AN ABSTRACT OF THE DISSERTATION OF

María Alejandra Martínez-Ghersa for the degree of Doctor of Philosophy in
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Ryegrass (Lolium multiflorum Lam.)

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Plant form is a compromise between resource gathering, reproduction and

the tolerance to physical demands of the abiotic and biotic environment. In

an agricultural field in addition to the natural factors causing stress, humans

also introduce physical and mechanical stresses, and chemical pesticides

into the environment. Many of these factors are hazardous, since they

represent stresses to which plants are unable to develop defense

mechanisms. However, weeds have persisted in the agricultural

environment despite the efforts to eradicate them. They have adapted to

environmental changes such as crop rotation and have developed tolerance

to stressors like pesticides in very short periods of times (less than 10

years), much less time than normally expected for evolutionary responses

to occur. Perhaps a key to why weeds persist in stress-dominated habitats

is the way they compromise between yield and survival. The mechanisms that explain which process is relevant in the control of seed production or seedling growth relate to the ecophysiology of the individual plants. However, trade-offs between plants physiological functions will have implications at both population and community levels.

Climate change, air pollution and water scarcity are examples of environmental stresses that particularly affect agriculture. Herbicides are a major technological tool for agriculture and are responsible, at least in part, for significant increases in crop production during the last quarter of the century. The research presented in this dissertation was developed to understand the extent that individual responses to multiple environmental stresses can be extrapolated to population-level responses in an annual weed species.

The specific objectives were to assess (1) the impact of three anthropogenic stresses (herbicide, UVB light and ozone) and their interactions on individual Italian ryegrass ontogeny and reproduction and (2) the potential evolutionary effect of these stresses and combinations on changes in population size and structure over time.

Plants were capable of growth and reproductive compensation under the studied stresses. Stress factors with similar biochemical mechanisms had different effects at the individual plant and population levels of organization. Compensation occurred at all levels of organization: as individuals modifying growth and allocation to different organs and as populations modifying birth, and death rates and density dependent responses. The ability to compensate sometimes decreased with the number of stress factors (e.g. herbicide and UVB). In other cases, compensation ability increased with the number of stress factors (e.g. ozone and herbicide).

Individual and Population Responses to Abiotic Stresses in Italian Ryegrass
(*Lolium multiflorum* Lam.)

by

María Alejandra Martínez-Ghersa

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I understand that my dissertation will become part of the permanent
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María Alejandra Martínez-Ghersa, Author

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Dave Olszyk provided guidance for the analysis of data and writing of
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To my Father, whom I miss every day

To Marco, Julie, Felipe and Josefina, for the pride they make me feel

To Claudio, for each and every day together

The beauty and genius of a work of art may be reconceived, though its first material expression be destroyed; a vanished harmony may yet again inspire the composer; but when the last individual of a race of living things breathes no more, another heaven and another earth must pass before such a one can be again.

William Beebe, 1906

INDIVIDUAL AND POPULATION RESPONSES TO ABIOTIC STRESSES IN ITALIAN RYEGRASS (*LOLIUM MULTIFLORUM* LAM)

CHAPTER 1

GENERAL INTRODUCTION

To design a plant for a particular environment, we would begin with the abiotic conditions that are likely to prevail: maximum and minimum temperatures, soil acidity, amount and distribution of rainfall, prevalence of fires, frequency and intensity of storm-force winds, likelihood of floods, the presence of toxics in the soil (Crawley, 1997). We should probably ask whether our ideal plant is a broadleaf grass, how long it lives, how long it takes to flower, how tall it will be, and how much biomass will be invested in roots, shoots, or support structures like stems and branches *versus* productive tissues like leaves and flowers. We would ask what kind of seeds it produces, how are they dispersed, if seeds germinate at once or, if they are dormant. These are questions of plant life history. Each of the 250,000 living vascular plant species possesses a unique combination of such traits which reflects both phylogeny and recent ecological circumstances (Crawley, 1997). The major traits of a plant species may have evolved over millions of years in quite different environments, surrounded by different competitors, facilitators, herbivores and pollinators.

