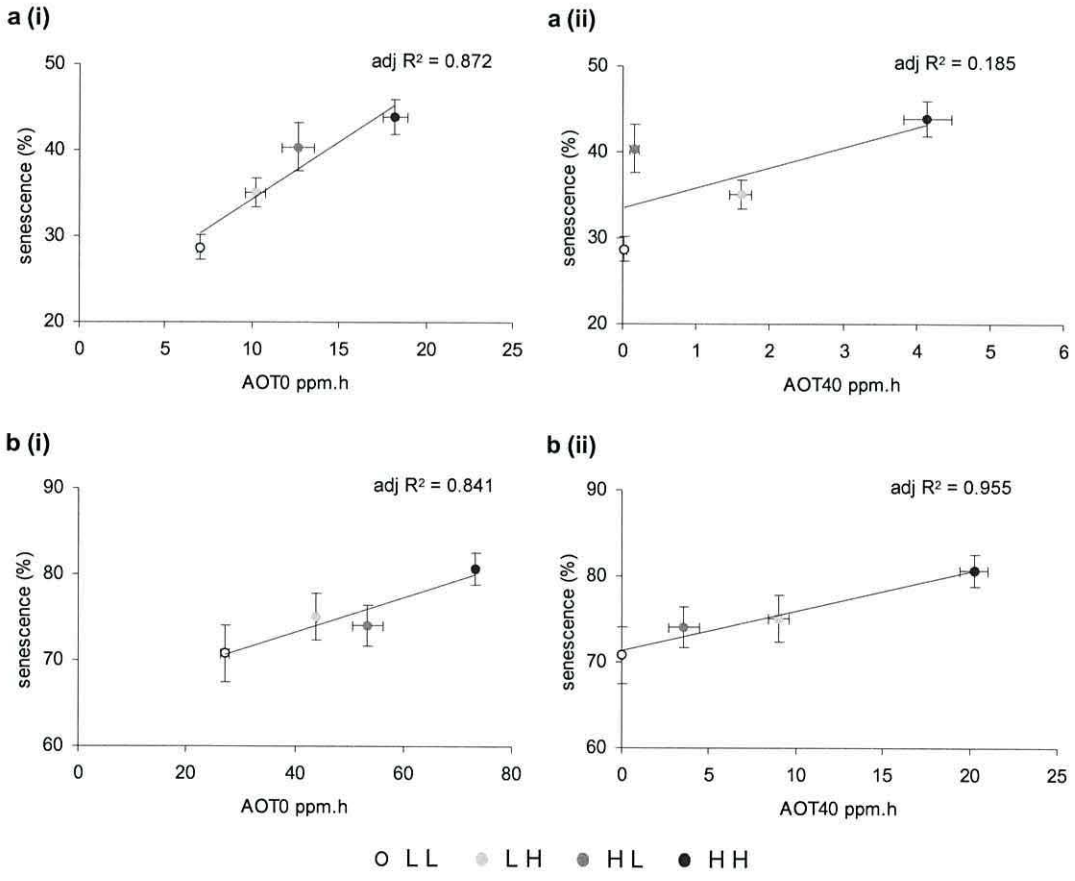

Figure 3.14 – Linear relationship between species level whole plant senescence and received ozone dose as accumulated ozone (ppm.h) over a threshold of i) 0 ppb and ii) 40 ppb after a) 3 weeks and b) 11 weeks exposure to low background*low peak (LL), low background*high peak (LH), high background*low peak (HL) and high background*high peak (HH) ozone treatments. Bars represent standard errors of the mean.

senescence, assessed after 11 ozone episodes, was significant with AOT40 (adj R^2=0.955, P=0.015) and almost significant with AOT0 (adj R^2=0.841, P=0.054) (Figure 3.14 b). The similar adjusted R^2 values for both thresholds may relate to the lower magnitude of difference in senescence among treatments, especially between the LH and HL treatments, compared to early season senescence and the inclusion of an additional day of peak episode in the two weeks prior to assessment. Although the most significant relationship is for AOT40, the assessment of which threshold value provides the best explanation for the response of late season senescence to received ozone dose is confounded by the low significance of ozone treatment effects in week 11 and a number of mid season factors which may have influenced the senescence data (see section 3.3.2.1).

Significant relationships occurred between species level whole plant senescence and the received ozone dose (AOTX) over the exposure period for both linear and quadratic regression. The relationships remained highly significant (P<0.01) regardless of the applied threshold value (0 ppb, 20 ppb or 40 ppb) or the hours per day (hd^{-1}) period applied (12 hour or 24 hour). However, the adjusted R^2 (adj R^2) values decreased with a 12 hd^{-1} period for AOT0 and AOT40 (Table 3.5) and those calculated over a 12 hd^{-1} were not considered further. The adj R^2 values are considered to allow comparison between linear and quadratic regression models. The regression model with the highest adj R^2 value differed for the different threshold values with a quadratic model providing the best fit for AOT0 and a linear model for both AOT20 and AOT40. Although significant relationships were found for all AOTXs the adj R^2 value decreased with

Table 3.5 – Linear and quadratic regression analysis of relationship between whole plant senescence and accumulated ozone over a threshold (AOT) of X ppb calculated for 12 and 24 hd^{-1} periods throughout the exposure period.

AOT(Xppb)	Period	Linear Regression				Quadratic regression			
		R^2	adj R^2	F value	*P* value	R^2	adj R^2	F value	*P* value
AOT0	24 hd^{-1}	0.760	0.752	95.00	<0.001	0.831	0.819	71.23	<0.001
	12 hd^{-1}	0.732	0.723	81.99	<0.001	0.794	0.780	55.89	<0.001
AOT20	24 hd^{-1}	0.468	0.451	26.43	<0.001	0.471	0.435	12.91	<0.001
	12 hd^{-1}	0.482	0.465	27.92	<0.001	0.485	0.449	13.63	<0.001
AOT40	24 hd^{-1}	0.363	0.342	17.09	<0.001	0.364	0.320	8.31	0.001
	12 hd^{-1}	0.350	0.329	16.19	<0.001	0.350	0.306	7.82	0.002

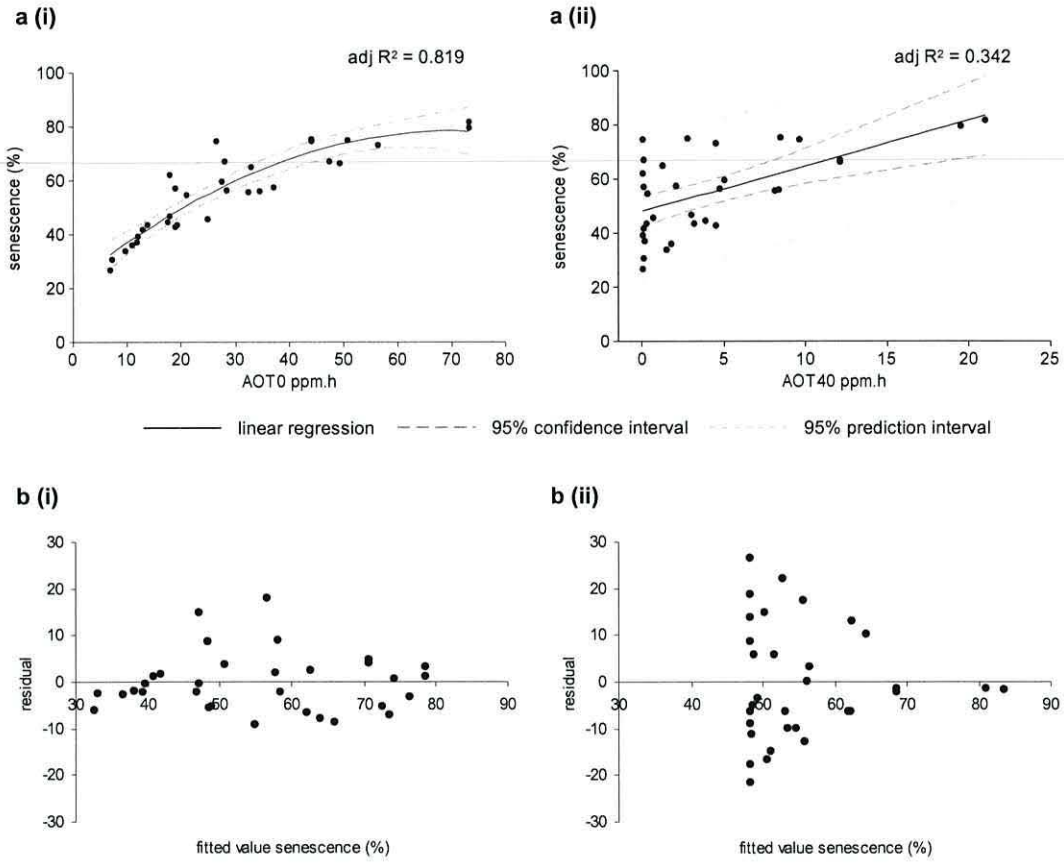

Figure 3.15 – Relationship between whole plant senescence and 24 hour per day accumulated ozone over a threshold (AOTX) of (i) 0 ppb and (ii) 40 ppb throughout the exposure period. Line of best fit described by a(i) quadratic regression for AOT0, a(ii) linear regression for AOT40 and b) resulting residual variation.

increasing threshold value with a quadratic regression model for AOT0 providing the best fit for the amount of observed whole plant senescence (adj R^2=0.819, P<0.001). The quadratic relationship was due to the development of senescence over time, an amount of regeneration with plants continuing to produce new leaves throughout the exposure period and the nature of measuring senescence as a percentage.

Comparison of the relationships for AOT0 and AOT40 showed a clustering of senescence data at low AOTX values for early season senescence and for ozone treatments with a low received ozone dose though the exposure period (Figure 3.15 a). This was greatest for AOT40 where there was no increase in AOT value for the LL treatment and less than 5 ppm.h received in the HL treatment over the exposure period as the ozone concentration rarely exceeded 40 ppb. Examination of the residual variation for the regression relationship showed a reasonable distribution for AOT0 while there was greater variation and a clustered distribution for AOT40 (Figure 3.15

b). This clustering and the low adj R^2 value both indicate that the regression model for AOT40 is not an appropriate descriptor of the relationship between senescence and ozone dose. Total accumulated ozone calculated for a period of 24 hd^{-1} without a threshold value imposed (AOT0) provided the best description for the response of whole plant senescence to ozone dose when considered across the entire exposure period.

3.3.1.5 Relative ozone sensitivity

The greatest response to elevated ozone in *A. odoratum* was in terms of increased/premature senescence resulting in a reduction in the percentage of healthy leaves (Table 3.6). *A. odoratum* was most sensitive to elevated background concentrations with peak episodes throughout the exposure period although relative sensitivity to the different ozone treatments varied over the exposure period both at the species level and among populations.

At the species level, plants exhibited greater relative sensitivity under elevated background concentrations without peak episodes (HL treatment) compared to peak episodes at low background (LH treatment) after three and five ozone episodes (Table 3.6). All four populations exhibited this trend although the extent of sensitivity differed. Over both assessment weeks the Aber and Birds Rock populations were the least sensitive to the LH treatment while Maltraeth exhibited the greatest sensitivity to the HL treatment. Maltraeth exhibited the greatest relative sensitivity to all elevated ozone treatments while Birds Rock was the least sensitive in terms of early season senescence.

The trend in comparative sensitivity altered for mid and late season senescence. Relative sensitivity for mid season senescence after eight ozone episodes was affected by a number of potentially confounding factors detailed in section 3.3.1.2 and may not provide an accurate assessment of response to ozone exposure. Species level relative sensitivity for late season senescence after eleven ozone episodes exhibited the opposite trend to early season senescence as plants were more sensitive to peak episodes at low background (LH treatment) than to elevated background alone (HL treatment) (Table 3.6). The Birds Rock and Maltraeth populations followed this trend while Aber and

Table 3.6 – Species and population level relative ozone sensitivity (RS) for foliar injury and plant growth assessment criteria. Relative sensitivities in relation to the low background*low peak treatment (LL) and denoted as RS_{LH} (low background*high peak treatment (LH)), RS_{HL} (high background*low peak treatment (HL)), RS_{HH} (high background*high peak treatment (HH)). *woe* denotes weeks of exposure. Significant differences (P_T) are indicated by **=P<0.01, * = P<0.05 and (*) = P<0.1 for background, peak and interaction effects (suffix B, P or I respectively). RS values of <1 and >1 represent sensitivity and resistance respectively.

Assessment criteria	woe	Species				Aber				Birds Rock				Cwm Idwal				Maltraeth			
		RS_{LH}	RS_{HL}	RS_{HH}	P_T	RS_{LH}	RS_{HL}	RS_{HH}	P_T	RS_{LH}	RS_{HL}	RS_{HH}	P_T	RS_{LH}	RS_{HL}	RS_{HH}	P_T	RS_{LH}	RS_{HL}	RS_{HH}	P_T
Foliar injury																					
% healthy leaves	3	0.91	0.84	0.79	**B (*)P	0.95	0.82	0.80	*B	0.95	0.92	0.84	*B (*)P	0.88	0.79	0.71	**B *P	0.87	0.80	0.80	*B (*)P *I
	5	0.92	0.84	0.74	*B	0.95	0.83	0.73	*B	0.98	0.94	0.80	*B (*)P	0.93	0.81	0.73	*B	0.83	0.75	0.71	*B
	8	1.04	0.96	0.82	*B (*)I	1.13	0.90	0.77	*B	1.08	1.03	0.82		1.03	0.93	0.87	*B	0.92	0.95	0.79	
	11	0.85	0.89	0.66	(*)B (*)P	0.95	0.79	0.61	*B	0.89	0.94	0.69	*P	0.85	0.83	0.63	*B (*)P	0.72	1.04	0.74	
Plant growth																					
AG dwt	12	1.04	1.04	0.98		1.06	1.05	0.97	(*)I	1.06	1.04	0.95		1.07	1.06	1.01		0.96	1.02	0.98	
R_{till}	12	0.98	1.01	1.00		1.08	1.05	0.89		1.05	1.03	1.03		0.89	1.14	0.90		0.89	0.91	1.06	*I
Tiller number	12	1.00	1.02	1.01	**B	1.02	1.03	1.02		1.03	1.04	1.01		0.97	1.02	1.01		0.96	0.98	1.03	(*)I

AG, above ground; dwt, dry weight; R_{till}, relative tillering

Cwm Idwal remained more sensitive to elevated background. Although the factors influencing mid season senescence may have also affected late season senescence, the shift in sensitivity in relation to peak episodes may be due to the inclusion of an extra day of peak episode in weeks 10 and 11.

A. odoratum was not sensitive to elevated ozone in relation to above ground plant growth in terms of dry weight biomass or tillering (Table 3.6). There was a slight trend for stimulated growth with elevated ozone although this was not significant or consistent across treatments. The populations differed in sensitivity to the different ozone treatments with no effect, stimulation and reduction in growth dependant on which assessment criteria and treatment were considered. However, the extent of stimulation or reduction was small and non-significant in the majority of cases.

The populations differed in relative sensitivity dependant upon the assessment criteria applied. All populations were considered sensitive for foliar injury as the percentage of healthy leaves although the relative sensitivity to peak episodes at low background (LH treatment) and elevated background alone (HL treatment) differed among population and between early and late season senescence. The sensitivity of *A. odoratum* in terms of foliar injury did not equate to a similar sensitivity in plant growth.

3.3.2 Stomatal conductance

Stomatal conductance (g_s) differed significantly between adaxial and abaxial leaf surfaces ($P<0.05$) with g_s highest on the adaxial surface for both leaf two and leaf three at the species and population level (Figure 3.16). There was a non significant trend for decreased adaxial stomatal conductance with increasing leaf age at the species level with 111.6 mmol m^{-2} s^{-1} and 92.4 mmol m^{-2} s^{-1} for leaves two and three respectively (Figure 3.16 a). A significant increase with leaf age occurred for abaxial g_s at the species level ($P<0.05$) with 17.0 mmol m^{-2} s^{-1} for leaf two and 26.9 mmol m^{-2} s^{-1} for leaf three.

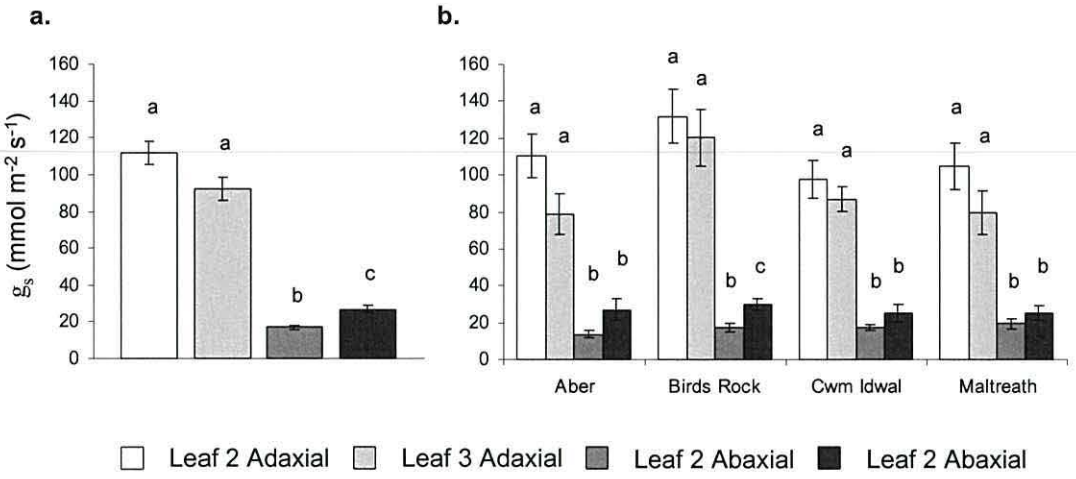

Figure 3.16 –Stomatal conductance for adaxial and abaxial surfaces on leaf 2 and leaf 3 at a) species level and b) population level. Letters a, b and c indicate significant differences at $P<0.05$. Bars represent standard errors of the mean.

All populations exhibited significantly higher g_s for the adaxial surface compared to the abaxial surface ($P<0.05$) and a trend for decreased adaxial g_s and increased abaxial g_s with leaf age (Figure 3.16 b). The difference in abaxial g_s with leaf age was significant in the Birds Rock population increasing from 17.4 mmol m^{-2} s^{-1} for leaf two to 29.7 mmol m^{-2} s^{-1} for leaf three ($P<0.05$). Populations differed significantly for leaf three adaxial g_s ($P<0.05$) with a significantly higher g_s of 120.5 mmol m^{-2} s^{-1} for the Birds Rock population than the g_s of 79.1, 87.0 and 79.7 mmol m^{-2} s^{-1} for the Aber, Cwm Idwal and Maltraeth populations respectively. No significant differences between populations were found for leaf two adaxial g_s or abaxial g_s for either leaf two or three.

3.4 Discussion

Exposure to elevated ozone concentrations exerts strong oxidative stress on plant species and can induce chronic responses including ozone-specific visible injury (Bungener *et al* 1999b), premature senescence (Bergmann *et al* 1999, Bermejo *et al* 2003), alterations in resource allocation (Grantz *et al* 2006) and reproductive output (Black *et al* 2000). Elevated tropospheric ozone concentrations have a potentially detrimental effect on semi-natural vegetation and increasing background concentrations are of major concern (Ashmore *et al* 2002). This study demonstrates the potential for

chronic ozone responses to elevated background concentrations with and without peak episodes in relation to ambient peak episodes and intraspecific variation in *A. odoratum*. The results are discussed first in relation to the experimental design and received ozone exposure, second in terms of the species level response and finally in terms of the intraspecific variation in response to ozone exposure and in relation to variation in stomatal conductance.

3.4.1 Experimental design and received ozone exposure

A number of factors relating to experimental design may have influenced the observed response of *A. odoratum* to elevated ozone including the solardome environment, pot related growth restrictions and edge effects as previously discussed in Chapter 2 (see Section 2.4.1). Plant response to ozone exposure may differ with pot size (Whitfield *et al* 1996) and between monocultures and communities due to differences in root restriction, canopy shading and competition. The relatively small pot size used in this study is likely to have restricted root growth although this was not suggested for *A. odoratum* during a short-term acute ozone exposure (Chapter 2). The extent of restriction is likely to have been greater in this study due to the longer period of plant establishment and exposure with plant age at the start of exposure the same as those harvested following the four week acute exposure experiment. This may have influenced the observed response in shoot growth although some degree of root restriction may result in a more accurate assessment of ozone effects as root restriction is expected under competition in the natural environment (Whitfield *et al* 1996). Plants in a pot-grown monoculture also experience greater edge effects and higher levels of ozone exposure than those grown in a community (Wilbourne *et al* 1995). This may have affected plant response although the close spacing of plant pots within trays may have reduced edge effects and provided some degree of canopy shading and canopy growth competition. The extent of edge effects will have differed among plant positions within trays with greater effects on those plants in edge positions and plants located closest to the air inlet tubing may have experienced higher levels of ozone exposure. However any confounding effects should be minimal in relation to population response

as the randomisation procedure applied should have prevented any localisation of effects among populations.

Plants were exposed to four ozone treatments with a combination of continuous background concentrations and peak episodes during daylight hours. Average concentrations through the exposure period were 14 ppb to 19 ppb for low background and 29 ppb to 36 ppb for high background while peak episodes averaged 52 ppb and 72 ppb at each background level respectively. However, interruptions in the ozone supply and alterations in the number of peak episode days resulted in three distinct periods during the 12 week exposure: early season with four peak episode days per week, mid season with one to two peak episode days and interrupted background concentrations, and late season with five peak episode days. Differing peak episode profiles may have altered the relative plant response to elevated background and peak episodes while ozone supply interruptions may have allowed some natural regeneration between the early and late season periods. Mid season nutrient treatment may have aided regeneration while aphid infestation and rust infection and the subsequent pesticide treatment may also have influenced the observed plant response. Despite equal treatment among solardomes, the nutrient application and pesticide treatment may have had some effect, although it is unlikely that there was any interaction between these and the different ozone treatments due to the interruptions in ozone supply during the weeks in which they were applied.

Received ozone dose was determined using the AOT40 concept (Fuhrer *et al* 1997) which is considered the most applicable measure of ozone dose for semi-natural vegetation species (LRTAP Convention, 2007). However, AOT40 may under represent the effect of elevated background concentrations falling below 40 ppb and maintained overnight concentrations (Ashmore *et al* 2002) as in the elevated background treatment (HL) where concentrations averaged 29 ppb to 36 ppb. Although a flux-based approach would be preferable the complexity of this approach precludes its use in many species and modification of the AOT index has been suggested as an acceptable alternative (Ashmore *et al* 2002). In this study, the AOT index was modified by using thresholds of 0 ppb, 20 ppb and 40 ppb over both 12 hour per day and 24 hour per day periods. The 24 hour per day period was applied to include overnight elevated background concentrations as although nocturnal stomatal conductance and ozone uptake are lower

than during daylight hours, significant ozone flux may still occur (Musselmann and Minnick 2000).

The combined elevated background with peak episodes (HH) treatment received the highest ozone dose while ozone dose differed among the other elevated ozone treatments dependant on the AOT index applied. The 24 hour AOT40 ascribed a greater importance to peak episodes at low background (LH treatment) than for elevated background (HL treatment) with 10.6 ppm.h and 4.1 ppm.h respectively after 12 weeks of exposure while the ozone doses were 49.7 ppm.h and 59.9 ppm.h for 24 hour AOT0 respectively, ascribing a greater ozone dose with elevated background. An equal accumulated ozone dose in the LH and HL treatments may have allowed a more direct comparison of the relative effects of peak episodes and elevated background. However, the relative ozone dose is entirely dependent upon the AOT index applied and comparison between different thresholds should provide an adequate assessment of relative response.

3.4.2 *A. odoratum* response to rural ozone profiles

Increased/premature whole plant senescence occurred under elevated ozone treatments with the greatest effects observed under elevated background with peak episodes although the magnitude of response differed through the exposure period. The largest responses were observed for early season senescence after three and five ozone episodes. The relatively fast development of premature senescence is consistent with previous results for this species under an episodic ozone regime (Hayes *et al* 2006) and acute ozone exposure (Chapter 2). The most notable result was the induction of higher levels of premature senescence with elevated background than observed with peak episodes at low background. However, this response did not remain consistent throughout the exposure period.

The magnitude of response decreased for mid and late season senescence. Reductions in the magnitude of response in terms of senescence during ozone exposure were also observed in *A. odoratum* and *Agrostis capillaris* by Hayes *et al* (2006) and were thought

to relate to the continual production of new leaves in these species. This may also relate to the natural progression of senescence through the growing season as observed in the control treatment and under acute ozone exposure (Chapter 2). A number of additional factors may have influenced this result including interruptions to the ozone supply, nutrient treatment and aphid and rust infection. The mid season ozone supply interruptions and nutrient treatment may have aided natural regeneration in the elevated ozone treatments slowing the rate of development of senescence. In addition, damage due to aphid infestation and rust infection may have resulted in the higher amount of senescence observed in the control treatment at this time and reduced the magnitude of response.

The trend observed for early season senescence in relative sensitivity to elevated background and peak episodes at low background was reversed for late season senescence with peak episodes inducing a larger response. This shift in response relates to the inclusion of an extra day of peak episode in the two weeks prior to assessment and may also have been influenced by carry over effects from the factors affecting mid season senescence. However, the observed increases in senescence under elevated ozone were small and non-significant. This may be due to the natural progression of senescence through the growing season as *A. odoratum* tends to lose the majority of leaves over winter (Schippers *et al* 1999) and whole plant senescence in the control treatment had reached 70.8 % at this time.

The amount of individual leaf senescence on the second and third fully expanded leaves also increased under elevated ozone although the relative importance of background levels and peak episodes on any increase in individual leaf senescence was difficult to discern. The effects were greatest at the third fully expanded leaf position under elevated background with peak episodes. The largest increase occurred for late season senescence with a trend for a greater effect of peak episodes than elevated background although this relates to the inclusion of the extra days of peak episode. The variation in response over the exposure period and among leaf positions may be due to leaf and tiller turnover as the plants continued to produce new leaves and tillers throughout the exposure period. The rates at which leaf two became leaf three and new assessment tillers were required as each tiller became fully senesced will have influenced the

relative age, amount ozone received and amount of natural and premature senescence observed for each individual leaf assessed at any time over the exposure period.

Increased/premature senescence is an unspecific form of ozone induced foliar injury thought to occur with the induction of senescence-associated genes and promoted cell death (Miller *et al* 1999, Pell *et al* 1997) and is considered a common response in many wild herb and grass species (Bergmann *et al* 1995, Bergmann *et al* 1999, Franzaring *et al* 2000, Bermejo *et al* 2003). A faster rate of development of senescence under elevated ozone, as observed both on whole plants and individual leaves in *A. odoratum*, may reduce the effective growing season and resource accretion as increased senescence results in decreased photosynthetic capacity (Gay and Thomas 1995, Vandermeinen *et al* 2005). This has implications for long term response with potentially detrimental effects on overwintering potential and competitive ability due to lower resource accumulation.

The most notable result in terms of foliar injury in this study was the induction of higher levels of premature early season senescence with elevated background than observed with peak episodes at low background. This contrasts with the response of *A. odoratum* when grown in a seven species model community under similar ozone profiles although elevated background with peak episodes did result in premature senescence (Hayes 2007). However, in the same study two other component species, *Potentilla erecta* and *Festuca ovina*, did exhibit a greater effect of elevated background (Hayes 2007). The effects of ozone exposure may be mediated for plants grown in communities compared to those grown individually due to reduced edge effects and canopy shading which may result in lower levels of ozone exposure (Wilbourne *et al* 1995) although this may vary with community structure and plant position within the canopy and ozone responses may also be affected by competitive interactions among species (Davison and Barnes 1998, Nussbaum and Fuhrer 2000, Bassin *et al* 2007). However, these results suggest that increased background ozone concentrations of around 35 ppb to 40 ppb predicted for across most of the UK by 2030 (Coyle *et al* 2003b) have the potential to induce foliar injury to a similar or greater extent than ambient peak episodes.

The occurrence of premature senescence under elevated ozone was not accompanied by any significant reductions in shoot growth in *A. odoratum* although a slight increase in

tiller number was observed. However, foliar injury without an associated growth reduction and in some cases growth responses without the development of foliar injury are not uncommon (Franzaring *et al* 2000, Inclan *et al* 1999, Woo and Hinckley 2005, Hayes *et al* 2006, Bender *et al* 2006). This may result from alterations in resource allocation to root and shoot components (Davison and Barnes 1998, Grantz *et al* 2006) and a trend for greater reduction in root biomass compared to shoot biomass was observed in *A. odoratum* under acute ozone exposure (Chapter 2). The lack response of shoot biomass is consistent with the results of Hayes *et al* (2006) following a 10 week chronic ozone exposure. However, significant reductions in cumulative biomass were observed in *A. odoratum* grown in a seven species model community under similar ozone profiles following two seasons of ozone exposure (Hayes 2007) suggesting the potential for a longer-term growth response to chronic ozone exposure in this perennial species.

The increase in tiller number under elevated background despite the lack of biomass response may relate to changes in tiller morphology with resources allocated to the production of a greater number of smaller tillers as the level of foliar injury increased. This supports the suggestion that alterations in tiller morphology may have occurred under acute ozone exposure (Chapter 2). However, the magnitude of response observed in this study was small and results from spring wheat cv. Minaret suggest there is very little effect (Ewert *et al* 1999). Further investigation of the leaf to sheath biomass ratio may provide a clearer indication of the response.

Ozone dose response relationships between whole plant senescence and AOTX were investigated for different threshold values and different periods of exposure. A significant relationship was observed between early season senescence and 24 hour AOT0 as this index provided the best explanation for the observed response to elevated background and peak episodes at low background. In comparison, late season senescence exhibited the most significant relationship with AOT40 although the relationship was similar between AOT40 and AOT0. The development of senescence throughout the exposure period exhibited a significant ozone dose relationship with all AOT indices applied although a quadratic regression relationship with AOT0 provided the best explanation of the observe response. The quadratic nature of this relationship relates to the effect of natural progression of senescence in the control treatment. This

result is comparable to the ozone dose response functions for *A. odoratum* under acute ozone exposure (Chapter 2) and *Tanacetum vulgare* and *Trifolium arvense* under chronic ozone exposure (Bergmann *et al* 1999).

3.4.3 Intraspecific variation in ozone sensitivity

The four *A. odoratum* populations studied differed significantly for all ozone response assessment criteria regardless of ozone treatment. The highest levels of natural senescence both for whole plants and individual leaves under control conditions were observed in the Aber and Maltraeth populations. Shoot growth was highest in the Cwm Idwal population while Birds Rock exhibited the highest rate of tillering. This among population variation was consistent with the highly polymorphic morphology of this species (Hubbard 1992). Levels of variation in stomatal conductance were lower as the only significant difference among populations was for stomatal conductance on the adaxial surface of the third fully expanded leaf which was highest in the Birds Rock population.

The populations differed in ozone sensitivity in terms of foliar injury although the greatest responses were observed under elevated background with peak episodes in each population. Cwm Idwal was the most sensitive population in terms of whole plant senescence throughout the exposure period while Birds Rock was the least sensitive in terms of whole plant and individual leaf senescence. All four populations exhibited the same trend in response for early season senescence with a greater sensitivity to elevated background than peak episodes at low background. This trend was maintained throughout the exposure period in the Aber and Cwm Idwal populations while the Birds Rock and Maltraeth populations were more sensitive to peak episodes for late season senescence.

The lower sensitivity of the Birds Rock population in terms of foliar injury does not relate to the potentially higher ozone flux expected with the higher stomatal conductance in this population (Pleijel *et al* 2000, Bungener *et al* 1999b). However, the difference in stomatal conductance among populations was only evident for one surface

of the third leaf and the potential inhibitory effect of ozone exposure on stomatal opening (Torsethaugen *et al* 1999) may have negated any effect of this difference. Reductions in stomatal conductance under elevated ozone have been reported in semi-natural species including *A. odoratum* (Hayes 2007, Reiling and Davison 1995, Power and Ashmore 2002) although at low concentrations ozone may stimulate stomatal conductance (Robinson *et al* 1998).

The populations did not exhibit any significant response to ozone exposure in terms of plant growth. There was a slight trend for stimulated shoot growth with elevated background and peak episodes at low background in three populations while the Maltraeth population exhibited a trend for decrease with peak episodes. The response in terms of relative tillering and tiller number were more variable among populations although there were no clear trends. The low magnitude of response indicates that the populations were relatively insensitive to ozone exposure for all plant growth assessment criteria.

The trends in among population relative sensitivity in terms of foliar injury were similar to those observed in these populations under acute exposure with Birds Rock exhibiting the lowest sensitivity (Chapter 2). As with previous studies there was no clear relationship between the development of foliar injury and the observed plant growth response (Bergmann *et al* 1995, Warwick and Taylor 1995, Power and Ashmore 2002, Hayes *et al* 2006). The populations also varied in plant growth relative sensitivity in this study although the level of response and variation were much lower than observed under acute exposure (Chapter 2). This may be due the perennial nature of this species and a longer-term chronic ozone exposure may be required to induce a significant growth response. The results for relative sensitivity in terms of foliar injury suggest that the populations may differ response to ambient peak episodes and elevated background concentrations however there was no clear pattern of population response in relation to altitude or predicted ambient ozone climate.

Chapter 4: Effects of simulated current and future upland ozone profiles on *Anthoxanthum odoratum*

4.1 Introduction

In the UK, annual mean background concentrations are currently around 25 ppb in lowland rural regions and increase with altitude to around 35 ppb in upland regions (NEGTAP 2001). The increase in concentration with altitude is due to the higher wind speeds and more turbulent air conditions which result in greater replacement of ozone removed through deposition (Coyle *et al* 2002, Ashmore *et al* 2002). This influences the extent of variation across the diurnal cycle as concentrations are often maintained overnight in upland areas with overnight ozone depletion approximately 25 % of that observed in lowland areas (Coyle *et al* 2002). The maintenance of elevated ozone concentrations overnight is also evident during peak episodes (Figure 4.1). This has implications for the adverse effects on upland vegetation as many plant species exhibit some nocturnal stomatal conductance, although usually lower than during the day, which may result in significant ozone flux into the plant (Musselman and Minnick 2000).

The effects of elevated night time ozone concentrations are of interest not just in terms of current conditions experienced by upland vegetation species but also in relation to future increases in background ozone concentrations. Trends in UK ozone concentrations over the last 30 to 40 years suggest that although peak concentrations are declining as a result of reduced emissions of NO and CO across Europe, background concentrations are rising due to increasing anthropogenic emissions at a global scale (NEGTAP 2001, Vingarzan 2004). Background ozone concentrations are predicted to reach around 35 ppb in lowland areas by 2030 comparable to those currently experienced in upland areas, while concentrations in upland areas will reach approximately 40 ppb and are expected to be in the range of 40 to 50 ppb across most of the UK by the end of the century (Coyle *et al* 2003b). Increased background

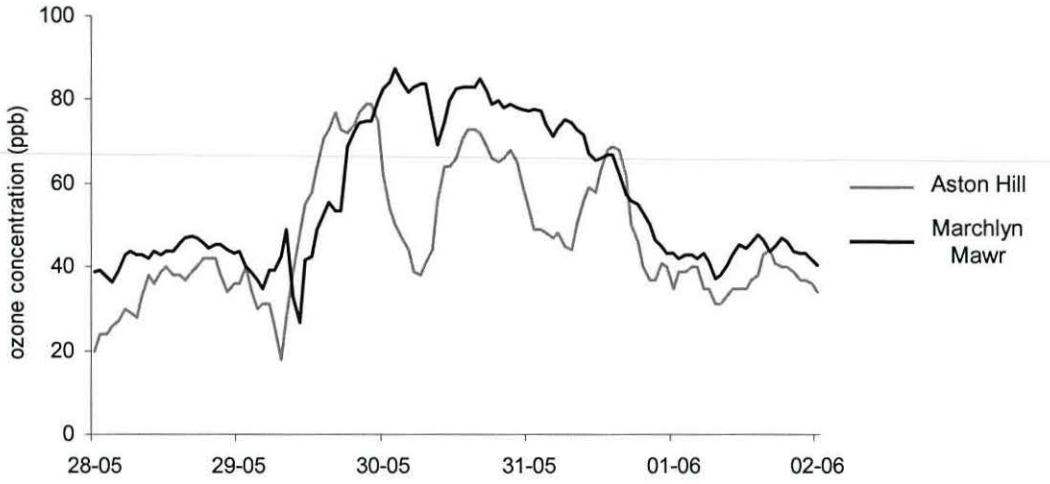

Figure 4.1 – Hourly ozone concentrations during a three day peak episode in May to June 2003 at lowland (Aston Hill) and upland (Marchlyn Mawr) rural monitoring sites.

concentrations are expected to have the greatest impact in upland areas where ozone levels are less depleted over night (Ashmore *et al* 2002).

Few studies have considered the effects of elevated ozone concentrations on upland vegetation species (Foot *et al* 1997, Hayes *et al* 2006, Hayes 2007) while the response of semi-natural vegetation to elevated background concentrations has been highlighted as an area requiring further investigation (Ashmore *et al* 2002, Coyle *et al* 2003a). The aim of this study was to investigate the response of the perennial grass species *Anthoxanthum odoratum* to ozone exposure profiles representative of upland areas under current and elevated ozone scenarios. Intraspecific variation in response among populations of *A. odoratum* from different altitude sites in North Wales was investigated in terms of visible injury, flowering and plant growth. *A. odoratum* has previously been shown to exhibit premature senescence in response to ozone exposure (Hayes *et al* 2006, Chapters 2 and 3) and a similar magnitude of response in terms of early season senescence was observed under elevated background as with peak episodes at low background concentrations in these populations (see Chapter 3). Upland ozone profiles were stylised from a peak episode which occurred during May to June 2003 at the Marchlyn Mawr upland rural ozone monitoring site in Snowdonia, UK (Figure 4.1) and included constant background concentrations and a three-day low magnitude peak episode with slow rise and fall in ozone concentration in the day before and after respectively.

4.2 Materials and Methods

4.2.1 Plant material

Fourteen rametes per individual were vegetatively propagated in March 2006 from stock plants collected from four populations in North Wales in June-July 2004 (see Chapter 1, section 1.4 for details of sample collection). Plants were grown in an ericaceous-sand mix compost (ratio 3:2, litres:kg) at the Pen-y-Ffridd Research Station, Bangor. Rametes were established from single tillers of similar size in a heated glasshouse (day 18 °C and night 16 °C) with supplemental lighting. After three weeks rametes were transplanted into 1 litre 10 cm square pots, lined with perforated black plastic to contain root growth within the pot, and transferred to a sheltered outside position under ambient conditions. At four weeks ramete size was determined by tiller number. Sixty four individuals, 16 per population, were selected on the basis of plant health and ramete size uniformity. Eight rametes per individual were paired by size and one ramete per pair was destructively harvested to soil level to determine pre-exposure biomass. Above ground biomass was removed, fresh weight determined and plant material then dried at 65 °C for at least four days for dry weight measurement. At five weeks the remaining four rametes per individual were transferred to the CEH ozone exposure facility, Abergwyngregyn.

4.2.2 Ozone exposure

4.2.2.1 Ozone fumigation system

Plants were exposed to ozone in eight replicate ventilated hemispherical glasshouses (solardomes) 2 metres high and 3 metres diameter receiving approximately two complete air changes per minute. Ozone was generated by a G11 ozone generator from oxygen supplied by a Workhorse 8 oxygen generator (both Dryden Aqua, UK Ltd) and

delivered to the solardomes by a computer controlled (Labview, version 7) mass-flow controller system. Ozone was added to charcoal filtered air and concentrations in one solardome were continuously monitored on a Model 49C ozone analyser (Thermo Electron) with ozone supply to all solardomes adjusted accordingly via a feedback control loop. Ozone concentrations in all solardomes were monitored on a 30 minute cycle by two API400 ozone analysers (Envirotech) of matched calibration. Hourly mean ozone concentrations (ppb) were calculated from half hourly measurements and used to calculate the accumulated ozone over a threshold of X ppb (AOTX ppm.h) for 12 daylight hours (7 am to 7 pm) and 24 hours per day over the experimental period. Weekly AOTXs were calculated from the start of ozone exposure to 9 am on the corresponding assessment day.

4.2.2.2 Ozone treatment profiles

Four ozone treatments were applied in a weekly episodic regime with background concentrations of 10 ppb (Control), 35 ppb (Low), 60 ppb (Medium) and 85 ppb (High) and peak concentrations elevated by 20 ppb above the background. Weekly ozone profiles consisted of two days at the background concentration and a five-day ozone episode with peak concentrations reached over 24 hours, maintained for a further 72 hours and gradually returned to the background concentration over 24 hours (Figure 4.2).