All of the traits are likely to be trade offs and compromises between conflicting costs and benefits for survival. These trade-offs and compromises are the subject of this thesis.

Organisms must allocate finite resources among competing demands (Cody, 1966). There must be trade offs between traits promoting fecundity and those promoting survival (Williams, 1966; Stearns, 1976) because all terrestrial plants must fulfill the basic requirements of survival, growth and reproduction. Vascular plants must intercept light efficiently, support the weight of the above ground parts, exchange gases with the external environment, and absorb and conduct liquids. Their roots must be securely anchored and capable of extracting mineral nutrients from the soil. They must also reproduce (Niklas, 1988). Thus, plant form is a compromise between resource gathering (leaves and roots), sex (pollination and seed production and dispersal) and the mechanisms of coping with physical constraints of the abiotic and biotic environment (protection against wind, ultraviolet radiation, pollutants, desiccation, pathogens and herbivores). For plant life to be sustained, this compromise is resolved in different ways by species coexisting in any particular environment over time. All of these compromises are expressed in morphology and represent the interaction between physical and developmental constraints on growth (Crawley, 1997).

If natural selection has molded plant function the way a corporate manager organizes a business, then investment in any function should cease when the return on that investment falls below the return of alternative investments. For example, the biochemical capacity for CO_2 fixation in plants should be curtailed whenever a limitation in the availability of any other resource prevents the efficient use of an additional capacity to photosynthesize. Constraints on efficient use might affect photosynthesis directly (e.g. low light) or indirectly, if sites are closer or more distant from the reactions of photosynthesis. Biochemical capacity for CO_2 fixation could be a good predictor of both resource availability and environmental stress.

How stress affects plants depends on the nature of the stress. Grime (1977) defined stress as any factor that restricts plant growth. It is useful, however, to distinguish between (1) stress caused by insufficient resources (e.g. water, lights, nutrients) to support maximal growth and (2) stress caused by direct physiological damage to the plant by the disruption of metabolism. Plant response to insufficient resources is quite predictable (Chapin et al., 1991). It involves compensation changes in allocation to maximize acquisition of the resources that most directly limit growth (Bloom et al., 1985) For example, light limited plants increase carbon allocation to leaf

and stem production at the expense of roots, whereas nutrient or water limited plants increase allocation to roots relative to that of shoots.

A plant's biotic environment also affects its performance. For example, predators, pathogens and competitors of the same and different species can cause reduced growth. Hence plant abundance as well as size under a set of field conditions is determined by both its relative competitive ability with other plants and its relative susceptibility to herbivore, pathogen and abiotic stress factors. Trade-offs between plant physiological functions must have implications at individual, population and community levels.

1.1 POPULATION REGULATION

There are only four factors that determine the number of individuals in a population: births (B), deaths (D), immigrants (i) and emigrants (E). The number of individuals in a new population at time t+1 are related to those in the old population at time t by the equation:

$$N_{t+1} = N_t + B - D + I - E.$$

If the numbers of births, deaths, immigrants and emigrants do not change with density, then a population will increase to infinity or decline to extinction (Watkinson, 1997). Observations on a large number of populations (e.g. Harper, 1977; Silvertown and Lovett Doust, 1993) show

that offspring number most often remains more or less constant from year to year. In other populations, however, the number of offspring may decrease as population size increases. Unless one or more of the demographic variables in the above equation is a function of plant density, population size (N) cannot be regulated and the population could not persist in the face of uncertain and fluctuating environments.

While density dependent control of one or all demographic variables is necessary to explain population regulation, it is the *interaction* between density-dependent and density-independent processes that determine the population actual size. For example, if we consider a population where the birth rate (i.e. seed production) is density dependent, and the death rate is both density independent and varies with environmental conditions, population size must vary depending on the interaction between abiotic and biotic conditions.

1.2 THE AGRICULTURAL ENVIRONMENT

All plants can, to some extent, counteract stresses from low resource supply, although their success in doing so depends on the range of resource availabilities to which they are adapted (Chapin III, 1988). The evolutionary response to insufficient resources in most plants is to

genetically fix the patterns of allocation that maximize acquisition of the limiting resource and to reduce overall resource requirements by reducing growth rate. On the other hand, changes in patterns of resource acquisition do not readily ameliorate stresses caused by direct damage to the plant (Chapin III, 1991).

In addition to the natural factors that cause stress, humans are responsible for physical and mechanical stresses in agricultural fields. Humans also introduce chemical pesticides as stressors into the agricultural environment. However, weeds persist in agricultural environments despite tremendous efforts to eradicate them. They adapt to swift environmental changes, like crop rotation, and have developed tolerance to herbicides over relatively short periods of times (i.e. less than 10 years), -much less time than normally expected for evolutionary responses to occur. Perhaps a key to why weeds persist in stress-dominated environments is the way they compromise between yield and survival.

Weed density is probably the most important factor determining effects of weeds on crop's yields (Radosevich et al., 1997). Immigration of weed seed and the extent of the soil seed bank are important demographic parameters for an annual weed population to persist. The seed bank is related to the life history of the weed during previous cropping seasons, and especially

dependent on the performance of the weed population during the previous season (Zimhdal, 1988). By controlling the number of seed each plant produces, growing conditions determine to a great extent weed abundance the following year (Harper, 1977; Sagar and Mortimer, 1976). Nevertheless, the mechanisms that explain which processes control seed production or seedling growth are not demographic but relate to the ecophysiology of the individual plants. For example, if any abiotic factor modifies the individual response to herbicide application, this will in turn impact demographic parameters such as death rate. The birth rate can also be affected by density of the plant population itself, which can in turn, be modified by the death rate caused by herbicide use.

Understanding processes at the scale of the individual becomes particularly important when environmental, agricultural, and genetic interactions occur. For example, the influence of herbicide application on demographic parameters usually has a genetic component, because some individuals in the population are naturally more susceptible to the chemical than others. Some plants are also more responsive to changes in ultraviolet light levels than others, while others are more sensitive to different kinds of physical stress. The consequence of this differential sensitivity within a plant species is that different genotypes will be favoured under differing environmental conditions.

Even in the absence of a demographic response (i.e. no change in population density), it is important to determine whether environmental factors caused no response at the individual level, or whether, in the presence of individual physiological response, birth and death rates were compensated, resulting in a constant population density.

For most weeds in most habitats, it is not possible to predict the dynamics of the populations (Cousens and Mortimer, 1995). To make such predictions we must ask not only how weed plants respond to combinations of new and old human-imposed and natural stresses, but also determine the consequences of theses stresses on plant population dynamics.

1.3 WEEDS AND ENVIRONMENTAL HAZARDS

Climate change, air pollution, water scarcity and loss of plant genetic diversity are examples of environmental stresses that particularly affect agriculture (Frisvold and Kuhn, 1999). Broad scale changes in environmental factors will probably alter productivity of agroecosystems, influence the distribution of agricultural pests, and impact the selection of crop varieties (Patterson, 1995).