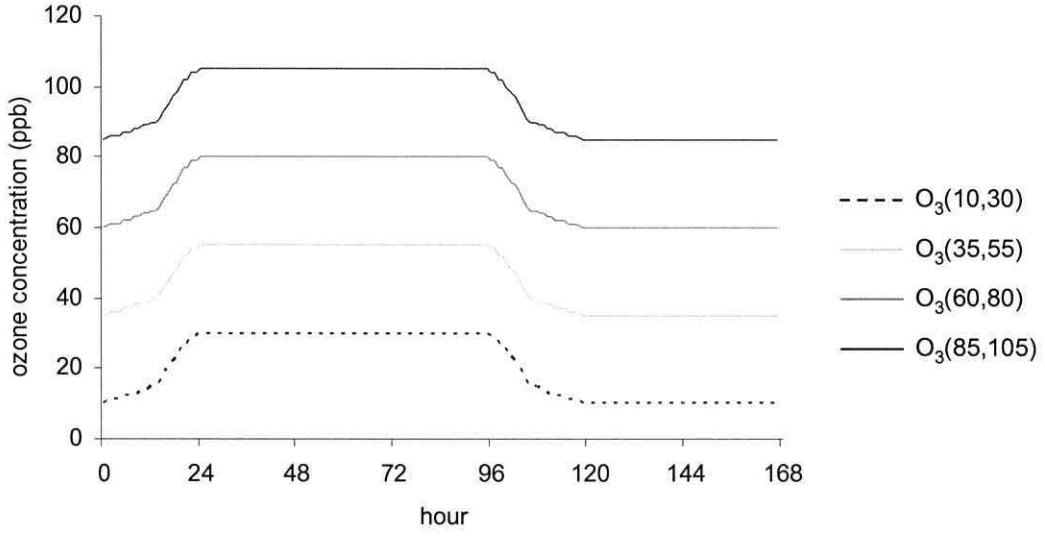

Figure 4.2 – Weekly ozone treatment profiles as O_3(background concentration, peak concentration) for Control O_3(10, 30), Low O_3(35, 55), Medium O_3(60, 80), and High O_3(85, 105) treatments.

4.2.2.3 Experimental design

Solardomes were split into two randomised blocks of four treatments with eight individuals per population per replicate block. Plants were randomly assigned to solardomes within each replicate with one ramete per individual in each solardome. Plants were arranged on trays (0.4 m x 0.3 m) lined with capillary matting and with drainage holes 10 mm above the base. Tray positions were duplicated among solardomes and plants were randomly assigned to 32 positions across six trays within each solardome. Plant pots were spaced approximately 5 cm apart on each tray and trays were rotated weekly within each solardome. Plants were transferred to the solardomes five weeks after propagation and allowed to acclimatise at 20 ppb for five days prior to ozone exposure. Plants were exposed to ozone treatments for eight weeks from 9th May to 3rd July 2006 receiving eight ozone episodes. Plants were irrigated with a timed automatic misting system for 30 to 60 minutes every night. The duration was adjusted to account for changes in canopy size and climatic conditions over the exposure period with supplemental watering by hand as necessary.

4.2.2.4 Visual assessment

All plants were visually assessed weekly for percentage of senescence and flowering prior to and throughout the exposure period. Whole plant senescence was assessed as the percentage of leaves with $\geq$ 25% necrotic senescence. For those plants which flowered during the exposure period, flower number and flower stage (emerging, flowering or finished) were recorded.

4.2.2.5 Plant growth assessment

Plants were destructively harvested to soil level after eight ozone episodes to determine post-exposure biomass. Above ground biomass was removed, fresh weight determined and plant material dried at 65 °C for at least four days for dry weight measurement. Relative growth rate (RGR) was calculated for dry weight total above ground biomass using pre- and post-exposure measurements as:

$$RGR = (\ln w_2 - \ln w_1)/(t_2 - t_1)$$

where w is weight (g), t is time (weeks) and subscript denotes harvest date.

4.2.2.6 Relative ozone sensitivity

A relative ozone sensitivity index was determined for comparison of ozone effects between different assessment criteria and populations. Foliar injury assessed as the percentage of whole plant senescence was converted to the percentage of healthy leaves for this purpose as:

$$\% \text{ healthy leaves} = 100 - \% \text{ whole plant senescence}$$

Relative sensitivity (RS) of plant growth and individual week whole plant senescence were calculated using treatment means as the proportion under elevated ozone low ($O_3(35,55)$), medium ($O_3(60,60)$) and high ($O_3(85,100)$ treatments relative to the control ($O_3(10,30)$) where:

$$RS = 1 + ((O_{3\ elevated} - O_{3\ control}) / O_{3\ control})$$

RS values of <1 or >1 are obtained with a reduction or stimulation respectively for plant growth and % healthy leaves under elevated ozone.

For the rate of development of foliar injury, assessed as $wpns_{coeff}$ (defined in section 4.2.3), the relative sensitivity was calculated using treatment means as the rate under control conditions relative to the rate under elevated ozone where:

$$RS = O_{3\ control} / O_{3\ elevated}$$

RS values of <1 and >1 are obtained for an increase or decrease respectively in the rate of development under elevated ozone.

The relative ozone sensitivity index describes the effects of elevated ozone in relation to the control with a relative sensitivity of 1 indicating no response, <1 indicating sensitivity or reduction and >1 indicating resistance or stimulation.

4.2.3 Statistical analysis

Arcsine transformation was performed on individual week senescence data and natural log transformation on post-exposure fresh and dry weight biomass data. Values for the eight plants per population per dome were averaged to provide two replicates per treatment per population for all variables except number of flowering plants, where the actual number provided the two replicates per treatment. Population dome averages were used for all subsequent analysis. Time-course data for whole plant necrotic senescence (wpns) was analysed by regression in Minitab version 14 and the resulting regression coefficients saved for subsequent analysis as variable $wpns_{coeff}$. Variables were analysed for the main and interaction effects of ozone treatment and population using an analysis of variance (ANOVA) model with a randomized block design, linear polynomial contrasts for ozone treatment and Post-hoc multiple comparisons using Tukey HSD test in Genstat version 8. Populations exhibited some inequality of variance which may have violated the assumptions of the analysis model and were reanalysed separately for ozone treatment as a main effect with linear polynomial contrasts for ozone treatment and post-hoc multiple comparisons using Tukey HSD test. ANOVA model for overall and population level analysis given in Table 4.1. Ozone dose response was analysed by regression of whole plant senescence against AOTX using treatment means from all assessment weeks throughout the exposure period in Minitab version 14.

Table 4.1. – ANOVA model for overall and individual population analysis.

Overall		Individual populations	
Source of variation	**DF**	**Source of variation**	**DF**
Block	1	**Block**	1
Treatment‡	3	**Treatment**‡	3
linear‡	*1*	*linear*‡	*1*
deviations‡	*2*	*deviations*‡	*2*
residual	*3*	*residual*	*3*
Population‡	3	**Total**	7
Treatment x Population‡	9		
linear x population‡	*3*		
deviations‡	*6*		
residual	*12*		
Total	31		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

4.3 Results

4.3.1 Received ozone exposure

Average total and weekly ozone concentrations received by all treatments were within 10 ppb of the target concentration with peak concentrations elevated by between 15 ppb and 25 ppb above the background concentration (Table 4.2). The differences between the expected and actual average background ozone concentrations were greatest in the third week of exposure for the elevated ozone treatments when the ozone supply was interrupted for 22 hours and all solardomes received charcoal filtered air with <10 ppb ozone. In the fifth week of exposure one replicate solardome for the O_3(60,80) treatment received over 250 ppb above the target peak concentration during the ozone episode. The hourly average ozone concentration steadily increased over 17 hours, reaching a maximum concentration of 355 ppb at 9 am on day 32, due to deterioration and failure of the mass flow controller supplying ozone to the solardome. The mean hourly ozone concentration for this treatment at that time reached 216 ppb (Figure 4.3). Overall, the average peak concentration for this week was 10 ppb lower than the target in this

Table 4.2 – Total and weekly average ozone concentrations (ppb) received for background and peak times during weekly profile for ozone treatments.

	Ozone Treatment							
	O_3(10,30)		O_3(35,55)		O_3(60,80)		O_3(85,105)	
Week	bgnd	peak	bgnd	peak	bgnd	peak	bgnd	peak
1	10.6 ±0.4	27.1 ±0.3	34.5 ±0.5	53.2 ±0.4	52.0 ±0.9	74.2 ±0.5	84.5 ±0.5	105.0 ±0.7
2	12.8 ±0.2	28.4 ±0.5	37.1 ±0.4	53.8 ±1.1	59.6 ±0.7	68.3 ±1.8	80.3 ±1.0	93.9 ±2.2
3	11.2 ±0.4	28.1 ±0.2	31.8 ±1.4	55.2 ±0.3	44.9 ±2.3	77.6 ±0.3	67.7 ±3.3	104.8 ±0.3
4	10.8 ±0.3	27.2 ±0.2	36.4 ±0.7	55.4 ±0.2	57.4 ±1.0	80.1 ±0.3	80.5 ±1.7	105.4 ±0.3
5	9.6 ±0.3	27.3 ±0.5	33.7 ±0.9	54.2 ±0.4	54.3 ±1.7	73.3 ±4.2	78.3 ±2.2	104.3 ±0.5
6	11.4 ±0.3	28.2 ±0.3	35.1 ±0.9	53.7 ±1.3	60.6 ±1.5	80.5 ±0.3	79.0 ±2.0	103.9 ±0.3
7	10.8 ±0.4	28.4 ±0.2	36.0 ±0.5	59.2 ±1.6	63.0 ±0.5	83.7 ±0.2	81.3 ±1.2	105.0 ±0.3
8	9.9 ±0.3	26.4 ±0.3	36.2 ±0.3	53.0 ±0.2	62.2 ±0.5	82.2 ±0.3	82.6 ±1.2	106.0 ±0.4
Total	10.9 ±0.1	27.6 ±0.1	35.1 ±0.3	54.7 ±0.3	56.6 ±0.5	77.5 ±0.6	78.9 ±0.7	103.5 ±0.3

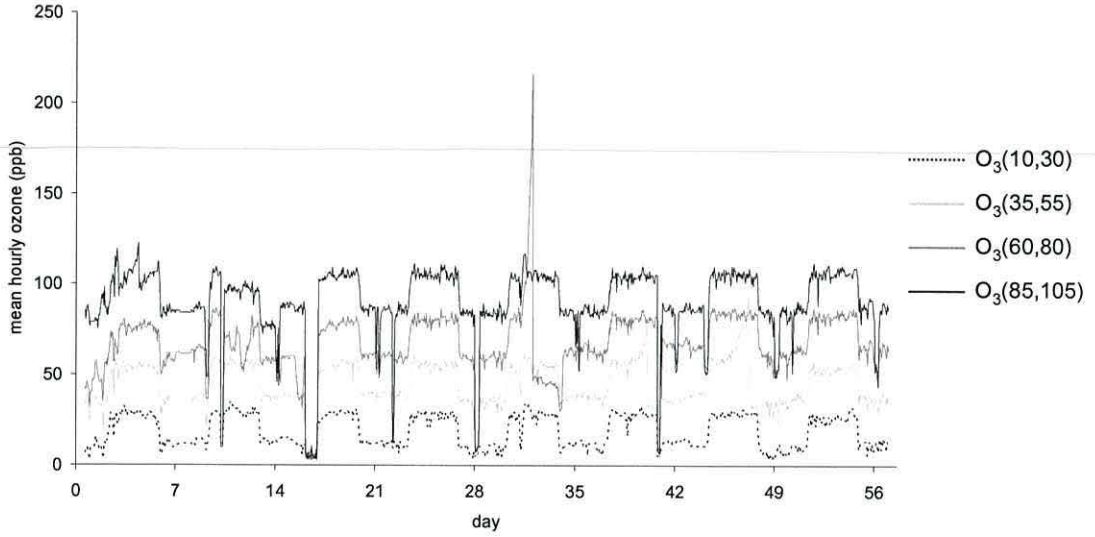

Figure 4.3 – Hourly mean ozone concentration (ppb) received over eight weeks of exposure for control (O_3(10,30)), low (O_3(35,55)), medium (O_3(60,60)) and high (O_3(85,100) ozone treatments.

treatment due to suspension of the ozone supply for 48 hours for maintenance during which time this replicate solardome received charcoal filtered air with < 10 ppb ozone. The intended weekly ozone profile was applied throughout the exposure period with the exception of the disruptions in weeks three and five and short periods when ozone supply was suspended or concentrations reduced to 50 ppb for routine access into the solardomes.

The received ozone dose, measured as accumulated ozone over a threshold of X ppb (AOTX), increased in a linear fashion throughout the exposure period in all treatments (Figure 4.4). After eight weeks of exposure the received ozone dose as AOT0 increased with regular intervals of around 32 ppm.h between successive ozone treatments (Figure 4.4 a). This was comparable to the predicted increment of 33.8 ppm.h expected for a 25 ppb elevation in ozone concentration between treatment levels. When a 40 ppb threshold was applied, the difference in AOT40 between O_3(35,55) and O_3(60,80), and between O_3(60,80) and O_3(85,105) were comparable at 28.2 ppm.h and 31.4 ppm.h respectively, while the difference between O_3(10,30) and O_3(35,55) was less than half these at 10.4 ppm.h (Figure 4.4 b). The AOT40 for O_3(10,30) was zero because the ozone concentration did not rise above 40 ppb in this treatment.

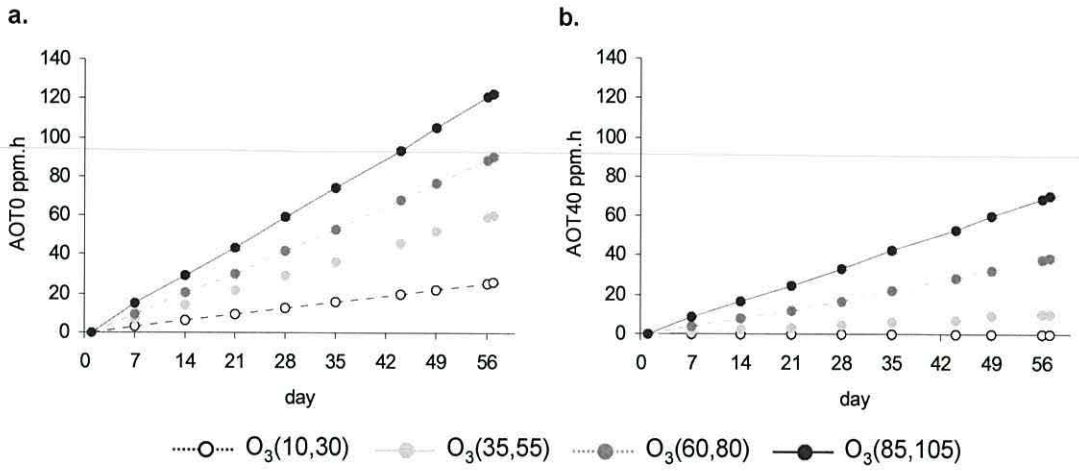

Figure 4.4 - Mean weekly accumulated ozone (ppm.h) over an eight week exposure period for control (O_3(20)), low (O_3(35,55)), medium (O_3(60,60)) and high (O_3(85,100) ozone treatments. Ozone accumulated over a threshold of a) 0 ppb and b) 40 ppb for 24 hours per day.

4.3.2 Visual assessment

4.3.2.1 Senescence

Increased/premature senescence with elevated ozone was observed at the species level after three ozone episodes and continued throughout the remaining five weeks of exposure (Figure 4.5). Summary of overall ANOVA results are given in Table 4.3 and full details in Appendix III. The greatest increases in senescence under elevated ozone were observed after six to eight weeks of exposure with average increases above the control treatment of 5.5 %, 11.1 % and 19.6 % for the O_3(35,55), O_3(60,80) and O_3(85,105) treatments respectively (Figure 4.5). Average senescence under the highest ozone treatment (O_3(85,105)) was double that observed in the control treatment for weeks three through eight (P<0.05). The extent of senescence in the O_3(10,30) and O_3(85,105) treatments after seven weeks of exposure are illustrated in Figure 4.6. Senescence in the O_3(35,55) and O_3(60,80) treatments began to diverge after six ozone episodes. This may have been due to the high peak of ozone which occurred during the fifth ozone episode in one replicate solardome of the O_3(60,80) treatment. However the standard errors of the means suggest that the variance is similar between treatments after this event and do not indicate any large change in one replicate solardome.

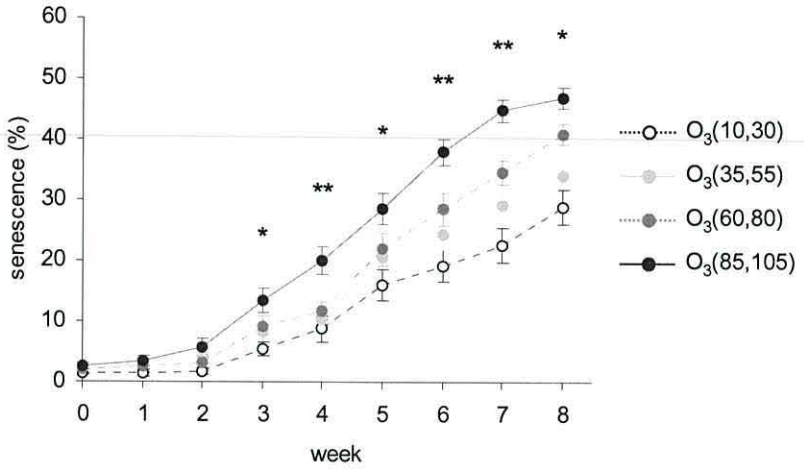

Figure 4.5 – The development of whole plant senescence over eight weeks exposure to control (O_3(10,30)), low (O_3(35,55)), medium (O_3(60,60)) and high (O_3(85,100) ozone treatments. Significant treatment effects are shown as **=P<0.01 and *= P<0.05.

Significant linear relationships, assessed by linear contrasts in the ANOVA model, between senescence and successive ozone treatments occurred in weeks three through eight (P<0.05) suggesting the increase in observed senescence followed the increase in applied ozone concentration for each assessment week. Significant deviations from this relationship occurred after seven ozone episodes (P<0.01) where the increment was similar between the lower three ozone treatments at 5.5 % to 6.4 %, but increased to 10.3 % between the O_3(60,80) and O_3(85,105) treatments. By week eight the differences between successive treatments had become more uniform ranging from 5.1 % to 6.9 % (Figure 4.5).

Populations differed significantly in the amount of whole plant senescence regardless of ozone treatment in weeks two through five (P<0.05) with the highest levels observed in the Aber population and the lowest in the Birds Rock population (Figure 4.7). This became non-significant for week six onwards as the variation increased both within and among treatments for each population. Differences in the response of individual populations to the applied ozone treatments were observed after two and four ozone episodes (P<0.05). Summary of population level ANOVA results are given in Table 4.4 and full details in Appendix III In week two the interaction effect was mainly due to the Birds Rock and Maltraeth populations, where the highest levels of senescence were exhibited in the O_3(35,55) and O_3(10,30) treatments respectively. Although there was a

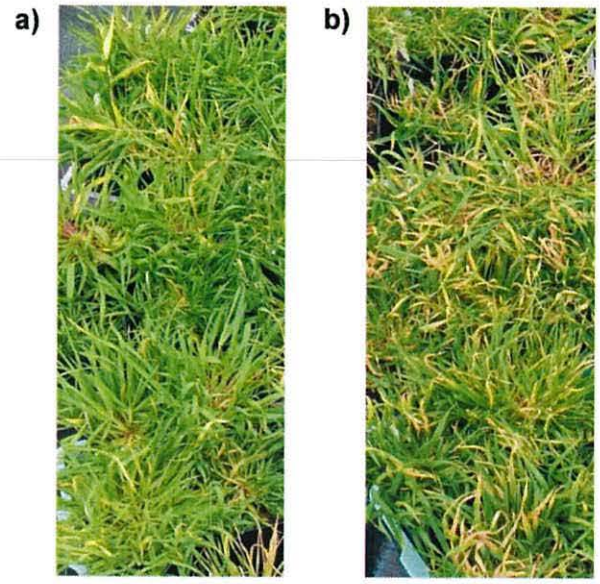

Figure 4.6 – Senescence after seven weeks exposure to a) O_3(10,30) and b) O_3(85,105) ozone treatments.

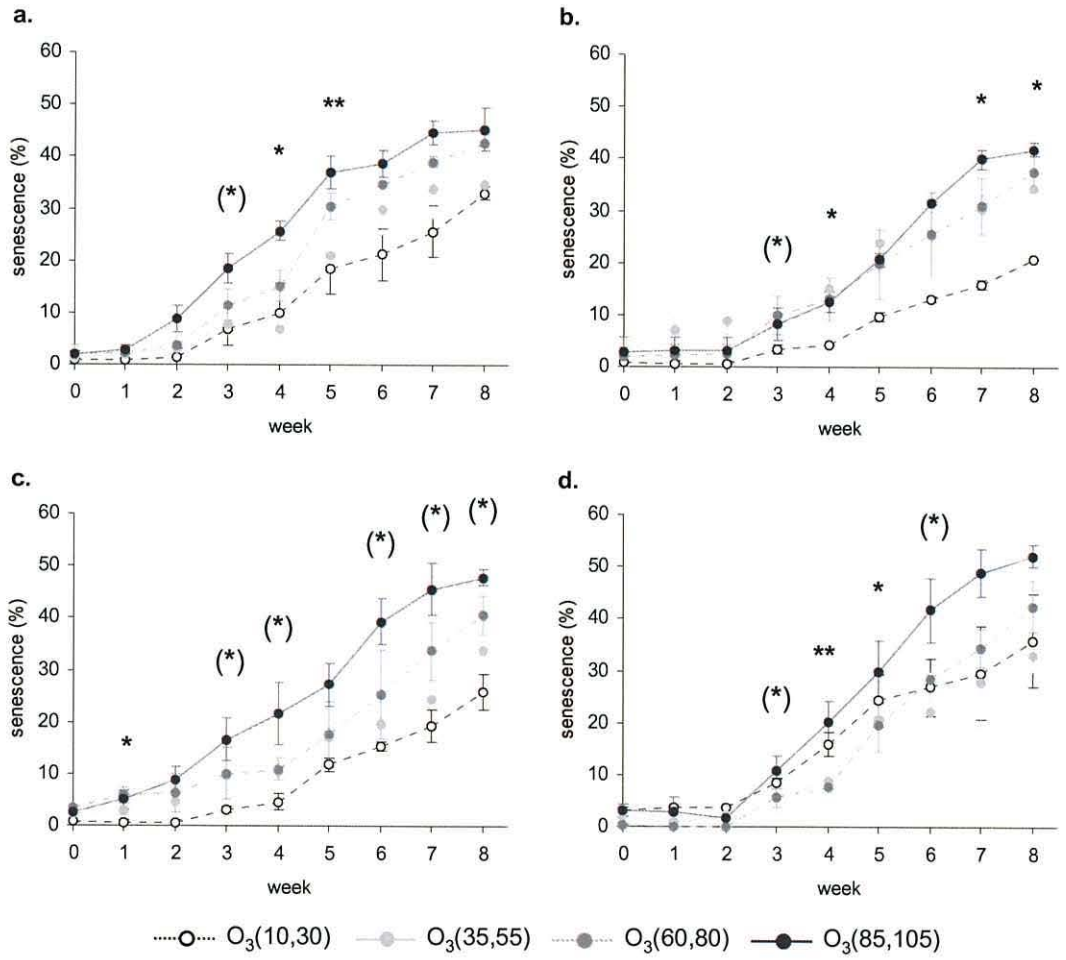

Figure 4.7 – The development of whole plant senescence for populations a) Aber, b) Birds Rock, c) Cwm Idwal and d) Maltraeth over eight weeks exposure to control (O_3(10,30)), low (O_3(35,55)), medium (O_3(60,60)) and high (O_3(85,100) ozone treatments. Significant treatment effects are shown as **= P<0.01 *= P<0.05 and (*)=P<0.1.

significant interaction effect there was no significant ozone treatment effect overall or for any individual population. The observed senescence levels were all below 10 % in this week and it is likely that most of this effect is attributable to pre-exposure differences affecting the development of senescence in the early stages of exposure. In week four there were significant treatment effects for the majority of populations and the interaction effect was apparent with each population exhibiting a different response (Figure 4.7). The biggest difference in response is shown by the Maltraeth population where the control treatment had double the amount of senescence observed in the $O_3(35,55)$ and $O_3(60,80)$ treatments.

The Aber, Birds Rock and Cwm Idwal populations exhibited the expected trend for increased senescence with increasing levels of elevated ozone. The Maltraeth population followed this trend between the three elevated ozone treatments but displayed higher senescence in the control treatment than both the $O_3(35,55)$ and $O_3(60,80)$ treatments for the first five weeks of exposure. All significant ozone treatment effects found for this population were between the $O_3(85,105)$ treatment and lower elevated ozone treatments (Figure 4.7). The highest levels of senescence were observed in the $O_3(85,105)$ treatment in three populations while the Birds Rock population exhibited no differentiation between the elevated ozone treatments until week six. Populations differed in the number of episodes received before any divergence was observed between the $O_3(35,55)$ and $O_3(60,80)$ treatments. Aber exhibited the earliest divergence occurring between weeks three and four, Cwm Idwal and Maltraeth diverged after six episodes and Birds Rock showed no indication of divergence between these treatments until week eight.

The Birds Rock and Cwm Idwal populations exhibited large variation in the $O_3(60,80)$ treatment in weeks five and six in comparison with the other treatments. This may be due to the high peak in ozone that occurred in one replicate solardome of this treatment during the fifth ozone episode. This had no impact on treatment divergence in Birds Rock but may have accelerated the divergence between the $O_3(35,55)$ and $O_3(60,80)$ treatments in the Cwm Idwal population which occurred 10 days after this event.

Populations exhibited linear relationships between senescence and successive ozone treatments, assessed by linear contrasts in the ANOVA model, in weeks two to eight of

the exposure period. The Aber and Cwm Idwal populations showed significant relationships for weeks two through seven ($P<0.05$) with no significant deviations and this extended into week eight for Cwm Idwal. The Birds Rock population showed a linear relationship for weeks three, four, seven and eight ($P<0.05$) but was not observed in weeks five and six due to the high variation in the $O_3(60,80)$ treatment. Although significant relationships were found in weeks three through six in the Maltraeth population, these differed significantly from a linear relationship in weeks three ($P<0.1$), four and five ($P<0.05$) due to the high levels of senescence in the control treatment.

After eight weeks of exposure the increase in senescence under elevated ozone was highest for the $O_3(85,105)$ treatment at the species level ($P<0.05$) and there is a significant linear relationship between the observed senescence and the level of elevated ozone applied ($P<0.01$, Figure 4.8 a). The same trend was observed in all populations although significant treatment effects only occurred in the Birds Rock and Cwm Idwal populations ($P<0.1$ and $P<0.05$ respectively) with a significant linear relationship for both ($P<0.05$). No significant treatment effects or linear relationships occurred in the Aber or Maltraeth populations due to the large within treatment variation observed in the $O_3(35,55)$ and $O_3(10,30)$ treatments respectively (Figure 4.8 b).

The rate of development of senescence at the species level, assessed by the coefficients of regression for whole plant senescence against days of exposure ($wpns_{coeff}$), increased

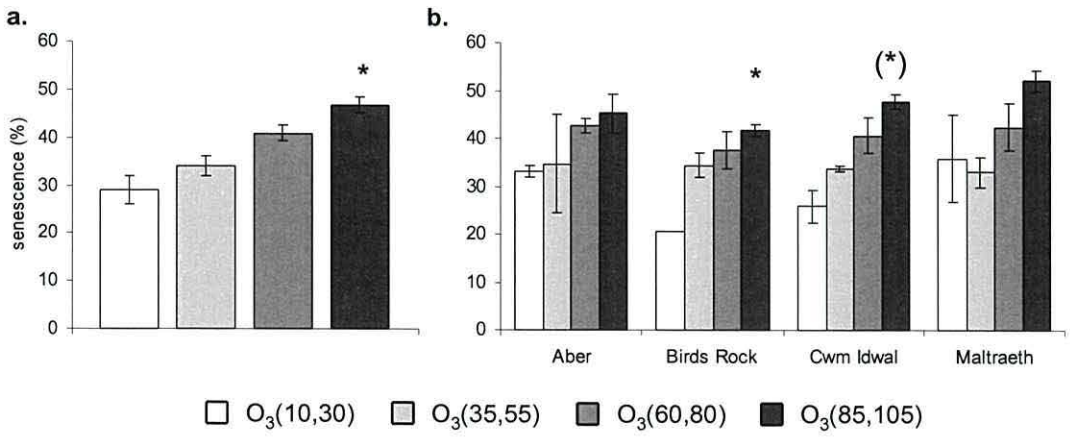

Figure 4.8 – Whole plant senescence after eight ozone episodes for a) species and b) population level. Significant differences from the control treatment ($O_3(10,30)$) are shown as *= $P<0.05$ and (*)=$P<0.1$

under elevated ozone by 17.2 %, 43.9 % and 74.1 % for $O_3(35,55)$, $O_3(60,80)$ and $O_3(85,105)$ treatments respectively ($P<0.01$, Figure 4.9 a). Summary of overall ANOVA results are given in Table 4.3. A highly significant linear relationship, assessed by linear contrasts in the ANOVA model, occurred between $wpns_{coeff}$ and successive ozone treatments ($P<0.001$) indicating that the increase in the rate of development of senescence followed the linear increase in the applied ozone concentrations. Although the increment between the treatments increased slightly at the higher levels of ozone exposure there was no significant deviation from the linear relationship.

Populations differed significantly in the rate of development of senescence regardless of ozone treatment ($P<0.05$) with the lowest rate in the Birds Rock population and the highest in the Maltraeth population (Figure 4.9 b). All populations followed the trend for an increased rate of development of senescence under elevated ozone, although ozone treatment effects were only significant for Cwm Idwal ($P<0.05$) and at the $P<0.1$ level for Birds Rock. Summary of population level ANOVA results are given in Table 4.4. The Aber and Maltraeth populations showed no significant effects of ozone treatment due to large within treatment variation in the $O_3(35,55)$ and $O_3(10,30)$ treatments respectively. Significant linear relationships between $wpns_{coeff}$ and successive ozone treatments occurred in the Birds Rock and Cwm Idwal populations ($P<0.05$) and at the $P<0.1$ level for Aber and Maltraeth. The increment between treatments varied among populations with the largest difference occurring between the $O_3(10,30)$ and $O_3(35,55)$ treatments in the Birds Rock population and the $O_3(60,80)$ and $O_3(85,105)$ treatments in Cwm Idwal (Figure 4.9 b). Despite the different increments between the

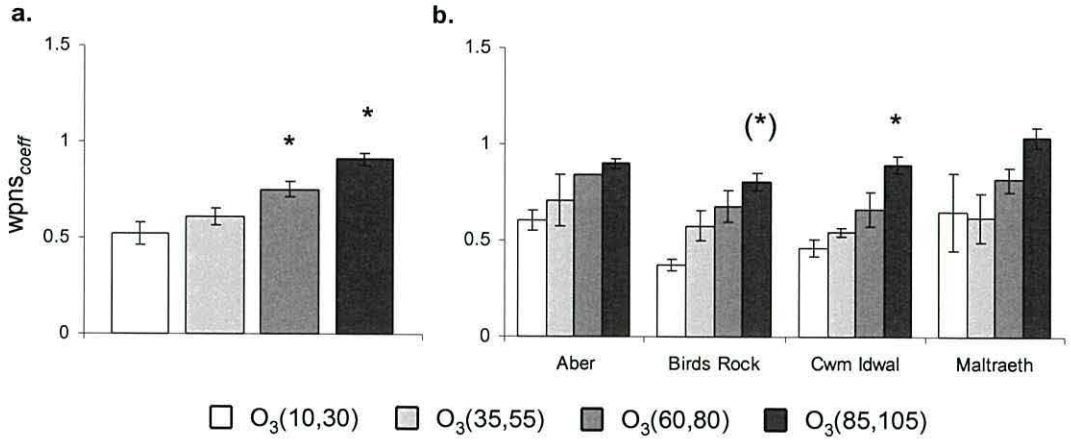

Figure 4.9 – Rate of development of senescence ($wpns_{coeff}$) over eight weeks of ozone exposure for a) species and b) population level. Significant differences from the control treatment ($O_3(10,30)$) are shown as *= $P<0.05$ and (*)=$P<0.1$

ozone treatments there were no significant deviations from the linear relationship.

4.3.2.1 Flowering

Ozone exposure had no significant effect on either the number of flowers produced per plant or the number of flowering plants when considered separately for the emerging and flowering stages or the combined total at the species level (Figure 4.10) and population level. Populations differed in flowering regardless of ozone treatment with significant differences in the number of flowers per plant for weeks zero though five ($P<0.05$) and the number of flowering plants throughout the exposure period ($P<0.05$). The Birds Rock population exhibited the greatest number of flowering plants and flowers per plant while the smallest numbers for both were observed in the Maltraeth population. It is unlikely that these results accurately represent the effect of elevated ozone on flowering as at least 50 % of the plants within this experiment did not produce flowers regardless of the applied ozone treatment. There was a large amount of variation between replicates with an average 35 % more plants flowering in replicate block two compared to replicate block one. This may be due to random variation in flowering between the source individuals allocated to each replicate block, as in the majority of cases where flowering was observed the rametes sourced from one individual produced flowers.

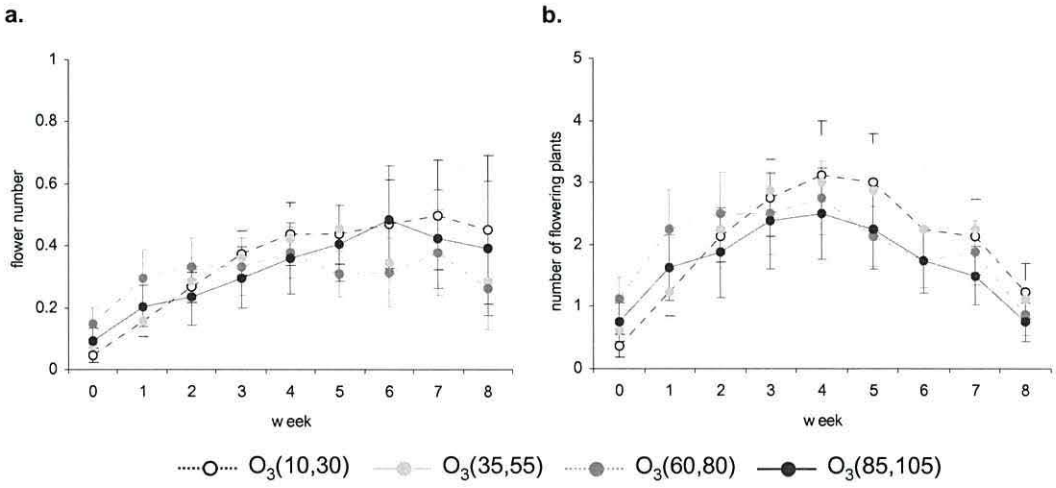

Figure 4.10 – Total flowering (emerging and flowering stages combined) at the species level as a) mean number of flowers per plant and b) number of flowering plants per dome over eight weeks of ozone exposure to control (O_3(10,30)), low (O_3(35,55)), medium (O_3(60,60)) and high (O_3(85,100) ozone treatments. No significant differences were observed. Bars represent standard errors of the mean.

4.3.3 Plant growth

Plant growth exhibited no significant ozone treatment effects or linear relationships with the level of elevated ozone applied at the species level after eight weeks of ozone exposure (Figure 4.11). Summaries of overall and population level ANOVA results are given in Tables 4.3 and 4.4 and full details in Appendix III. Populations differed significantly for above ground fresh and dry weight biomass regardless of ozone treatment ($P<0.01$) with plant growth highest for the Aber population and lowest in the

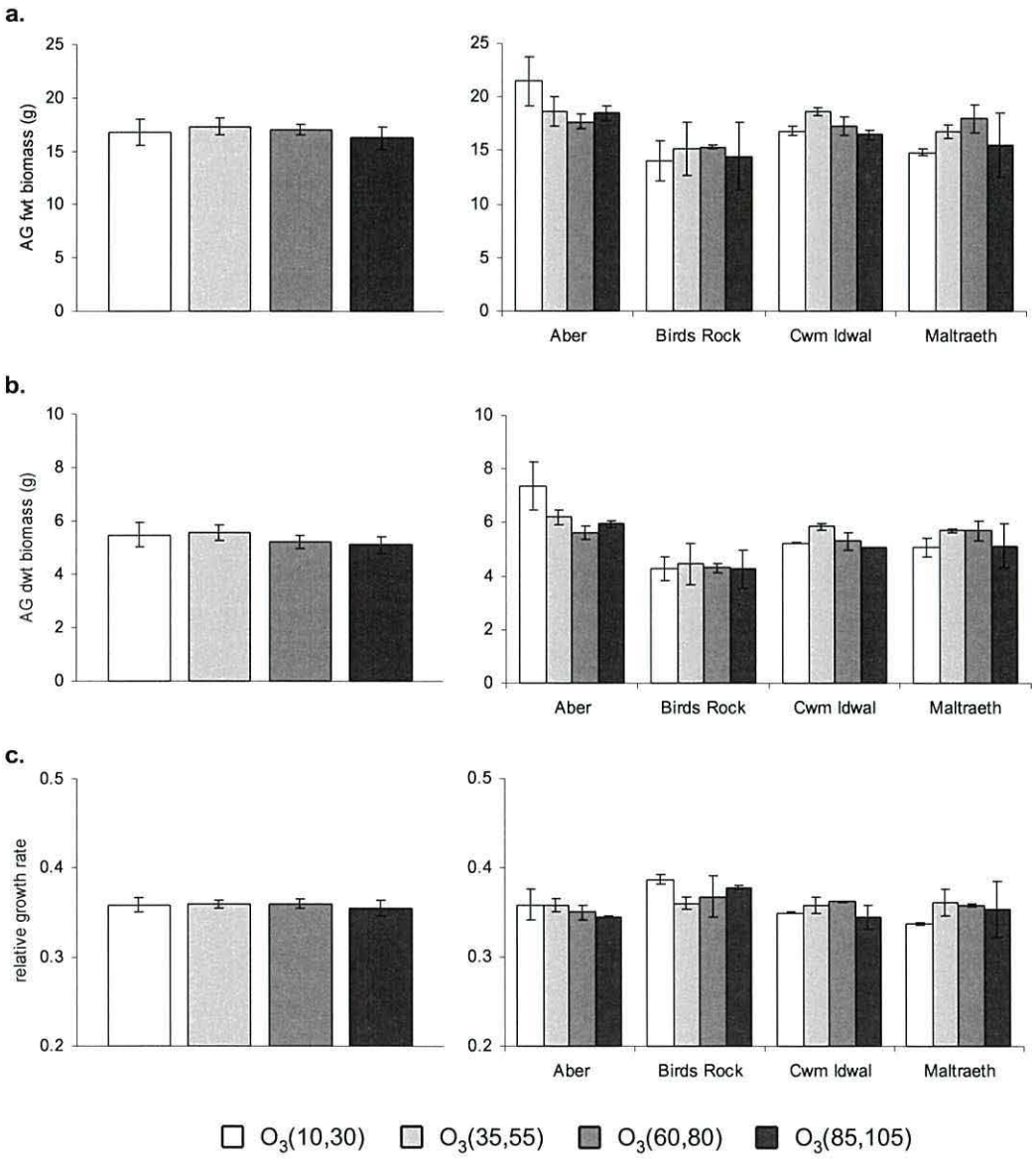

Figure 4.11 – Species and population level response for above ground biomass a) fresh weight b) dry weight and c) relative growth rate after eight weeks of ozone exposure to control (O_3(10,30)), low (O_3(35,55)), medium (O_3(60,60)) and high (O_3(85,100) ozone treatments. No significant differences were observed. Bars represent standard errors of the mean.

Birds Rock population. Although there were no significant treatment effects there was a slight trend for decreased growth under elevated ozone in Aber and for an increase at lower levels of elevated ozone, O_3(35,55) and O_3(60,80) treatments, for Cwm Idwal and Maltraeth (Figure 4.11). Relative growth rate follows a similar trend although the control treatment exhibited a higher growth rate than the elevated ozone treatments in the Birds Rock population.

The dry weight to fresh weight ratio (dwt:fwt) showed a non significant trend for decrease under the O_3(60,80) and O_3(85,105) treatments at the species level (Figure 4.12 a). The larger decrease for the O_3(60,80) treatment may be due to the high peak in ozone that occurred in one replicate solardome of this treatment during the fifth ozone episode. The populations differed significantly regardless of ozone treatment (P<0.05) with the highest ratio in the Maltraeth population and the lowest in the Birds Rock and Cwm Idwal populations (Figure 4.12 b). The Cwm Idwal population exhibited a significant treatment effect for dwt:fwt ratio (P<0.05) with the biggest difference between the O_3(35,55) and O_3(60,80) treatments. The Aber population followed the same trend for decrease with elevated ozone as for all other biomass variables. The Maltraeth population exhibited a different trend for dwt:fwt ratio compared to the other biomass variables with the ratio higher in the O_3(10,30) and O_3(85,105) treatments than the other two treatments.

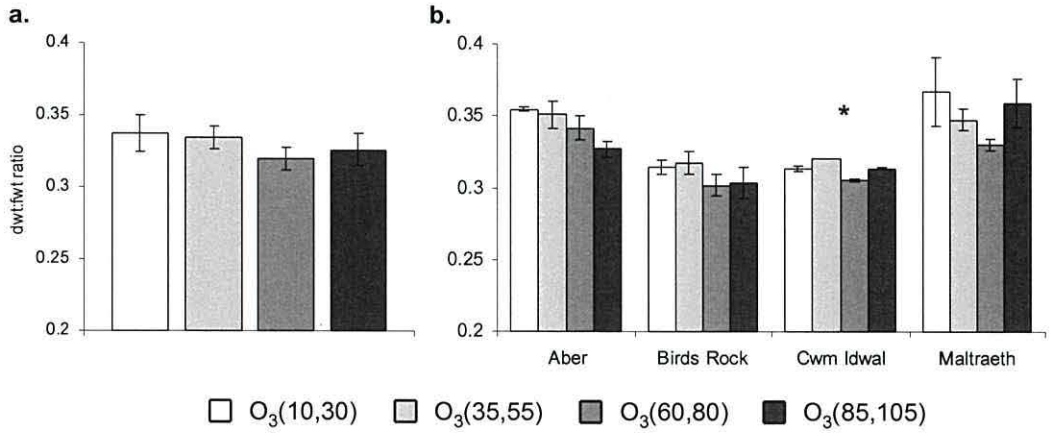

Figure 4.12 – Above ground biomass dry weight to fresh weight (dwt:fwt) ratio for a) species and b) population level after eight weeks exposure to control (O_3(10,30)), low (O_3(35,55)), medium (O_3(60,60)) and high (O_3(85,100) ozone treatments. Significant treatment effects are indicated by *= P<0.05.

Table 4.3 – Summary of overall ANOVA analysis results.

Source of variation	DF	wpns week 2			wpns week 4			wpns week 6			wpns week 8		
		S.S.	M.S.	*F value*	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*
Block	1	0.0031	0.0031	0.74	0.0139	0.0139	10.36	0.0282	0.0282	13.64	0.0001	0.0001	0.03
Treatment‡	3	0.0513	0.0171	4.1	0.1925	0.0642	47.86**	0.2329	0.0776	37.58**	0.1832	0.0611	15.93*
linear‡	*1*	*0.0384*	*0.0384*	*9.18(*)*	*0.1791*	*0.1791*	*133.65***	*0.2286*	*0.2286*	*110.66***	*0.1823*	*0.1823*	*47.55***
deviations‡	*2*	*0.0130*	*0.0065*	*1.55*	*0.0133*	*0.0067*	*4.97*	*0.0043*	*0.0022*	*1.05*	*0.0009*	*0.0005*	*0.12*
residual	*3*	*0.0125*	*0.0042*	*2.06*	*0.0040*	*0.0013*	*0.57*	*0.0062*	*0.0021*	*0.38*	*0.0115*	*0.0038*	*0.68*
Population‡	3	0.0253	0.0084	4.16*	0.0283	0.0094	4.02*	0.0492	0.0164	3.04(*)	0.0302	0.0101	1.78
Treatment x Population‡	9	0.0606	0.0067	3.32*	0.0916	0.0102	4.33*	0.0396	0.0044	0.82	0.0288	0.0032	0.57
linear x population‡	*3*	*0.0423*	*0.0141*	*6.96***	*0.0247*	*0.0082*	*3.5**	*0.0064*	*0.0021*	*0.4*	*0.0062*	*0.0021*	*0.37*
deviations‡	*6*	*0.0183*	*0.0031*	*1.51*	*0.0669*	*0.0111*	*4.74**	*0.0332*	*0.0055*	*1.03*	*0.0226*	*0.0038*	*0.67*
residual	*12*	*0.0243*	*0.0020*		*0.0282*	*0.0024*		*0.0646*	*0.0054*		*0.0677*	*0.0056*	
Total	31	0.1772			0.3584			0.4207			0.3215		

Source of variation	DF	$wpns_{coeff}$			Above ground fwt			Above ground dwt			Relative growth rate		
		S.S.	M.S.	*F value*	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*
Block	1	0.0204	0.0204	10.14	0.1745	0.1745	7.43	0.0920	0.0920	8.17	0.0014	0.0014	23.21
Treatment‡	3	0.6841	0.2280	113.48**	0.0088	0.0029	0.12	0.0348	0.0116	1.03	0.0001	0.0000	0.53
linear‡	*1*	*0.6747*	*0.6747*	*335.77***	*0.0075*	*0.0075*	*0.32*	*0.0326*	*0.0326*	*2.89*	*0.0000*	*0.0000*	*0.53*
deviations‡	*2*	*0.0094*	*0.0047*	*2.34*	*0.0013*	*0.0006*	*0.03*	*0.0022*	*0.0011*	*0.1*	*0.0001*	*0.0000*	*0.53*
residual	*3*	*0.0060*	*0.0020*	*0.12*	*0.0705*	*0.0235*	*0.77*	*0.0338*	*0.0113*	*0.51*	*0.0002*	*0.0001*	*0.2*
Population‡	3	0.1811	0.0604	3.63*	0.5703	0.1901	6.26**	0.8860	0.2953	13.42**	0.0025	0.0008	2.83(*)
Treatment x Population‡	9	0.0549	0.0061	0.37	0.2336	0.0260	0.85	0.2076	0.0231	1.05	0.0021	0.0002	0.79
linear x population‡	*3*	*0.0104*	*0.0035*	*0.21*	*0.0488*	*0.0163*	*0.54*	*0.0533*	*0.0178*	*0.81*	*0.0005*	*0.0002*	*0.55*
deviations‡	*6*	*0.0445*	*0.0074*	*0.45*	*0.1848*	*0.0308*	*1.01*	*0.1543*	*0.0257*	*1.17*	*0.0016*	*0.0003*	*0.91*
residual	*12*	*0.1997*	*0.0166*		*0.3647*	*0.0304*		*0.2641*	*0.0220*		*0.0035*	*0.0003*	
Total	31	1.1462			1.4224			1.5183			0.0097		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

Table 4.4 – Summary of population level ANOVA analysis results.

			wpns week 8			$wpns_{coeff}$			Above ground dwt			Relative growth rate		
Population	**Source of variation**	**DF**	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***
Aber	**Block**	1	0.0149	0.0149	2.37	0.0060	0.0060	0.48	0.0083	0.0083	0.45	0.0000	0.0000	0.01
	Treatment‡	3	0.0279	0.0093	1.48	0.1067	0.0356	2.82	0.0772	0.0257	1.40	0.0003	0.0001	0.33
	linear‡	*1*	*0.0246*	*0.0246*	*3.91*	*0.1043*	*0.1043*	*8.27(*)*	*0.0604*	*0.0604*	*3.29*	*0.0003*	*0.0003*	*0.89*
	deviations‡	*2*	*0.0034*	*0.0017*	*0.27*	*0.0024*	*0.0012*	*0.10*	*0.0168*	*0.0084*	*0.46*	*0.0000*	*0.0000*	*0.05*
	residual	*3*	*0.0188*	*0.0063*		*0.0378*	*0.0126*		*0.0551*	*0.0184*		*0.0008*	*0.0003*	
	Total	7	0.0617			0.1506			0.1405			0.0011		
Birds Rock	**Block**	1	0.0002	0.0002	0.09	0.0040	0.0040	0.42	0.2091	0.2091	43.53	0.0005	0.0005	2.21
	Treatment‡	3	0.0735	0.0245	10.74*	0.1995	0.0665	6.93(*)	0.0730	0.0243	5.07	0.0008	0.0003	1.21
	linear‡	*1*	*0.0639*	*0.0639*	*27.97**	*0.1947*	*0.1947*	*20.29**	*0.0000*	*0.0000*	*0.01*	*0.0000*	*0.0000*	*0.16*
	deviations‡	*2*	*0.0097*	*0.0048*	*2.12*	*0.0048*	*0.0024*	*0.25*	*0.0730*	*0.0365*	*7.60(*)*	*0.0008*	*0.0004*	*1.74*
	residual	*3*	*0.0069*	*0.0023*		*0.0288*	*0.0096*		*0.0144*	*0.0048*		*0.0007*	*0.0002*	
	Total	7	0.0806			0.2323			0.2965			0.0021		
Cwm Idwal	**Block**	1	0.0000	0.0000	0.01	0.0063	0.0063	1.02	0.0006	0.0006	0.15	0.0002	0.0002	2.48
	Treatment‡	3	0.0615	0.0205	9.02(*)	0.2120	0.0707	11.48*	0.0476	0.0159	3.95	0.0004	0.0001	1.34
	linear‡	*1*	*0.0614*	*0.0614*	*27.02**	*0.1996*	*0.1996*	*32.42**	*0.0243*	*0.0243*	*6.06(*)*	*0.0000*	*0.0000*	*0.15*
	deviations‡	*2*	*0.0001*	*0.0000*	*0.02*	*0.0124*	*0.0062*	*1.01*	*0.0233*	*0.0116*	*2.90*	*0.0004*	*0.0002*	*1.93*
	residual	*3*	*0.0068*	*0.0023*		*0.0185*	*0.0062*		*0.0120*	*0.0040*		*0.0003*	*0.0001*	
	Total	7	0.0683			0.2367			0.0602			0.0009		
Maltraeth	**Block**	1	0.0066	0.0066	0.79	0.0485	0.0485	1.91	0.0012	0.0012	0.04	0.0012	0.0012	2.94
	Treatment‡	3	0.0491	0.0164	1.95	0.2208	0.0736	2.90	0.0445	0.0148	0.50	0.0007	0.0002	0.54
	linear‡	*1*	*0.0388*	*0.0388*	*4.62*	*0.1865*	*0.1865*	*7.34**	*0.0012*	*0.0012*	*0.04*	*0.0002*	*0.0002*	*0.50*
	deviations‡	*2*	*0.0104*	*0.0052*	*0.62*	*0.0342*	*0.0171*	*0.67*	*0.0434*	*0.0217*	*0.73*	*0.0005*	*0.0002*	*0.55*
	residual	*3*	*0.0252*	*0.0084*		*0.0762*	*0.0254*		*0.0894*	*0.0298*		*0.0013*	*0.0004*	
	Total	7	0.0809			0.3455			0.1351			0.0031		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

4.3.4 Ozone dose response

There was a significant linear relationship between species level whole plant senescence and the received ozone dose, measured as accumulated ozone over a threshold of X ppb (AOTX), across the exposure period (Table 4.5). The relationship remained highly significant ($P<0.001$) regardless of the applied threshold value (0 ppb, 20 ppb or 40 ppb). There was no difference in the observed relationship for AOTXs calculated over a 24 hour or 12 hour per day (hd^{-1}) period with a threshold value of 0 ppb. However the R^2 value decreased with a 12 hd^{-1} period when threshold values of 20 ppb and 40 ppb were applied and those calculated over a 12 hd^{-1} period were not considered further. Although significant relationships were found for all AOTXs, the R^2 value decreased with increasing threshold values with a threshold of 0 ppb providing the best fit for the amount of observed whole plant senescence ($R^2=0.842$, $P<0.001$).

Comparison of the relationships for AOT0 and AOT40 showed a clustering of senescence data at low AOTX values for measurements made in the early weeks of exposure and for the lower ozone treatments through the exposure period (Figure 4.13 a). This was greatest for AOT40 where there was no increase in AOTX value over the exposure period in the $O_3(10,30)$ treatment as the ozone concentration never exceeded the 40 ppb threshold and only a small increase for the $O_3(35,55)$ treatment. The

Table 4.5 –Linear regression analysis of relationship between whole plant senescence and accumulated ozone over a threshold (AOT) of X ppb calculated for 12 and 24 per day (hd^{-1}) periods throughout the exposure period.

AOT(Xppb)	Period	Linear regression			
		R^2	adj R^2	F value	*P* value
AOT0	**24 hd^{-1}**	0.842	0.837	181.31	<0.001
	12 hd^{-1}	0.841	0.837	180.27	<0.001
AOT20	**24 hd^{-1}**	0.714	0.706	85.00	<0.001
	12 hd^{-1}	0.710	0.702	83.41	<0.001
AOT40	**24 hd^{-1}**	0.588	0.576	48.62	<0.001
	12 hd^{-1}	0.582	0.570	47.37	<0.001

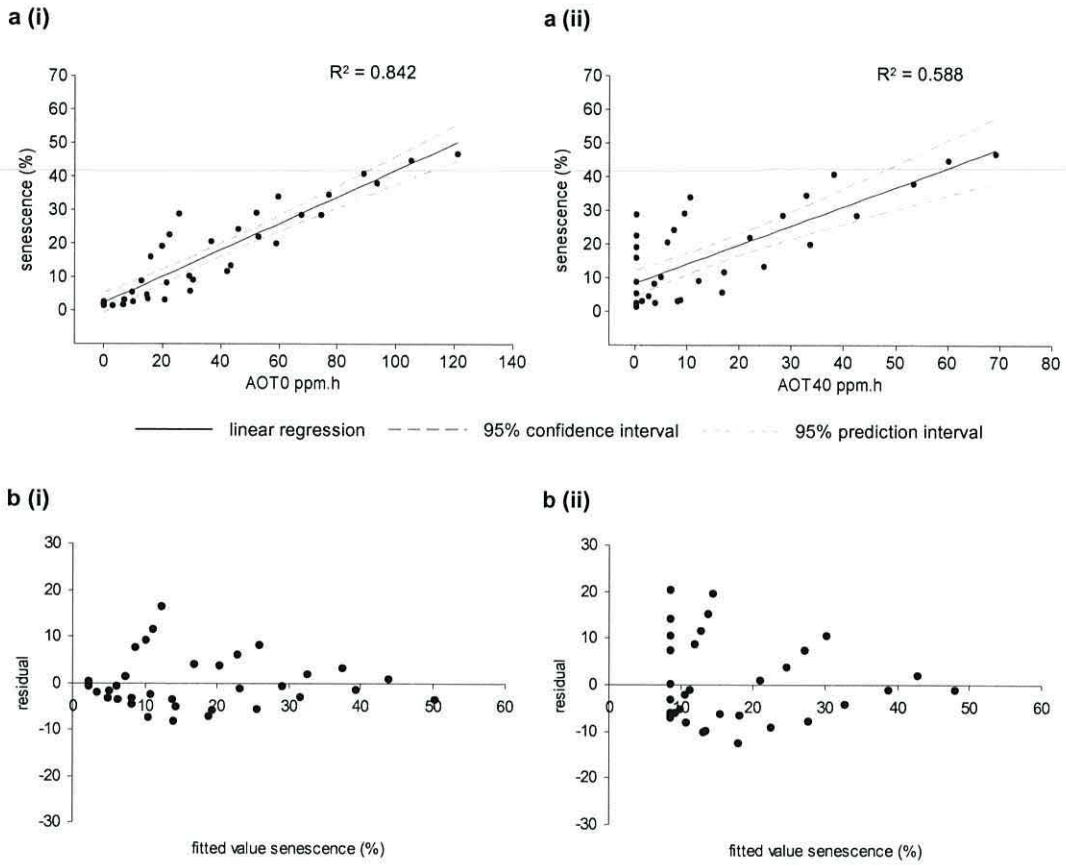

Figure 4.13 - Relationship between whole plant senescence and 24 hour per day accumulated ozone over a threshold (AOT) throughout the exposure period described by a) linear regression and b) resulting residual variation for ozone thresholds of (i) 0 ppb and (ii) 40 ppb.

development of senescence over the exposure period in the $O_3(10,30)$ and $O_3(35,55)$ treatments can clearly be differentiated for AOT40. Examination of the residual variation for the linear regression relationship showed a reasonable distribution for AOT0 while there was greater variation and a definite clustered distribution for AOT40 (Figure 4.13 b). This and the low R^2 value indicate that the regression model for AOT40 is not an appropriate descriptor of the relationship between senescence and ozone dose. Total accumulated ozone calculated for a period of $24hd^{-1}$ without a threshold value imposed (AOT0) provided the best description for the response of whole plant senescence to ozone dose when considered across the entire exposure period.

4.3.5 Relative ozone sensitivity

The greatest response to elevated ozone in *A. odoratum* was in terms of increased/premature senescence resulting in a reduction in the percentage of healthy leaves (Table 4.6). The populations all exhibited sensitivity to ozone for this assessment criterion with relative sensitivity increasing with level of ozone exposure and the highest relative sensitivities observed for the high (O_3(85,105)) ozone treatment. Ozone sensitivity increased through the exposure period although the comparative relative sensitivity of the populations varied over time. Maltraeth exhibited the lowest sensitivity through the first six weeks of exposure although this was due to the high levels of senescence in the control treatment. Overall the Cwm Idwal population exhibited the fastest response although Birds Rock was the most sensitive in terms of the rate of development of senescence ($wpns_{coeff}$, Table 4.6).

As with foliar injury, relative sensitivity for plant growth variables were generally greatest in the O_3(85,105) ozone treatment. Above ground dry weight biomass was most sensitive to elevated ozone although all plant growth responses were non-significant. The populations differed in plant growth responses with Aber exhibiting the greatest sensitivity for both shoot dry weight and fresh weight while the other three populations exhibited some degree of growth stimulation. In terms of relative growth rate (RGR) the Aber and Birds Rock populations were most sensitive with reductions under elevated ozone while some increase in RGR occurred in Cwm Idwal and Maltraeth. Populations differed for dry weight to fresh weight ratio sensitivity although only Aber exhibited a clear trend for reduction with elevated ozone.

The populations differed in relative sensitivity dependant upon the assessment criteria applied. All populations were considered sensitive for foliar injury as the percentage of healthy leaves although this did not equate to a similar sensitivity in plant growth. The greatest variation in relative sensitivity occurred among plant growth variables both within and among populations.

Table 4.6 – Species and population level relative ozone sensitivity (RS) for foliar injury and plant growth assessment criteria. Relative sensitivities in relation to the control treatment (O_3(10,30)) denoted as RS_L (low treatment, O_3(35,55)), RS_M (medium treatment, O_3(60,80)), RS_H (high treatment, O_3(85,100)). *woe* denotes weeks of exposure. Significant differences are indicated by **=P<0.01, * = P<0.05 and (*) = P<0.1 for differences from the control and overall treatment effects (P_T). RS values of <1 and >1 represent sensitivity and resistance respectively.

Assessment criteria	woe	Species				Aber				Birds Rock				Cwm Idwal				Maltraeth			
		RS_L	RS_M	RS_H	P_T	RS_L	RS_M	RS_H	P_T	RS_L	RS_M	RS_H	P_T	RS_L	RS_M	RS_H	P_T	RS_L	RS_M	RS_H	P_T
Foliar injury																					
% healthy leaves	1	0.98	0.99	0.98		0.99	0.99	0.98		0.93	0.98	0.97		0.98	0.95	0.96 *	*	1.03	1.04	1.01	
	2	0.97	0.99	0.96		0.98	0.98	0.93		0.92	0.98	0.97		0.96	0.94	0.92		1.02	1.04	1.02	
	3	0.97	0.96	0.92 *	*	0.99	0.95	0.88	(*)	0.93	0.93	0.95	(*)	0.93	0.93	0.86	(*)	1.03	1.03	0.97	(*)
	4	0.98	0.97 *	0.88 *	**	1.03	0.94	0.83	*	0.89 *	0.91 *	0.92 *	*	0.94	0.93	0.82	(*)	1.08	1.10	0.95 *	**
	5	0.95	0.93	0.85 *	*	0.97 *	0.85 *	0.77 *	**	0.84	0.89	0.88		0.94	0.94	0.83		1.05	1.06	0.93	*
	6	0.94	0.88 *	0.77 *	**	0.89	0.93	0.78		0.86	0.86	0.79		0.95	0.88	0.72	(*)	1.06	0.98	0.80	(*)
	7	0.92 *	0.85 *	0.71 *	**	0.89	0.82	0.74		0.83	0.82	0.71 *	*	0.94	0.82	0.67	(*)	1.03	0.93	0.73	
	8	0.93	0.83	0.75 *	*	0.98	0.86	0.82		0.83	0.79	0.73 *	*	0.89	0.80	0.70 (*)	(*)	1.04	0.90	0.75	
$wpns_{coeff}$		0.85	0.70 *	0.57 *	**	0.85	0.72	0.67		0.65	0.55	0.46 (*)	(*)	0.85	0.70	0.52 *	*	1.04	0.79	0.62	
Plant growth																					
AG fwt	8	1.03	1.02	0.97		0.87	0.82	0.86		1.08	1.09	1.03		1.11	1.03	0.98		1.13	1.21	1.05	
AG dwt	8	1.01	0.95	0.93		0.84	0.76	0.81		1.04	1.01	1.00		1.12	1.01	0.97		1.13	1.13	1.02	
RGR	8	1.00	1.00	0.99		1.00	0.98	0.96		0.93	0.95	0.98		1.02	1.03	0.99		1.07	1.06	1.05	
dwt:fwt ratio	8	0.99	0.95	0.97		0.99	0.96	0.92		1.01	0.96	0.97		1.02	0.97	1.00	*	0.95	0.90	0.98	

AG, above ground; BG, below ground; fwt, fresh weight; dwt, dry weight

4.4 Discussion

Increasing tropospheric background ozone concentrations are expected to have the greatest impact on upland areas where ozone levels are less depleted over night (Ashmore *et al* 2002) although declining peak concentrations in combination with increased background may result in ozone profiles in lowland areas resembling those currently experienced in upland areas (NEGTAP 2001). This study demonstrates the potential for detrimental effects of elevated ozone exposure in profiles typical of upland areas on *A. odoratum* in terms of foliar injury and plant growth. The results are discussed first in relation to the experimental design and received ozone exposure, second in terms of the species level response and finally in terms of the intraspecific variation in response to ozone exposure.

4.4.1 Experimental design and received ozone exposure

Several factors relating to experimental design may have influenced the observed response of *A. odoratum* to elevated ozone including solardome related chamber effects and pot related growth restrictions as previously discussed in Chapters 2 and 3. Edge effects may also have influenced response as the spacing of plant pots 5 cm apart will have allowed greater air flow around each plant and potentially higher levels of ozone exposure than for plants within a community (Wilbourne *et al* 1995). However, these effects should be consistent between solardomes and the randomisation and rotation procedures applied should minimise any confounding effects of within solardome position. Under this assumption comparison between treatments should provide an adequate assessment of relative ozone sensitivity.

Plants were exposed to four ozone treatments of incrementally increased background concentrations combined with a low magnitude episodic regime. Background concentrations were maintained throughout the weekly profile and elevated by 20 ppb to peak levels over five days. Average ozone concentrations were maintained within 10 ppb of the target concentration for the majority of the eight week exposure period. Plants received an maximum accumulated 24h AOT0 of 122.9 ppm.h and 24h AOT40

of 70.0 ppm.h after eight weeks exposure in the O_3(85,105) treatment. The 24 hour per day period was applied to include ozone concentrations maintained overnight and the received ozone dose assessed as 24 hour AOT0 followed the linear increase in ozone concentrations among treatment levels.

The system failure in the fifth week of exposure resulted in a high peak of ozone received overnight in one replicate solardome of the O_3(60,80) treatment. The potential effect of such high concentrations on the observed plant responses may have been mitigated by low nocturnal ozone uptake. However, considerable nocturnal ozone flux can still occur despite the lower levels of stomatal conductance compared to those during daylight hours (Musselman and Minnick (2000) and foliar injury has been reported following overnight ozone exposure in *Trifolium subterraneum* (Sild *et al* 1999) and *Betula pendula* (Matyssek *et al* 1995). A large effect of this peak would have been expected to result in greater variation in the O_3(60,80) treatment compared to the control. The results for foliar injury and plant growth do not indicate any such increased variation and the system error is therefore considered to have had a minimal impact on the observed responses.

4.4.2 *A. odoratum* response to upland ozone profiles

The greatest response of *A. odoratum* to elevated ozone exposure was in terms of premature senescence as observed in previous studies in this species (Hayes *et al* 2006, Hayes 2007, see Chapters 2 and 3). Significant premature senescence occurred after three weeks and the amount of senescence increased in a linear fashion with increased ozone exposure. This response was observed throughout the exposure period with the rate of development of senescence increased by 17.2 %, 43.9 % and 73.1 % in successive ozone treatments. This is consistent with the accelerated rate of senescence observed under acute exposure (Chapter 2) and a chronic episodic ozone regime (Hayes *et al* 2006). The linear increase in senescence with successive ozone treatment was supported by the significant ozone dose response relationship observed for whole plant senescence throughout the exposure period with received ozone assessed as AOT0. Although threshold levels of 20 ppb and 40 ppb also exhibited significant relationships

a threshold of 0 ppb provided the best explanation of the observed senescence levels and may be attributed to the linear increase in accumulated ozone dose among treatments when assessed as AOT0.

The slight decrease in extent of senescence under elevated ozone at the end of exposure may relate to the continual production of new leaves (Hayes *et al* 2006) and the natural progression of senescence under control conditions through the exposure period. Lower levels of natural senescence were observed in this study compared with previous exposure experiments (Chapters 2 and 3). This may be due to differences in the timing of exposure in relation to the growing season and plant age. Higher rates of senescence from late summer onwards are typical of many perennial grass species including *A. odoratum* (Schippers *et al* 1999, Grant *et al* 1996) and this is consistent with the 10 % and 25 % higher amounts of natural senescence observed in the late season acute and rural episodic exposure experiments described in Chapters 2 and 3 respectively. The higher levels in the rural episodic ozone experiment (Chapter 3) also relate to greater plant age as rametes were established for an additional four weeks prior to exposure.

The development of senescence appears to follow a logistic curve through the exposure period with the initial lag, acceleration and logistic phases evident and a slight reduction in the rate of senescence exhibited under the highest ozone treatment at the end of exposure. This supports the suggestion that ozone exposure may alter the shape of this curve with the logarithmic and stationary phases reached at earlier dates (Chapter 2) and may result in a reduced effective growing season. Senescence results in reduced photosynthetic capacity and lower resource accumulation (Gay and Thomas 1995, Vandermeinen *et al* 2005) and has implications for overwintering potential and competitive ability. Although premature senescence is a common symptom of ozone exposure in many wild herb and grass species (Bergmann *et al* 1995, Bergmann *et al* 1999, Franzaring *et al* 2000, Bermejo *et al* 2003) the ecological significance of this response has been questioned as it often occurs without any accompanying growth reductions (Franzaring *et al* 2000, Inclan *et al* 1999, Woo and Hinckley 2005, Hayes *et al* 2006, Bender *et al* 2006).

The premature senescence observed in this study was not accompanied by any significant effects on above ground plant growth although a slight trend for reductions

in dry weight and dry weight to fresh weight ratio were observed. This lack of significant response is consistent with the previous results for this species under chronic episodic ozone exposure (Chapter 3, Hayes *et al* 2006) but not under acute ozone exposure despite a higher total accumulated ozone dose (Chapter 2). This may relate to alterations in growth rate with plant age (Duru and Ducrocq 2000) as younger plants often have a higher growth rate and may be more sensitive to ozone exposure (Lyons and Barnes 1998) although this is unlikely as the relative growth rate under control conditions was only slightly lower than that observed when plants were harvested four weeks earlier (Chapter 2). It is more likely that alterations in root to shoot resource allocation resulted in the maintenance of shoot growth at the expense of root growth (Grantz *et al* 2006) and this is supported by the trend for a greater reduction in root growth than shoot growth under acute ozone exposure (Chapter 2). Lower resource allocation to root growth may result in longer-term reductions in plant vitality and competitive ability especially in perennial species. No reduction in shoot growth during ozone exposure but reductions following overwintering in *Galium sacatile* and *Saxifraga stellaris* were thought to have resulted from a reduction in root reserves (Hayes *et al* 2006). The occurrence of premature senescence and possible reductions in root reserves in *A. odoratum* may result in a slower longer-term shoot growth response to chronic ozone exposure as suggested by reductions in cumulative above ground biomass in *A. odoratum* grown in a seven species model community following two seasons of ozone exposure (Hayes 2007).

The slight reduction in dry weight to fresh weight ratio is consistent with the trend observed under acute exposure (Chapter 2) and indicates greater water content under elevated ozone. This may relate to alterations in tiller morphology such as an increase in leaf to sheath ratio which may result in lower dry matter density and greater water content (Maurice *et al* 1997). An increased leaf to sheath ratio has been linked to smaller tiller size (Sugiyama 1995) and resource allocation to the production of new tillers rather than the growth of existing tillers with increased foliar injury may result in a greater number of small tillers. Increased tiller number was previously observed in *A. odoratum* under chronic rural episodic ozone exposure (Chapter 3) although tiller size was not determined. Further assessment of tillering and leaf to sheath ratio may provide insights into the mechanisms of shoot growth maintenance despite accelerated senescence rates under elevated ozone exposure.

4.4.3 Intraspecific variation in ozone sensitivity

The four *A. odoratum* populations studied differed significantly for the majority of assessment criteria regardless of ozone treatment and was consistent with the highly polymorphic morphology of this species (Hubbard 1992). The lowest levels of natural senescence were observed in the Birds Rock population while Maltraeth and Aber exhibited the highest levels. In terms of plant growth Aber exhibited the highest shoot biomass while Birds Rock had the highest relative growth rate under control conditions and this was consistent with previous results for these populations (Chapter 2).

All four populations were sensitive to ozone exposure in terms of premature senescence but differed in the extent and timing of response through the exposure period. Aber and Cwm Idwal exhibited the fastest response although the rate of senescence decreased towards the end of exposure and Aber was the least sensitive after eight weeks of exposure. This decrease in sensitivity relates to the shape of the logistic curve for development of senescence as discussed above (section 4.4.2). Maltraeth exhibited the lowest relative sensitivity throughout the exposure period which contrasts with the results under chronic episodic exposure where this population was the most sensitive (Chapter 3). However, this may be due to the high levels of senescence observed under control conditions in the current experiment which had a confounding effect on the calculation of relative sensitivity.

In terms of the rate of development of senescence ($wpns_{coeff}$), Aber was the least sensitive and the Birds Rock population exhibited the greatest sensitivity despite the slower response to elevated ozone. This difference may be attributed to the use of linear rather than non-linear regression to assess the rate of senescence. An early response followed by a reduction in the extent of senescence has previously been observed in this species under both acute and chronic exposure (Chapter 2, Chapter 3, Hayes *et al* 2006) and is thought to relate to the continual production of new leaves (Hayes *et al* 2006) and the progression of senescence following a logistic curve. These results demonstrate that peaks in response may occur at different times during exposure in different populations and a lower magnitude of response at the end of exposure may not equate to the lowest sensitivity. An earlier response in terms of senescence may result in a greater degree of

reduction in resource accretion throughout the growing season (Vandermeinen *et al* 2005) and may indicate a greater long-term sensitivity.

The earlier response in terms of premature senescence in the Aber population corresponded to the greatest reductions in plant growth although this was not significant. The populations differed in plant growth response exhibiting varying sensitivity for the different assessment criteria although only Aber exhibited any clear trend in response to successive elevated ozone treatments. The trend for reduction in above ground dry weight at the species level reflects the greater response in Aber than the other four populations. This variation among populations is consistent with response to acute ozone exposure although significant ozone effects were obtained with the higher level of exposure (Chapter 2). Previous studies have demonstrated that one season of chronic ozone exposure does not result in significant effects on plant growth in this species (Hayes *et al* 2006, Chapter 3). However, reductions may occur over a longer time period as observed by Hayes (2007) where reductions in cumulative biomass occurred following two seasons of ozone exposure for *A. odoratum* grown in a seven species model community.

The level of intraspecific variation in plant growth response was lower than previously observed under acute exposure (Chapter 2) and the lack of clear trends in these assessment criteria is consistent with previous chronic ozone exposure (Chapter 3). However, the populations differed in the magnitude and timing of response in terms of premature senescence and these results highlight the importance of considering the potential effects of alterations in the progression of senescence throughout the growing season. The early development of premature senescence and greater trend for reduction in shoot growth observed in the Aber population and combined with a potential reduction in root growth, as observed under acute exposure (Chapter 2), suggests that this population is potentially the most sensitive to ozone exposure in the long term.

Chapter 5: Efficiency of a streptavidin-biotin enrichment method for microsatellite isolation in *Anthoxanthum odoratum* L.

5.1 Introduction

Microsatellites, also known as simple sequence repeats (SSRs), short tandem repeats (STRs) and variable number tandem repeats (VNTRs), are short sequences of DNA containing tandemly repeated units of 1 to 6 nucleotide base pairs. Microsatellites have become one the most popular molecular markers for studies of intraspecific genetic variation due to their abundance within eukaryotic genomes, characteristically high levels of polymorphism and co-dominant nature (Li *et al* 2002, Zane *et al* 2002). Microsatellites have been employed in studies ranging from parentage assignment (Gerber *et al* 2000, Saltonstall 2003), dispersal and population structuring (Heuertz *et al* 2003, Lian *et al* 2003) to genome mapping and quantitative trait analysis (Andaya and Mackill 2003, Shen *et al* 2003).

Despite the wide range of applications, the use of microsatellites is limited by the requirement for isolation within many new study species due to the relatively species specific nature of these markers (Zane *et al* 2002). Cross-species amplification of homologous loci has been observed in marine turtles (Fitzsimmons *et al* 1995), fish species (Rico *et al* 1996) and among some ryegrass and fescue species (Jones *et al* 2001) although in plant species cross-species amplification is largely restricted to closely related taxa (Peakall *et al* 1998, Yamamoto *et al* 2002, Kolliker *et al* 2001, Jump *et al* 2002). Microsatellite isolation can be achieved through traditional genomic library screening or using Random amplified polymorphic DNA (RAPD) based, primer extension or selective hybridization enrichment methods (reviewed in Zane *et al* 2002). However, microsatellite isolation is often costly, time consuming and inefficient (Squirrell *et al* 2003, Zane *et al* 2002) and enrichment methods are therefore considered the most appropriate for species with large genomes or relatively low microsatellite abundance such as plant species.

Microsatellite isolation using selective hybridization is a popular enrichment technique which has been employed in a wide range of species including fish and mammals (Refseth *et al* 1997, Karagyozov *et al* 1993), reptiles (Gardener *et al* 1999, Gow *et al* 2006) and plant species (Edwards *et al* 1996, Dayanandan *et al* 1998, Cordeiro *et al* 1999, Cai *et al* 2003). These techniques involve hybridization of fragmented DNA with filter bound (Karagyozov *et al* 1993, Armour *et al* 1994) or biotinylated (Kandpal *et al* 1994, Kijas *et al* 1994, Refseth *et al* 1997) repeat containing probes which are then washed to remove non-target DNA fragments with resulting enrichment efficiency ranging from 20 % to 90 % (Zane *et al* 2002).

In this study, a streptavidin-biotin selective hybridization enrichment method was employed for microsatellite isolation in the tufted, perennial grass species *Anthoxanthum odoratum* L. This short-lived tetraploid grassland species is widespread throughout northern Europe (Hedberg 1990), morphologically variable (Hubbard 1992) with reproduction predominantly through wind pollinated outcrossing (Silvertown *et al* 2002). Previous studies have inferred population differentiation in *A. odoratum* based on morphological, physiological and phenological traits (Snaydon 1970, Snaydon and Davies 1972, 1976, Davies and Snaydon 1976, Sims and Kelley 1998). However, few studies have employed molecular markers to assess genetic variation in *A. odoratum*. Earlier studies utilised isozymes and allozymes (Wu and Jain 1980, Silvertown *et al* 2002) while more recently, Silvertown *et al* (2005) demonstrated genetic divergence between adjacent populations following reinforcement of reproductive isolation using chloroplast microsatellites and inter-simple-sequence-repeat (ISSR) markers.

The development of nuclear microsatellite markers would provide additional molecular tools for assessment of genetic diversity, population differentiation and population-environment interaction in *A. odoratum*. Recent developments in both microsatellite isolation techniques (Zane *et al* 2002) and the analysis of polyploid genotype profiles (Thrall and Young 2000, Esselink *et al* 2004, Markwith *et al* 2006) have made the application of microsatellite markers in such species a more viable option. The enrichment efficiency of modifications to a streptavidin-biotin selective hybridization enrichment method for isolation of di-, tri- and tetranucleotide microsatellite repeats in *A. odoratum* and the viability of isolated microsatellite loci for population genetic analysis are considered.

5.2 Methods

Genomic libraries were constructed and enriched for microsatellite repeats using streptavidin-biotin enrichment based on a modified enrichment method described by Gardner *et al* (1999). Modifications to the protocol were investigated for enrichment efficiency for CT dinucleotide microsatellite repeats. Oligonucleotides employed in microsatellite library construction and loci isolation are given in Table 5.1. The eleven enrichment methods applied are detailed below with the modifications used in each method summarized in Table 5.2. The two most successful techniques were further investigated for enrichment efficiency for CA dinucleotide repeats, GAT and AAT trinucleotide repeats and tetranucleotide AAAG and TCAG repeats.

Table 5.1 – Oligonucleotides for enriched library construction and microsatellite isolation

Oligonucleotide type	Primer	Sequence (5'–3')
Linker	S61	GGCCAGAGACCCCAAGCTTCG
	S62	GATCCGAAGCTTGGGGTCTCTGGCC*
Biotinylated	AAAG-biot	$(AAAG)_6$GCAC[Biotin]A
	TCAG-biot	$(TCAG)_6$GCAC[Biotin]A
	GAT-biot	$(GAT)_8$GCAC[Biotin]A
	AAT-biot	$(AAT)_8$GCAC[Biotin]A
	CA-biot	$(CA)_{12}$GCAC[Biotin]A
	CT-biot	$(CT)_{12}$GCAC[Biotin]A
Vector	M13-20F	GTAAAACGACGGCCAG
	M13-R	CAGGAAACAGCTATGAC
Repeat motif	AAAG-Int	$(AAAG)_6$B
	TCAG-Int	$(TCAG)_6$V
	GAT-Int	$(GAT)_8$H
	AAT-Int	$(AAT)_8$B
	CA-Int	$(CA)_{12}$D
	CT-Int	$(CT)_{12}$D

*, phosphorylated at 3' end.

5.2.1 Microsatellite library construction

5.2.1.1 DNA preparation

Genomic DNA was extracted from 100 mg fresh or 20 mg dried leaf tissue using a DNeasy Plant mini kit (Qiagen) according to the manufacturer's protocol. 5 µg DNA was pooled from extracts of one to three individuals (Table 5.2) and concentrated by sodium acetate precipitation. 5 µg DNA was fragmented by restriction digest with 10 U *Sau*3A and 1x *Sau*3A reaction buffer (Promega) in a 20 µl reaction incubated at 37 °C for 3 or 12 hours followed by heat inactivation at 65 °C for 30 minutes (Table 5.2).

Adapters were generated by hybridization of equimolar amounts (1.5 ηmol) of the linker oligonucleotides, S61 and S62 (Table 5.1), incubated at 80 °C for 2 minutes then slowly cooled to 20 °C over 1 hour. 0.9 ηmol adapter was ligated to the digested DNA in 1x Quick Ligation buffer with 84 Weiss Units T4 DNA Ligase (New England Biolabs). The ligation reaction was centrifuged briefly, incubated at room temperature for 5 minutes and chilled on ice. Ligated DNA fragments were ethanol precipitated, resuspended in 20 µl TE buffer and visualized on a 2.5 % agarose gel alongside a GeneRuler™ 100 base pair (bp) DNA ladder (Fermentas). Fragments in the size range 200 bp to 1000 bp were excised and gel purified using a QIAquick Gel Extraction kit (Qiagen) following the manufacturer's protocol and eluted in 50 µl double distilled (dd) H_2O.

A polymerase chain reaction (PCR) amplification step prior to selective hybridization was performed in a number of enrichment methods (Table 5.2). 50 µl PCR reactions consisted of 1 µl ligated and size selected DNA (approximately 0.1 µg), 3.5 mM $MgCl_2$, 0.2 mM each dNTP, 20 pmol S61 linker oligonucleotide, 1.25 U Thermo-Start® DNA polymerase and 1x Thermo-Start® Reaction buffer (ABgene). Thermal cycling conditions consisted of an initial enzyme activation at 95 °C for 15 minutes, 35 cycles of denaturation at 94 °C, annealing at 55 °C and extension at 72 °C with 1 minute duration at each step, and a final extension at 72 °C for 8 minutes. PCR products were visualized on a 1 % agarose gel (Figure 5.1 a).

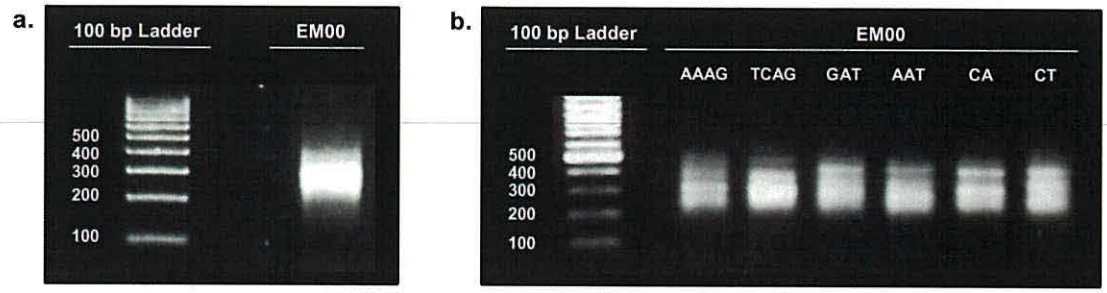

Figure 5.1 – PCR amplification during microsatellite library construction by enrichment method EM00. a) PCR prior to selective hybridization and b) PCR of captured DNA fragments after enrichment for each microsatellite repeat motif.

5.2.1.2 Selective hybridization

Streptavidin Magnesphere® Paramagnetic Particles (SA-PMPs, Promega) were prepared according to the manufacturers instructions. 100 µl SA-PMPs were resuspended in 100 µl 5x SSC (0.75 M NaCl, 75 mM trisodium citrate) with or without 200 ρmol biotinylated primer (Table 5.2). Where the biotinylated primer was added the SA-PMPs were incubated at room temperature for 20 minutes. SA-PMPs were washed three times in 100 µl 5x SSC and resuspended in 50 µl 1x Hybridization Solution (HS: 0.5 M NaCl, 4% w/v polyethylene glycol 8000) at 50°C and stored at 50°C.