1.3.1 Herbicides

Crop production usually requires that the competitive impact of weeds be minimized to optimise yields. Thus weed control has become one of the most important activities in crop production (Radosevich et al., 1997). Herbicides are a major technological tool for agriculture and are responsible, at least in part, for significant increases in crop production during the last quarter century. The effects of herbicide application have genetic, stage of growth and environmental components. Selectivity of an applied herbicide depends on the inherent resistance or susceptibility of the particular species to the chemical. The stage of growth of the plant and the environment under which the plant has grown, and is growing at the time of treatment and will grow following treatment, are also important for effective weed control (Muzik, 1976). The complexity of responses to herbicide application results from the interaction of components, since no one operate singly under any field condition. However, study of individual components has been most common. Research has been devoted to relating the genetic make up of weed populations to herbicide response. A large volume of information is available on the genetics and physiology of herbicide resistance (Powles and Holtum, 1994). The stage of plant growth at which weeds are most susceptible to herbicides has also been extensively studied. Growing young plants under optimal conditions are the most susceptible to herbicides (Aberg, 1964). This generality also implies

that there is an optimum level for every environmental condition, e.g.

temperature, light, moisture, wind, radiation, which plays a role in herbicide

sensitivity. An understanding of how environment affects a plant's response

to herbicide depends on how well its response to environment is

understood (Musik, 1976).

It is recognized that plant environments are dynamic complexes of

interrelationships between the physical environment and plant (Billings,

1952). The plant environment is almost always less than ideal, typically

having components that diverge from the optimum. Plants are rarely

exposed to a single stress, with adjustments to several concurrent stresses

at one time being required (Mooney and Winner, 1991). Hence the

response of a plant to a chemical will, in part, be related to all the other

stresses to which it has been subjected during its lifetime or will be

subjected to following the treatment.

1.3.2. Ultraviolet B light

One of the most striking recent environmental global changes is the

significant downward trend in column stratospheric ozone outside of tropical

regions (Kerr, 1991, 1993). Information obtained between 1979 and 1989

with the Total Ozone Mapping Spectrometer (TOMS) aboard satellite

Nimbus-7 shows an average decrease in ozone column of 4.5 percent

annually in the Northern hemisphere between 30 and 60° N. Ozone has

declined from 3 to 11 percent in the same latitude of Southern hemisphere

(Madronich 1992). Due to the protective function of ozone against ultraviolet

light, a concomitant increase in surface ultraviolet B (UVB) light irradiance

in some regions has been reported (Federick et al., 1991).

Using data from the TOMS system and the action spectra for DNA damage

(Setlow, 1974), Madronich (1992) calculated the increase in biologically

active UVB radiation dose that occurred between 1979 and 1989 as a

consequence of thinning the ozone column. Calculations indicate that the

largest relative increases in UVB occurred at high latitudes and that

changes in the tropics are of less importance. There is international

consensus about the need to evaluate and eventually mitigate the effects of

UVB light increase on biological systems (UNEP, 1991; SCOPE, 1993).

There is no information relating environmental UVB changes with the

response of plants to herbicide application.

Because of the high quantum energy, the greatest part of the UVB radiation

penetrates cells, is adsorbed and causes acute injuries. UVB light causes

photooxidative action in cells, and photolesions, particularly in bio-

membranes. UVB damage to plants can be identified by changes in

enzyme activity (increase peroxidase activity, inhibition of cytochrome

oxidase), poor energy status of cells, lower photosynthetic yield and by disturbed growth (reduced extension growth and pollen tube elongation) (Tevini and Teramura, 1989). Due to the effective absorption of UVB by epicuticular wax and by protective pigments (flavonoids) dissolved in the cell sap, the protoplasm in the cells of higher plants is largely protected from radiation injuries. Synthesis of these protective pigments is induced by UVB, and they accumulate as stress increases (Bornman and Teramura, 1993.

There is abundant information on the effects of UVB light on higher plants, especially from short term experiments with cultivated species. These studies show that high levels of UVB light can reduce photosynthetic rate, growth rate, increase synthesis of protective pigments and alter plant morphology (Caldwell et al., 1989; Teramura and Sullivan, 1994).