10 µl ligated and size selected DNA or PCR product were added to 40 µl of 1x HS containing 20 ρmol S61 linker oligonucleotide and with the addition of 200 ρmol biotinylated primer in enrichment methods where the biotinylated primer was not added to the SA-PMPs (Table 5.2). The solution was denatured at 95 °C for 5 minutes and cooled to 55 °C. The prepared DNA and SA-PMPs were combined and incubated at 37 °C or 55 °C for 20 minutes with or without constant agitation at 350 rpm (Table 5.2).

5.2.1.3 Magnetic capture

To remove unbound DNA fragments the SA-PMPs were washed eight times with 100 µl 1x SSC, except for method EM00 where the first four washes were performed with 2x SSC. Each 100 µl SSC wash contained 10 ρmol S61 linker oligonucleotide to limit the formation of concatamers by blocking terminal primer sites (Gardner *et al* 1999).

Wash stringency was varied between enrichment methods with wash temperatures of between 20 °C and 80 °C. In general, four lower temperature and four higher temperature washes were performed and the specific temperatures applied in each method are detailed in Table 5.2.

Captured DNA fragments were recovered by resuspension of SA-PMPs in 20 µl 0.15 NaOH incubated at room temperature for 20 minutes with regular, gentle agitation. The supernatant was removed and neutralised by addition of 1.3 µl 1.25 M acetic acid and 2.2 µl 10x TE (pH 8.0). Captured DNA fragments were purified using a QIAquick PCR Purification kit (Qiagen) following the manufacturer's protocol and eluted in 50 µl Buffer EB (10 mM Tris-Cl, pH 8.5).

5.2.1.4 PCR amplification of captured fragments and cloning

Captured DNA fragments were PCR amplified using 2.5 µl captured DNA template, 4 mM $MgCl_2$, 0.2 mM each dNTP, 30 pmol S61 linker oligonucleotide, 1.25 U Thermo-Start® DNA polymerase and 1x Thermo-Start® Reaction buffer (ABgene) in a 50 µl volume. PCR reactions were performed with an initial enzyme activation at 95 °C for 15 minutes, one cycle of 3 minutes denaturation at 94 °C, 45 seconds annealing at 60 °C and 2 minutes extension at 72 °C, 39 cycles of 94 °C for 45 seconds, 60 °C for 45 seconds and 72 °C for 2 minutes, and a final extension of 72 °C for 10 minutes. PCR products were visualized on a 1 % agarose gel (Figure 5.1 b). PCR products were purified prior to cloning in several enrichment methods (Table 5.2), using a QIAquick PCR Purification kit (Qiagen) following the manufacturer's protocol and eluted in 30 µl Buffer EB (10 mM Tris-Cl, pH 8.5).

Cloning was performed using a TOPO TA Cloning® Kit (Invitrogen) with PCR product cloned into pCR®2.1-TOPO® vector and transformed into TOP10F' *E. coli* competent cells according to the manufacturer's protocol. Cells were plated onto 37 °C prewarmed ampicillin selective LB agar plates, spread with 32 µl 50 mg/ml X.gal and 40 µl 100 mg/ml IPTG and incubated at 37 °C overnight. Recombinant colonies were detected by blue/white screening, individual colonies transferred into 20 µl 10 mM Tris-HCl (pH 8.5) and incubated at 95 °C for 10 minutes. The resulting lysed colony solution was stored at -20 °C.

Table 5.2 – Summary of enrichment method modifications for microsatellite library construction

	Enrichment										
	EM00	**EM01**	**EM02**	**EM03**	**EM04**	**EM05**	**EM06**	**EM07**	**EM08**	**EM09**	**EM10**
DNA Preparation											
DNA pooled from *N* individuals	1	1	2	2	2	2	2	2	3	3	3
Restriction digest incubation (hours)	3	3	3	3	3	3	3	3	12	12	12
PCR amplification	Y	Y	N	N	N	N	N	N	N	N	Y
Selective hybridization											
Biotinylated primer hybridization with SA-PMPs or DNA	SA-PMP	SA-PMP	SA-PMP	DNA	DNA	DNA	DNA	DNA	DNA	SA-PMP	SA-PMP
DNA-Biotin-Bead incubation temperature (°C)	55	55	55	55	55	37	37	55	55	55	55
DNA-biotin-bead incubation 350 rpm agitation	N	N	N	N	Y	N	Y	Y	Y	Y	Y
Magnetic capture											
2x SSC washes	4x 20°C	N	N	N	N	N	N	N	N	N	N
1x SSC washes	4x 30°C	1x RT 1x 30°C 1x 35°C 1x 40°C 1x 45°C 1x 50°C 1x 55°C 1x 60°C	3x 50°C 3x 60°C 1x 70°C 1x 80°C	4x 50°C 4x 60°C	4x 50°C 4x 60°C	4x 50°C 4x 60°C	4x 50°C 4x 60°C	4x 40°C 4x 50°C	4x 40°C 4x 50°C	4x 40°C 4x 50°C	4x 40°C 4x 50°C
PCR amplification and cloning											
PCR purification prior to cloning	N	N	N	N	N	N	N	N	Y	Y	Y

EMxx, enrichment method; Y, applied; N, not applied.

5.2.2 Isolation of microsatellite loci

5.2.2.1 PCR screening of recombinant colonies

Recombinant colonies likely to contain microsatellites were identified by PCR screening. Two screening PCR reactions were performed: one with the M13-20F and M13-R vector primers (Table 5.1) and a motif primer, and the other with the M13-R vector primer and a motif primer. An unlabelled repeat motif primer (Table 5.2) was included to detect microsatellite repeats by hybridization with insert sequences containing the enriched repeat motif. PCR amplification was performed with 0.5 µl colony solution as template, 4 mM $MgCl_2$, 0.2 mM each dNTP, 2 pmol each primer, 0.25 U Thermo-Start® DNA polymerase and 1x Thermo-Start® Reaction buffer (ABgene) in a 10 µl volume. Thermal cycling conditions were one cycle of enzyme activation at 95 °C for 15 minutes, 45 seconds annealing at 60 °C and 2 minutes extension at 72 °C, 34 cycles of 45 seconds denaturation at 94 °C, 60 °C for 45 seconds and 72 °C for 2 minutes, and a final extension of 72 °C for 5 minutes. Presence of a microsatellite repeat was indicated by PCR products with two or more bands in the former and an amplification product in the latter after visualization on a 1.5 % agarose gel.

5.2.2.2 Sequencing and primer design

Colonies with microsatellite containing inserts were PCR amplified for sequencing with 0.5 µl lysed colony solution, 4 mM $MgCl_2$, 0.2 mM each dNTP, 5 pmol each vector primer, 0.625 U Thermo-Start® DNA polymerase and 1x Thermo-Start® Reaction buffer (ABgene) in a 25 µl volume. Amplification reactions were performed using touch-down PCR with an initial enzyme activation at 95 °C for 15 minutes, 14 cycles of 45 seconds denaturation at 94 °C, 45 seconds annealing at 67 °C Δ -0.5°C per cycle and 2 minutes extension at 72 °C, 15 cycles of 94 °C for 45 seconds, 60 °C for 45 seconds and 72 °C for 2 minutes, and a final extension of 72 °C for 5 minutes. PCR products were visualized on a 1.5 % agarose gel and successful amplifications ExoSap purified with the addition of 20 U Exonuclease I and 1 U shrimp alkaline phosphotase to 10 µl PCR product incubated at 37 °C for 40 minutes and 80 °C for 15 minutes. Purified PCR

products were sequenced using dye terminator chemistry (Applied Biosystems) and resolved on an ABI 377 DNA sequencer (Applied Biosystems) or by Macrogen Inc. (Korea) on an ABI 3730xl DNA analyser.

Vector and adapter regions were removed and the insert sequences were aligned in CodonCode Aligner version 1.6.2. Duplicate sequences were identified as those with greater than 95 % similarity. Enrichment method efficiency was determined as the percentage of PCR screened colonies containing potential microsatellite loci as unique insert sequences with a repeat region of at least 14 bp in length. Primers were designed in PRIMER3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi/) and AmplifX version 1.3.7 selecting for primer length between 18 bp and 22 bp and GC content between 40 % and 60 % with all other conditions set to default. Microsatellite locus sequences are given in Appendix IV. An 18 bp universal M13(-21) sequence tail was added at the 5' end of each forward primer (Table 5.3) to enable fluorescent labelling of PCR fragments using the tailed primer method (Schuelke 2000).

5.2.3 Primer evaluation and loci polymorphism

Microsatellite primers were tested for PCR amplification, production of interpretable genotype bands and level of polymorphism in sixteen individuals of *A. odoratum* from four North Wales populations using the tailed primer method of fluorescent labelling. Primer specific PCR amplification reactions were optimised using a gradient of magnesium chloride ($MgCl_2$) from 1.5 mM to 4.0 mM, and thermal cycling conditions with annealing temperatures from 50°C to 60°C, step durations from 30 seconds to 2 minutes and final extensions of 10 minutes to 30 minutes. Optimised PCR amplification reactions were performed in a 10 µl volume with 1 - 5 ng genomic DNA template, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1 pmol tailed forward primer, 4 pmol reverse primer, 4 pmol M13(-21) fluorescent labelled primer, 0.25 U Thermo-Start® DNA polymerase and 1x Thermo-Start® Reaction buffer (ABgene). Reaction conditions included an initial enzyme activation step of 95 °C for 15 minutes, 25 cycles of loci specific thermal cycling, 10 cycles of M13(-21) fluorescent label incorporation with 94 °C for 30 seconds, 52 °C for 45 seconds and 72 °C for 45 seconds, and a loci specific final

Table 5.3 – Primer details for isolated microsatellite loci and tailed primer fluorescent labelling

Locus	Primer Sequence (5'-3') (F, forward; R, reverse) [M13(-21) tail]		Thermal cycling
AoM00B1	F	[M13]CAGCAGTAGGTGCTCCATGA	A
	R	GATCTGGTTGTGGAGGAGGA	
AoM00C1	F	[M13]TGATGAGCCAATGTCGTTGT	A
	R	CTCGGAGAGTGGAGATGAGG	
AoM00F1	F	[M13]CACATCAAAGGTCGTATCCC	B
	R	GCCATGAACCCTAGAATTCC	
AoM01B1	F	[M13]TTACCCAAATTCAGTCGATCA	A
	R	ACATCACCTCATTCCCGAAG	
AoM01F1	F	[M13]CTCGCACAACAAAGGTCGTA	A
	R	GGGAAAATTGGAGTGAGTGC	
AoM01F2	F	[M13]TAGCTCCAGTCGTATATCCC	B
	R	GTGAGACTCGATTGGCAGTA	
AoM01F3	F	[M13]CGAGCTAACATGCAGAAGCA	A
	R	GCACCCACTCATCTCACTCA	
AoM01F4	F	[M13]ATCGGTCCATTTGTTCTTGG	A
	R	CAGGAGACGAGCAATGACAG	
AoM03F1	F	[M13]CCCATAAGCTTGTGACTTGT	B
	R	GTGCTGCCCAAATCTTCAAC	
AoM03F2	F	[M13]TCCAGGTACACGGCCATCT	B
	R	GCTCGAATCATTGAGCCAGT	
AoM06F1	F	[M13]TTATGAACGGGTTGCTGGCT	B
	R	CCCAAAGGGGAAAATCGAGA	
AoM07F1	F	[M13]TCGAGTAAGCCTCGATTCGC	B
	R	CTCGCACAGCAAAGGTCGTA	
AoM07F2	F	[M13]GCACGGTCATTGTCAACCGA	A
	R	TAACTAGGCATGGCTGTGGT	
AoM07F3	F	[M13]ATGCCGATGCCGGCATAGAA	B
	R	ATGCCATTGTCGGCATGCTC	
AoM07F4	F	[M13]GCATCGTGGTCATGCCGATA	B
	R	TCATGCCATTGTCGGCATGT	

Tailed primer fluorescent labelling

M13(-21) primer	Cy5 – TGTAAAACGACGGCCAGT

A: thermal cycles of 94 °C for 1 minute, 56 °C for 1 minute and 72°C for 1 minute, final extension at 72 °C for 10 minutes

B: thermal cycles of 94 °C for 30 seconds, 60 °C for 30 seconds and 72°C for 2 minutes, final extension at 72 °C for 30 minutes

extension step at 72°C. Primer sequences and loci specific thermal cycling conditions are detailed in Table 5.3. Successful PCR amplification was determined by visualization on a 1.5 % agarose gel, genotype profiles were resolved on a CEQ 8000 Genetic Analysis System (Beckman Coulter) with the CEQ DNA Size Standard Kit-400 as an internal size standard and microsatellite fragment sizes were determined using the CEQ 8000 Genetic Analysis System software version 9.0.25 (Beckman Coulter).

The polyploid nature of *A. odoratum* negated the production of complete genotype data due to the difficulty in determining allele copy number from peak heights obtained using PCR based microsatellite markers (Becher *et al* 2000, Markwith *et al* 2006). An allele presence/absence matrix was used to determine the number of allele phenotypes per locus (Becher *et al* 2000, Saltonstall 2003) and observed heterozygosity (H_O) was calculated as the proportion of individuals exhibiting a heterozygote allele phenotype. Hardy-Weinberg expected heterozygosity (H_E) statistics were calculated using partial genotype data in the computer program TETRASAT (Markwith *et al* 2006) under the assumption of allotetraploidly. Per-locus H_O and H_E were calculated from the representative samples of each population and then averaged as the number of heterozygotes in the pooled sample exceeded the computational capability of the TETRASAT program (Markwith *et al* 2006, Markwith and Scanlon 2007).

5.3 Results

5.3.1 Enrichment efficiency

Eleven streptavidin-biotin enrichment methods were investigated for isolation of CT dinucleotide microsatellite repeats with 38 to 192 recombinant colonies PCR screened per method (Table 5.4). The number of colonies identified by PCR screening as likely to contain microsatellite repeats ranged from 0 % to 21.3 %. Two enrichment methods, EM02 and EM05, yielded no positively screened colonies. This can be attributed to wash temperatures exceeding 60°C and a low incubation temperature without agitation during selective hybridization respectively. The high wash temperatures may have disrupted the DNA - Biotin primer - SA-PMP complex prior to elution removing both bound and unbound DNA fragments while the low incubation temperature may have resulted in reduced DNA - Biotin primer hybridization. In both methods, sufficient DNA fragments were obtained after elution and PCR amplification to warrant cloning, indicating insufficient removal of unbound DNA fragments during magnetic capture.

Table 5.4 – Enrichment method efficiency as a percentage of PCR screened colonies for isolation of CT dinucleotide microsatellite repeats

Enrichment	No. of PCR screened colonies	% positively screened and sequenced	% unique sequences	% unique repeats
EM00	47	21.3	21.3	6.4
EM01	38	21.1	15.8	15.8
EM02	192	0.0	0.0	0.0
EM03	192	2.1	2.1	1.0
EM04	56	1.8	1.8	1.8
EM05	136	0.0	0.0	0.0
EM06	72	1.4	1.4	1.4
EM07	104	13.5	8.7	3.9
EM08	192	13.5	7.8	0.0
EM09	96	5.2	2.1	0.0
EM10	96	4.2	3.1	0.0

EMxx, enrichment method

The level of redundancy in terms of duplicate insert sequences ranged from 0 % to 59.6 % of sequenced colonies. The addition of a PCR amplification step prior to selective hybridization is a potential source for increased duplication although the results do not indicate any trend for increased redundancy between enrichment methods with or without this step (Table 5.4). However, only three of the nine methods for which positively screened colonies were obtained included this step. The highest levels of redundancy were observed for enrichment methods EM08 (42.2 %), EM09 (59.6 %) and EM10 (26.2%) where genomic DNA was pooled from three individuals, suggesting that isolation of microsatellite repeats from pooled DNA may increase the chance of obtaining duplicate sequences. The level of attrition due to both duplicate insert sequences and the lack of a microsatellite repeat averaged 48.1 % of sequenced colonies ranging from 0 % to 100 %.

Enrichment method efficiency, as the percentage of PCR screened colonies containing *unique* microsatellite repeats, ranged from 0 % to 15.8 % (Table 5.4). Six enrichment methods isolated microsatellite repeats with EM00 and EM01 the most successful with enrichment efficiencies of 6.4 % and 15.8 % respectively. Of the five remaining methods, two did not yield any positively screened colonies and three yielded no microsatellite repeats despite having 4.2 % to 13.5 % of colonies positively screened by

Table 5.5 – Comparison of EM00 and EM01 enrichment method efficiencies as a percentage of PCR screened colonies for isolation of tetra-, tri-, and dinucleotide microsatellite repeats

Enrichment motif	Enrichment	No. of PCR screened colonies	% positively screened and sequenced	% unique sequences	% unique repeats
AAAG	**EM00**	48	2.1	2.1	0.0
	EM01	47	8.5	8.5	0.0
TCAG	**EM00**	48	18.8	10.4	2.1
	EM01	31	22.6	22.6	3.2
GAT	**EM00**	48	6.3	6.3	4.2
	EM01	22	0.0	0.0	0.0
AAT	**EM00**	35	8.6	5.7	0.0
	EM01	31	0.0	0.0	0.0
CA	**EM00**	47	2.1	2.1	0.0
	EM01	16	0.0	0.0	0.0
CT	**EM00**	47	21.3	21.3	6.4
	EM01	38	21.1	15.8	15.8

EMxx, enrichment method

PCR. The latter three methods, EM08, EM09 and EM10, involved a 12 hour restriction digest step and had the highest levels of redundancy due to duplicate sequences. These results indicate the relative inefficiency of the PCR screening method for detection of microsatellite containing colonies. However, the efficiency using PCR screening is likely to be higher than that obtained for randomly sequenced colonies although this was not tested.

The two most successful enrichment methods for isolation of CT motifs, EM00 and EM01, were further investigated for isolation of CA, GAT, AAT, AAAG and TCAG repeats (Table 5.5). Unique microsatellite repeats were isolated by enrichment for GAT and TCAG with an efficiency of 4.2 % and 2.1 % for EM00, and 0 % and 3.2 % for EM01 respectively. No unique microsatellite repeats were isolated for CA, AAT or AAAG with either enrichment method. The variation in enrichment efficiency observed between repeat motifs is likely to be a product of specific repeat abundance within the genome. The highest enrichment efficiencies for both methods were obtained for isolation of CT microsatellite repeats indicating that, of those repeat motifs investigated for isolation, CT repeats are the most prevalent in *A. odoratum*.

Table 5.6 – Enrichment methods and motifs yielding microsatellite repeats

Enrichment	Enrichment motif	Unique microsatellite repeats	% suitable for primer design	Total loci	Loci
EM00	TCAG	1	100	1	AoM00B1
	GAT	2	50	1	AoM00C1
	CT	3	33.3	1	AoM00F1
EM01	TCAG	1	100	1	AoM01B1
	CT	6	66.7	4	AoM01F1 AoM01F2 AoM01F3 AoM01F4
EM03	CT	2	100	2	AoM03F1 AoM03F2
EM04	CT	1	0	0	-
EM06	CT	1	100	1	AoM06F1
EM07	CT	4	100	4	AoM07F1 AoM07F2 AoM07F3 AoM07F4

EMxx, enrichment method

Twenty one unique microsatellite repeats were isolated from all enrichments, two for each GAT and TCAG, and 17 for CT (Table 5.6). Of these six (28.6 %) were unsuitable for primer design due to inadequate flanking region length or base composition and primer pairs were designed for 15 microsatellite loci. Di-, tri-, tetra- and pentanuclotide microsatellite loci were developed with perfect, imperfect, interrupted and interrupted compound repeat types (Table 5.7).

5.3.2 Locus polymorphism

Fifteen microsatellite loci were screened for amplification and polymorphism in *A. odoratum* (Table 5.7). Of these four loci did not amplify interpretable genotype profiles (26.7 %), eight loci were monomorphic (53.3 %) and three loci, the pentanucleotide AoM00B1, tetranucleotide AoM01B1 and dinucleotide AoM06F1 exhibited polymorphism (20.0 %). Three loci exhibited possible fixed heterozygosity with a

Table 5.7 – Microsatellite locus amplification and polymorphism screening in *A. odoratum*. Locus allele number per sample, total allele number, allele size range and polymorphism are described. Total number of allele phenotypes as the number of multiallelic profiles observed, observed (H_O) and expected (H_E) heterozygosities are given for polymorphic loci.

			***A. odoratum* screening**								
Locus	**Repeat motif**	**Repeat type**	***n***	**No. alleles per sample**		**No. alleles**	**size range (bp)**	**Polymorphic**	**No. allele phenotypes**	**H_O (±SD)**	**H_E (±SD)**
				min	**max**						
AoM00B1	$(TCCGA)_4$	perfect	16	1	2	3	222-230	Y[a m]	4	n/a	n/a
AoM00C1	$(ATC)_5$	perfect	16	1	1	1	186	N	n/a	n/a	n/a
AoM00F1	$(CT)_7$	perfect	4	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
AoM01B1	$(CTGA)_5$	perfect	16	1	2	7	176-204	Y[m]	8	0.69 (0.24)	0.67 (0.05)
AoM01F1	$(CT)_4TCC(CT)_5$	interrupted	16	2[f]	2	2	199-209	N	n/a	n/a	n/a
AoM01F2	$(CT)_2G(CT)_7$	imperfect	4	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
AoM01F3	$(AG)_8$	perfect	16	1	1	1	170	N	n/a	n/a	n/a
AoM01F4	$(CT)_7$	perfect	16	1	1	1	181	N	n/a	n/a	n/a
AoM03F1	$(CT)_7$	perfect	4	1	1	1	179	N	n/a	n/a	n/a
AoM03F2	$(CT)_3CCCTAT(CT)_7GC(CT)_4$	interrupted	4	1	1	1	241	N	n/a	n/a	n/a
AoM06F1	$(CT)_8CG(CT)_4(GT)_3$	interrupted compound	16	2[f]	3	22	203-336	Y[m]	14	1.00 (0.00)	0.79 (0.11)
AoM07F1	$(GA)_{11}$	perfect	4	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
AoM07F2	$(AG)_2G(AG)_{20}$	imperfect	4	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
AoM07F3	$(TC)_7$	perfect	4	2[f]	2	2	191-193	N	n/a	n/a	n/a
AoM07F4	$(CT)_8$	perfect	4	1	1	1	205	N	n/a	n/a	n/a

n, number of individuals screened; SD, standard deviation; n/a, not applicable; [f], possible fixed heterozygosity; Y, yes; N, no; [a], evidence of null alleles or allelic dropout; [m], alleles not corresponding to expected repeat;

minimum of two alleles per sample and locus AoM06F1 exhibited a polyploid profile with a maximum of three alleles per sample. Inconsistent allele amplification was observed in AoM00B1 indicating allelic dropout or the presence of null alleles and this locus was not considered further. All polymorphic markers exhibited alleles which did not correspond to the expected repeat motif by 1 to 2 bp suggesting mutation in the microsatellite flanking region or repeat region. All observed alleles were included for determination of locus genetic diversity.

At the polymorphic AoM01B1 and AoM06F1 loci genetic diversity was relatively high with 7 and 22 alleles per locus, 8 and 14 allele phenotypes (see section 5.2.3), observed heterozygosities of 0.69 and 1.00, and expected heterozygosities of 0.67 and 0.79 respectively (Table 5.7). The observed heterozygosity of 1.00 at AoM06F1 was due to all sampled individuals exhibiting heterozygous allele phenotypes. However, this is based on a small number of screened individuals. No inferences of inbreeding could be made based on heterozygote excess or deficiency for these tetraploid loci (Markwith *et al* 2006).

5.4 Discussion

The highest enrichment efficiencies of 6.4 % to 15.8 %, as the percentage of PCR screened colonies containing unique microsatellite repeats, were obtained for isolation of CT dinucleotide repeats using enrichment methods EM00 and EM01 respectively. Only two other motifs, GAT and TCAG, isolated microsatellite repeats with enrichment efficiencies of up to 4.2 % and 3.2 % respectively. The higher enrichment efficiencies obtained for isolation of CT repeats is consistent with the greater abundance of these sequences in the Gramineae (Gupta and Varshney 2000) and other Embrophyta (Zane *et al* 2002).

The obtained microsatellite enrichment efficiencies are lower than those reported for isolation of tetranucleotide repeats by very similar enrichment methods in the lizard species *Egernia stokesii* (16.7 %, Gardner *et al* 1999) and *Anolis roquet* (53.5 %, Gow *et al* 2006) and the majority of those reported for plant species with colony screening

prior to sequencing (10 % to 100 %, reviewed in Squirrell *et al* 2003). The enrichment efficiency of this technique in *A. odoratum* is more comparable with the 6.4 % and 9.0 % reported for *Pelargonium* spp with positive colonies detected using random sequencing (Becher *et al* 2000). This suggests that PCR screening may not be an accurate method for the detection of microsatellite containing colonies and was highlighted in several enrichments where up to 13.5 % of colonies were PCR screened as positive although none contained microsatellite repeats.

The efficiency of streptavidin-biotin enrichment is dependant upon the ability to recover microsatellite containing DNA after selective hybridization. This is affected by the success of adapter ligation as DNA fragments are recovered by PCR amplification using the adapter as a primer annealing site, the efficiency of biotinylated primer hybridization to repeat containing DNA and the stringency of washes during magnetic capture (Zane *et al* 2002). Adapter ligation was performed in the same manner for all enrichment methods and is unlikely to be the source of the varying enrichment efficiencies obtained. The selective hybridization step was most affected by the incubation temperature with 37 °C resulting in a very low efficiency. The most influential factor was the wash temperature during magnetic capture with temperatures exceeding 60 °C resulting in no positively screened colonies. This is consistent with the 40 % decrease in enrichment efficiency observed in sugarcane, *Saccharum* spp, with an increase in wash temperature from 50 °C to 71 °C (Cordeiro *et al* 1999).

Redundancy due to duplicate insert sequences averaged 21.0 % (ranging from 0 % to 59.6 %) of sequenced colonies with the greatest levels, 26.2 % to 59.6 %, observed when DNA was pooled from three individuals. However, pooled DNA may not be the cause of such high levels of redundancy as this is not consistent with the 16.1 % and 17.2 % duplicate sequences reported by Gardner *et al* (1999) and Gow *et al* (2006) where DNA was pooled from 7 and 8 individuals respectively. The level of attrition due to both the lack of a microsatellite repeat and duplicate inserts was on average 48.1 %. This is more comparable with results for microsatellite isolation using non-enriched libraries (48.7 %) rather than enriched libraries (36.0 %) in plant species (Squirrell *et al* 2003).

A further source of attrition is the number of unique microsatellite loci isolated for which the flanking regions are unsuitable for primer design due to base composition or length. Gardner *et al* (1999) reported the isolation of microsatellite repeats with complete flanking regions by the incorporation of the biotin label and a noncomplementary region at the 3' end of the biotinylated primer. Although the number of loci for which base composition precluded primer design was not directly reported, primers were designed for only 55 % of the isolated loci. Utilising the same biotinylated primer method the attrition rate due to unsuitable flanking regions was 28.6 % for *A. odoratum* in this study and the same was obtained in *Anolis roquet* (Gow *et al* 2006) and is lower than the mean 46 % for plant species (Squirrell *et al* 2003).

Fifteen microsatellite loci were screened in *A. odoratum* and three loci exhibited polymorphism. One locus was excluded due to inconsistent amplification and potential null alleles. Two polymorphic loci, AoM01B1 and AoM06F1, exhibited 7 to 22 alleles and 8 to 14 allele phenotypes per locus. AoM06F1 exhibited a polyploid profile with up to three alleles per individual and possible fixed heterozygosity as no homozygotes were observed. A larger sample size is necessary to characterize both the polyploid genotypes and heterozygosity at these loci although the variability observed with a small sample size indicates the potential of these markers for assessment of population genetic diversity. However, allele sizes at both loci did not always conform to the expected repeat motif indicating mutation within the flanking or repeat regions. Similar allele size discrepancies to those observed in *A. odoratum* have been reported in many species, including polyploid *Ipomoea* spp (Buteler *et al* 1999), *Pelargonium* spp (Becher *et al* 2000), and *Phragmites australis* (Saltonstall 2003). Indel mutation can result in size homoplasy (Grimaldi and CrouauRoy 1997, Estoup *et al* 2002) with same size alleles containing different numbers of microsatellite repeat units or alleles of different size containing the same number of repeat units. Allele sequence characterization is therefore required to determine the underlying nucleotide variation.

The isolation of microsatellite markers in *A. odoratum* using streptavidin-biotin enrichment was largely unsuccessful with low enrichment efficiency and yield of polymorphic loci. In many cases, modifications to the enrichment procedure did not provide any useful information regarding optimisation of this technique. Repetition of enrichments and mean efficiency may remove some of the stochastic variation observed

and provide a more comprehensive assessment of enrichment method efficiency. Further optimisation is necessary to provide an efficient enrichment method and a working set of microsatellite markers in this species.

Chapter 6: Genetic diversity of *Anthoxanthum odoratum* populations in North Wales

6.1 Introduction

Patterns of genetic diversity within and among populations can provide insights into the ecological and evolutionary processes that influence the genetic composition of populations such as dispersal (Ouborg *et al* 1999), mating system (Ng *et al* 2004, Hodgins and Barrett 2007), population bottlenecks (Luikart *et al* 1998), and adaptation (Skot *et al* 2002). The quantity of information relating to genetic variation in plant species has greatly increased in recent years with the growing application of molecular markers in the study of population structuring (Ge *et al* 2005, Gomez *et al* 2003), habitat fragmentation (Young *et al* 1996, Yamagishi *et al* 2007), metapopulation dynamics (Navascues *et al* 2006), conservation management (Kreivi *et al* 2005, Ueno *et al* 2005), hybridization and polyploidy (Salmon *et al* 2005, Abbott *et al* 2007), intra- and inter-specific phylogenetics and phylogeography (Despres *et al* 2002, Katayama and Ogihara 1996, Catalan *et al* 1997) and quantitative traits (Andaya and Mackill 2003, Kim *et al* 2004).

Levels of genetic diversity are thought to relate to evolutionary potential as genetic variation provides the basis for adaptation and differentiation through the mechanisms of mutation, genetic drift and selection. Low levels of genetic diversity are often associated with rare or endangered species (Cole 2003), small isolated populations (Ouborg *et al* 2006) and bottleneck events (Amos and Harwood 1998) with such species or populations usually considered to be of conservation importance. Species and populations exhibiting high levels of genetic diversity are expected to have a greater evolutionary potential for adaptation in relation to environmental change (Ackerman and Ward 1999) however this may not always be the case (Amos and Harwood 1998). In addition, species which are highly variable for morphological, physiological and phenological traits are often expected to exhibit corresponding high levels of underlying genetic variation, as observed in the orchid *Tolumnia variegata* (Ackerman and Ward

1999). Phenotypic traits have been used to infer population diversity, differentiation and adaptation in relation to heterogeneity in environmental conditions such as light intensity, temperature and metal pollution (Linhart and Grant 1996). However, observed variation in phenotypic traits may be attributable to genotype-environment interactions and represent phenotypic plasticity rather than the underlying genetic variation (Schlichting and Smith 2002). The application of molecular markers can provide information regarding genetic diversity and differentiation underlying observed phenotypic variation.

Plant species provide an interesting model for the study of genetic variation due to the sedentary nature of adult individuals and differential transmission modes of the nuclear and cytoplasmic genomes. The biparentally inherited nuclear genome is transmitted through dispersal of both pollen and seeds. In angiosperms the two organelle genomes, mitochondrial and plastid (chloroplast), are usually maternally inherited with dispersal through seeds, although some evidence of paternal leakage has been observed in some species (Ouborg *et al* 1999, McCauley *et al* 2007). Of the two organelles variation in the chloroplast genome is most widely assessed due to the relatively conserved nature of this genome which has facilitated the development of universal primers and the presence of intraspecific variation (Dumolin-Lapegue *et al* 1997, Weising and Gardner 1999, Provan *et al* 2001, Saltonstall 2001, Grivet *et al* 2001, Provan *et al* 2004). Genetic diversity in the different genomes may reveal information on the magnitude of pollen-mediated and seed-mediated gene flow and therefore the relative importance of each dispersal mechanism in determining population genetic diversity, differentiation and structuring (Petit *et al* 2005).

Anthoxanthum odoratum is a short-lived perennial tetraploid (2n = 4*x* = 20), morphologically highly variable, wind pollinated and predominantly outcrossing species (Wu and Jain 1980, Silvertown *et al* 2002) which reproduces primarily through seed production with vegetative propagation of minor importance (Antonovics 1972). The origin of this polyploid species has been suggested as autotetraploid (Felber 1987), however, allotetraploidy was considered highly probable based on ecology, morphology and cytology (Hedberg 1990) and supported by phylogenetics (Pimental *et al* 2007). Allopolyploidy was assumed in this study based on the greater support for an allopolyploid origin of this species (Hedberg 1990, Pimentel *et al* 2007).

Previous studies have suggested local adaptation of this species within relatively short time scales over small geographic distances in response to fertilizer and liming treatments (Snaydon 1970, Snaydon and Davies 1972, Snaydon and Davies 1976, Davies and Snaydon 1976, Silvertown *et al* 2005) and zinc pollution (Antonovics 2006). These observations were based on differentiation among adjacent populations in morphological and physiological traits and flowering phenology across sharp environmental boundaries. Pre-zygotic reproductive isolation has been suggested as a possible mechanism behind this differentiation as a result of limited pollen flow due to differences in flowering time (Silvertown *et al* 2005, Antonovics 2006). However, other studies have suggested that the responses to environmental conditions may be related to phenotypic plasticity rather than to genotype-environment interactions and population differentiation (Platenkamp 1991, Platenkamp and Shaw 1992).

In this study, genetic diversity and differentiation were estimated among four *A. odoratum* populations from North Wales which had previously been screened for ozone sensitivity (see Chapters 2, 3 and 4) as a preliminary screen for genetic variation which may relate to observed differences in ozone sensitivity. Genetic variation in the nuclear genome was primarily determined using the Amplified Fragment Length Polymorphism technique (AFLP, Vos *et al* 1995). These markers require no prior sequence knowledge and produce a large number of reproducible loci providing a genome wide scan of genetic variation (Vos *et al* 1995, Bensch and Akesson 2005, Jones *et al* 1997). In addition, two nuclear microsatellite markers developed in this species (see Chapter 5) were also employed. Genetic variation in the chloroplast genome was determined using universal primer based Polymerase chain reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) markers characterised in these populations and two chloroplast microsatellite loci previously shown to exhibit intraspecific variation in this species (Provan *et al* 2004). The aims of this study were to determine (1) whether these populations differ in the level of genetic diversity, (2) are genetically differentiated and (3) the relative contribution of pollen-mediated and seed-mediated gene flow to dispersal.

6.2 Methods

6.2.1 Sample collection and DNA extraction

Anthoxanthum odoratum L. individuals were collected from four populations in North Wales in June-July 2004 (see Chapter 1, section 1.4 for details of sample collection). Tissue samples were taken from twenty individuals per population which had been utilised in at least one ozone exposure experiment (see Chapters 2, 3 and 4). Genomic DNA was extracted from 100 mg fresh or 20 mg dried leaf tissue using a DNeasy Plant mini kit (Qiagen) according to the manufacturer's protocol. DNA quantification was performed using a NanoDrop® ND-1000 UV-Vis Spectrophotometer.

6.2.2 Nuclear DNA markers

6.2.2.1 AFLP fingerprinting

AFLP was performed as described in Vos *et al* (1995) using the rare cutter *Eco*RI and frequent cutter *Mse*I restriction endonucleases with minor modifications. The double stranded adapters were generated by hybridization of equimolar amounts of the adapter oligonucleotides, Ead1 and Ead2 for *Eco*RI and Mad1 and Mad2 for *Mse*I (Table 6.1), incubated at 90 °C for 2 minutes then slowly cooled to 20 °C over 1 hour. 0.35 µg DNA was fragmented by restriction digest with 5 U of each *Eco*RI and *Mse*I, 1x *Eco*RI reaction buffer (New England Biolabs (UK) Ltd) and 100 µg/ml Bovine Serum Albumin (BSA) in a 40 µl volume incubated at 37 °C for 1 hour. Adapter ligation was performed in a 50 µl reaction with the 40 µl restricted DNA, 5 pmol *Eco*RI adapter, 50 pmol *Mse*I adapter, 1 U T4 DNA Ligase (Promega) and 1x DNA Ligase buffer incubated at 16 °C overnight. The restriction ligation mixture (RL) was visualized on a 2 % agarose gel alongside a 100 bp DNA ladder (Promega) to determine successful

Table 6.1 – Oligonucleotides for AFLP fingerprinting

Oligonucleotide	Sequence (5'-3')
Adapters	
Ead1	CTCGTAGACTGCGTACC
Ead2	AATTGGTACGCAGTCTAC
Mad1	GACGATGAGTCCTGAG
Mad2	TACTCAGGACTCAT
Preamplification	
E00	GACTGCGTACCAATTC
M00	GATGAGTCCTGAGTAA
Selective amplification	
E32	*GACTGCGTACCAATTC AAC
E33	*GACTGCGTACCAATTC AAG
E35	*GACTGCGTACCAATTC ACA
E40	*GACTGCGTACCAATTC AGC
M49	GATGAGTCCTGAGTAA CAG
M60	GATGAGTCCTGAGTAA CTC
M61	GATGAGTCCTGAGTAA CTG

E, *Eco*RI; M, *Mse*I; *, FAM fluorescent label

restriction digestion and adapter ligatation. The RL mixture was then diluted ten-fold with ddH_2O and stored at -20 °C.

A preamplification (PA) step was performed with *Eco*RI and *Mse*I primers complementary to the adapter and restriction site with no selective bases (E00 and M00, Table 6.1). Preamplification PCR reactions were performed in a 20 µl volume with 5 µl of diluted RL as DNA template, 3 mM $MgCl_2$, 0.2 mM each dNTP, 5 ρmol of each E00 and M00 primer, 0.5 U Go Taq® DNA Polymerase and 1x Go Taq® reaction buffer (Promega). Thermal cycling conditions consisted of an initial denaturation at 95 °C for 2 minutes, 30 cycles of 30 seconds denaturation at 95 °C, 1 minute annealing at 56 °C, 1 minute extension at 72 °C, and a final extension at 72 °C for 10 minutes. Preamplification PCR products were visualized on a 2 % agarose gel alongside a 100 bp DNA ladder (Promega). The PA PCR products were diluted ten-fold with ddH_2O and stored at -20 °C.

Selective amplification (SA) PCR reactions included a fluorescently labelled *Eco*RI primer and unlabelled *Mse*I primer, each with 3 additional selective bases, with four primer combinations, E32/M60, E33/M60, E35/M49 and E40/M61 (Table 6.1) applied.

Selective amplification PCR reactions were performed in a 20 µl volume with 5 µl of diluted PA template, 3 mM $MgCl_2$, 0.2 mM each dNTP, 4 ρmol *Eco*RI primer, 5 ρmol *Mse*I primer, 0.5 U Go Taq® DNA Polymerase and 1x Go Taq® reaction buffer (Promega). Thermal cycling conditions consisted of an initial denaturation at 95 °C for 2 minutes, 13 cycles of 30 seconds denaturation at 95 °C, 30 seconds annealing at 65 °C Δ -0.7°C per cycle and 1 minute extension at 72 °C, 23 cycles of 30 seconds denaturation at 95 °C, 30 seconds annealing at 56 °C, 1 minute extension at 72 °C, and a final extension at 72 °C for 10 minutes. Selective amplification PCR products were visualized on a 2 % agarose gel alongside a 100 bp DNA ladder (Promega).

1 µl SA PCR product was resolved on an ABI 3130xl genetic analyser (Applied Biosystems) with 10 µl formamide and 0.25 µl GeneScan™ 600 LIZ® internal size standard (Applied Biosystems) included to size fragments following the manufacturer's instructions. The generated AFLP profiles were analysed by ABI PRISM® GeneMapper® Software (Applied Biosystems) scoring presence and absence of fragments between 80 bp and 400 bp in a binary presence/absence genotype table. The AFLP protocol was repeated twice for two randomly chosen individuals to determine fragment reproducibility.