In addition, direct yield losses due to increased UVB (simulating ozone column erosion) have been observed in half of studied species under field conditions (Bornman and Teramura, 1993). Also experiments with solar UVB exclusion have shown yield and growth increase in some grass and woody species (Tevini and Teramura, 1989; Bornman and Teramura, 1993), while others have found no effects at all (Dai et al., 1997). A consistent observation from UVB light supplied by fluorescent lamps is the

induction of photomorphogenic responses. For example, exposure to UVB light causes an increase in leaf width (Cen and Bornman, 1993), tillering (Barnes et al., 1990) and accumulation of flavonoid pigments (Beggs and Wellmann, 1985) in many species.

1.3.3. Tropospheric ozone

In the troposphere, ozone is created by a complex set of chemical reactions involving trace air pollutants (Firor, 1990). The average concentration of ozone in the lower atmosphere is increasing. Its increase is attributable to releases in the air of nitrogen oxides and hydrocarbons, which are by-products from a wide range of industrial and agricultural activities, e.g. driving cars fuelled by fossil fuels and biomass burning. Tropospheric ozone is produced during the oxidation of volatile hydrocarbons in sunlight when nitric oxide concentrations are above a critical level (Vitousek, 1992). Under background conditions nitric oxides concentration is low, but due to industrial combustion or extensive biomass burning, large areas of the northern temperate zone and seasonally dry tropics experience nitric oxide concentrations above critical levels much of the year. Ozone generation is linearly proportional to nitric oxide concentration over much of the Earth (Jacob and Wofsy, 1988). Tropospheric ozone is the most damaging air pollutant worldwide, causing substantial reductions in productivity and

mortality in natural ecosystems and significant economic losses in agriculture (Reich and Amundsen, 1985).

Ozone enters leaves through open stomata, and dissociates rapidly in plant tissues to form atmospheric oxygen and peroxides (Larcher, 1995). The peroxides first adversely affect the plasmalemma, and then other biomembranes, so that ion transfer processes are impaired, producing "leakiness" of cell membranes to important cations like potassium. While there are some indications of cellular repair, sites of injury in damaged cells are unspecific. Only when levels of O_3 are low, or present for short periods, is subsequent recovery of normal cellular transport capability possible (Wellburn, 1994). Prolonged experimental exposure of trees, wild herbs and grasses to ozone concentrations similar to those measured in the atmosphere cause a significant reduction in growth and yields of these plants (Matyssek et al., 1992).

The possibility of interactions between different air pollutants, other chemicals that plants are exposed to, and climate change, cannot be ignored. Ozone is the foremost example. Depletion of ozone in the stratosphere increases the flux of UVB, but tropospheric ozone near the earth's surface is rising in many places. Tropospheric ozone absorbs some of the UVB, but in doing so adds to the problem of global warming.

1.4 THE CASE OF ITALIAN RYEGRASS

Italian ryegrass (*Lolium multiflorum* Lam) is a major grass seed crop in the Willamette Valley and an important weed in cereal fields west of the Cascade Mountains (Appleby et al., 1976; Burril et al., 1988). Italian ryegrass is highly competitive with winter wheat, with as much as 60 percent loss in grain yield being reported (Appleby et al., 1976). Cultural practices and selective herbicides (mainly diclofop-methyl) are the most common means used to control this weed. Diclofop-methyl (Hoelon) is an herbicide used in cereal production in the United States and throughout the world. *L. multiflorum* has developed resistance to diclofop-methyl. Populations of resistant ryegrass were first observed in Oregon in 1987, after less than a decade of intensive use of the herbicide for weed control in wheat (Brewster and Appleby, 1988; Stanger and Appleby, 1989). These naturally occurring resistant populations of *L. multiflorum* have already been used for a number of physiological, genetic, and ecological investigations of the processes regulating herbicide resistance (Ghersa et al., 1994a, b; Roush et al., 1991). However information relating plant responses to specific environmental hazards and resulting weed population responses is non-existent.