6.2.2.2 Nuclear microsatellites

Individuals were genotyped for two nuclear microsatellite loci, the tetranucleotide AoM01B1 and dinucleotide AoM06F1 developed in *A. odoratum* (see Chapter 5), using the tailed primer method of fluorescent labelling (Schuelke 2000). Primer sequences and locus specific thermal cycling conditions are given in Table 6.2. PCR amplification reactions were performed in a 10 µl volume with 5 ng genomic DNA template, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1 ρmol tailed forward (F) primer, 4 ρmol reverse (R) primer, 4 ρmol M13 fluorescent labelled primer, 0.25 U Go Taq® DNA Polymerase and 1x Go Taq® reaction buffer (Promega). Thermal cycling conditions consisted of an initial denaturation at 95 °C for 2 minutes, 25 cycles of loci specific thermal cycling (Table 6.x), 10 cycles of M13 fluorescent label incorporation with 95 °C for 30 seconds, 52 °C for 45 seconds and 72 °C for 45 seconds, and a loci specific final extension step at 72°C. PCR products were visualized on a 2 % agarose gel alongside a 100 bp DNA ladder (Promega). Microsatellite genotype profiles were resolved on an ABI 3130xl

Table 6.2 – *Anthoxanthum odoratum* nuclear microsatellite loci primer sequences and thermal cycling conditions

			Thermal cycling			
Locus	**Primer**	**Primer Sequence (5'-3')**	***D***	***A***	***E***	***FE***
AoM01B1	F	[M13]TTACCCAAATTCAGTCGATCA	95°C	56°C	72°C	72°C
	R	ACATCACCTCATTCCCGAAG	1m	1m	1m	10m
	M13(ned)	NED – TAGAAGGCACAGTCGAGG				
AoM06F1	F	[M13]TTATGAACGGGTTGCTGGCT	95°C	60°C	72°C	72°C
	R	CCCAAAGGGGAAAATCGAGA	30s	30s	2m	30m
	M13(fam)	FAM – TGTAAAACGACGGCCAGT				

F, forward primer; R, reverse primer; [M13], M13 tail; NED & FAM, fluorescent labels;
Thermal cycling: *D*, denaturation; *A*, annealing; *E*, extension; *FE*, final extension; s, seconds; m, minutes

genetic analyser (Applied Biosystems) with 10 µl formamide and 0.25 µl GeneScan™ 600 LIZ® internal size standard (Applied Biosystems) included to size fragments following the manufacturer's instructions.

The genotype profiles were analysed by ABI PRISM® GeneMapper® Software (Applied Biosystems). Both loci amplified alleles which exhibited 1 bp differences from the expected repeat motif, suggesting mutation other than changes in repeat number causing length variation. However, for the purpose of analysis such alleles were grouped with the closest allele size fitting the repeat motif.

6.2.3 Cytoplasmic DNA markers

6.2.3.1 Chloroplast PCR-RFLP

Universal primers designed for the large single copy (LSC) region of the Eudicot chloroplast genome were adopted from Grivet *et al* (2001) (Table 6.3). The *trn*T/*trn*F (TF) and *psb*C/*trn*S (CS) regions were screened for nucleotide polymorphism in sixteen individuals of *Anthoxanthum odoratum* from across four populations. PCR amplification reactions were performed in a 25 µl volume with 1 - 5 ng genomic DNA template, 2 mM $MgCl_2$, 0.2 mM each dNTP, 5 pmol each primer, 0.625 U Thermo-Start® DNA polymerase and 1x Thermo-Start® Reaction buffer (ABgene). Thermal

Table 6.3 – Chloroplast primers for PCR-RFLP and cpSSR loci

Locus	Primer	Sequence (5' – 3')
PCR-RFLP		
psbC/*trnS* (CS)	*F	GGTCGTGACCAAGAAACCAC
	*R	GGTTCGAATCCCTCTCTCTC
trnT/*trnF* (TF)	*F	CATTACAAATGCGATGCTCT
	*R	ATTTGAACTGGTGACACGAG
trnT/*trnL* (TL)	*F	CATTACAAATGCGATGCTCT
	R	ACGTAGtGTAGTCTAGCAATTC
cpSSR		
rpoC2/*rps2*	†F	[M13]TTATTTATTTCAAGCTATTTCGG
	†R	AATATCTTCTTGTCATTTTTTCC
	§M13(fam)	FAM – TGTAAAACGACGGCCAGT
atpB/*rbcL*	†F	[M13]GATTGGTTCTCATAATTATCAC
	†R	TATTGAATTAACTAATTCATTTCC
	M13(ned)	NED – TAGAAGGCACAGTCGAGG

F, forward primer; R, reverse primer; [M13], M13 tail; NED & FAM, fluorescent labels; Primer source: *, Grivet *et al* (2001); †, Provan *et al* (2004); §, Schuelke (2000)

cycling conditions consisted of an initial enzyme activation step of 95 °C for 15 minutes, 30 cycles of 45 seconds denaturation at 94 °C, 45 seconds annealing at 50 °C and 2 minutes extension at 72 °C, and a final extension at 72 °C for 10 minutes. PCR products were visualized on a 2 % agarose gel alongside a 100 bp DNA ladder (Promega). PCR products were purified by the addition of 20 U Exonuclease I and1 U shrimp alkaline phosphotase to 10 µl PCR product, incubated at 37 °C for 40 minutes and 80 °C for 15 minutes. Purified PCR products were sequenced using dye terminator chemistry by Macrogen Inc. (Korea) on an ABI 3730xl DNA analyser (Applied Biosystems).

A. odoratum sequences were aligned by eye, polymorphic sites determined and sequence authenticity confirmed by performing a BLASTn search on the GenBank website (http://www.ncbi.nlm.nih.gov/). The CS region exhibited no polymorphism and was not considered further. TF region sequences were aligned with the Poaceae species *Phalaris arundinacea* (GenBank accession number: AY589138) and *Zea Mays* (GenBank accession number: NC001666) to determine intergenic spacer and exon positions. Nucleotide polymorphisms were identified within restriction enzyme recognition sites for *Bst*BI and *Hpy*CH4IV in the *trn*T/*trn*L (TL) intergenic spacer in *A. odoratum* and a *trn*L reverse primer designed in AmplifX version 1.3.7.

TL intergenic spacer PCR amplification reaction and thermal cycling conditions were performed as for the TF and CS regions described above. Cytotypes were determined by PCR-RFLP in 20 individuals per population. Restriction digests were performed separately in 10 µl reactions. 5 µl PCR product was digested with 1 U *Bst*BI in 1x NEBuffer 4 reaction buffer (New England Biolabs (UK) Ltd) incubated at 65 °C for 3 hours. 5 µl PCR product was digested with 1 U *Hpy*CH4IV in 1x NEBuffer 1 reaction buffer (New England Biolabs (UK) Ltd) incubated at 37 °C for 3 hours and heat inactivated at 65 °C for 20 minutes. PCR-RFLP fragments were visualised on a 2 % agarose gel alongside a 100 bp DNA ladder (Promega) and polymorphisms detected by band presence and size.

6.2.3.2 Chloroplast microsatellites

Two universal chloroplast microsatellite (cpSSR) loci primer pairs, *rpo*C2/*rps*2 and *atp*B/*rbc*L (Table 6.3), designed for use in Poaceae species and previously shown to exhibit polymorphism in *A. odoratum* (Provan *et al* 2004) were employed in this study. PCR products were fluorescently labelled using the tailed primer method (Schuelke 2000). PCR amplification reactions were performed in a 10 µl volume with 5 ηg genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1.25 ρmol tailed forward (F) primer, 5 ρmol reverse (R) primer, 5 ρmol M13 fluorescent labelled primer, 0.25 U Go Taq® DNA Polymerase and 1x Go Taq® reaction buffer (Promega). Thermal cycling conditions consisted of an initial denaturation at 95 °C for 2 minutes, 25 cycles of denaturation at 95 °C, annealing at 56 °C and extension at 72 °C with 1 minute duration at each step, 10 cycles of M13 fluorescent label incorporation with 95 °C for 30 seconds, 52 °C for 45 seconds and 72 °C for 45 seconds, and a final extension step at 72°C for 10 minutes. PCR products were visualized on a 2 % agarose gel alongside a 100 bp DNA ladder (Promega). CpSSR length variants were resolved on an ABI 3130xl genetic analyser (Applied Biosystems) with the GeneScan™ 600 LIZ® internal size standard (Applied Biosystems) included to size fragments following the manufacturer's instructions.

6.2.4 Data analysis

6.2.4.1 AFLP analysis

AFLP data was analysed using both band-based and allele frequency-based approaches under the assumptions that AFLP markers behave as dominant markers, comigrating fragments are homologous loci and the additional assumption of populations in Hardy-Weinberg Equilibrium (HWE) for allele frequency-based analysis (Després *et al* 2002, Bonin *et al* 2007). Methods for the estimation of allele frequencies from dominant biallelic markers usually rely on the assumption of HWE under a diploid model (Bonin *et al* 2007). Although allele frequency-based methods have been applied to dominant biallelic marker data in polyploid species (Perez-Collazos and Catalan 2006, Silvertown *et al* 2005 and Pimentel *et al* 2007), any inferences regarding population genetic diversity and differentiation using such methods should be viewed with caution due to the violation of the diploid assumption. However, for the purpose of comparison with previous studies of *A. odoratum* using ISSR (Silvertown *et al* 2005) and AFLP (Pimentel *et al* 2007) markers, allele frequency-based as well as band-based analyses were included in this study. Only polymorphic markers were included in the analyses.

i) Band-based analysis

Diversity parameters estimated included the percentage of the total polymorphic loci present in a population (%P), the total number of fragments per population (T_{pop}), mean (A_{pop}) and minimum-maximum (N-X) number of fragments per individual per population, and the percentage of polymorphic loci within the population (%P_{pop}). The number of rare fragments (f_r) and private fragments (f_{pr}) per population were also calculated. Rare fragments were defined as those which occurred with a frequency of less than 10 % in the dataset and private fragments were confined to a single population (Tribsch *et al* 2002, Martinez-Otega *et al* 2004). Nei's (1987) gene diversity (h) was calculated from marker frequencies in AFLPdat (Ehrich 2006) with 95 % confidence intervals estimated from bootstrapping 1000 times over markers. To determine if marker frequencies differed significantly among populations Fisher's exact tests

(Raymond and Rousset 1995) were performed on marker frequencies at each locus between all pairs of populations in TFPGA version 1.3 (Miller 1997).

Genetic distances between individual pairs were calculated using the Nei and Li (1979) similarity coefficient and relationships among individual samples recovered through Neighbour Joining (NJ) clustering in PHYLIP version 3.67 (Felsenstein 2007) with bootstrap support estimated from 1000 bootstrap replicates. Unrooted NJ phylograms were drawn in Treeview version 1.6.6. Principal coordinates analysis (PCoA) was performed on Nei and Li genetic distances using the computer program PCO (Anderson 2003). Partitioning of total genetic diversity among and within populations was further evaluated by analysis of molecular variance (AMOVA, Excoffier *et al* 1992) on Euclidian pairwise distances in Arlequin version 3.1 (Excoffier *et al* 2006) with data treated as haplotypic and 1000 permutations to obtain significance levels of the variance. Among population isolation by distance was tested by correlation between average population pairwise Nei and Li genetic distances and geographic distance (km) using the Mantel test implemented in Arlequin version 3.1 (Excoffier *et al* 2006) with significance levels assessed through 1000 permutations.

ii) Allele frequency-based analysis

Genetic differentiation (θ^B) among populations and population pairs was tested using the Bayesian package Hickory version 1.1 (Holsinger and Lewis 2007, Holsinger *et al* 2002). All options were set to the default values with a burn-in of 5000 iterations, 100 000 iterations in each Markov chain Monte Carlo (MCMC) sample chain and values retained at every 20th iteration. Posterior distributions of θ^B were compared to determine if there were significant differences in genetic differentiation between all pairwise population combinations. Population structure was inferred using the Bayesian population assignment package STRUCTURE version 2.2 (Falush *et al* 2007). Individuals were clustered into 1 to 4 populations (K) using the admixture model with a burn-in of 10 000 iterations, simulation of 50 000 MCMC iterations and 10 replicates. Isolation by distance was tested by correlation between pairwise population θ^B estimated in Hickory and geographic distance (km) using the Mantel test implemented in Arlequin version 3.1 (Excoffier *et al* 2006) with significance levels assessed through 1000 permutations.

6.2.4.2 Nuclear microsatellites

The tetraploid genotypes obtained for the two microsatellite loci were maintained as homozygote, full heterozygote or partial heterozygote genotypes as the genotype profiles did not allow the determination of allele copy number and the inheritance pattern was unknown. The total number of alleles and allele phenotypes, as the number of different multiallelic profiles observed, and the percentage occurrence of each tetraploid genotype were calculated per locus. Genetic diversity and differentiation was analysed using the computer program TETRASAT under the assumption of allotetraploidy (Markwith *et al* 2006) and by the analysis of individual pairwise genetic distances.

Population genetic diversity and differentiation were estimated using the program TETRASAT (Markwith *et al* 2006) which analyses tetraploid microsatellite genotypes by calculating every combination of allele frequencies using iterative substitution of all possible allele configurations based on the alleles found for each partial heterozygote. Diversity parameters estimated for each population included the mean number of alleles per locus (A_P), multilocus observed heterozygosity (H_O) defined as the proportion of heterozygotes, including partial and full heterozygotes, averaged across loci, multilocus expected heterozygosity (H_E) and multilocus Shannon–Wiener diversity index (H'). Pairwise population genetic differentiation was estimated as Nei's G_{ST} (1986). The mean multilocus H_E and H' were calculated from a 10 000 value subset of all possible multilocus values for each population. G_{ST} values were calculated for each randomly selected configuration from a maximum of 10 000 allele configurations per locus. Mean multilocus population values were calculated from a 10 000 value subset of all possible multilocus values.

However, the number of partial heterozygotes per population for both loci in this dataset exceeded the computational capability of the program requiring the analysis of subpopulations to determine population level parameters (Markwith *et al* 2006, Markwith and Scanlon 2007). As the sampling strategy did not provide a clear method for defining subpopulations 10 replicate datasets were analysed. Each replicate consisted of 10 randomly assigned individuals per population and each individual was represented at least twice across the replicates. Genetic diversity and differentiation

parameters were averaged across the 10 replicate analyses. Among population isolation by distance was tested by correlation between pairwise population average G_{ST} and geographic distance (km) using the Mantel test implemented in Arlequin version 3.1 (Excoffier *et al* 2006) with significance levels assessed through 1000 permutations.

Genetic distances between individual pairs were calculated using the Bruvo *et al* (2004) approach. This method was designed for the calculation of relative distances between microsatellite genotypes in polyploids taking into account stepwise mutation processes and allowing the generation of genetic distances involving partial heterozygotes. The genetic distances (Bruvo *et al* 2004) were used to recover relationships among individual samples through principal coordinates analysis (PCoA) performed using the computer program PCO (Anderson 2003) and Neighbour Joining (NJ) clustering in PHYLIP version 3.67 (Felsenstein 2007). An unrooted NJ phylogram was drawn in Treeview version 1.6.6. Among population isolation by distance was tested by correlation between average population pairwise Bruvo *et al* (2004) genetic distances and geographic distance (km) using the Mantel test implemented in Arlequin version 3.1 (Excoffier *et al* 2006) with significance levels assessed through 1000 permutations.

6.2.4.3 Cytoplasmic DNA markers

Cytotypes were defined by combining cp PCR-RFLP and cp SSR data. Genetic diversity and differentiation was estimated using the computer program PERMUT (http://www.pierroton.inra.fr/genetics/labo/Software) as described in Pons and Petit (1995, 1996). The parameters estimated included the mean within population genetic diversity (h_S), total gene diversity (h_T) and among population genetic differentiation (G_{ST}) for unordered alleles and the equivalent parameters (v_S, v_T, N_{ST}) for ordered alleles taking into account the similarities between cytotypes. $N_{ST} > G_{ST}$ was tested to indicate the presence of phylogeographic structure by comparison of observed N_{ST} (N_{STobs}) with permutated N_{ST} (N_{STper}) obtained after 1000 random permutations of cytotype identities in PERMUT following Burban *et al* (1999). The relationship among cytotypes was represented by a statistical parsimony network generated using the software TCS version 1.21 (Clement *et al* 2000). Pairwise population genetic differentiation (F_{ST}) was estimated in TFPGA version 1.3 (Miller 1997). Among population isolation by distance was tested by correlation between pairwise population

θ and geographic distance (km) using the Mantel test implemented in Arlequin version 3.1 (Excoffier *et al* 2006) with significance levels assessed through 1000 permutations.

6.2.4.4 Pollen-to-seed migration ratio

The relative contribution of pollen and seed dispersal to the observed levels of gene flow among populations was assessed by the pollen-to-seed migration ratio (r) calculated following Petit *et al* (2005) and Ennos (1994) as:

$$r = m_p/m_s = [(1/F_{STnr} - 1)(1+F_{IS}) - 2(1/F_{STc} - 1)] / (1/F_{STc} - 1)$$

where F_{STnr} is the overall nuclear AFLP AMOVA F_{ST} estimate and F_{STc} is overall chloroplast F_{ST} estimate and F_{IS} was set to zero.

6.3 Results

6.3.1 AFLP fingerprinting

The four primer combinations used generated 448 unambiguously scorable polymorphic fragments between 80 and 400 base pairs (bp). Marker frequencies were low with fragments present in an average 20.5 % of samples and a small proportion of loci (21.1 %) present per sample. The majority of fragments were classified as rare with 52.7 % occurring in less than 10 % of the samples. Private fragments, those confined to a single population, constituted 19.0 % of the loci and were also classified as rare, occurring in less than eight individuals within the population. The percentage of total polymorphic loci (%P) observed in a single population ranged from 69.6 % to 74.1 % (Table 6.4). High levels of within population variation were observed with over 96 % of loci within each population exhibiting polymorphism (%P_{pop}) and less than 35 % of loci present in a single individual. Nei gene diversity did not differ between populations as all values

Table 6.4 – AFLP genetic diversity parameters in four *A. odoratum* populations.

Population	%*P*	T_{pop}	A_{pop}	*N-X*	$\%P_{pop}$	f_r	f_{pr}	*h*
Aber	74.1	343	99.7	87-112	96.8	135	23	0.201 (0.186,0.218)
Birds Rock	69.9	321	92.9	79-106	97.5	114	18	0.191 (0.175,0.209)
Cwm Idwal	72.1	333	92.7	77-109	97.0	129	19	0.190 (0.175,0.208)
Maltraeth	69.6	323	92.8	78-110	96.6	115	25	0.193 (0.175,0.211)

%*P*, percentage total polymorphic loci; T_{pop}, total number fragments per population; A_{pop}, mean number fragments per individual per population; *N-X*, minimum-maximum number fragments per individual per population; $\%P_{pop}$, percentage loci polymorphic within a population; f_r, number rare fragments; f_{pr}, number private fragments; *h*, Nei gene diversity with 95% confidence intervals indicated between parentheses

fell within the 95 % confidence intervals. All diversity parameters indicated similar levels of polymorphism and diversity within the four populations (Table 6.4). Pairwise Fisher's exact tests found no significant difference in marker frequencies among populations.

Individuals exhibited low levels of genetic distance for AFLP markers with pairwise Nei and Li (1979) genetic distances in the range 0.010 to 0.027. Neighbour Joining (NJ) representation of genetic distances by unrooted phylograms revealed no clustering of individuals by population (Figure 6.1). Bootstrap support was low with only two nodes supported by bootstrap values greater than 70 % under the extended majority rule consensus method (Figure 6.1 b). Both nodes represent clusters of two individuals while only one groups individuals from the same population. However no inference can be made regarding these samples as when clustering may occur even at low levels of genetic differentiation when large numbers of loci are analysed by Neighbour Joining (Hollingsworth and Ennos 2004). Additionally, this node support was not observed when a NJ phylogram was produced under the strict consensus method (data not shown).

Table 6.5 – Analysis of molecular variance (AMOVA) of Euclidian pairwise distances based on 448 AFLP loci.

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation	*P*-value
Among populations	3	182.888	0.87783	1.98 %	<0.01
Within populations	76	3298.850	43.40592	98.02 %	

d.f, degrees of freedom

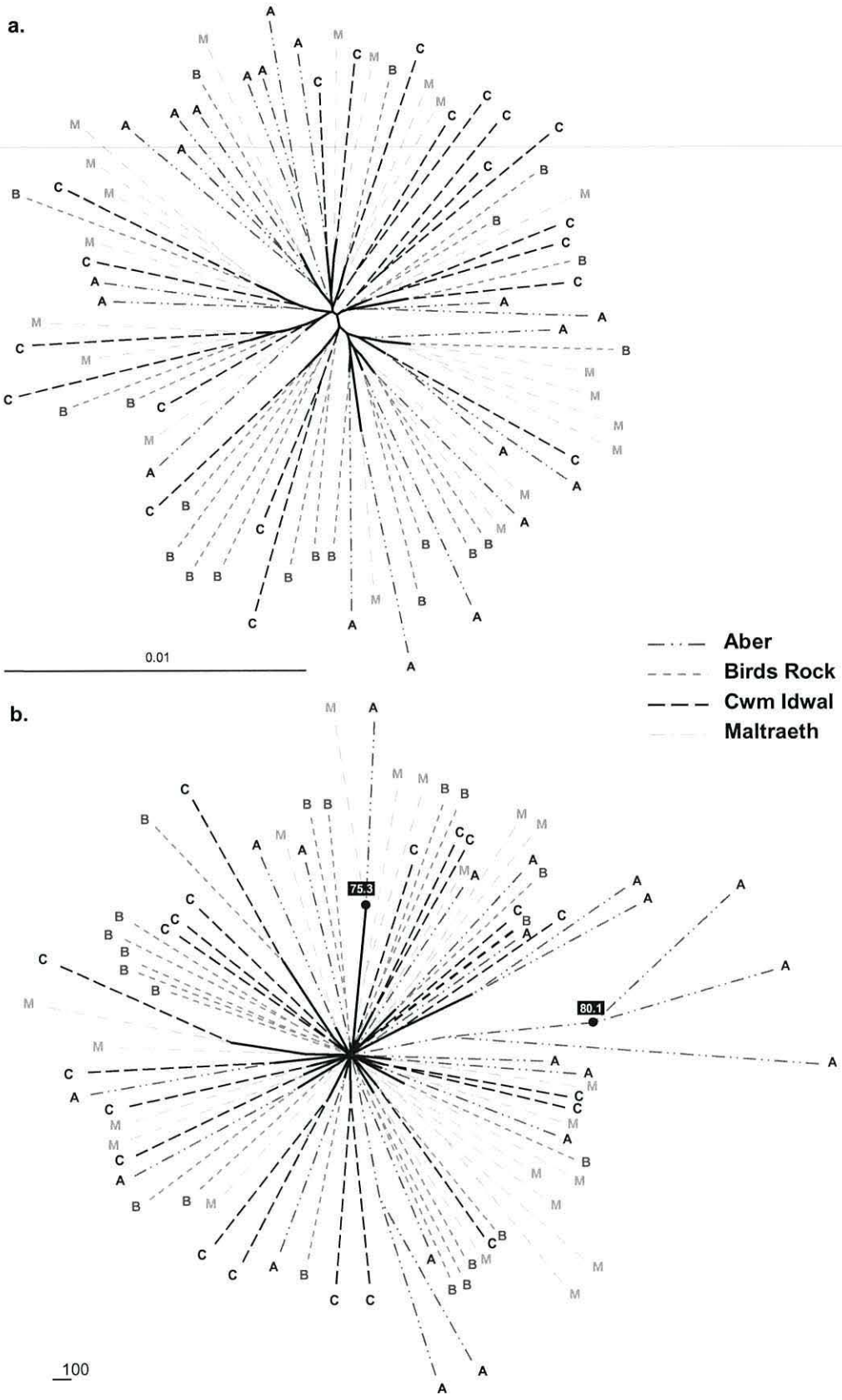

Figure 6.1 – Neighbour Joining analysis of among individual pairwise Nei and Li genetic distances based on 448 AFLP markers as a) Unrooted phylogram where scale represents genetic distance of 0.01 and b) unrooted extended majority rule consensus phylogram with bootstrap values >70% shown. Letters represent population of origin.

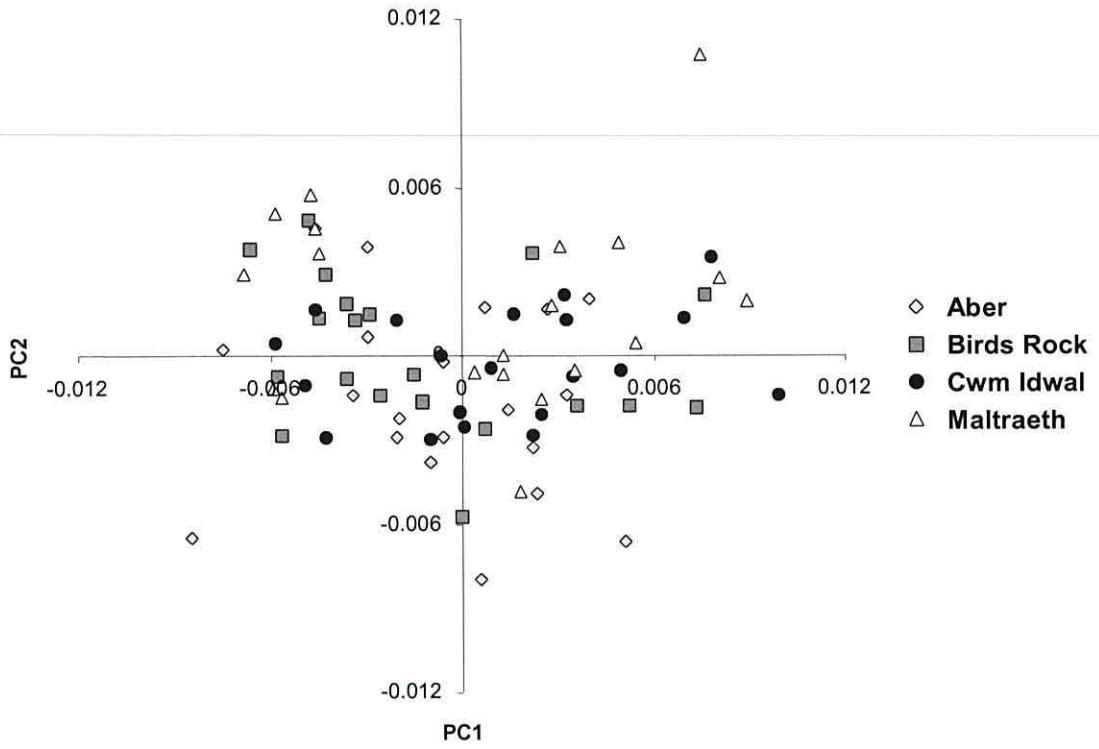

Figure 6.2 – Principal coordinates analysis (PCoA) of among individual pairwise Nei and Li genetic distances based on 448 AFLP markers. The first two axes PC1 (horizontal) and PC2 (vertical) extracting 20.6 % of the total variation are plotted.

The results of the Principle Coordinates Analysis of pairwise Nei and Li (1979) genetic distances were concordant with those obtained through the Neighbour Joining analysis. The first three axes extracted 27.0 % of the total variation with 13.7 %, 6.9 % and 6.4 % for the first, second and third axes respectively. The variation explained within the first two axes (20.6 %) is not partitioned among populations with individuals from all localities widely distributed within the plot (Figure 6.2). The lack of clustering observed in both the NJ analysis and PCoA were further supported by the Analysis of molecular variance (AMOVA) results (Table 6.5). The majority of the genetic variability was found within populations (98.02 %) while among population variation only accounted for 1.98 % of the total variation ($P < 0.01$). All of the band-based analyses illustrate the high level of variability among individuals and the lack of population differentiation resolved by the AFLP markers.

Allele frequency-based analyses were also employed in the estimation of genetic differentiation and population structure. Both analysis packages employed, Hickory version 1.1 (Holsinger and Lewis 2007) and STRUCTURE version 2.2 (Falush *et al* 2007), perform Bayesian analysis using (MCMC) algorithms and required the

Table 6.6 – Pairwise population estimates of θ^B genetic differentiation (bold) and average Nei and Li genetic distance estimated from AFLP markers (lower left diagonal) and geographic distance (km) (upper right diagonal). Standard deviations are given in brackets.

Population	Aber	Birds Rock	Cwm Idwal	Maltraeth
Aber		64.123	11.434	25.812
Birds Rock	**0.0096 (0.0049)** 0.0167 (0.0023)		52.903	64.640
Cwm Idwal	**0.0101 (0.0056)** 0.0168 (0.0023)	**0.0037*[a] (0.0027)** 0.0166 (0.0024)		24.292
Maltraeth	**0.0112 (0.0044)** 0.0167 (0.0024)	**0.0162[a] (0.0051)** 0.0171 (0.0021)	**0.0030*[a] (0.0021)** 0.0168 (0.0022)	

*, lower bound of the 95 % credible interval close to zero; [a], significant differentiation determined by comparison of θ^B posterior distributions.

assumption of Hardy-Weinberg Equilibrium and diploidy for the estimation of allele frequencies from the dominant AFLP marker data.

Overall genetic differentiation was low with $\theta^B = 0.0154$ (SD 0.0027). However, θ^B was significantly greater than zero as the lower bound (2.5%) of the credible interval, the Bayesian equivalent to the 95 % confidence interval, was not negative (0.0103). Pairwise population estimates of θ^B (Table 6.6) indicate that the Birds Rock and Maltraeth populations show the greatest level of differentiation ($\theta^B = 0.016$). The lowest levels of differentiation occur between Cwm Idwal and both Birds Rock ($\theta^B = 0.0037$) and Maltraeth ($\theta^B = 0.0030$) with the lower bound of the credible interval close to zero (0.0001 and 0.0002 respectively). Differences between the pairwise θ^B estimates were tested by comparison of the posterior distributions. The Birds Rock and Maltraeth were significantly more differentiated than Cwm Idwal from either of these populations as the minimum credible intervals for these comparisons were not negative. In all other comparisons the θ^B estimates were equivalent indicating similar levels of differentiation.

The results of the population assignment analysis in STRUCTURE found that all samples exhibited mixed ancestry for each of the K inferred clusters (Figure 6.3). The greatest support was observed for partitioning samples into two clusters with the estimated Ln probability value closest to zero ($K = 2$, Ln Pr($X|K$) = -15331.9, Figure 6.3 a). However, individuals showing a greater affinity to one of the $K = 2$ inferred clusters

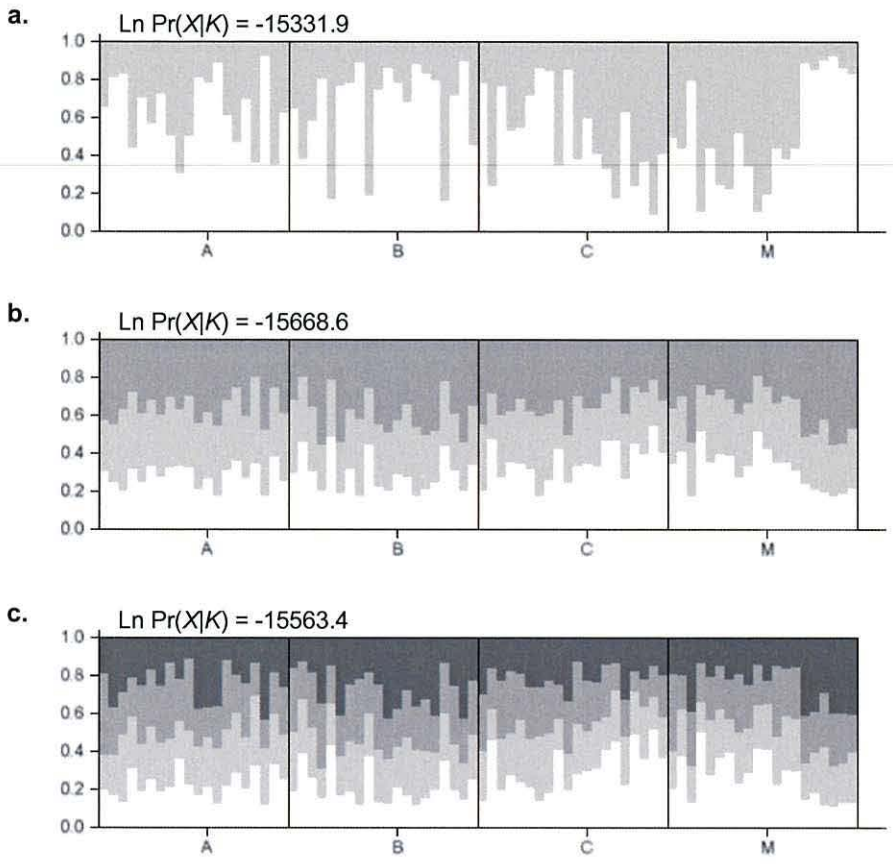

Figure 6.3 – Summary plots of estimated cluster (*K*) membership inferred from 448 AFLP markers in STRUCTURE v 2.2 for a) $K = 2$, b) $K = 3$ and c) $K = 4$ population clusters. Bars represent individual samples and shading indicates different clusters. Samples are grouped by population of origin where A is Aber, B is Birds Rock, C is Cwm Idwal and M is Maltraeth. The estimated Ln probability of the data (Ln Pr (*X*|*K*)) is given above each plot.

did not group by population of origin. These results further corroborate the high levels of within population variation and lack of population differentiation observed in this dataset.

Among population isolation by distance was tested using the Mantel test for both band-based average Nei and Li (1979) genetic distances and allele frequency-based θ^B estimates. No significant correlation between genetic differentiation and geographic distance was observed for either Nei and Li genetic distance ($r = 0.197$, $P = 0.338$) or θ^B estimates ($r = 0.274$, $P = 0.376$). The significant pairwise population differences observed for θ^B estimates (Table 6.6) had no relation to the geographic distance between the populations.

6.3.2 Nuclear microsatellites

The two nuclear microsatellites genotyped in *A. odoratum* exhibited relatively high levels of polymorphism (Table 6.7, Selkoe and Toonen 2006). The tetranucleotide AoM01B1 locus exhibited a total of six alleles and 11 allele phenotypes. Heterozygotes constituted 60 % of the assayed individuals however none of the samples exhibited more than two alleles at this locus. The dinucleotide locus AoM06F1 exhibited much higher levels of variation with a total of 37 alleles, unique allele phenotypes observed in 87.5 % of the samples and heterozygotes constituting 92.5 % of the assayed samples. Seventy five percent and 16.25 % of individuals were partial heterozygotes with two and three alleles respectively, while only one individual exhibited a full heterozygote tetraploid genotype (1.25 %, Table 6.7).

Population level genetic diversity statistics were estimated over ten replicate analyses in TETRASAT (Table 6.8). The majority of population variation in the mean number of alleles per locus (A_P) was explained by locus AoM06F1 due to the higher levels of variation at this locus. The Aber and Maltraeth populations exhibited 16 and 21 alleles at this locus respectively, which correspond to the lowest (A_P = 7.15) and highest (A_P = 9.75) mean number of alleles per locus. The multilocus observed heterozygosity (H_O) ranged from 0.710 to 0.825 and the variation among population was explained by locus AoM01B1. H_O was lowest in Birds Rock and Maltraeth which had double the number of homozygous individuals at this locus compared to Aber and Cwm Idwal. Multilocus expected heterozygosities (H_E) did not differ greatly between populations, ranging from 0.735 to 0.757, and did not correspond to the variation in population H_O. However, no inferences of inbreeding could be made based on heterozygote deficiency estimated in

Table 6.7 – Nuclear microsatellite locus polymorphism in *A. odoratum* (n = 80).

Locus	Repeat motif	Size range (bp)	A_N	A_{Phen}	Tetraploid genotype (%)			
					HM	$PH_{(2)}$	$PH_{(3)}$	*FH*
AoM01B1	$(CTGA)_5$	170-198	6	11	40	60	0	0
AoM06F1	$(CT)_8CG(CT)_4(GT)_4$	198-278	37	70	7.5	75	16.25	1.25

A_N, total number of alleles; A_{Phen}, total number of allele phenotypes; *HM*, homozygote; $PH_{(2)}$, partial heterozygote with 2 alleles, $PH_{(3)}$, partial heterozygote with 3 alleles; *FH*, full heterozygote with 4 alleles

Table 6.8 - Genetic diversity statistics averaged over ten replicate TETRASAT analyses for *Anthoxanthum odoratum* populations based on two nuclear microsatellite markers. Standard deviations of the mean are given in brackets.

Population	A_P	Multilocus H_O	Multilocus H_E	Multilocus H'
Aber	7.15 (0.67)	0.820 (0.054)	0.735 (0.033)	2.301 (0.167)
Birds Rock	8.85 (1.03)	0.710 (0.078)	0.757 (0.019)	2.545 (0.149)
Cwm Idwal	8.50 (0.88)	0.825 (0.049)	0.755 (0.030)	2.491 (0.178)
Maltraeth	9.75 (0.68)	0.715 (0.047)	0.740 (0.042)	2.610 (0.119)

A_P, mean number of alleles per locus; H_O, multilocus observed heterozygosity; H_E, multilocus expected heterozygosity; H', multilocus Shannon Weiner diversity index

TETRASAT due to the tetraploid nature of these loci (Markwith *et al* 2006). The multilocus Shannon-Weiner diversity index (H') corresponded to the variation observed in A_P with the lowest levels of diversity observed in Aber (H' = 2.301) and the highest in Maltraeth (H' = 2.610). Pairwise estimates of among population genetic differentiation (G_{ST}) were relatively low (Table 6.9). Birds Rock and Maltraeth exhibited the lowest level of differentiation (G_{ST} = 0.0267) while Cwm Idwal and Birds Rock were the most differentiated (G_{ST} = 0.0592). The majority of populations were differentiated to a similar extent when the standard deviations of average G_{ST} were considered, however, the Birds Rock and Maltraeth populations were less differentiated than all other populations.

Pairwise individual Bruvo *et al* (2004) genetic distances averaged 0.662 (SD 0.143) and ranged from 0.083 to 0.957, where a distance of zero signified complete similarity with identical profiles across all loci. Neighbour Joining (NJ) analysis represented by an unrooted phylogram (Figure 6.4) and the Principle Coordinates Analysis (Figure 6.5) of

Table 6.9 – Average pairwise population estimates of Nei's (1986) G_{ST} genetic differentiation (bold) and average Bruvo *et al* (2004) genetic distance estimated from two nuclear microsatellite markers (lower left diagonal) and geographic distance (km) (upper right diagonal). Standard deviations are given in brackets.

Population	Aber	Birds Rock	Cwm Idwal	Maltraeth
Aber		64.123	11.434	25.812
Birds Rock	**0.0548 (0.0135)** 0.6730 (0.1428)		52.903	64.640
Cwm Idwal	**0.0418 (0.0099)** 0.6636 (0.1297)	**0.0592 (0.0120)** 0.6863 (0.1295)		24.292
Maltraeth	**0.0530 (0.0204)** 0.6607 (0.1473)	**0.0267 (0.0079)** 0.6519 (0.1435)	**0.0561 (0.0170)** 0.6688 (0.1497)	

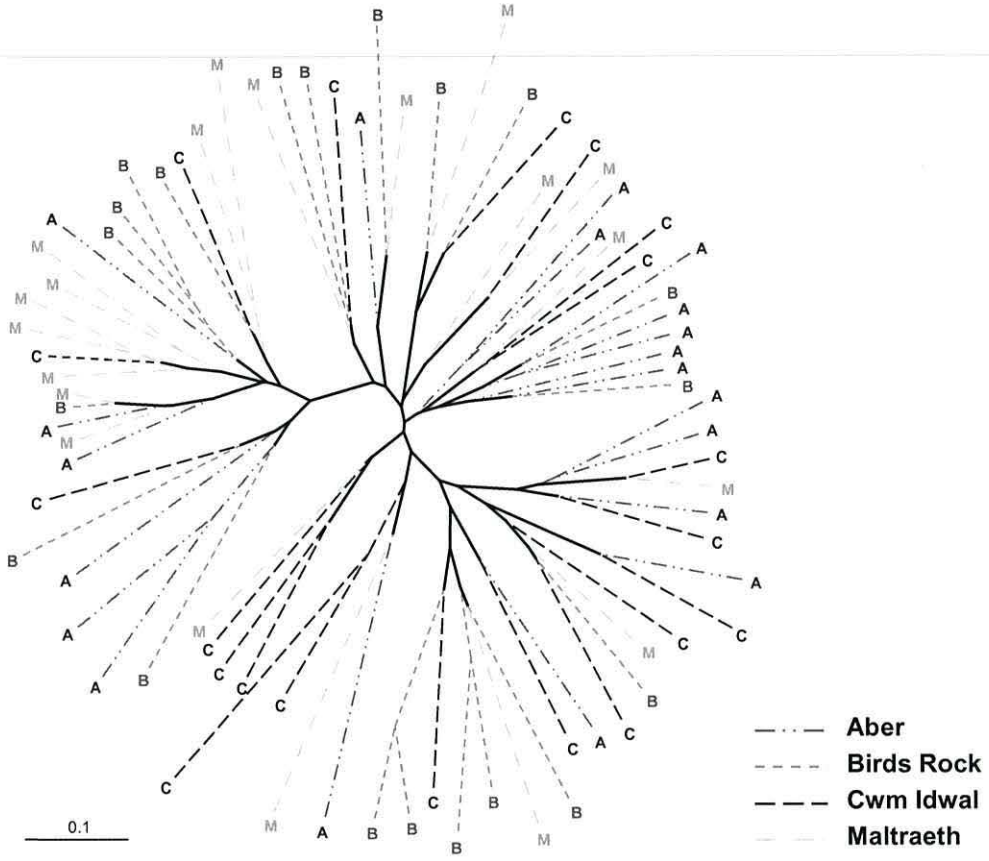

Figure 6.4 – Neighbour Joining analysis of among individual pairwise Bruvo *et al* (2004) genetic distances based on two nuclear microsatellite markers. Letters represent population of origin.

Bruvo genetic distances revealed no clustering of individuals by population. Node support within the NJ phylogram was not estimated as it was not possible to produce bootstrap replicates of the genotype data from which the distance matrix was calculated. The PCoA extracted 53.6 % of the total variation on the first three axes with 29.0 %, 13.7 % and 10.9 % for the first, second and third axes respectively. Despite the relatively high levels of variation explained by the first two axes (42.7 %) there was no partitioning among populations with individuals from all localities widely distributed within the plot (Figure 6.5). Both the NJ analysis and PCoA illustrate a lack of population differentiation using the Bruvo *et al* (2004) method of calculating relative genetic distances between microsatellite genotypes.

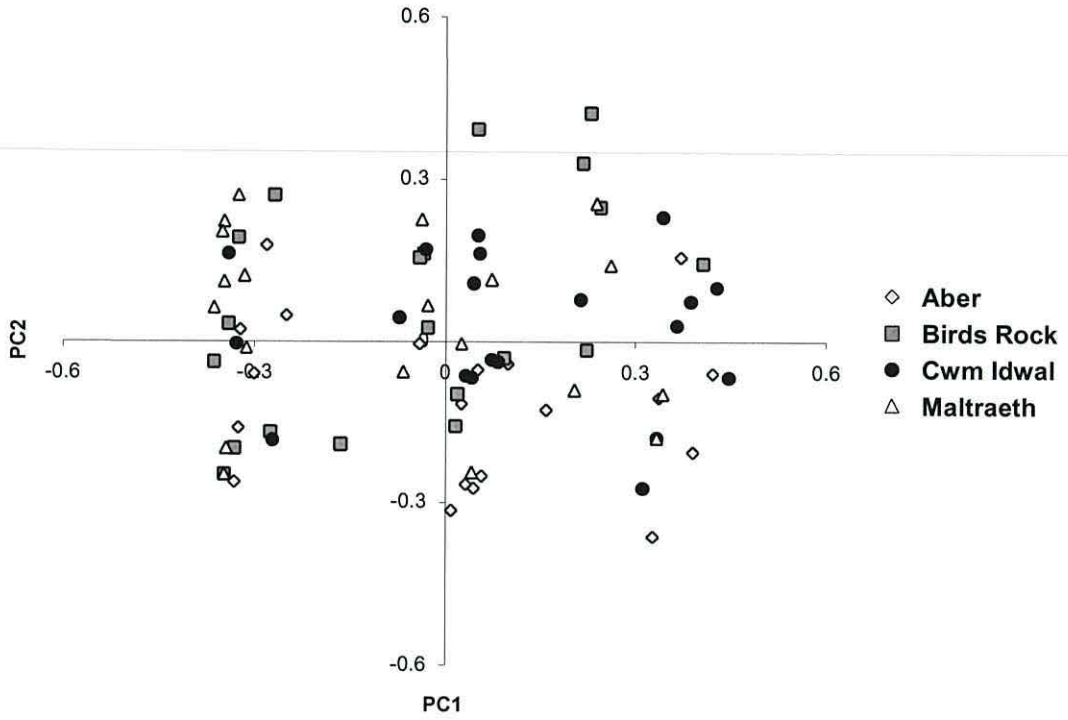

Figure 6.5 – Principal coordinates analysis (PCoA) of among individual pairwise Bruvo *et al* (2004) genetic distances based on two nuclear microsatellite markers. The first two axes PC1 (horizontal) and PC2 (vertical) extracting 42.7 % of the total variation are plotted.

Among population isolation by distance was tested using the Mantel test for both G_{ST} estimates and average Bruvo *et al* (2004) genetic distances (Table 6.9). No significant correlation between genetic differentiation and geographic distance was observed for either G_{ST} (r = -0.171, P = 0.698) or Bruvo genetic distance (r = 0.133, P = 0.469).

6.3.2 Cytoplasmic DNA markers

Three variants of the chloroplast *trn*T/*trn*L (TL) region segregating at two polymorphic nucleotide sites were detected by cp PCR-RFLP with the *Bst*BI and *Hpy*CH4IV restriction endonucleases. The chloroplast microsatellite loci, *rpo*C2/*rps*2 and *atp*B/*rbc*L, amplified two and three alleles respectively. The combined cp PCR-RFLP and cp SSR data distinguished five cytotypes within the four *A. odoratum* populations (Table 6.10).The two main cytotypes, Cyt 3 (n = 41) and Cyt 4 (n = 28), were present in all four populations and represented 86.3 % of *A. odoratum* individuals (Figure 6.6). Cyt 1 (n = 9) was restricted to the Aber and Maltraeth populations. Two rare cytotypes

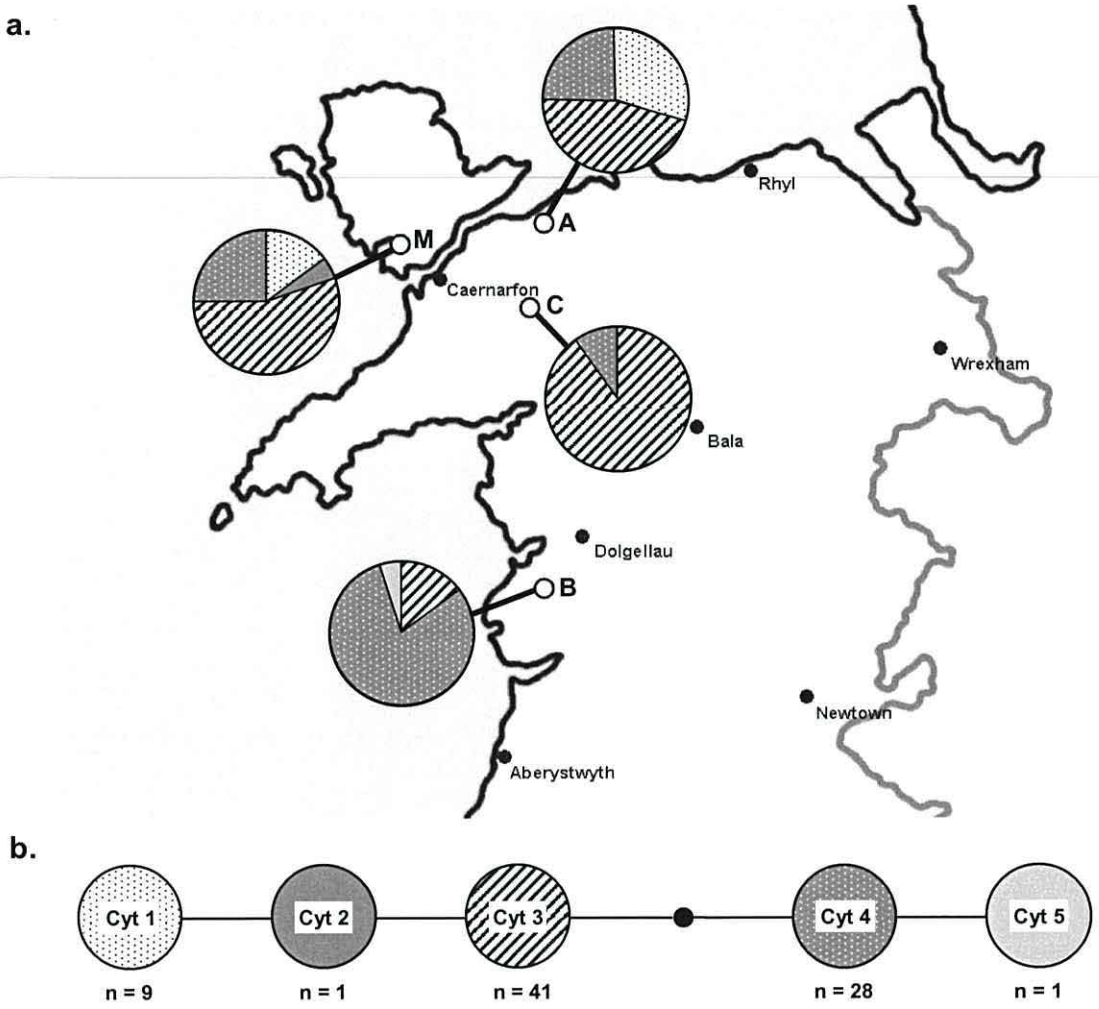

Figure 6.6 – a) Distribution and frequency of cytotypes in North Wales *Anthoxanthum odoratum* populations: Aber (A), Birds Rock (B), Cwm Idwal (C) and Maltraeth (M). b) Statistical parsimony network of among cytotype relationships with number of individuals exhibiting each cytotype indicated below. Small black circle represents an intermediate cytotype which was no detected.

Table 6.10 – Description of chloroplast PCR-RFLP and cp SSR polymorphic fragments and cytotypes identified in *Anthoxanthum odoratum* populations.

	cp PCR-RFLP		cp SSR	
Cytotype	**TL *Bst*BI** band 2	**TL *Hpy*CH4IV** band 2	***rpo*C2/*rps*2** (bp)	***atp*B/*rbc*L** (bp)
cyt 1	2	2	144	156
cyt 2	2	1	144	156
cyt 3	2	1	144	157
cyt 4	1	1	145	157
cyt 5	1	1	145	158

band, number of bands after restriction digest; bp, base pairs

Table 6.11 –Cytoplasmic genetic diversity and differentiation estimated in PERMUT for ordered and unordered alleles. Standard errors are given in brackets.

Diversity parameter		Cytoplasmic genetic diversity
Unordered alleles	h_s	0.466 (0.118)
	h_T	0.655 (0.067)
	G_{ST}	0.289 (0.207)
Ordered alleles	v_S	0.450 (0.135)
	v_T	0.660 (0.104)
	N_{ST}	0.317 (0.214)
Percentage of $N_{STper} > N_{STobs}$		20.8 %

h_S & vs, intrapopulation diversity; h_T & v_T, total gene diversity; G_{ST} & N_{ST}, among population genetc differentiation; N_{STper}, permutated N_{ST}; N_{STobs}, observed N_{ST}.

were detected in the Birds Rock and Maltraeth populations, Cyt 5 and Cyt 2 respectively, which occurred in only one individual from each population. The Cwm Idwal population exhibited the least cytoplasmic variation with only the two main cytotypes detected and dominated by Cyt 3 which occurred in 90 % of individuals (Figure 6.6 a).

Cytoplasmic diversity measured for unordered (h_S, h_T, G_{ST}) and ordered alleles taking into account similarities between haplotypes (v_S, v_T, N_{ST}) demonstrated that the majority of the total diversity (h_T = 0.655, v_T = 0.660) was contained within populations (h_S = 0.466, v_S = 0.450). Population genetic differentiation was relatively low with G_{ST} = 0.289 and N_{ST} = 0.317 (Table 6.11). The permutation test found that N_{ST} was not significantly higher than G_{ST} with the mean permutated N_{ST} (N_{STper} = 0.274) similar to the observed G_{ST} and 20.8 % of the permutated N_{ST} values greater than the observed N_{ST}. This suggests that there is no phylogeographic structure among populations.

Population cytoplasmic genetic differentiation estimated as F_{ST} in TFPGA (Miller 1997) was equal to the observed N_{ST} (F_{ST} = 0.317, N_{STobs} = 0.317) and pairwise population F_{ST} estimates ranged from -0.030 to 0.669 (Table 6.12). The Aber and Maltraeth populations exhibited no differentiation (F_{ST} = -0.030, SD 0.015) as each population contained three cytotypes at similar frequency. The highest levels of differentiation were observed between the Birds Rock population and all other populations, F_{ST} ranging from 0.433 to 0.669, due to the higher frequency of Cyt 4 in

Table 6.12 – Pairwise population cytoplasmic genetic differentiation (F_{ST}) estimated by jacknifing over loci in TFPGA (lower left diagonal) and geographic distances (km) (upper right diagonal). Standard deviations are given in brackets.

Population	Aber	Birds Rock	Cwm Idwal	Maltraeth
Aber		64.123	11.434	25.812
Birds Rock	0.433 (0.074)		52.903	64.640
Cwm Idwal	0.145 (0.068)	0.669 (0.032)		24.292
Maltraeth	-0.030 (0.015)	0.443 (0.089)	0.062 (0.032)	

this population. Birds Rock and Cwm Idwal were the most differentiated (F_{ST} = 0.669, SD 0.032) as each population was dominated by a different cytotype, Cyt 4 and Cyt 3 respectively (Figure 6.6). Pairwise population estimates of F_{ST} separated the more Northern populations (Aber, Cwm Idwal and Maltraeth) from the Birds Rock population. The Mantel test for among population isolation by distance revealed no significant correlation between genetic differentiation and geographic distance despite the relatively high correlation coefficient (r = 0.791, P = 0.245).

6.3.2 Pollen-to-seed migration ratio

The relative contribution of pollen and seed dispersal to the observed among population gene flow was calculated from the overall nuclear AFLP AMOVA F_{ST} and chloroplast F_{ST} estimates 0.0198 and 0.317 respectively. Comparison of these genetic differentiation estimates resulted in a pollen-to-seed migration ratio of 20.97. This demonstrates higher levels of pollen-mediated gene flow in the bi-parentally inherited nuclear genome than seed-mediated gene flow in the maternally inherited chloroplast genome among these populations. Although the F_{ST} estimates derived from the different markers may not be directly comparable the magnitude of the difference and resulting pollen-to-seed migration ratio are quite conclusive.

6.4 Discussion

Population genetics is the basis for understanding how genetic units such as individuals and populations behave and interact. The assessment of population genetic parameters such as genetic diversity, differentiation and levels of gene flow can aid the understanding of whether phenotypic characteristics such as morphology, physiology and differential response to environmental stress have an underlying genetic basis or are purely environmental in their plasticity. The results presented here show that the four North Wales *A. odoratum* populations sampled in this study are characterised by high levels of intrapopulation genetic diversity and low levels of interpopulation genetic differentiation in the nuclear genome. Cytoplasmic markers also revealed relatively high levels of intrapopulation genetic diversity and population differentiation was substantially higher than for the nuclear markers. These results suggest that the four North Wales populations may form part of a more or less continuous population maintained by higher levels of pollen mediated gene flow than seed mediated gene flow.

The majority of the total genetic diversity at AFLP loci (98%) was observed at the intrapopulation level. This percentage is high compared to other studies on the same species which provide estimates at 18.7% (Pimentel *et al* 2007) and 94.1% (Odat 2004). Intrapopulation Nei's gene diversity estimates (h = 0.190-0.201) were at least double those observed within European populations of *A. odoratum* and were more comparable to the within region estimates (h = 0.160-0.38) obtained in the genus level phylogenetic study by Pimentel *et al* (2007). The difference in these estimates may be attributable to sample size as each European population consisted of only five individuals while within region estimates were based on 10 to 20 individuals from across two to four populations. This assertion is corroborated by an intraspecific study of *A. odoratum* in Central Germany which used an intermediate sample size of 13 individuals and obtained higher Nei's gene diversity estimates of h = 0.262-0.384 (Odat 2004). In addition to sample size, the number of intraspecific polymorphic markers employed, 79 (Pimentel *et al* 2007) and 216 (Odat 2004), may also have affected the gene diversity estimates. The estimates obtained in this study are lower than those obtained with half the loci in Odat (2004) across a similar geographical range, indicating a lower level of diversity in the North Wales populations. However, this may be attributable to increased

noise within the dataset when large numbers of polymorphic loci (>250 AFLP) are employed (Hollingsworth and Ennos 2004) and differences in sample size as more than 20 individuals per population are preferable for the accurate estimation of population genetic diversity (Krauss 2000).

The two microsatellite markers revealed similarly high levels of intrapopulation genetic diversity as the AFLPs with average multilocus observed hererozygosity of H_O = 0.768, expected heterozygosity of H_E = 0.747 and Shannon-Weiner diversity of H' = 2.487. The H_O and H_E values obtained are higher than average for species with similar life history traits (H_O = 0.55-0.65, H_E = 0.53-0.63) although direct comparison with these estimates is unwise as they were obtained in a survey of microsatellite data from predominantly diploid species (Nybom 2004). Comparison with other tetraploid species is limited due to the relatively small number of studies employing co-dominant microsatellite markers in polyploid species and variation in the diversity statistics reported. However, average multilocus observed heterozygosity (H_O = 0.768) was greater than that obtained in the polyploid perennial wetland grass *Phragmites austalis* (H_O = 0.43) over 719 individuals sampled worldwide (Saltonstall 2003) while the average Shannon-Weiner diversity (H' = 2.487) was slightly lower than observed in populations of the hexaploid salt-marsh grass *Elymus athericus* (H' = 2.82) (Bockelman *et al* 2003). The relatively small range of H' (2.301 to 2.610) in the *A. odoratum* populations compared with *Elymus athericus* (H' = 1.41-4.07) (Bockelman *et al* 2003) indicates homogeneity of genetic diversity among the populations. The microsatellite based diversity estimates obtained in this study may not be accurate due to the small number of loci (Nybom 2004) and sub-sampling method employed for analysis with the computer program TETRASAT (Markwith *et al* 2006, Markwith and Scanlon 2007). Despite these limitations, the microsatellite loci revealed a similar trend for high levels of intrapopulation diversity and low levels of among population heterogeneity in genetic diversity as the AFLP markers.

The high levels of polymorphism and genetic diversity observed within the North Wales populations were associated with very low levels of genetic differentiation among populations at AFLP markers (AMOVA F_{ST} = 0.0198, overall θ^B = 0.0154) and nuclear microsatellites (pairwise G_{ST} of <0.06). The higher level of differentiation observed at microsatellite loci compared to AFLP data may be expected due to the low number of

loci used (Nybom 2004). The pattern of population differentiation varied between these marker sets with the Birds Rock and Maltraeth populations most differentiated at AFLP (θ^B = 0.0162) markers and least differentiated at microsatellites loci (G_{ST} = 0.0267). This difference may be due to the low number of microsatellite loci and the sub-sampling analysis method employed in the study. These low levels of population differentiation were corroborated by the results of both the band-based Neighbour Joining and Pricipal Coordinates analyses and the allele frequency-based analysis in STRUCTURE, where no clustering of individuals based on population of origin were observed. The level of differentiation observed at AFLP loci is lower than that observed among *A. odoratum* populations over a similar geographic range in central Germany (AMOVA Fst = 0.059) (Odat 2004), and substantially smaller than estimates among regions across mainland Europe (AMOVA Fst = 0.3) (Pimentel *et al* 2007). The results presented in this study are most comparable to those observed at a much smaller spatial scale among plots at the Park Grass Experiment (PGE, Rothamstead, UK), using allele frequency-based analysis of ISSR markers (θ^B = 0.0152-0.0203) (Silvertown *et al* 2005). Although genetic differentiation estimates based on ISSR and AFLP should be comparable, ISSR markers may over-emphasize differences between closely related populations (Nybom 2004, Qian *et al.* 2001). The low level of genetic differentiation observed for both AFLP and microsatellite loci suggest a high level of connectivity through pollen-mediated gene flow among the *A. odoratum* populations.

Cytoplasmic markers revealed five cytotypes within the four North Wales *A. odoratum* populations with a total genetic diversity of h_T = 0.655. Levels of intraspecific cytoplasmic diversity have not been published for other studies with this species, however this value falls within the range previously observed in two other grass species *Lolium perenne* (h_T = 0.821) and *L. rigidum* (h_T = 0.463) (Balfourier *et al* 2000). The majority of the total genetic diversity at chloroplast markers was observed at the intrapopulation level (71%) and slightly higher than the 60 % observed in the two *Lolium* species. The observed genetic differentiation in *A. odoratum* did not reveal any phylogeographic structure in the North Wales populations as N_{ST} (0.317) was not significantly higher than G_{ST} (0.289) (Pons and Petit 1995, Pons and Petit 1996, Burban *et al* 1999). Overall population differentiation was lower than that observed in *L. perenne* (G_{ST} = 0.401), *L. rigidum* (G_{ST} = 0.463) (Balfourier *et al* 2000) and the average for angiosperm species (G_{ST} = 0.637) (Petit *et al* 2005). Although lower, this may be

attributable to the inclusion of populations from a wider geographic range in these studies. The effect of geographical distance on levels of cytoplasmic differentiation is corroborated by the low levels observed in *A. odoratum* among plots at the PGE (F_{ST} = 0.0074) (Silvertown *et al* 2005). The pairwise estimates of among population differentiation (F_{ST}) revealed the greatest differences between the Birds Rock population and all other populations. This pattern was associated with larger geographical distance although no significant correlation was observed.

The results discussed above demonstrate low levels of population genetic differentiation at nuclear markers and higher levels revealed by cytoplasmic markers suggesting greater pollen-mediated compared to seed-mediated gene flow. Higher levels of gene flow may be expected for the nuclear genome compared to the chloroplast genome due to differences in the mode of inheritance and dispersal mechanism. The nuclear genome is inherited bi-parentally through both pollen and seeds while the chloroplast is maternally inherited through seeds alone in angiosperm species (Ouborg *et al* 1999). Although the chloroplast is predominantly maternally inherited some evidence of paternal leakage has been observed in some angiosperm species (McCauley *et al* 2007). In this study the pollen-to-seed migration ratio (r), calculated from the overall AFLP AMOVA F_{ST} and chloroplast F_{ST} estimates, was r = 20.97. The level of pollen-mediated compared to seed-mediated gene flow observed among these populations is not unusual and is similar to the median value of r = 17 obtained in a survey of 93 plant species by Petit *et al* (2005). These results indicate that although the *A. odoratum* populations are highly connected by pollen flow, seed flow is more restricted.

The four North Wales populations exhibit relatively high levels of genetic diversity in both the nuclear and chloroplast genomes although the amount of genetic diversity is similar among populations. The low level of population differentiation suggests that these populations may form part of a continuous distribution across North Wales and this can be considered likely due to the prevalence of suitable grassland habitats throughout this region. The Snowdonia mountains and Menai Straits, which may have provided barriers to gene flow among these populations, do not appear to have hampered pollen-mediated gene flow although they may have provided some restriction to seed-mediated gene flow. An additional factor which may have increased population genetic similarity is the historical inclusion of this species for pasture and hay meadow

seed mixtures (Hubbard 1992). Although these are unlikely to have been applied at the sample sites included in this study, gene flow among natural and seeded populations may have aided genetic similarity.

In relation to ozone sensitivity, the genetic similarity of these populations is concordant with the similar trend of response to ozone exposure for most variables among populations although the magnitude of effect differed (see Chapters 2, 3 and 4). The chloroplast genome revealed the greatest population differentiation and may provide the greatest potential for relating ozone sensitivity and genetic variation at the population level. Although antioxidant gene expression involved in plant response to oxidative stresses such as ozone pollution is predominantly nuclear based, the chloroplast genome may be involved in the regulation of signalling pathways controlling such responses (Baier *et al* 2005). In addition, these populations exhibited high levels of both intrapopulation genetic variability and variation in response to ozone exposure. As such, investigation of the relationship between genetic variation and ozone sensitivity may be best targeted at the individual rather than population level.

Chapter 7: General Discussion

The effects of ozone pollution on the perennial grass species *Anthoxanthum odoratum* were investigated in terms of ozone sensitivity to contrasting ozone pollution scenarios and intraspecific variation in relation to population genetic diversity. This discussion considers the ecological significance of the observed responses to ozone exposure, the potential impacts of future increases in tropospheric ozone concentration and the potential for the evolution of ozone resistance in relation to intraspecific variation in ozone response and the observed population genetic diversity and differentiation in this species. Recommendations for future work are also included.

7.1 Ozone sensitivity in *Anthoxanthum odoratum*

The response of *A. odoratum* to ozone pollution was investigated under three different ozone exposure profiles representing acute exposure (Chapter 2) and simulated current and future scenarios for both rural (Chapter 3) and upland (Chapter 4) areas. The key findings for the response of *A. odoratum* under these contrasting ozone scenarios are summarised in Table 7.1. The most significant and consistent response of this species to ozone exposure was for the development of premature senescence which occurred under each ozone profile applied, while significant growth responses only occurred under the highest elevated ozone levels. These results were consistent with previous studies of ozone sensitivity in this species (Nebel and Fuhrer 1994, Hayes *et al* 2006, Hayes 2007).

The extent of premature senescence and the rate of development differed among the contrasting ozone profiles, although a relatively fast response was observed in each case with significant increases after one week under acute exposure and three weeks under the chronic rural and upland episodic exposure regimes. Comparison of the extent of senescence at the end of the chronic exposures revealed that elevated background concentrations in the range predicted for much of the UK in the next 50 years (Coyle *et*

Table 7.1 – Summary of *A. odoratum* response to three simulated ozone exposure profiles: acute, rural and upland, in terms of premature senescence, the rate of development of senescence ($wpns_{coeff}$) and shoot and root biomass components.

Ozone Exposure		Assessment Criteria				
		Senescence[†]				
Profile	Treatment	maximum response[§]	at end of exposure	$wpns_{coeff}$[††]	Shoot biomass[††]	Root biomass[††]
Acute	O_3(150)	+ 21.8 ** (*4w*)	+ 21.8 **	+ 145.7 **	- 8.4 *	- 15.0 Ns
Rural	O_3(20,75)[a]	+ 6.3 (*) (*3w*)	+ 4.2 (*)	n/a	+ 3.7 ns	n/a
	O_3(45,50)[b]	+ 11.6 ** (*3w*)	+ 3.2 (*)	n/a	+ 4.3 ns	n/a
	O_3(45,100)[c]	+ 15.1 ** (*3w*)	+ 9.9 (*)	n/a	- 2.1 ns	n/a
Upland[‡‡]	O_3(35,55)	+ 6.4 * (*7w*)	+ 5.1 ns	+ 17.3 ns	+ 1.2 ns	n/a
	O_3(60,80)	+ 12.0 ns (*8w*)	+ 12.0 ns	+ 43.9 *	- 4.6 ns	n/a
	O_3(85,105)	+ 22.2 * (*7w*)	+ 17.9 *	+ 74.1 *	- 6.9 ns	n/a

[†], response as the increase in senescence above the control; [††], response as percentage change from the control; [§], maximum response observed with week of exposure given in brackets (x*w*); [a], significant differences for peak effects; [b], significant differences for background effects, [c], significant differences for peak or background effects; [‡‡], significant differences from the control assessed by post hoc comparisons

al 2003b) for both rural (O_3(45,50)) and upland (O_3(35,55)) profiles induced a similar response to ambient peak episodes (O_3(20,75)). However, the magnitude of response varied throughout the exposure periods and the greatest response did not always occur at the end of the exposure period. This was especially apparent under the rural profile as the greatest responses in all treatments were observed after three to five weeks. At this time, elevated background concentrations resulted in double the increase in senescence observed under ambient peak episodes. Although this suggests that increased background concentrations may have a larger effect on vegetation than current peak episodes, under the upland profile a longer exposure and greater accumulated ozone dose were required to elicit a similar magnitude of response

A number of factors which may have affected the comparative response under rural and upland profiles included differences in plant age and the timing of exposure in relation to the growing season. The upland profile exposure was applied in early summer from May to July, while the rural profile exposure was applied in late summer from July to

September with plants that had been established for an additional four weeks prior to exposure. Both of these factors will have influenced plant growth rate and the natural rate of senescence above which the ozone effect was measured. Relative growth rate (RGR) and stomatal conductance tend to decrease with increased plant age (Bassin *et al* 2007) as do various plant architecture traits including specific leaf area (SLA) (Schippers and Olff 2000). Higher RGR and SLA are thought to relate to greater ozone sensitivity (Bassin *et al* 2007) and in *Plantago major* ozone sensitivity was found to be higher for plants in the seedling stage (Reiling and Davison 1992b, Lyons and Barnes 1998). However, in the present study a greater magnitude of premature senescence was observed at an earlier date for older plants under the rural profile. This difference in senescence is more likely to relate to the measurement of ozone effect as the magnitude of percentage senescence above the control, in relation to the timing of exposure during the growing season and the development of natural senescence.

The greatest effects of rural profile ozone exposure in terms of premature senescence occurred after three weeks and decreased through the exposure period. A slight trend for decreased magnitude of effect was also observed under the upland profile at the end of the exposure period. This decrease resulted from a relatively fast initial response to elevated ozone followed by a decrease in rate which was accompanied by an increasing rate of natural senescence under control conditions. The decrease in rate may relate to the continual production of leaves throughout the exposure period in this species (Hayes *et al* 2006). The greater decrease in ozone effect in the rural exposure relates to the higher rates of natural senescence towards the end of the growing season and the greater plant age. An increase in senescence with plant age and increased rates of senescence from late summer onwards are typical of many perennial grass species including *A. odoratum* (Schippers *et al* 1999, Schippers and Olff 2000, Grant *et al* 1996). Determination of ozone sensitivity based on end of exposure senescence alone would underestimate the effects of rural profiles on *A. odoratum*.

The results of this study demonstrate that the assessment of ozone sensitivity based on premature senescence is largely dependant upon the timing of assessment and exposure in relation to plant age, growing season and the accompanying natural rate of senescence. The rate of development of senescence was assessed using linear regression coefficients ($wpns_{coeff}$) for the acute and upland profile exposures (Table 7.1). In the

upland profile exposure the significant linear increase among treatments indicated that there was a clear relationship between incremental increases in background concentrations and the rate of senescence in *A. odoratum* (Chapter 4). The large and highly significant increase under the acute exposure was greater than would have been expected following the trend observed in the upland exposure. The assessment of the progression of senescence over time using linear regression was adequate for the acute exposure but may not have been appropriate for the upland exposure as the development of senescence appeared to follow a logistic curve through the exposure period (Chapter 4).

A logistic curve often provides the best description for the progression of natural senescence through the growing season and with increasing plant age. This is illustrated in Figure 7.1, where senescence data from the control treatments of the three exposures were combined and demonstrates the progression of senescence with plant age after vegetative propagation. As previously discussed in Chapters 2 and 4, exposure to elevated ozone may result in a shift in this curve with the acceleration, logarithmic, negative acceleration and stationary phases reached at an earlier date and may result in a reduction in the effective growing season. Such a shift in development curve may explain the decrease in the rate of senescence and magnitude of response above the

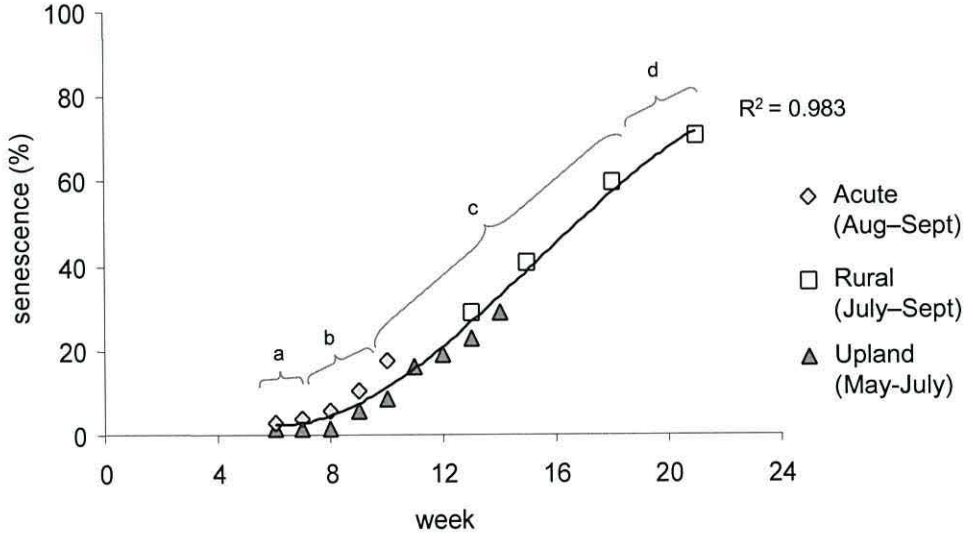

Figure 7.1 – The development of whole plant senescence under control conditions with plant age in weeks from vegetative propagation. Polynomial regression and logistic curve relationship illustrated with a) lag, b) acceleration, c) logarithmic and d) negative acceleration phases highlighted. Data combined from three ozone exposure experiments.

control towards the end of exposure in the rural profile. Between ozone treatment comparisons of the logarithmic phase may provide the best assessment of differing rates of senescence while the time and ozone dose required to reach each phase would provide additional information regarding the rapidity of response.

Premature senescence, as observed in *A. odoratum* under all three ozone profiles, is a non-specific form of ozone induced foliar injury and a common response in many semi-natural vegetation species (Bergmann *et al* 1995, Bergmann *et al* 1999, Franzaring *et al* 2000, Bermejo *et al* 2003), although the ecological significance of foliar injury has been questioned as it often occurs without any accompanying growth reductions (Franzaring *et al* 2000, Inclan *et al* 1999, Woo and Hinckley 2005, Hayes *et al* 2006, Bender *et al* 2006). However, senescence is associated with reductions in photosynthetic capacity and resource accumulation (Gay and Thomas 1995, Vandermeinen *et al* 2005) and has implications for overwintering potential and competitive ability.

Shifts in the development of senescence in response to elevated ozone, especially early in the growing season as observed under the upland profile exposure, have implications for long-term plant vitality. Predicted future background ozone concentrations are expected to show the greatest increases in winter and early spring (Ashmore *et al* 2002) resulting in greater levels of ozone-induced oxidative stress throughout the growing season. Increased early season background ozone concentrations may have a greater impact on resource accumulation, especially in combination with continued elevated ozone concentrations through the growing season, than late season peak episodes alone.

In this study, premature senescence was accompanied by significant growth responses only under acute ozone exposure (Table 7.1). Significant reductions in above ground shoot growth occurred with a trend for greater reduction in root growth. This, in combination with a trend for decrease in the below ground to above ground biomass ratio suggests an alteration in resource allocation to shoot growth. Chronic ozone exposure did not result in any significant plant growth response in *A. odoratum* although a trend for stimulation under low levels of elevated ozone and reduction with higher levels was observed under both the rural and upland exposures. The maintenance of shoot growth despite significant premature senescence under chronic ozone exposure may result from alterations in root to shoot resource allocation as suggested under acute

exposure and reductions in the root:shoot allocation coefficient (k) were observed in over 50 % of the species included in a meta-analysis by Grantz *et al* (2006) and in 9 of the 14 monocot species included.

The maintenance of shoot growth may also be related to alterations in tiller morphology and resource allocation among the leaf and sheath shoot components. The trend for reduction in dry weight to fresh weight biomass ratio observed under acute and upland profiles indicates a greater water content under elevated ozone. This may result from a greater leaf to sheath ratio (Maurice *et al* 1997) and the production of a greater number of smaller tillers (Sugiyama 1995) as suggested by an increase in tiller number under rural profile exposure. Alterations in tiller morphology may effect not only plant development, biomass production and resource allocation but physiology as tiller size has been related to within plant carbon availability (Sugiyama 1995). However, few studies have considered tiller morphology or tillering in response to ozone and results from spring wheat cv. Minaret suggest there is very little effect (Ewert *et al* 1999).

Several factors relating to experimental design may have influenced the observed response of *A. odoratum* to elevated ozone including solardome related chamber effects, edge effects and pot related growth restrictions, as previously discussed in Chapters 2, 3 and 4. These factors may limit the extrapolation of observed responses to responses under field conditions. An additional factor is water availability, as plants under experimental conditions are often kept well watered while plants under field conditions may experience drought, resulting in reduced stomatal conductance and lower potential ozone uptake. However, responses to drought and ozone exposure may be species specific and drought may not confer any general protection against ozone effects (Bungener *et al* 1999a). In relation to among exposure comparisons, solardome related chamber effects should be consistent and pot size remained constant. However, edge effects may have differed among the three exposures as the spacing of plant pots 5 cm apart in the acute and upland profile exposures will have allowed greater air flow around each plant compared with the rural profile exposure where plant pots were adjacent and provided some canopy shading and competition. Plants in the acute and upland exposures may therefore have received higher ozone levels than those in the rural exposure (Wilbourne *et al* 1995).

7.2 Intraspecific variation in ozone sensitivity

The four *A. odoratum* populations exhibited differing responses to the three different ozone exposure profiles and the key findings are summarised in Table 7.2. All populations exhibited premature senescence under each of the contrasting ozone profiles although the timing and extent of response differed while the greatest intraspecific variation in response was observed for plant growth under acute exposure.

In terms of premature senescence, the Aber population exhibited the fastest responses although the magnitude of response decreased by the end of each exposure period due the high rates of natural senescence in this population. Cwm Idwal exhibited the greatest magnitude of response to all three exposure profiles. The magnitude of response in the Birds Rock population differed with ozone profile, exhibiting the lowest responses overall to the acute and rural profiles and a greater response to the upland profile. This difference relates to the timing of exposure during the growing season as the upland exposure occurred in early summer and the acute and rural exposures occurred in late summer. Comparison of response to similar background concentration treatments suggests that this population was most sensitive to ozone exposure in early summer and this increased sensitivity may relate to phenological stage as the upland exposure coincided with the flowering period of this species, and plants in this growth stage may be more sensitive to ozone (Bassin *et al* 2007). The greater difference among profiles observed in Birds Rock compared to the other populations corresponds to the greater number of flowering plants and flowers per plant in this population. The response of Maltraeth to the upland profile treatments was confounded by high levels of senescence in the control treatment and this negates the comparison of among profile variation in response for this population.

All four populations exhibited a greater response to elevated background than increased ambient peaks in the first five weeks under the rural profile exposure. However, the populations differed in response by the end of exposure with the Birds Rock and Maltraeth populations exhibiting the opposite trend with a greater effect of ambient peak episodes. This suggests that these populations may be more sensitive to ambient peak episodes

Table 7.2 - Summary of population responses to three simulated ozone exposure profiles: acute, rural and upland, in terms of premature senescence, the rate of development of senescence ($wpns_{coeff}$) and shoot and root biomass components.

Exposure	Assessment criteria	Treatment	Population							
			Aber		Birds Rock		Cwm Idwal		Maltraeth	
Acute	**Senescence maximum response**[†§]	**O_3(150)**	+ 24.4 *(3w)*	**	+ 19.5 *(4w)*	**	+ 23.8 *(4w)*	**	+ 22.3 *(4w)*	**
	Senescence at end of exposure[†]	**O_3(150)**	+ 21.6	**	+ 19.5	**	+ 23.8	**	+ 22.3	**
	$wpns_{coeff}$[††]	**O_3(150)**	+ 101.0	**	+ 162.4	*	+ 163.8	**	+ 209.0	**
	Shoot biomass[††]	**O_3(150)**	- 7.6	ns	- 22.5	**	+ 3.1	ns	- 8.4	ns
	Root biomass[††]	**O_3(150)**	- 13.3	(*)	- 23.4	ns	- 9.1	ns	- 15.3	ns
Rural	**Senescence maximum response**[†§]	**O_3(20,75)**[a]	- 3.8 *(8w)*	ns	+ 4.1 *(3w)*	(*)	+ 8.6 *(3w)*	*	+ 9.5 *(5w)*	ns
		O_3(45,50)[b]	+ 12.1 *(3w)*	*	+ 5.8 *(3w)*	*	+ 14.9 *(3w)*	**	+ 13.9 *(3w)*	*
		O_3(45,100)[c]	+ 14.2 *(5w)*	*	+ 13.6 *(5w)*	*	+ 21.0 *(3w)*	**	+ 16.0 *(5w)*	*
	Senescence at end of exposure[†]	**O_3(20,75)**[a]	+ 1.3	ns	+ 3.8	*	+ 6.1	(*)	+ 5.9	ns
		O_3(45,50)[b]	+ 4.7	*	+ 1.9	ns	+ 6.9	*	- 0.8	ns
		O_3(45,100)[c]	+ 8.9	*	+ 10.3	*	+ 14.9	*	+ 5.5	ns
	Shoot biomass[††]	**O_3(20,75)**[a]	+ 6.2	ns	+ 6.2	ns	+ 7.1	ns	- 4.2	ns
		O_3(45,50)[b]	+ 5.2	ns	+ 3.6	ns	+ 6.3	ns	+ 2.2	ns
		O_3(45,100)[c]	- 3.2	ns	- 4.6	ns	+ 0.7	ns	- 1.7	ns
Upland[‡‡]	**Senescence maximum response**[†§]	**O_3(35,55)**	+ 8.8 *(6w)*	ns	+ 14.4 *(7w)*	ns	+ 7.8 *(8w)*	ns	- 4.7 *(6w)*	ns
		O_3(60,80)	+ 13.4 *(6w)*	ns	+ 17.0 *(8w)*	ns	+ 14.7 *(8w)*	ns	+ 6.6 *(8w)*	ns
		O_3(85,105)	+ 19.1 *(7w)*	ns	+ 24.1 *(7w)*	*	+ 26.3 *(7w)*	ns	+ 19.3 *(7w)*	ns
	Senescence at end of exposure[†]	**O_3(35,55)**	+ 1.6	ns	+ 13.8	ns	+ 7.8	ns	- 2.8	ns
		O_3(60,80)	+ 9.6	ns	+ 17.0	ns	+ 14.7	ns	+ 6.6	ns
		O_3(85,105)	+ 12.2	ns	+ 21.3	*	+ 21.9	(*)	+ 16.2	ns
	$wpns_{coeff}$[††]	**O_3(35,55)**	+ 17.3	ns	+ 55.0	ns	+ 17.2	ns	- 4.2	ns
		O_3(60,80)	+ 39.7	ns	+ 82.3	ns	+ 42.7	ns	+ 26.5	ns
		O_3(85,105)	+ 48.9	ns	+ 115.9	(*)	+ 93.1	*	+ 60.1	ns
	Shoot biomass[††]	**O_3(35,55)**	- 15.8	ns	+ 4.0	ns	+ 11.6	ns	+ 12.8	ns
		O_3(60,80)	- 23.6	ns	+ 0.7	ns	+ 1.2	ns	+ 12.6	ns
		O_3(85,105)	- 19.1	ns	- 0.5	ns	- 3.2	ns	+ 1.5	ns

[†], response as the increase in senescence above the control; [§], maximum response observed with week of exposure given in brackets (x*w*); [††], response as percentage change from the control; [a], significant differences for peak effects; [b], significant differences for background effects, [c], significant differences for peak or background effects; [‡‡], significant differences from the control assessed by post hoc comparisons

while Aber and Cwm Idwal are more sensitive to elevations in background concentration.