Recent literature provides evidence for interactions between biotic and abiotic environmental factors resulting in increased tolerance to herbicide application by ryegrass plants (Devine and Shimabukuro, 1994). Drought stress was shown to increase herbicide tolerance in susceptible plants, even when high doses were applied (Pastori, 1995). It was also shown that ryegrass plants infected with endophyte fungi had increased tolerance to low doses of diclofop-methyl (Vila Aiub and Ghersa, 2000).

Morphological changes produced by exposure of ryegrass to ultraviolet light could modify the tolerance of susceptible plants to herbicide application. The differential tolerance of ryegrass plants to changing background environments can result in different survival levels from herbicide application. Hence different final densities of the weed could result, which might, in turn, have further demographic impacts in the crop-weed community through regulation of density-dependent seed production of Italian ryegrass.

Herbicide mode of action of diclofop methyl is at the acetyl-coA carboxylase (ACCase) enzyme, an enzyme catalyzing precursor of pigments that provide protection to UVB light. A combination of stress factors may reinforce, weaken, mask or reverse responses of plants to a single stress (Larcher, 1995). Enhancement of an effect in combination with other factors

is often observed, especially with combination of chemical stresses. On the other hand, woody plants under the influence of frost not only become freezing tolerant, but also become resistant to desiccation (Larcher, 1995). It seems possible that the combined effects of stresses to plants could be synergistic or antagonistic depending on the nature of the stressors and the particular ecological environment.

Ryegrass response to herbicide application is dose dependent. Heterozygous individuals with the herbicide resistance gene survive normal, label doses of diclofop-methyl, but die if double herbicide doses are applied. Genetic variation in the ryegrass population determines characteristics associated with: (1) phenology and morphology (ability of the population to limit the amount of herbicide absorbed); (2) physiology (ability to alter translocation, activity, or metabolism of the herbicide). If acclimation to ultraviolet light or ozone modifies the response to herbicide in individuals not carrying genes for herbicide resistance, a change in phenotypic characteristic of the population will result that should be evident in further progeny. In the presence of the second stressor (UVB light or ozone) ryegrass survivors of the herbicide application contributing to progeny will be herbicide resistant and herbicide susceptible, whereas in the absence of the second stressor, mainly the phenotypes carrying resistant genes will be represented in the subsequent generation of progeny.

Herbicide application has two main effects: (1) decrease in density by mortality and (2) decrease in growth of surviving susceptible individuals. Selectivity of herbicide and subsequent growth responses of surviving individuals could then be affected by environmental stressors to which the plants were subjected both prior to and after herbicide treatment. Barnes et al. (1988) suggest that photomorphogenic responses of plants to ultraviolet light could determine changes in competitive balance. By using exclusion experiments, Becwar et al., (1982) demonstrated that solar UVB affects stem elongation of wheat plants. More recently, Searles et al. (1995) detected effects of UVB in tropical areas on the morphology of native plants (e.g. *Cecropia obtusifolia, Tetragastris panamensis, Icalophysllum longifolium*). It could be that such responses have a cost in terms of resources used that would otherwise be allocated to different physiological functions like a rapid growth. However, these same responses could give advantages to species like Italian ryegrass if plants are subjected to high herbicide selection pressure, if they confer an increase in herbicide tolerance, through morphological changes or the induction of heritable mutations.

1.5 RESEARCH OBJECTIVES AND THESIS STRUCTURE

1.5.1 Objectives

The research presented in this dissertation was developed to understand the extent that individual responses to multiple environmental stresses can be extrapolated to population-level responses in an annual weed species. The general hypothesis is that the effect of abiotic stressors on biological systems decreases from the lowest to higher levels of organization because of compensation mechanisms acting in opposite direction (Fig. 1.1).

The specific objective is to assess the impact of three anthropogenic stresses (herbicide, UVB light and ozone) and their interactions on individual Italian ryegrass ontogeny growth and reproduction. The potential evolutionary effect of these stresses and combinations on changes in population size and structure over time is also of interest in this thesis.