The only significant plant growth response was for a reduction in shoot biomass in the Birds Rock population under acute ozone exposure. However, a trend for reduction in root biomass was observed in all four populations and was almost significant for Aber. The Aber, Cwm Idwal and Maltraeth populations all exhibited a trend for greater reduction in root biomass than shoot biomass under acute exposure while the Birds Rock population exhibited a similar reduction for both biomass components. High relative growth rate (RGR) and stomatal conductance are thought to infer greater ozone sensitivity (Bassin *et al* 2007) and the significant growth reductions in Birds Rock correspond to the highest RGR and stomatal conductance among the populations. However, this response was not replicated under the chronic ozone exposures. The Cwm Idwal population exhibited a trend for stimulation of shoot growth under elevated ozone in the acute and rural exposures while the Aber population exhibited large but non-significant reductions in shoot growth under the upland profile exposure. Although the populations exhibited differing trends for shoot growth response under chronic ozone exposure, multi-season exposure and assessment of root growth response are necessary to determine the longer-term responses of these populations.

The intraspecific variation in ozone response observed among these *A. odoratum* populations was similar to those found among populations of other grass species. Foliar injury was also ubiquitous among populations of *Spartina alterniflora*, with similar small variations in the timing and magnitude of ozone effect (Taylor *et al* 2002). In terms of growth responses, both *Elymus glaucus* (Yoshida *et al* 2001) and *Spartina alterniflora* (Taylor *et al* 2002) also exhibited a lack of above ground biomass response to chronic ozone exposure in any population and this was accompanied by reductions in root mass in both species. The maintenance of shoot growth was accompanied by morphological variations including reduced leaf area and increased tiller number in *Elymus glaucus* (Yoshida *et al* 2001) and reductions in shoot and leaf number in *Spartina alterniflora* (Taylor *et al* 2002). Shifts in morphology may also have occurred in *A. odoratum*, as suggested by the increase in tiller number under rural exposure. Although root biomass responses in *A. odoratum* were only assessed under acute ozone exposure, the greater reduction in root than shoot biomass suggested that this may have

occurred under chronic exposure when shoot growth was not significantly affected. Populations of *Elymus glaucus* (Yoshida *et al* 2001) and *Spartina alterniflora* (Taylor *et al* 2002) were found to vary in the extent of ozone response under chronic exposure, however as with *A. odoratum*, the direction of response was usually similar among populations.

The relatively small differences in population response in these grass species are also similar to those observed in *Fragaria vesca* (Manninen *et al* 2003) and *Centaurea jacea* (Ramo *et al* 2006b). None of these species have exhibited as significant ozone effects nor such large variations in ozone sensitivity among populations as observed in studies of *Plantago major* (e.g. Reiling and Davison 1992a, 1992c, Lyons *et al* 1997) which may in part relate to the greater plant age during exposure in the majority of these studies (Manninen *et al* 2003, Ramo *et al* 2006b, Taylor *et al* 2002), including those presented here as plant age and development stage can be important factors in determining ozone sensitivity (Bassin *et al* 2007). Reductions in ozone sensitivity with increasing plant age have been reported in *P. major* (Lyons and Barnes 1998) and the higher levels of ozone sensitivity may relate to the exposure of young seedlings in the majority of inter-population studies in this species. In comparison with *P. major*, *A. odoratum* and the other species described above exhibit lower sensitivity and a smaller degree of intraspecific variation in ozone response. However, even subtle differences in ozone response may have implications for population vitality and survival, and if such differences alter comparative reproductive output, may have effects on the genetic diversity and composition of populations.

7.3 Population genetic diversity in relation to ozone sensitivity

Genetic diversity and differentiation were estimated among the four *A. odoratum* populations as a preliminary screen for genetic variation which may relate to observed differences in ozone sensitivity. Genetic variation in the nuclear genome was determined using Amplified Fragment Length Polymorphism (AFLP) fingerprinting and two microsatellite loci developed in this study (Chapter 5). In the chloroplast genome genetic variation was determined using Polymerase chain reaction – Restriction

Fragment Length Polymorphism (PCR-RFLP) and two chloroplast microsatellites (cpSSR). The four populations were characterised by high levels of intrapopulation genetic diversity at both nuclear and cytoplasmic markers, while interpopulation genetic differentiation differed, with low levels in the nuclear genome and substantially higher levels in the chloroplast genome (Chapter 6).

Relatively high levels of polymorphism and genetic diversity were observed within the populations for AFLP and nuclear microsatellite loci although the populations did not differ for any of the diversity parameters assessed. The Birds Rock and Maltraeth populations exhibited the greatest amount of genetic differentiation at AFLP loci and the lowest differentiation at microsatellite loci. However, the microsatellite based estimate may not be accurate due to the low number of microsatellite loci employed (Nybom 2004). The lack of population differentiation at nuclear markers indicates a high level of connectivity through pollen-mediated gene flow among the *A. odoratum* populations.

Cytoplasmic markers also revealed relatively high levels of intrapopulation genetic diversity although the populations differed for presence and frequency of the five cytotypes. The Cwm Idwal population exhibited the lowest variation with two cytotypes while the Maltraeth population was the most variable with four. Cytoplasmic markers revealed the greatest population genetic differentiation indicating some restriction to seed flow among the populations due to the maternal inheritance of the chloroplast genome (Ouborg *et al* 1999). The Birds Rock population was most genetically differentiated from all other populations and this pattern was associated with larger geographical distance although no significant correlation was observed.

These results indicate that these populations may form part of a more or less continuous population in North Wales maintained by higher levels of pollen mediated gene flow than seed mediated gene flow. High levels of pollen mediated gene flow may be expected for widespread, predominantly outcrossing, wind pollinated species such as *A. odoratum*, resulting in low levels of population divergence and the maintenance of genetic variation within populations compared to rare, narrowly distributed species (Loveless and Hamrick 1984). A continuous distribution of *A. odoratum* across North

Wales can be considered likely due to the prevalence of suitable grassland habitats throughout this region.

In relation to ozone sensitivity, the genetic similarity of these populations is concordant with the similar broad trends in response to ozone exposure for most variables among populations although the magnitude and timing of effects differed (Chapters 2, 3 and 4). The chloroplast genome revealed the largest population differentiation and may provide the greatest potential for relating ozone sensitivity and genetic variation at the population level as the chloroplast genome may be involved in the regulation of signalling pathways controlling antioxidant gene expression involved in plant response to oxidative stresses such as ozone pollution (Baier *et al* 2005). The Birds Rock population was the most divergent from the other populations based on cytoplasmic markers. This population differed from the others for physiological traits including higher relative growth rate and stomatal conductance. In terms of ozone sensitivity, the Bird's Rock population exhibited the lowest levels of premature senescence under elevated ozone, the greatest biomass reduction under acute ozone exposure and a similar reduction for both root and shoot components while the other populations exhibited a greater reduction in root biomass.

It may be possible to correlate population level marker frequencies with relative ozone sensitivity as previously examined in *Plantago major* and *P. intermedia* (Wolff *et al* 2000). However, several factors would limit this approach for the *A. odoratum* populations used in this study including the small number of populations investigated and the lack of significant differences in AFLP marker frequencies among populations when assessed by Fisher's exact tests. The assessment of genetic diversity and ozone sensitivity in a greater number of populations from a wider geographical area may increase the validity of this approach in *A. odoratum*.

7.4 Conclusions

A. odoratum is sensitive to ozone exposure in terms of premature senescence exhibiting a relatively fast response under all ozone profiles investigated. Exposure to rural and

upland chronic ozone profiles demonstrated that elevated background ozone concentrations may result in a similar or greater premature senescence response to increased ambient peak episodes. Elevated ozone concentrations resulted in shifts in the rate of development of senescence following a logistic curve with the timing of assessment and exposure during the growing season influencing the observed magnitude of response. The development of premature senescence was only accompanied by significant reductions in above ground biomass under acute exposure. However, a trend for greater reduction in root growth was also observed indicating a shift in resource allocation. The maintenance of shoot growth observed under chronic ozone exposure may result from a reduction in resource allocation to root growth. The occurrence of premature senescence, possible reductions in root reserves and alterations in tiller morphology in *A. odoratum* may result in a slower longer-term shoot growth response to chronic ozone exposure. Multi-season chronic ozone exposures are required to determine the longer-term responses of perennial species such as *A. odoratum*.

Intraspecific variation in response was observed with the four populations differing in the response to elevated ozone dependant on the assessment criteria applied and the timing of exposure in relation to both the growing season and phenological stage. The relatively fast response of the Aber population in terms of premature senescence, reduced root biomass under acute exposure and trends for reductions in shoot growth under early season upland exposure suggest that this population may be the most sensitive population and has the greatest potential for long-term detrimental effects in relation to the predicted increases in early season background ozone concentrations.

The four populations exhibited high levels of intrapopulation genetic diversity and low levels of population genetic differentiation maintained by high levels of pollen-mediated gene flow. In contrast, greater restrictions on seed-mediated gene flow resulted in higher levels of population differentiation assessed by chloroplast markers. The lack of population structuring and small number of populations investigated in this study precluded the detailed assessment of the relationship between population level marker frequencies and ozone sensitivity.

7.5 Further work

The results from this study have demonstrated the importance of assessing intraspecific variation in response to ozone exposure. This study would be improved by the inclusion of a greater number of populations covering a greater geographic range and encompassing a wider altitudinal cline. This would allow the assessment of intraspecific variation in ozone response in relation to altitude and correlation with genetic diversity and differentiation. Determination of the ambient ozone climate at sample sites using passive sampling would also provide a greater understanding of the observed population responses to imposed natural ozone regimes.

Premature senescence is a common ozone response among many wild species and the predominant response for *A. odoratum*. Modelling of the rate of development of senescence as a logistic curve at the species and population level in relation to plant age and growing season under control conditions would allow the determination of key times for comparison of senescence rates. Quantification of the effect of senescence on photosynthetic capacity and resource allocation in combination with the senescence curve would enable extrapolation of the effects of ozone induced shifts in relation to the timing of exposure during the growing season. This may be particularly useful when considering the impacts of future increases in background ozone concentrations early in the growing season.

In terms of plant growth, the response of root growth to elevated ozone requires further investigation to determine if the maintenance of shoot growth under elevated ozone results in a reduction in resource allocation to the roots. Sequential biomass harvests throughout the exposure period may also provide an indication of whether growth responses are consistent throughout ozone exposure. Tillering and tiller morphology were highlighted as areas requiring further investigation in relation to the development of foliar injury, leaf turnover, specific leaf area and the leaf to sheath ratio. The separation of biomass into senescent and green biomass following harvest may also provide elucidation of the maintenance of shoot biomass despite high levels of senescence under elevated ozone. The potential carry-over effects from one years ozone exposure on overwintering and regrowth requires greater attention.

Investigation of the effect of ozone exposure on both daytime and nocturnal stomatal conductance would provide useful insights into the potential for ozone flux in relation to elevated background concentrations. Exposure of plants to three elevated ozone profiles of constant, daylight hours only or overnight only concentrations of 50 ppb would determine the relative effects of nocturnal ozone uptake. The timing of ozone exposure through the growing season may have significant effects on plant response especially when elevated ozone coincides with periods of high relative growth rate and flowering. These influences on the dynamics of response to ozone could be investigated further by the development of ozone flux-effect relationships, using varying flux thresholds and day/night time periods for flux accumulation.

In terms of linking genetic diversity with ozone sensitivity, assessment of response at the individual level would allow the determination of relative sensitivity for each genotype. Pairwise differences in ozone sensitivity could be related to pairwise genetic distances to determine if individuals with similar sensitivity are more closely related. Estimation of genetic diversity could include assessment of additional chloroplast regions and screening of markers developed for other grass such as microsatellites and sequence-tagged sites developed for *Lolium perenne* (Jones *et al* 2001, Lem and Lallemand 2003).

Table AI.1 – Details of ANOVA analysis results for Acute ozone exposure described in Chapter 2.

			wpns week 1			wpns week 2			wpns week 3			wpns week 4		
	Source of variation	DF	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*
Overall	Block	3	0.0076	0.0025	0.85	0.0151	0.0050	5.65	0.0095	0.0032	6.05	0.0402	0.0134	17.03
	Treatment‡	1	0.3661	0.3661	122.98**	0.6723	0.6723	755.85**	0.5929	0.5929	1137.38**	0.5679	0.5679	721.54**
	residual	*3*	0.0089	0.0030	1.43	0.0027	0.0009	0.34	0.0016	0.0005	0.17	0.0024	0.0008	0.18
	Population‡	3	0.0098	0.0033	1.57	0.0539	0.0180	6.86**	0.1906	0.0635	21.05**	0.1459	0.0486	11.32**
	Treatment x Population‡	3	0.0096	0.0032	1.54	0.0073	0.0024	0.92	0.0035	0.0012	0.39	0.0014	0.0005	0.11
	residual	*18*	0.0375	0.0021		0.0472	0.0026		0.0543	0.0030		0.0774	0.0043	
	Total	31	0.4395			0.7983			0.8523			0.8351		
Aber	Block	3	0.0032	0.0011	0.20	0.0044	0.0015	1.22	0.0056	0.0019	2.21	0.0428	0.0143	7.71
	Treatment‡	1	0.0667	0.0667	12.53*	0.1642	0.1642	136.79**	0.1635	0.1635	192.67**	0.1265	0.1265	68.43**
	residual	*3*	*0.0160*	*0.0053*		*0.0036*	*0.0012*		*0.0026*	*0.0009*		*0.0056*	*0.0019*	
	Total	7	0.0859			0.1721			0.1717			0.1749		
Birds Rock	Block	3	0.0080	0.0027	3.22	0.0117	0.0039	8.37	0.0263	0.0088	8.02	0.0049	0.0016	1.30
	Treatment‡	1	0.1180	0.1180	142.5**	0.1972	0.1972	422.62**	0.1141	0.1141	104.19**	0.1298	0.1298	104.68**
	residual	*3*	*0.0025*	*0.0008*		*0.0014*	*0.0005*		*0.0033*	*0.0011*		*0.0037*	*0.0012*	
	Total	7	0.1285			0.2103			0.1437			0.1384		
Cwm Idwal	Block	3	0.0098	0.0033	2.80	0.0159	0.0053	3.27	0.0038	0.0013	3.74	0.0088	0.0029	1.00
	Treatment‡	1	0.1286	0.1286	110.39**	0.2011	0.2011	124.28**	0.1723	0.1723	508.13**	0.1550	0.1550	53.18**
	residual	*3*	*0.0035*	*0.0012*		*0.0049*	*0.0016*		*0.0010*	*0.0003*		*0.0088*	*0.0029*	
	Total	7	0.1419			0.2218			0.1771			0.1725		
Maltraeth	Block	3	0.0073	0.0024	2.00	0.0208	0.0069	9.14	0.0219	0.0073	26.36	0.0338	0.0113	2.89
	Treatment‡	1	0.0624	0.0624	51.07**	0.1171	0.1171	154.51**	0.1466	0.1466	529.53**	0.1580	0.1580	40.49**
	residual	*3*	*0.0037*	*0.0012*		*0.0023*	*0.0008*		*0.0008*	*0.0003*		*0.0117*	*0.0039*	
	Total	7	0.0733			0.1402			0.1693			0.2035		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

Table AI.1 –Continued

			SPAD week 1			SPAD week 2			SPAD week 3			SPAD week 4		
	Source of variation	DF	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*
Overall	**Block**	3	15.902	5.3010	8.62	56.028	18.676	3.05	63.359	21.120	3.93	37.215	12.405	5.12
	Treatment‡	1	0.9840	0.9840	1.60	16.560	16.560	2.71	0.6820	0.6820	0.13	22.212	22.212	9.17(*)
	residual	*3*	1.8460	0.6150	0.13	18.344	6.1150	0.94	16.131	5.3770	0.91	7.2710	2.4240	0.31
	Population‡	3	70.662	23.554	4.97*	80.033	26.678	4.10*	149.54	49.846	8.44**	305.49	101.83	13.1**
	Treatment x Population‡	3	3.5710	1.1900	0.25	10.997	3.6660	0.56	20.307	6.7690	1.15	18.145	6.0480	0.78
	residual	*18*	85.306	4.7390		117.12	6.5060		106.35	5.9080		139.95	7.7750	
	Total	31	178.27			299.07			356.36			530.28		
Aber	**Block**	3	7.1960	2.3990	2.21	19.433	6.4780	5.42	65.715	21.905	9.93	89.470	29.820	1.88
	Treatment‡	1	4.0020	4.0020	3.69	0.3810	0.3810	0.32	18.590	18.590	8.43(*)	0.7300	0.7300	0.05
	residual	*3*	*3.2520*	*1.0840*		*3.5880*	*1.1960*		*6.6160*	*2.2050*		*47.500*	*15.830*	
	Total	7	14.450			23.401			90.921			137.70		
Birds Rock	**Block**	3	42.875	14.292	11.64	61.258	20.419	3.88	21.651	7.2170	3.43	8.0160	2.6720	1.80
	Treatment‡	1	0.0000	0.0000	0.00	1.0710	1.0710	0.20	0.7200	0.7200	0.34	3.6090	3.6090	2.44
	residual	*3*	*3.6820*	*1.2270*		*15.783*	*5.2610*		*6.3140*	*2.1050*		*4.4460*	*1.4820*	
	Total	7	46.557			78.112			28.686			16.072		
Cwm Idwal	**Block**	3	4.5700	1.5230	0.32	20.225	6.7420	4.32	19.514	6.3850	1.52	7.3630	2.4540	0.98
	Treatment‡	1	0.2840	0.2840	0.06	2.7300	2.7300	1.75	0.5870	0.5870	0.14	12.367	12.367	4.95
	residual	*3*	*14.473*	*4.8240*		*4.6830*	*1.5610*		*12.620*	*4.2070*		*7.4900*	*2.4970*	
	Total	7	19.327			27.637			32.361			27.221		
Maltraeth	**Block**	3	19.100	6.3670	2.42	54.502	18.167	4.54	48.449	16.150	9.11	19.967	6.6560	114.75
	Treatment‡	1	0.2690	0.2690	0.10	23.376	23.376	5.84(*)	1.0920	1.0920	0.62	23.652	23.652	407.78**
	residual	*3*	*7.9050*	*2.6350*		*12.007*	*4.0020*		5.3180	1.7730		*0.1740*	*0.0580*	
	Total	7	27.274			89.884			54.859			43.793		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

Table AI.1 –Continued

			$wpns_{coeff}$			$SPAD_{coeff}$			Above ground fwt			Above ground dwt		
	Source of variation	DF	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*
Overall	**Block**	3	0.4266	0.1422	14.15	0.0648	0.0216	8.77	0.5594	0.1865	18.57	0.4734	0.1578	17.43
	Treatment‡	1	4.7660	4.7660	474.18**	0.0138	0.0138	5.60(*)	0.0079	0.0079	0.78	0.0989	0.0989	10.92*
	residual	*3*	0.0302	0.0101	0.30	0.0074	0.0025	0.19	*0.0301*	*0.0100*	*0.23*	*0.0272*	*0.0091*	*0.15*
	Population‡	3	2.0529	0.6843	20.52**	0.2248	0.0749	5.75**	0.1702	0.0567	1.30	0.5511	0.1837	3.02(*)
	Treatment x Population‡	3	0.1189	0.0396	1.19	0.0274	0.0091	0.70	0.0749	0.0250	0.57	0.0950	0.0317	0.52
	residual	*18*	0.6002	0.0334		0.2346	0.0130		*0.7857*	*0.0437*		*1.0957*	*0.0609*	
	Total	31	7.9949			0.5727			1.6281			2.3410		
Aber	**Block**	3	0.4398	0.1466	8.42	0.1765	0.0588	6.70	0.2744	0.0915	5.04	0.3912	0.1304	10.52
	Treatment‡	1	1.5402	1.5402	88.52**	0.0061	0.0061	0.69	0.0130	0.0130	0.72	0.0296	0.0296	2.39
	residual	*3*	*0.0522*	*0.0174*		*0.0263*	*0.0088*		*0.0544*	*0.0181*		*0.0372*	*0.0124*	
	Total	7	2.0322			0.2089			0.3417			0.4579		
Birds Rock	**Block**	3	0.0384	0.0128	0.59	0.0257	0.0086	3.60	0.1016	0.0339	1.20	0.1356	0.0452	13.91
	Treatment‡	1	0.6517	0.6517	29.83*	0.0074	0.0074	3.11	0.0484	0.0484	1.71	0.1481	0.1481	45.58**
	residual	*3*	*0.0656*	*0.0219*		*0.0071*	*0.0024*		*0.0849*	*0.0283*		*0.0098*	*0.0033*	
	Total	7	0.7556			0.0403			0.2349			0.2935		
Cwm Idwal	**Block**	3	0.1385	0.0462	2.64	0.0369	0.0123	15.53	0.4093	0.1364	386.37	0.5257	0.1752	60.80
	Treatment‡	1	1.4803	1.4803	84.65**	0.0066	0.0066	8.35(*)	0.0213	0.0213	60.27**	0.0022	0.0022	0.75
	residual	*3*	*0.0525*	*0.0175*		*0.0024*	*0.0008*		*0.0011*	*0.0004*		*0.0087*	*0.0029*	
	Total	7	1.6713			0.0458			0.4317			0.5365		
Maltraeth	**Block**	3	0.2213	0.0738	4.53	0.0114	0.0038	0.56	0.3500	0.1167	3.52	0.3876	0.1292	3.85
	Treatment‡	1	1.2128	1.2128	74.43**	0.0211	0.0211	3.11	0.0001	0.0001	0.00	0.0140	0.0140	0.42
	residual	*3*	*0.0489*	*0.0163*		*0.0204*	*0.0068*		*0.0995*	*0.0332*		*0.1006*	*0.0335*	
	Total	7	1.4830			0.0529			0.4497			0.5023		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

Table AI.1 –Continued

	Source of variation	DF	Below ground dwt S.S.	Below ground dwt M.S.	Below ground dwt *F value*	Total dwt S.S.	Total dwt M.S.	Total dwt *F value*	Above ground RGR S.S.	Above ground RGR M.S.	Above ground RGR *F value*	Below ground RGR S.S.	Below ground RGR M.S.	Below ground RGR *F value*
Overall	**Block**	3	0.7210	0.2403	2.67	0.4441	0.1480	7.42	0.0140	0.0047	10.87	0.0335	0.0112	2.47
	Treatment‡	1	0.4062	0.4062	4.52	0.1495	0.1495	7.50(*)	0.0048	0.0048	11.12*	0.0220	0.0220	4.86
	residual	*3*	*0.2697*	*0.0899*	*1.24*	*0.0598*	*0.0199*	*0.34*	0.0013	0.0004	0.11	0.0136	0.0045	1.42
	Population‡	3	0.3815	0.1272	1.75	0.4065	0.1355	2.34	0.0163	0.0054	1.39	0.1448	0.0483	15.17**
	Treatment x Population‡	3	0.0552	0.0184	0.25	0.0767	0.0256	0.44	0.0061	0.0020	0.52	0.0025	0.0008	0.26
	residual	*18*	*1.3048*	*0.0725*		*1.0441*	*0.0580*		0.0702	0.0039		0.0573	0.0032	
	Total	31	3.1384			2.1808			0.1126			0.2736		
Aber	**Block**	3	0.9597	0.3199	10.42	0.4731	0.1577	11.26	0.0426	0.0142	106.47	0.0176	0.0059	6.65
	Treatment‡	1	0.2034	0.2034	6.62(*)	0.0479	0.0479	3.42	0.0007	0.0007	5.12	0.0086	0.0086	9.69(*)
	residual	*3*	*0.0921*	*0.0307*		*0.0420*	*0.0140*		*0.0004*	*0.0001*		*0.0027*	*0.0009*	
	Total	7	1.2552			0.5630			0.0437			0.0288		
Birds Rock	**Block**	3	0.3585	0.1195	3.15	0.1651	0.0550	5.23	0.0005	0.0002	0.88	0.0017	0.0006	0.24
	Treatment‡	1	0.1673	0.1673	4.40	0.1543	0.1543	14.67*	0.0093	0.0093	45.58**	0.0105	0.0105	4.40
	residual	*3*	*0.1139*	*0.0380*		*0.0316*	*0.0105*		*0.0006*	*0.0002*		*0.0071*	*0.0024*	
	Total	7	0.6397			0.3509			0.0104			0.0193		
Cwm Idwal	**Block**	3	0.2547	0.0849	2.53	0.3889	0.1296	14.54	0.0155	0.0052	28.63	0.0168	0.0056	2.67
	Treatment‡	1	0.0251	0.0251	0.75	0.0001	0.0001	0.01	0.0001	0.0001	0.75	0.0016	0.0016	0.75
	residual	*3*	*0.1005*	*0.0335*		*0.0267*	*0.0089*		*0.0005*	*0.0002*		*0.0063*	*0.0021*	
	Total	7	0.3804			0.4157			0.0161			0.0247		
Maltraeth	**Block**	3	0.3560	0.1187	5.93	0.3524	0.1175	5.17	0.0193	0.0064	3.16	0.0485	0.0162	13.20
	Treatment‡	1	0.0657	0.0657	3.28	0.0240	0.0240	1.06	0.0008	0.0008	0.37	0.0039	0.0039	3.18
	residual	*3*	*0.0600*	*0.0200*		*0.0682*	*0.0227*		*0.0061*	*0.0020*		*0.0037*	*0.0012*	
	Total	7	0.4817			0.4446			0.0261			0.0561		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

Table AI.1 –Continued

			Total RGR			BG:AG ratio			dwt:fwt ratio		
	Source of variation	DF	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*
Overall	**Block**	3	0.0169	0.0057	6.47	0.0559	0.0187	1.41	0.0040	0.0013	1.43
	Treatment‡	1	0.0075	0.0075	8.60(*)	0.0095	0.0095	0.72	0.0021	0.0021	2.27
	residual	*3*	0.0026	0.0009	0.26	0.0398	0.0133	4.92	0.0028	0.0009	0.77
	Population‡	3	0.0405	0.0135	4.10*	0.0753	0.0251	9.32**	0.0339	0.0113	9.42**
	Treatment x Population‡	3	0.0048	0.0016	0.49	0.0017	0.0006	0.22	0.0005	0.0002	0.13
	residual	*18*	0.0593	0.0033		0.0485	0.0027		0.0216	0.0012	
	Total	31	0.1318			0.2308			0.0649		
Aber	**Block**	3	0.0299	0.0100	45.20	0.0165	0.0055	3.16	0.0125	0.0042	3.05
	Treatment‡	1	0.0014	0.0014	6.11(*)	0.0033	0.0033	1.89	0.0000	0.0000	0.01
	residual	*3*	*0.0007*	*0.0002*		*0.0052*	*0.0017*		*0.0041*	*0.0014*	
	Total	7	0.0319			0.0250			0.0166		
Birds Rock	**Block**	3	0.0005	0.0002	0.27	0.0190	0.0063	1.15	0.0028	0.0009	0.66
	Treatment‡	1	0.0096	0.0096	14.67	0.0002	0.0002	0.04	0.0009	0.0009	0.61
	residual	*3*	*0.0020*	*0.0007*		*0.0165*	*0.0055*		*0.0043*	*0.0014*	
	Total	7	0.0122			0.0357			0.0079		
Cwm Idwal	**Block**	3	0.0149	0.0050	8.89	0.0434	0.0145	3.08	0.0019	0.0006	2.92
	Treatment‡	1	0.0000	0.0000	0.01	0.0051	0.0051	1.09	0.0006	0.0006	2.89
	residual	*3*	*0.0017*	*0.0006*		*0.0141*	*0.0047*		*0.0006*	*0.0002*	
	Total	7	0.0165			0.0626			0.0031		
Maltraeth	**Block**	3	0.0253	0.0084	6.16	0.0119	0.0040	0.67	0.0016	0.0005	2.35
	Treatment‡	1	0.0014	0.0014	0.99	0.0026	0.0026	0.44	0.0011	0.0011	4.91
	residual	*3*	*0.0041*	*0.0014*		*0.0177*	*0.0059*		*0.0007*	*0.0002*	
	Total	7	0.0307			0.0322			0.0033		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

Table AII.1 –Details of overall ANOVA analysis results for Rural ozone exposure described in Chapter 3.

		wpns week 3			wpns week 5			wpns week 8			wpns week 11		
Source of variation	**DF**	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***
Block	1	0.0078	0.0078	3.05	0.0032	0.0032	0.71	0.0169	0.0169	9.57	0.0072	0.0072	2.26
Background‡	1	0.0985	0.0985	38.39**	0.0897	0.0897	20.03*	0.0281	0.0281	15.89*	0.0193	0.0193	6.05(*)
Peak‡	1	0.0257	0.0257	10.00(*)	0.0229	0.0229	5.12	0.0034	0.0034	1.92	0.0309	0.0309	9.66(*)
Background x Peak‡	1	0.0022	0.0022	0.85	0.0002	0.0002	0.05	0.0105	0.0105	5.93(*)	0.0013	0.0013	0.40
residual	*3*	*0.0077*	*0.0026*	*4.53*	*0.0134*	*0.0045*	*6.09*	*0.0053*	*0.0018*	*1.03*	*0.0096*	*0.0032*	*4.42*
Population‡	3	0.0801	0.0267	47.15**	0.1996	0.0665	90.51**	0.4842	0.1614	93.78**	0.2310	0.0770	106.59**
Background x Population‡	3	0.0047	0.0016	2.75(*)	0.0016	0.0005	0.72	0.0033	0.0011	0.64	0.0126	0.0042	5.8*
Peak x Population‡	3	0.0024	0.0008	1.38	0.0005	0.0002	0.23	0.0045	0.0015	0.86	0.0030	0.0010	1.36
Background x Peak x Population‡	3	0.0032	0.0011	1.90	0.0102	0.0034	4.64*	0.0048	0.0016	0.93	0.0012	0.0004	0.53
residual	*12*	*0.0068*	*0.0006*		*0.0088*	*0.0007*		*0.0207*	*0.0017*		*0.0087*	*0.0007*	
Total	31	0.2391			0.3502			0.5816			0.3247		

		Leaf 2 senescence week 2			Leaf 2 senescence week 4			Leaf 2 senescence week 6			Leaf 2 senescence week 8		
Source of variation	**DF**	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***
Block	1	0.0028	0.0028	1.59	0.0399	0.0399	9.33	0.0090	0.0090	0.70	0.0250	0.0250	0.64
Background‡	1	0.0015	0.0015	0.84	0.0039	0.0039	0.91	0.0004	0.0004	0.03	0.0288	0.0288	0.73
Peak‡	1	0.0121	0.0121	6.88(*)	0.0010	0.0010	0.24	0.0140	0.0140	1.09	0.0215	0.0215	0.55
Background x Peak‡	1	0.0500	0.0500	28.53*	0.0047	0.0047	1.09	0.0121	0.0121	0.93	0.0078	0.0078	0.20
residual	*3*	*0.0053*	*0.0018*	*1.37*	*0.0128*	*0.0043*	*1.57*	*0.0388*	*0.0129*	*1.95*	*0.1180*	*0.0393*	*3.50*
Population‡	3	0.0370	0.0123	9.65**	0.1256	0.0419	15.40**	0.6749	0.2250	33.86**	1.1302	0.3767	33.53**
Background x Population‡	3	0.0036	0.0012	0.95	0.0032	0.0011	0.39	0.0085	0.0028	0.42	0.0069	0.0023	0.20
Peak x Population‡	3	0.0089	0.0030	2.33	0.0060	0.0020	0.73	0.0487	0.0162	2.44	0.1156	0.0385	3.43(*)
Background x Peak x Population‡	3	0.0036	0.012	0.94	0.0378	0.0126	4.64*	0.0119	0.0040	0.60	0.0047	0.0016	0.14
residual	*12*	*0.0154*	*0.0013*		*0.0326*	*0.0027*		*0.0797*	*0.0066*		*0.1348*	*0.0112*	
Total	31	0.1402			0.2675			0.8980			1.5732		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

Table AII.1 –Continued

Source of variation	DF	Leaf 2 senescence week 10			Leaf 2 senescence week 12			Leaf 3 senescence week 4			Leaf 3 senescence week 6		
		S.S.	M.S.	*F value*	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*
Block	1	0.0355	0.0355	2.21	0.0818	0.0818	2.29	0.0184	0.0184	9.47	0.0361	0.0361	1.57
Background‡	1	0.0845	0.0845	5.27	0.0441	0.0441	1.24	0.0439	0.0439	22.60*	0.0455	0.0455	1.98
Peak‡	1	0.1696	0.1696	10.57*	0.0504	0.0504	1.41	0.0579	0.0579	29.81*	0.0609	0.0609	2.66
Background x Peak‡	1	0.0039	0.0039	0.25	0.0216	0.0216	0.60	0.0164	0.0164	8.44(*)	0.0112	0.0112	0.49
residual	*3*	*0.0482*	*0.0161*	*1.64*	*0.1071*	*0.0357*	*6.15*	*0.0058*	*0.0019*	*0.37*	*0.0688*	*0.0229*	*3.18*
Population‡	3	0.7394	0.2465	25.18**	0.9442	0.3147	54.23**	0.2846	0.0949	18.20**	1.2145	0.4048	56.21**
Background x Population‡	3	0.1291	0.0430	4.40*	0.0324	0.0108	1.86	0.0516	0.0172	3.30(*)	0.0418	0.0139	1.93
Peak x Population‡	3	0.0234	0.0078	0.80	0.0316	0.0105	1.82	0.0284	0.0095	1.81	0.0368	0.0123	1.70
Background x Peak x Population‡	3	0.0146	0.0049	0.50	0.0143	0.0048	0.82	0.0227	0.0076	1.45	0.0100	0.0033	0.46
residual	*12*	*0.1175*	*0.0098*		*0.0696*	*0.0058*		*0.0625*	*0.0052*		*0.0864*	*0.0072*	
Total	31	1.3657			1.3971			0.5921			1.6120		

Source of variation	DF	Leaf 3 senescence week 8			Leaf 3 senescence week 10			Leaf 3 senescence week 12		
		S.S.	M.S.	*F value*	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*
Block	1	0.0072	0.0072	0.37	0.0132	0.0132	2.69	0.0064	0.0064	0.24
Background‡	1	0.0104	0.0104	0.53	0.0551	0.0551	11.22*	0.0891	0.0891	3.26
Peak‡	1	0.1048	0.1048	5.40	0.1373	0.1373	27.98*	0.0953	0.0953	3.49
Background x Peak‡	1	0.0070	0.0070	0.36	0.0114	0.0114	2.32	0.0039	0.0039	0.14
residual	*3*	*0.0582*	*0.0194*	*1.51*	*0.0147*	*0.0049*		*0.0820*	*0.0273*	*5.79*
Population‡	3	1.5395	0.5132	39.93**	0.8150	0.2717	32.91**	0.6432	0.2144	45.39**
Background x Population‡	3	0.0198	0.0066	0.51	0.1191	0.0397	4.81*	0.0318	0.0106	2.24
Peak x Population‡	3	0.0146	0.0049	0.38	0.0067	0.0022	0.27	0.0179	0.0060	1.26
Background x Peak x Population‡	3	0.0691	0.0230	1.79	0.0570	0.0190	2.30	0.0146	0.0049	1.03
residual	*12*	*0.1542*	*0.0129*		*0.0991*	*0.0083*		*0.0567*	*0.0047*	
Total	31	1.9848			1.3284			1.0410		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

Table AII.1 –Continued

Source of variation	DF	Above ground dwt			R_{till}			Tiller number		
		S.S.	M.S.	*F value*	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*
Block	1	0.0004	0.0004	0.02	0.00000	0.00000	0.13	8.2100	8.2100	17.87
Background‡	1	0.0003	0.0003	0.02	0.00000	0.00000	0.34	22.190	22.190	48.34**
Peak‡	1	0.0005	0.0005	0.03	0.00000	0.00000	0.21	0.7300	0.7300	1.59
Background x Peak‡	1	0.0275	0.0275	1.62	0.00000	0.00000	0.03	0.0400	0.0400	0.09
residual	*3*	*0.0509*	*0.0170*	*10.06*	0.00003	0.00001	*1.87*	*1.3800*	*0.4600*	*0.02*
Population‡	3	0.2080	0.0693	41.14**	0.00859	0.00286	539.57**	9738.1	3246.0	151.91**
Background x Population‡	3	0.0049	0.0016	0.97	0.00001	0.00000	0.61	2.8500	0.9500	0.04
Peak x Population‡	3	0.0012	0.0004	0.24	0.00003	0.00001	1.88	6.9600	2.3200	0.11
Background x Peak x Population‡	3	0.0199	0.0066	3.94*	0.00006	0.00002	3.80*	48.390	16.130	0.75
residual	*12*	*0.0202*	*0.0017*		*0.00006*	*0.00001*		*256.42*	*21.370*	
Total	31	0.3338			0.00879			10085.3		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

Table AII.2 –Details of population level ANOVA analysis results for Rural ozone exposure described in Chapter 3.

			wpns week 3			wpns week 5			wpns week 8			wpns week 11		
Population	**Source of variation**	**DF**	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***
Aber	**Block**	1	0.0068	0.0068	5.68	0.0002	0.0002	0.11	0.0046	0.0046	3.76	0.0015	0.0015	2.71
	Background‡	1	0.0296	0.0296	24.74*	0.0221	0.0221	13.63*	0.0150	0.0150	12.26*	0.0128	0.0128	23.32*
	Peak‡	1	0.0020	0.0020	1.66	0.0037	0.0037	2.27	0.0001	0.0001	0.08	0.0018	0.0018	3.30
	Background x Peak‡	1	0.0001	0.0001	0.11	0.0012	0.0012	0.71	0.0033	0.0033	2.70	0.0007	0.0007	1.25
	residual	*3*	*0.0036*	*0.0012*		*0.0049*	*0.0016*		*0.0037*	*0.0012*		*0.0017*	*0.0006*	
	Total	7	0.0420			0.0320			0.0267			0.0185		
Birds Rock	**Block**	1	0.0024	0.0024	3.25	0.0013	0.0013	1.87	0.0027	0.0027	0.81	0.0002	0.0002	0.27
	Background‡	1	0.0117	0.0117	15.82*	0.0156	0.0156	22.61*	0.0066	0.0066	1.97	0.0043	0.0043	5.49
	Peak‡	1	0.0075	0.0075	10.15*	0.0065	0.0065	9.39(*)	0.0023	0.0023	0.68	0.0087	0.0087	11.00*
	Background x Peak‡	1	0.0001	0.0001	0.12	0.0038	0.0038	5.45	0.0108	0.0108	3.21	0.0014	0.0014	1.80
	residual	*3*	*0.0022*	*0.0007*		*0.0021*	*0.0007*		*0.0101*	*0.0034*		*0.0024*	*0.0008*	
	Total	7	0.0239			0.0291			0.0325			0.0170		
Cwm Idwal	**Block**	1	0.0025	0.0025	2.62	0.0042	0.0042	2.07	0.0020	0.0020	3.12	0.0048	0.0048	4.03
	Background‡	1	0.0404	0.0404	42.46**	0.0325	0.0325	15.90*	0.0080	0.0080	12.29*	0.0143	0.0143	12.12*
	Peak‡	1	0.0126	0.0126	13.25*	0.0050	0.0050	2.44	0.0000	0.0000	0.04	0.0103	0.0103	8.72(*)
	Background x Peak‡	1	0.0005	0.0005	0.56	0.0001	0.0001	0.04	0.0011	0.0011	1.65	0.0003	0.0003	0.22
	residual	*3*	*0.0029*	*0.0010*		*0.0061*	*0.0021*		*0.0020*	*0.0007*		*0.0036*	*0.0012*	
	Total	7	0.0589			0.0479			0.0131			0.0332		
Maltraeth	**Block**	1	0.0000	0.0000	0.03	0.0007	0.0007	0.33	0.0090	0.0090	3.09	0.0023	0.0023	0.74
	Background‡	1	0.0215	0.0215	33.10*	0.0211	0.0211	10.50*	0.0018	0.0018	0.60	0.0004	0.0004	0.14
	Peak‡	1	0.0059	0.0059	9.12(*)	0.0083	0.0083	4.13	0.0054	0.0054	1.86	0.0130	0.0130	4.25
	Background x Peak‡	1	0.0047	0.0047	7.17(*)	0.0055	0.0055	2.73	0.0001	0.0001	0.04	0.0001	0.0001	0.02
	residual	*3*	*0.0020*	*0.0007*		*0.0060*	*0.0020*		*0.0088*	*0.0029*		*0.0092*	*0.0031*	
	Total	7	0.0341			0.0415			0.0251			0.0249		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

Table AII.2 – Continued.