These experiments advance knowledge in several areas of agricultural science: (1) the impact of environmental stresses on plants, (2) evolution of resistance in weed populations, (3) considerations in the development of biotechnology. The question of how different stress factors interact is not academic. One of the main scientific challenges of this century is to assess

the impact of people on the environment, including climate, and impacts on other living organisms. A key consideration here is the ability of plants to adapt to human-caused change. Thus, it seems important to study how organisms, populations and communities react to environmental stresses.

1.5.2 Structure of the thesis

The thesis is composed of five chapters. Chapters 2 through 4 contain the results of experiments to address thesis objectives (Fig. 1.1). There is a final chapter of general conclusions. First, experiments were performed that combined a stress factor of localized effects (ozone) with another with more systemic effect (herbicide). In this way, I examined the growth and yield of Italian ryegrass at the individual level to a combination of diclofop-methyl herbicide and tropospheric ozone doses in Chapter 2. The use of isolines of ryegrass that are susceptible and resistant to the herbicide allowed me to assess the fitness cost of resistance to each of the abiotic stressors. At the population level, in Chapter 3, I present results on the combined effects of ozone, herbicide and density on seed production of Italian ryegrass. In Chapter 4 I consider the effects of herbicide dose on both the efficacy of control and the plant species' evolution towards herbicide resistance. I explore the relation between demographic and evolutionary processes in this stressor-weed system. I present evidence from experiments in which *L. multiflorum* was used as a model system to test whether an environmental

stressor derived from global changes, such as UVB light, can influence the

success of herbicides to control weeds.
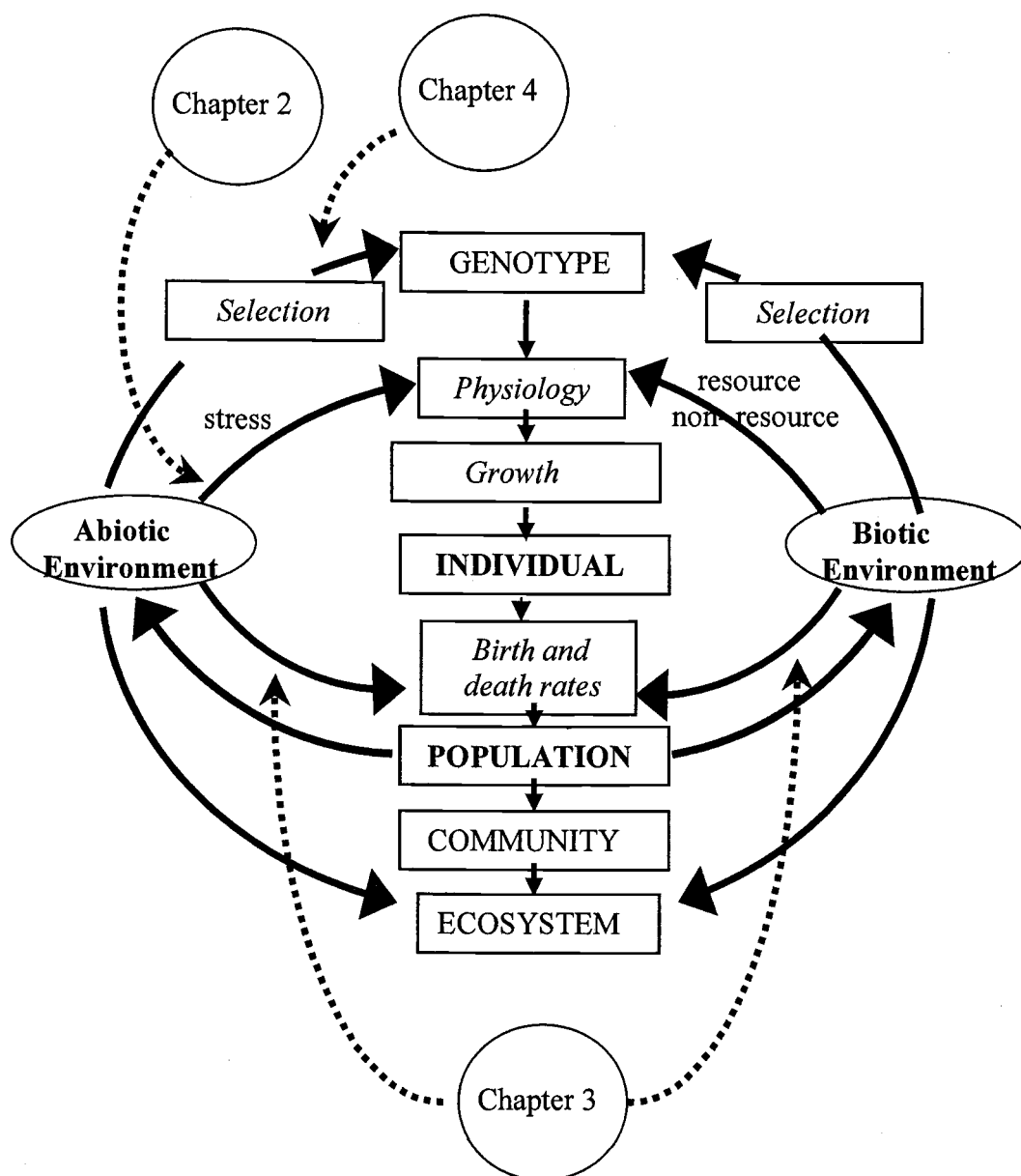
Figure 1.1. Complexity scales of biological systems and processes connecting scales. Thick arrows represent relationships of biological systems with environmental factors at each scale. Circles indicate processes explored in each chapter of this thesis.