Population	Source of variation	DF	Leaf 2 senescence week 2			Leaf 2 senescence week 4			Leaf 2 senescence week 6			Leaf 2 senescence week 8		
			S.S.	M.S.	*F value*	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*
Aber	**Block**	1	0.0017	0.0017	1.53	0.0030	0.0030	1.15	0.0000	0.0000	0.00	0.0003	0.0003	0.06
	Background‡	1	0.0005	0.0005	0.45	0.0059	0.0059	2.24	0.0063	0.0063	0.53	0.0054	0.0054	0.99
	Peak‡	1	0.0117	0.0117	10.68*	0.0001	0.0001	0.05	0.0049	0.0049	0.41	0.0236	0.0236	4.32
	Background x Peak‡	1	0.0248	0.0248	22.59*	0.0005	0.0005	0.17	0.0000	0.0000	0.00	0.0004	0.0004	0.07
	residual	*3*	*0.0033*	*0.0011*		*0.0078*	*0.0026*		*0.0360*	*0.0120*		*0.0164*	*0.0055*	
	Total	7	0.0419			0.0173			0.0472			0.0461		
Birds Rock	**Block**	1	0.0005	0.0005	0.12	0.0267	0.0267	4.10	0.0097	0.0097	3.48	0.0385	0.0385	1.94
	Background‡	1	0.0007	0.0007	0.19	0.0001	0.0001	0.02	0.0003	0.0003	0.10	0.0006	0.0006	0.03
	Peak‡	1	0.0013	0.0013	0.33	0.0009	0.0009	0.13	0.0141	0.0141	5.09	0.0471	0.0471	2.37
	Background x Peak‡	1	0.0054	0.0054	1.42	0.0155	0.0155	2.38	0.0056	0.0056	2.01	0.0000	0.0000	0.00
	residual	*3*	*0.0113*	*0.0038*		*0.0195*	*0.0065*		*0.0083*	*0.0028*		*0.0595*	*0.0198*	
	Total	7	0.0191			0.0627			0.0380			0.1457		
Cwm Idwal	**Block**	1	0.0009	0.0009	0.55	0.0098	0.0098	7.25	0.0023	0.0023	0.39	0.0007	0.0007	0.04
	Background‡	1	0.0038	0.0038	2.43	0.0000	0.0000	0.01	0.0021	0.0021	0.35	0.0183	0.0183	0.96
	Peak‡	1	0.0079	0.0079	5.08	0.0045	0.0045	3.37	0.0111	0.0111	1.84	0.0198	0.0198	1.04
	Background x Peak‡	1	0.0121	0.0121	7.78(*)	0.0144	0.0144	10.64*	0.0183	0.0183	3.05	0.0034	0.0034	0.18
	residual	*3*	*0.0047*	*0.0016*		*0.0041*	*0.0014*		*0.0180*	*0.0060*		*0.0572*	*0.0191*	
	Total	7	0.0293			0.0327			0.0519			0.0995		
Maltraeth	**Block**	1	0.0002	0.0002	0.61	0.0068	0.0068	2.65	0.0022	0.0022	0.13	0.0123	0.0123	0.40
	Background‡	1	0.0001	0.0001	0.36	0.0011	0.0011	0.42	0.0001	0.0001	0.01	0.0114	0.0114	0.37
	Peak‡	1	0.0002	0.0002	0.51	0.0015	0.0015	0.57	0.0326	0.0326	1.92	0.0466	0.0466	1.50
	Background x Peak‡	1	0.0114	0.0114	36.57**	0.0122	0.0122	4.79	0.0001	0.0001	0.01	0.0086	0.0086	0.28
	residual	*3*	*0.0009*	*0.0003*		*0.0076*	*0.0026*		*0.0510*	*0.0170*		*0.0929*	*0.0310*	
	Total	7	0.0128			0.0291			0.0860			0.1718		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

Table AII.2 – Continued.

Population	Source of variation	DF	Leaf 2 senescence week 10			Leaf 2 senescence week 12			Leaf 3 senescence week 4			Leaf 3 senescence week 6		
			S.S.	M.S.	*F value*	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*
Aber	**Block**	1	0.0042	0.0042	0.69	0.0177	0.0177	2.34	0.0004	0.0004	0.10	0.0002	0.0002	0.02
	Background‡	1	0.1106	0.1106	18.03*	0.0053	0.0053	0.70	0.0002	0.0002	0.07	0.0015	0.0015	0.16
	Peak‡	1	0.0886	0.0886	14.44*	0.0016	0.0016	0.21	0.0001	0.0001	0.03	0.0346	0.0346	3.66
	Background x Peak‡	1	0.0012	0.0012	0.19	0.0000	0.0000	0.00	0.0031	0.0031	0.85	0.0055	0.0055	0.58
	residual	*3*	*0.0184*	*0.0061*		*0.0228*	*0.0228*		*0.0108*	*0.0036*		*0.0283*	*0.0094*	
	Total	7	0.2230			0.0474			0.0146			0.0701		
Birds Rock	**Block**	1	0.0195	0.0195	2.26	0.0446	0.0446	6.31	0.0150	0.0150	4.51	0.0092	0.0092	1.36
	Background‡	1	0.0119	0.0119	1.37	0.0006	0.0006	0.08	0.0133	0.0133	4.00	0.0013	0.0013	0.19
	Peak‡	1	0.0502	0.0502	5.81(*)	0.0329	0.0329	4.65	0.0178	0.0178	5.35	0.0089	0.0089	1.32
	Background x Peak‡	1	0.0150	0.0150	1.74	0.0085	0.0085	1.20	0.0332	0.0332	9.97(*)	0.0138	0.0138	2.03
	residual	*3*	*0.0259*	*0.0086*		*0.0212*	*0.0071*		*0.0100*	*0.0033*		*0.0204*	*0.0068*	
	Total	7	0.1225			0.1078			0.0893			0.0536		
Cwm Idwal	**Block**	1	0.0154	0.0154	1.03	0.0374	0.0374	1.19	0.0079	0.0079	3.15	0.0100	0.0100	1.06
	Background‡	1	0.0873	0.0873	5.82(*)	0.0663	0.0663	2.10	0.0807	0.0807	32.30*	0.0796	0.0796	8.41(*)
	Peak‡	1	0.0073	0.0073	0.49	0.0208	0.0208	0.66	0.0598	0.0598	23.95*	0.0004	0.0004	0.04
	Background x Peak‡	1	0.0023	0.0023	0.15	0.0014	0.0014	0.05	0.0008	0.0008	0.30	0.0004	0.0004	0.04
	residual	*3*	*0.0450*	*0.0150*		*0.0945*	*0.0315*		*0.0075*	*0.0025*		*0.0284*	*0.0095*	
	Total	7	0.1572			0.2205			0.1566			0.1187		
Maltraeth	**Block**	1	0.0023	0.0023	0.10	0.0012	0.0012	0.18	0.0016	0.0016	0.15	0.0286	0.0286	1.30
	Background‡	1	0.0039	0.0039	0.17	0.0044	0.0044	0.69	0.0012	0.0012	0.11	0.0050	0.0050	0.22
	Peak‡	1	0.0470	0.0470	2.00	0.0267	0.0267	4.18	0.0085	0.0085	0.76	0.0538	0.0538	2.44
	Background x Peak‡	1	0.0001	0.0001	0.00	0.0259	0.0259	4.06	0.0021	0.0021	0.19	0.0017	0.0017	0.07
	residual	*3*	*0.0704*	*0.0235*		*0.0192*	*0.0064*		*0.0335*	*0.0112*		*0.0661*	*0.0220*	
	Total	7	0.1236			0.0773			0.0470			0.1552		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

Table AII.2 – Continued.

Population	Source of variation	DF	Leaf 3 senescence week 8			Leaf 3 senescence week 10			Leaf 3 senescence week 12		
			S.S.	M.S.	*F value*	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*
Aber	**Block**	1	0.0001	0.0001	0.02	0.0013	0.0013	0.72	0.0033	0.0033	0.50
	Background‡	1	0.0000	0.0000	0.00	0.0372	0.0372	20.72*	0.0042	0.0042	0.65
	Peak‡	1	0.0496	0.0496	10.49*	0.0169	0.0169	9.42(*)	0.0059	0.0059	0.91
	Background x Peak‡	1	0.0031	0.0031	0.66	0.0000	0.0000	0.00	0.0005	0.0005	0.08
	residual	*3*	*0.0142*	*0.0047*		*0.0054*	*0.0018*		*0.0195*	*0.0065*	
	Total	7	0.0671			0.0608			0.0334		
Birds Rock	**Block**	1	0.0022	0.0022	0.19	0.0438	0.0438	4.51	0.0126	0.0126	0.61
	Background‡	1	0.0001	0.0001	0.01	0.0078	0.0078	0.80	0.0091	0.0091	0.44
	Peak‡	1	0.0040	0.0040	0.36	0.0268	0.0268	2.76	0.0653	0.0653	3.15
	Background x Peak‡	1	0.0208	0.0208	1.84	0.0019	0.0019	0.19	0.0179	0.0179	0.86
	residual	*3*	*0.0339*	*0.0113*		*0.0291*	*0.0097*		*0.0623*	*0.0208*	
	Total	7	0.0610			0.1094			0.1671		
Cwm Idwal	**Block**	1	0.0058	0.0058	0.21	0.0001	0.0001	0.01	0.0009	0.0009	0.09
	Background‡	1	0.0297	0.0297	1.04	0.1291	0.1291	11.63*	0.0878	0.0878	9.43(*)
	Peak‡	1	0.0272	0.0272	0.96	0.0459	0.0459	4.14	0.0284	0.0284	3.05
	Background x Peak‡	1	0.0514	0.0514	1.81	0.0665	0.0665	5.99(*)	0.0001	0.0001	0.01
	residual	*3*	*0.0853*	*0.0284*		*0.0333*	*0.0111*		*0.0279*	*0.0093*	
	Total	7	0.1995			0.2749			0.1452		
Maltraeth	**Block**	1	0.0441	0.0441	3.89	0.0000	0.0000	0.01	0.0015	0.0015	0.26
	Background‡	1	0.0004	0.0004	0.04	0.0000	0.0000	0.01	0.0198	0.0198	3.46
	Peak‡	1	0.0385	0.0385	3.39	0.0544	0.0544	11.67*	0.0136	0.0136	2.38
	Background x Peak‡	1	0.0009	0.0009	0.08	0.0000	0.0000	0.00	0.0000	0.0000	0.0
	residual	*3*	*0.0340*	*0.0113*		*0.0140*	*0.0047*		*0.0172*	*0.0057*	
	Total	7	0.1179			0.0684			0.0520		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

Table AII.2 – Continued.

Population	Source of variation	DF	Above ground dwt S.S.	Above ground dwt M.S.	Above ground dwt *F value*	R_{till} S.S.	R_{till} M.S.	R_{till} *F value*	Tiller number S.S.	Tiller number M.S.	Tiller number *F value*
Aber	**Block**	1	0.0009	0.0009	0.22	0.00001	0.00001	2.88	12.087	12.087	16.49
	Background‡	1	0.0011	0.0011	0.26	0.00000	0.00000	0.68	2.2874	2.2874	3.12
	Peak‡	1	0.0000	0.0000	0.00	0.00000	0.00000	0.24	0.2041	0.2041	0.28
	Background x Peak‡	1	0.0233	0.0233	5.85(*)	0.00001	0.00001	1.89	2.5313	2.5313	3.45
	residual	*3*	*0.0120*	*0.0040*		*0.00001*	*0.00000*		*2.1987*	*0.7329*	
	Total	7	0.0372			0.00003			19.308		
Birds Rock	**Block**	1	0.0005	0.0005	0.12	0.00002	0.00002	4.32	63.850	63.850	1.58
	Background‡	1	0.0013	0.0013	0.31	0.00000	0.00000	0.06	2.7600	2.7600	0.07
	Peak‡	1	0.0001	0.0001	0.03	0.00000	0.00000	0.82	0.0100	0.0100	0.00
	Background x Peak‡	1	0.0128	0.0128	3.03	0.00000	0.00000	0.39	19.220	19.220	0.48
	residual	*3*	*0.0126*	*0.0042*		*0.00002*	*0.00001*		*121.13*	*40.380*	
	Total	7	0.0273			0.00005			206.96		
Cwm Idwal	**Block**	1	0.0002	0.0002	0.04	0.00000	0.00000	0.57	0.1000	0.1000	0.01
	Background‡	1	0.0002	0.0002	0.04	0.00001	0.00001	0.77	13.060	13.060	0.69
	Peak‡	1	0.0000	0.0000	0.00	0.00003	0.00003	3.87	7.1400	7.1400	0.38
	Background x Peak‡	1	0.0102	0.0102	2.13	0.00000	0.00000	0.52	2.0000	2.0000	0.11
	residual	*3*	*0.0143*	*0.0048*		*0.00002*	*0.00001*		*56.850*	*18.950*	
	Total	7	0.0249			0.00006			79.150		
Maltraeth	**Block**	1	0.0004	0.0004	0.04	0.00000	0.00000	0.74	0.8130	0.8130	0.27
	Background‡	1	0.0026	0.0026	0.25	0.00001	0.00001	1.81	6.9380	6.9380	2.32
	Peak‡	1	0.0016	0.0016	0.16	0.00000	0.00000	0.29	0.3400	0.3400	0.11
	Background x Peak‡	1	0.0012	0.0012	0.12	0.00005	0.00005	16.19*	24.675	24.675	8.24(*)
	residual	*3*	*0.0306*	*0.0102*		*0.00001*	*0.00000*		*8.9810*	*2.9940*	
	Total	7	0.0364			0.00007			41.747		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

Table AIII.1 - Details of overall ANOVA analysis results for Upland ozone exposure described in Chapter 3.

		wpns week 1			wpns week 2			wpns week 3			wpns week 4		
Source of variation	**DF**	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***
Block	1	0.0029	0.0029	2.6	0.0031	0.0031	0.74	0.0221	0.0221	6.33	0.0139	0.0139	10.36
Treatment‡	3	0.0067	0.0022	2.0	0.0513	0.0171	4.1	0.1493	0.0498	14.28*	0.1925	0.0642	47.86**
linear‡	*1*	*0.0053*	*0.0053*	*4.73*	*0.0384*	*0.0384*	*9.18(*)*	*0.1452*	*0.1452*	*41.68***	*0.1791*	*0.1791*	*133.65***
deviations‡	*2*	*0.0014*	*0.0007*	*0.64*	*0.0130*	*0.0065*	*1.55*	*0.0041*	*0.0021*	*0.59*	*0.0133*	*0.0067*	*4.97*
residual	*3*	*0.0033*	*0.0011*	*0.5*	*0.0125*	*0.0042*	*2.06*	*0.0105*	*0.0035*	*2.06*	*0.0040*	*0.0013*	*0.57*
Population‡	3	0.0076	0.0025	1.15	0.0253	0.0084	4.16*	0.0463	0.0155	9.15**	0.0283	0.0094	4.02*
Treatment x Population‡	9	0.0298	0.0025	1.49	0.0606	0.0067	3.32*	0.0365	0.0041	2.4(*)	0.0916	0.0102	4.33*
linear x population‡	*3*	*0.0093*	*0.0031*	*1.39*	*0.0423*	*0.0141*	*6.96***	*0.0174*	*0.0058*	*3.43(*)*	*0.0247*	*0.0082*	*3.5**
deviations‡	*6*	*0.0205*	*0.0034*	*1.54*	*0.0183*	*0.0031*	*1.51*	*0.0191*	*0.0032*	*1.88*	*0.0669*	*0.0111*	*4.74**
residual	*12*	*0.0266*	*0.0022*		*0.0243*	*0.0020*		*0.0203*	*0.0017*		*0.0282*	*0.0024*	
Total	31	0.0768			0.1772			0.2848			0.3584		

		wpns week 5			wpns week 6			wpns week 7			wpns week 8		
Source of variation	**DF**	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***
Block	1	0.0495	0.0495	17.16	0.0282	0.0282	13.64	0.0161	0.0161	130.88	0.0001	0.0001	0.03
Treatment‡	3	0.1333	0.0444	15.41*	0.2329	0.0776	37.58**	0.2835	0.0945	766.63**	0.1832	0.0611	15.93*
linear‡	*1*	*0.1284*	*0.1284*	*44.54***	*0.2286*	*0.2286*	*110.66***	*0.2808*	*0.2808*	*2278.34***	*0.1823*	*0.1823*	*47.55***
deviations‡	*2*	*0.0049*	*0.0024*	*0.84*	*0.0043*	*0.0022*	*1.05*	*0.0027*	*0.0013*	*10.77**	*0.0009*	*0.0005*	*0.12*
residual	*3*	*0.0087*	*0.0029*	*1.14*	*0.0062*	*0.0021*	*0.38*	*0.0004*	*0.0001*	*0.02*	*0.0115*	*0.0038*	*0.68*
Population‡	3	0.0811	0.0270	10.7**	0.0492	0.0164	3.04(*)	0.0358	0.0119	1.94	0.0302	0.0101	1.78
Treatment x Population‡	9	0.0529	0.0059	2.33(*)	0.0396	0.0044	0.82	0.0295	0.0033	0.53	0.0288	0.0032	0.57
linear x population‡	*3*	*0.0215*	*0.0072*	*2.84(*)*	*0.0064*	*0.0021*	*0.4*	*0.0066*	*0.0022*	*0.36*	*0.0062*	*0.0021*	*0.37*
deviations‡	*6*	*0.0314*	*0.0052*	*2.07*	*0.0332*	*0.0055*	*1.03*	*0.0229*	*0.0038*	*0.62*	*0.0226*	*0.0038*	*0.67*
residual	*12*	*0.0303*	*0.0025*		*0.0646*	*0.0054*		*0.0738*	*0.0062*		*0.0677*	*0.0056*	
Total	31	0.3556			0.4207			0.4391			0.3215		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

Table AIII.1 - Continued.

Source of variation	DF	$wpns_{coeff}$ S.S.	$wpns_{coeff}$ M.S.	$wpns_{coeff}$ *F value*	Above ground fwt S.S.	Above ground fwt M.S.	Above ground fwt *F value*	Above ground dwt S.S.	Above ground dwt M.S.	Above ground dwt *F value*	Relative growth rate S.S.	Relative growth rate M.S.	Relative growth rate *F value*
Block	1	0.0204	0.0204	10.14	0.1745	0.1745	7.43	0.0920	0.0920	8.17	0.0014	0.0014	23.21
Treatment‡	3	0.6841	0.2280	113.48**	0.0088	0.0029	0.12	0.0348	0.0116	1.03	0.0001	0.0000	0.53
linear‡	*1*	*0.6747*	*0.6747*	*335.77***	*0.0075*	*0.0075*	*0.32*	*0.0326*	*0.0326*	*2.89*	*0.0000*	*0.0000*	*0.53*
deviations‡	*2*	*0.0094*	*0.0047*	*2.34*	*0.0013*	*0.0006*	*0.03*	*0.0022*	*0.0011*	*0.1*	*0.0001*	*0.0000*	*0.53*
residual	*3*	*0.0060*	*0.0020*	*0.12*	*0.0705*	*0.0235*	*0.77*	*0.0338*	*0.0113*	*0.51*	*0.0002*	*0.0001*	*0.2*
Population‡	3	0.1811	0.0604	3.63*	0.5703	0.1901	6.26**	0.8860	0.2953	13.42**	0.0025	0.0008	2.83(*)
Treatment x Population‡	9	0.0549	0.0061	0.37	0.2336	0.0260	0.85	0.2076	0.0231	1.05	0.0021	0.0002	0.79
linear x population‡	*3*	*0.0104*	*0.0035*	*0.21*	*0.0488*	*0.0163*	*0.54*	*0.0533*	*0.0178*	*0.81*	*0.0005*	*0.0002*	*0.55*
deviations‡	*6*	*0.0445*	*0.0074*	*0.45*	*0.1848*	*0.0308*	*1.01*	*0.1543*	*0.0257*	*1.17*	*0.0016*	*0.0003*	*0.91*
residual	*12*	*0.1997*	*0.0166*		*0.3647*	*0.0304*		*0.2641*	*0.0220*		*0.0035*	*0.0003*	
Total	31	1.1462			1.4224			1.5183			0.0097		

Source of variation	DF	dwt:fwt ratio S.S.	dwt:fwt ratio M.S.	dwt:fwt ratio *F value*
Block	1	0.0019	0.0019	2.79
Treatment‡	3	0.0015	0.0005	0.73
linear‡	*1*	*0.0010*	*0.0010*	*1.38*
deviations‡	*2*	*0.0006*	*0.0003*	*0.41*
residual	*3*	*0.0021*	*0.0007*	*1.17*
Population‡	3	0.0107	0.0036	6.0*
Treatment x Population‡	9	0.0015	0.0002	0.28
linear x population‡	*3*	*0.0003*	*0.0001*	*0.17*
deviations‡	*6*	*0.0012*	*0.0002*	*0.33*
residual	*12*	*0.0071*	*0.0006*	
Total	31	0.0248		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

Table AIII.2 - Details of population level ANOVA analysis results for Upland ozone exposure described in Chapter 3.

			wpns week 1			wpns week 2			wpns week 3			wpns week 4		
Population	**Source of variation**	**DF**	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***	**S.S.**	**M.S.**	***F value***
Aber	**Block**	1	0.0001	0.0001	0.09	0.0002	0.0002	0.07	0.0009	0.0009	0.36	0.0009	0.0009	0.25
	Treatment‡	3	0.0017	0.0006	0.40	0.0445	0.0148	5.24	0.0612	0.0204	8.11(*)	0.1036	0.0345	9.28(*)
	linear‡	*1*	*0.0016*	*0.0016*	*1.18*	*0.0381*	*0.0381*	*13.46**	*0.0587*	*0.0587*	*23.32**	*0.0870*	*0.0870*	*23.37**
	deviations‡	*2*	*0.0000*	*0.0000*	*0.01*	*0.0064*	*0.0032*	*1.13*	*0.0026*	*0.0013*	*0.51*	*0.0167*	*0.0083*	*2.24*
	residual	*3*	*0.0041*	*0.0014*		*0.0085*	*0.0028*		*0.0076*	*0.0025*		*0.0112*	*0.0037*	
	Total	7	0.0059			0.0531			0.0697			0.1157		
Birds Rock	**Block**	1	0.0080	0.0080	3.35	0.0038	0.0038	1.43	0.0103	0.0103	11.04	0.0072	0.0072	6.88
	Treatment‡	3	0.0114	0.0038	1.59	0.0186	0.0062	2.33	0.0176	0.0059	6.26(*)	0.0464	0.0155	14.82*
	linear‡	*1*	*0.0023*	*0.0023*	*0.97*	*0.0015*	*0.0015*	*0.57*	*0.0137*	*0.0137*	*14.62**	*0.0274*	*0.0274*	*26.26**
	deviations‡	*2*	*0.0091*	*0.0045*	*1.90*	*0.0171*	*0.0085*	*3.21*	*0.0039*	*0.0020*	*2.08*	*0.0190*	*0.0095*	*9.10(*)*
	residual	*3*	*0.0071*	*0.0024*		*0.0080*	*0.0027*		*0.0028*	*0.0009*		*0.0031*	*0.0010*	
	Total	7	0.0265			0.0304			0.0307			0.0567		
Cwm Idwal	**Block**	1	0.0016	0.0016	4.07	0.0001	0.0001	0.03	0.0041	0.0041	0.94	0.0012	0.0012	0.26
	Treatment‡	3	0.0137	0.0046	11.64*	0.0410	0.0137	4.01	0.0746	0.0249	5.69(*)	0.0815	0.0272	5.92(*)
	linear‡	*1*	*0.0091*	*0.0091*	*23.11**	*0.0394*	*0.0394*	*11.57**	*0.0723*	*0.0723*	*16.54**	*0.0781*	*0.0781*	*17.03**
	deviations‡	*2*	*0.0046*	*0.0023*	*5.90(*)*	*0.0016*	*0.0008*	*0.23*	*0.0023*	*0.0012*	*0.27*	*0.0034*	*0.0017*	*0.37*
	residual	*3*	*0.0012*	*0.0004*		*0.0102*	*0.0034*		*0.0131*	*0.0044*		*0.0138*	*0.0046*	
	Total	7	0.0165			0.0513			0.0918			0.0964		
Maltraeth	**Block**	1	0.0049	0.0049	2.53	0.0021	0.0021	0.89	0.0102	0.0102	8.28	0.0074	0.0074	16.77
	Treatment‡	3	0.0097	0.0032	1.68	0.0079	0.0026	1.12	0.0323	0.0108	8.71(*)	0.0525	0.0175	39.55**
	linear‡	*1*	*0.0015*	*0.0015*	*0.77*	*0.0017*	*0.0017*	*0.71*	*0.0180*	*0.0180*	*14.52**	*0.0114*	*0.0114*	*25.66**
	deviations‡	*2*	*0.0082*	*0.0041*	*2.13*	*0.0063*	*0.0031*	*1.33*	*0.0144*	*0.0072*	*5.81(*)*	*0.0411*	*0.0206*	*46.49***
	residual	*3*	*0.0058*	*0.0019*		*0.0071*	*0.0024*		*0.0037*	*0.0012*		*0.0013*	*0.0004*	
	Total	7	0.0203			0.0171			0.0463			0.0612		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

Table AIII.2 – Continued.

Population	Source of variation	DF	wpns week 5			wpns week 6			wpns week 7			wpns week 8		
			S.S.	M.S.	*F value*	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*	S.S.	M.S.	*F value*
Aber	**Block**	1	0.0110	0.0110	121.23	0.0001	0.0001	0.03	0.0006	0.0006	0.13	0.0149	0.0149	2.37
	Treatment‡	3	0.0758	0.0253	278.61**	0.0514	0.0171	4.83	0.0488	0.0163	3.85	0.0279	0.0093	1.48
	linear‡	*1*	*0.0752*	*0.0752*	*828.75***	*0.0490*	*0.0490*	*13.8**	*0.0481*	*0.0481*	*11.39**	*0.0246*	*0.0246*	*3.91*
	deviations‡	*2*	*0.0006*	*0.0003*	*3.54*	*0.0025*	*0.0012*	*0.34*	*0.0007*	*0.0004*	*0.08*	*0.0034*	*0.0017*	*0.27*
	residual	*3*	*0.0003*	*0.0001*		*0.0107*	*0.0036*		*0.0127*	*0.0042*		*0.0188*	*0.0063*	
	Total	7	0.0871			0.0622			0.0621			0.0617		
Birds Rock	**Block**	1	0.0035	0.0035	0.59	0.0065	0.0065	1.0	0.0048	0.0048	2.12	0.0002	0.0002	0.09
	Treatment‡	3	0.0419	0.0140	2.39	0.0691	0.0230	3.56	0.0920	0.0307	13.66*	0.0735	0.0245	10.74*
	linear‡	*1*	*0.0263*	*0.0263*	*4.51*	*0.0620*	*0.0620*	*9.59*(*)	*0.0833*	*0.0833*	*37.11***	*0.0639*	*0.0639*	*27.97**
	deviations‡	*2*	*0.0156*	*0.0078*	*1.33*	*0.0071*	*0.0035*	*0.55*	*0.0087*	*0.0043*	*1.93*	*0.0097*	*0.0048*	*2.12*
	residual	*3*	*0.0175*	*0.0058*		*0.0194*	*0.0065*		*0.0067*	*0.0023*		*0.0069*	*0.0023*	
	Total	7	0.0629			0.0949			0.1035			0.0806		
Cwm Idwal	**Block**	1	0.0077	0.0077	2.25	0.0081	0.0081	1.53	0.0032	0.0032	0.77	0.0000	0.0000	0.01
	Treatment‡	3	0.0485	0.0162	4.72	0.0944	0.0315	5.94(*)	0.1037	0.0346	8.31(*)	0.0615	0.0205	9.02(*)
	linear‡	*1*	*0.0431*	*0.0431*	*12.59**	*0.0886*	*0.0886*	*16.71**	*0.1017*	*0.1017*	*24.43**	*0.0614*	*0.0614*	*27.02**
	deviations‡	*2*	*0.0054*	*0.0027*	*0.78*	*0.0058*	*0.0029*	*0.55*	*0.0020*	*0.0010*	*0.25*	*0.0001*	*0.0000*	*0.02*
	residual	*3*	*0.0103*	*0.0034*		*0.0159*	*0.0053*		*0.0125*	*0.0042*		*0.0068*	*0.0023*	
	Total	7	0.0665			0.1184			0.1194			0.0683		
Maltraeth	**Block**	1	0.0374	0.0374	137.93	0.0306	0.0306	11.89	0.0230	0.0230	2.57	0.0066	0.0066	0.79
	Treatment‡	3	0.0199	0.0067	24.51*	0.0576	0.0192	7.46(*)	0.0685	0.0228	2.55	0.0491	0.0164	1.95
	linear‡	*1*	*0.0053*	*0.0053*	*19.46**	*0.0354*	*0.0354*	*13.76**	*0.0543*	*0.0543*	*6.07*(*)	*0.0388*	*0.0388*	*4.62*
	deviations‡	*2*	*0.0147*	*0.0073*	*27.03**	*0.0222*	*0.0111*	*4.31*	*0.0142*	*0.0071*	*0.79*	*0.0104*	*0.0052*	*0.62*
	residual	*3*	*0.0008*	*0.0003*		*0.0077*	*0.0026*		*0.0269*	*0.0090*		*0.0252*	*0.0084*	
	Total	7	0.0581			0.0960			0.1184			0.0809		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

Table AIII.2 – Continued.

Population	Source of variation	DF	$wpns_{coeff}$ S.S.	$wpns_{coeff}$ M.S.	$wpns_{coeff}$ *F value*	Above ground fwt S.S.	Above ground fwt M.S.	Above ground fwt *F value*	Above ground dwt S.S.	Above ground dwt M.S.	Above ground dwt *F value*	Relative growth rate S.S.	Relative growth rate M.S.	Relative growth rate *F value*
Aber	**Block**	1	0.0060	0.0060	0.48	0.0036	0.0036	0.14	0.0083	0.0083	0.45	0.0000	0.0000	0.01
	Treatment‡	3	0.1067	0.0356	2.82	0.0449	0.0150	0.60	0.0772	0.0257	1.40	0.0003	0.0001	0.33
	linear‡	*1*	*0.1043*	*0.1043*	*8.27*(*)	*0.0275*	*0.0275*	*1.09*	*0.0604*	*0.0604*	*3.29*	*0.0003*	*0.0003*	*0.89*
	deviations‡	*2*	*0.0024*	*0.0012*	*0.10*	*0.0174*	*0.0087*	*0.35*	*0.0168*	*0.0084*	*0.46*	*0.0000*	*0.0000*	*0.05*
	residual	*3*	*0.0378*	*0.0126*		*0.0754*	*0.0251*		*0.0551*	*0.0184*		*0.0008*	*0.0003*	
	Total	7	0.1506			0.1239			0.1405			0.0011		
Birds Rock	**Block**	1	0.0040	0.0040	0.42	0.3290	0.3290	45.52	0.2091	0.2091	43.53	0.0005	0.0005	2.21
	Treatment‡	3	0.1995	0.0665	6.93(*)	0.0716	0.0239	3.30	0.0730	0.0243	5.07	0.0008	0.0003	1.21
	linear‡	*1*	*0.1947*	*0.1947*	*20.29**	*0.0036*	*0.0036*	*0.49*	*0.0000*	*0.0000*	*0.01*	*0.0000*	*0.0000*	*0.16*
	deviations‡	*2*	*0.0048*	*0.0024*	*0.25*	*0.0680*	*0.0340*	*4.71*	*0.0730*	*0.0365*	*7.60*(*)	*0.0008*	*0.0004*	*1.74*
	residual	*3*	*0.0288*	*0.0096*		*0.0217*	*0.0072*		*0.0144*	*0.0048*		*0.0007*	*0.0002*	
	Total	7	0.2323			0.4223			0.2965			0.0021		
Cwm Idwal	**Block**	1	0.0063	0.0063	1.02	0.0001	0.0001	0.03	0.0006	0.0006	0.15	0.0002	0.0002	2.48
	Treatment‡	3	0.2120	0.0707	11.48*	0.0400	0.0133	3.51	0.0476	0.0159	3.95	0.0004	0.0001	1.34
	linear‡	*1*	*0.1996*	*0.1996*	*32.42**	*0.0198*	*0.0198*	*5.20*	*0.0243*	*0.0243*	*6.06*(*)	*0.0000*	*0.0000*	*0.15*
	deviations‡	*2*	*0.0124*	*0.0062*	*1.01*	*0.0202*	*0.0101*	*2.66*	*0.0233*	*0.0116*	*2.90*	*0.0004*	*0.0002*	*1.93*
	residual	*3*	*0.0185*	*0.0062*		*0.0114*	*0.0038*		*0.0120*	*0.0040*		*0.0003*	*0.0001*	
	Total	7	0.2367			0.0515			0.0602			0.0009		
Maltraeth	**Block**	1	0.0485	0.0485	1.91	0.0368	0.0368	0.84	0.0012	0.0012	0.04	0.0012	0.0012	2.94
	Treatment‡	3	0.2208	0.0736	2.90	0.0858	0.0286	0.65	0.0445	0.0148	0.50	0.0007	0.0002	0.54
	linear‡	*1*	*0.1865*	*0.1865*	*7.34**	*0.0055*	*0.0055*	*0.12*	*0.0012*	*0.0012*	*0.04*	*0.0002*	*0.0002*	*0.50*
	deviations‡	*2*	*0.0342*	*0.0171*	*0.67*	*0.0804*	*0.0402*	*0.91*	*0.0434*	*0.0217*	*0.73*	*0.0005*	*0.0002*	*0.55*
	residual	*3*	*0.0762*	*0.0254*		*0.1318*	*0.0439*		*0.0894*	*0.0298*		*0.0013*	*0.0004*	
	Total	7	0.3455			0.2544			0.1351			0.0031		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

Table AIII.2 – Continued.

			dwt:fwt ratio		
Population	**Source of variation**	**DF**	**S.S.**	**M.S.**	***F value***
Aber	**Block**	1	0.0003	0.0003	0.56
	Treatment‡	3	0.0009	0.0003	0.67
	linear‡	*1*	*0.0008*	*0.0008*	*1.88*
	deviations‡	*2*	*0.0001*	*0.0000*	*0.07*
	residual	*3*	*0.0013*	*0.0005*	
	Total	7	0.0025		
Birds Rock	**Block**	1	0.0020	0.0020	45.53
	Treatment‡	3	0.0004	0.0001	2.69
	linear‡	*1*	*0.0002*	*0.0002*	*5.07*
	deviations‡	*2*	*0.0001*	*0.0001*	*1.51*
	residual	*3*	*0.0001*	*0.0001*	
	Total	7	0.0025		
Cwm Idwal	**Block**	1	0.0000	0.0000	3.34
	Treatment‡	3	0.0002	0.0001	16.0*
	linear‡	*1*	*0.0000*	*0.0000*	*4.66*
	deviations‡	*2*	*0.0002*	*0.0001*	*21.67**
	residual	*3*	*0.0000*	*0.0000*	
	Total	7	0.0003		
Maltraeth	**Block**	1	0.0040	0.0040	3.49
	Treatment‡	3	0.0015	0.0005	0.45
	linear‡	*1*	*0.0002*	*0.0002*	*0.15*
	deviations‡	*2*	*0.0014*	*0.0007*	*0.60*
	residual	*3*	*0.0034*	*0.0011*	
	Total	7	0.0089		

DF, degrees of freedom; ‡, aspects of analysis for which *F value* probabilities were obtained.

Appendix IV: Microsatellite locus sequences

Table A1V.1 – *A. odoratum* microsatellite locus sequences. Microsatellite repeats highlighted in bold and primer sites underlined.

Locus	Sequence (5' – 3')	Size (bp)
AoM00B1	CAGCAGTAGGTGCTCCATGAAGAATCGATCCAGTAATCTAGCTTGGT ATAATCTATTTTGTGACTGAGCACTGGCGTGTCATACCGAGTGCTCAC CGCGCTCCTCCG**TCCGATCCGATCCGATCCGA**CGCCTCACGCGGTC ACGCCGCCCACCAGTCTTTTCTTATCCACTTCTCACCTAGTCACCATC CTCCTCCACAACCAGATC	207
AoM00C1	TGATGAGCCAATGTCGTTGTAGAGCTCTATGGTCTCCATGCTTGGGG AGTCATTGTCGGTGTAGTACTCCATGTCACCCAAGCATGAAGAGCTAT TGTCGATGGAGTGCTCTATGGAGTCGTC**ATCATCATCATCATC**TTGT GGGATATCCTCATCTCCACTCTCCGAG	169
AoM00F1	CACATCAAAGGTCGTATCCCCGGTGTGGGTTTCCCTTTGCTGGCGAC CACCGCCTTCTCCTCCATTTCCGTCCACCATGGCTCGGCCACGATGG TCCAGGTAAGTGGTCCCACTACTA**CTCTCTCTCTCTCT**CAGGCTGCAA ATCGAGGCTTACTCGATTTTTCCCCTCTGGGAATTCTAGGGTTCATGG C	191
AoM01B1	TTACCCAAATTCAGTCGATCATCTATAAAACACTAGACGTATTCCGAC ATCTGTCTG**CTGACTGACTGACTGACTGA**CGATATTGCTGCTGCTGG TGCGTGCGCAGGTTGGTGACAGATATTCCTGGGACAACTGCTGCAAA CTTCGGGAATGAGGTGATGT	162
AoM01F1	CTCGCACAACAAAGGTCGTATCCCCGGCGTGGGCTTCCCTTTGCTGG CGGCCACCGCCTTCTCCTCTATCTCCGGCCACCGTGGCTCGGCCAC GTTGGTCAAGGTAAGTGGCTCAGCCACCA**CTCTCTCTTCCCTCTCTCT CT**GCCCTTACTGCTCTCGATCGAGCACTCACTCCAATTTTCCC	184
AoM01F2	TAGCTCCAGTCGTATATCCCGGCGTGGATATCCTGGCGCTGTTGACC ACCGCCCTCTCCGCGTCTCCGGCCACCGTGAGCTGGGCTCACGTGG TCCAGGTAGTCTCCTACCC**CTCTGCTCTCTCTCTCTCT**ACTGCTACTG CTACTGCCAATCGAGTCTCAC	162
AoM01F3	CGAGCTAACATGCAGAAGCAATGGCGGAGGTGTTAAAATCACCAGAT CATGCCATTGTCGGCATGCTTGCTAGTTCATCAAGCTCCAGAGGGTT **AGAGAGAGAGAGAGAG**GTAGCTTGGGCGTGTCCGGGAGAGTGAGT GAGATGAGTGGGTGC	154
AoM01F4	ATCGGTCCATTTGTTCTTGGTCTTCATCTGCAGACAATCAAGCGTTAC ATGCAAGCGAGTATCCTGCGGACGGCACCCAGGAAAGAGAGGAGTC ATCCCGTCTTTCTCCATTTGCGCCAGCTTGG**CTCTCTCTCTCTCT**GCA GCTCTGTCATTGCTCGTCTCCTG	165
AoM03F1	CCCATAAGCTTGTGACTTGTGGTGTTCCTCCTTGTAGAGGATGCACaA GTTCTTGCTTGTGTGTGTGCTACCAAA**CTCTCTCTCTCTCT**TACTGAA ATTAAAGCTTGAAAATAAcCCTtTGAAgATTCCTAAAaGTGCTTGTTGAA GATTTGGGCAGCAC	160

bp, base pairs

Table AIV.1 continued – *A. odoratum* microsatellite loci sequences. Microsatellite repeats highlighted in bold and primer sites underlined.

Locus	Sequence (5' – 3')	Size (bp)
AoM03F2	TCCAGGTACACGGCCATCTA**CTCTCTCCCTATCTCTCTCTCTCTGC CTCTCTCT**GGCTGCTAATTGAGTCCTTGCTCGATTTTCCCCTTTGAGG AATTAGGGTTCATTTGACTGAAcAAATCTTCAGTCCTAGATGCTATTGG GCTAGATGATGCAnTGGCTGGTGaTACTTTGATTCTACACTTACTACT GCTTCTACTGGCTCAATGATTCGAGC	219
AoM06F1	TTATGAACGGGTTGCTGGCTCAACTAATTATTCTATAATACTACGCTTT GGCCATCTCTCCGCTCTCCGGCCACCGTGAGCTGGGCTCACATGGT CCAGGTACACAGCCTC**CTCTCTCTCTCTCTCTCGCTCTCTCTGTGTGT** GctCCGGATCaTTGCTAATCGaGTATTTCTCGATTTTCCCCTTTGGG	190
AoM07F1	TCGAGTAAGCCTCGATTCGCAGCCT**GAGAGAGAGAGAGAGAGAGA GA**GTAGTGGTGGGACCACTTACCTAGACCAACGTGGCCGAGCCACG GTGGCCGGAGATGGAGGAGAAGGCGGTGGCCGCCAGCAAAGGGAA ACCCACGCCGGGGATACGACCTTTGCTGTGCGAG	170
AoM07F2	GCACGGTCATTGTCAACCGAATCAGGTACAAATTAT**AGAGGAGAGAG AGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG**TGAGTAGCAG CACACACTCATCACTGGCTCACTATGCATCCTCCACAGAGGAACACC ACAGCCATGCCTAGTTA	155
AoM07F3	ATGCCGATGCCGGCATAGAAATACCAAGCACCACCCCTGGACCCTCT CCCTCTCTCATTTCGTGTCCACCCGCATCCACTCACCTCACTCACTCT CCCAGACACGCCCAAGTTAT**CTCTCTCTCTCTCT**AAACCTTTGGAGCA CCATGAACTGGTGAGCATGCCGACAATGGCAT	175
AoM07F4	GCATCGTGGTCATGCCGATACCAGCATACAAATGCCAAGCACCACCC CTGGACTCTCTCCCTCTCTCATTTCGTGTCTACCCACTCCCTCTCACC CCACTCACTCTCCCAGACACGCCCTAGCTAC**CTCTCTCTCTCTCTCT**A TTCTTCTGGAGCACGACGAACTCGAGAACATGCCGACAATGGCATGA	190

bp, base pairs

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