In the last chapter, Chapter 5, I discuss the influence of combinations of abiotic and biotic stress factors on the structure and function of annual plant populations. In this final chapter I (1) put the main results of my thesis in the context of the ongoing research on the subject, (2) describe the scenarios where these results might have relevance, (3) describe how these results might contribute to management of weed populations, and (4) discuss the direction that future research in stress ecophysiology should take.

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CHAPTER 2

GROWTH AND YIELD RESPONSES OF ITALIAN RYEGRASS TO DICLOFOP-METHYL AND OZONE

M. Alejandra Martínez-Ghersa, David Olszyk and Steven R. Radosevich

2.1 ABSTRACT

Fitness of herbicide resistant individuals (R) is increased relative to that of susceptible individuals under herbicide application. However if the resistance gene brings negative physiological consequences to the plant, R plants might have lower growth or reproduction in herbicide-free environments, incurring significant fitness costs. In this study, effects of exposures to tropospheric ozone (O_3) on the response to diclofop-methyl application were examined for a herbicide resistant and a herbicide susceptible biotype of *Lolium multiflorum*. Italian ryegrass plants of both genotypes were grown in individual pots in control and ozone chambers. Plants were sprayed with diclofop-methyl herbicide at the recommended label dose, 1/8, or 1/16 the dose or left unsprayed. Plants of all treatments were harvested in six opportunities and growth analysis was performed. Total biomass attained by Italian ryegrass plants was larger the first year of the experiment when plants were planted during normal growing conditions. Ozone alone did not affect maximum biomass produced, but affected the timing at which maximum biomass was reached. Ozone affected most vegetative measures and seed production, in both S and R plants. The effect of ozone on biomass production of plants exposed to herbicide depended on herbicide dose and harvest date, and was reflected in the

different growth attained in each harvest period. Herbicide affected seed production in susceptible plants, but had a positive effect on seed production in R plants especially when plants were planted late in the season. Late planting decreased seed production of susceptible plants. When plants were exposed to ozone, herbicide intensified the negative effect of ozone on seed production of susceptible plants, but eliminated it in the resistant plants. If the apparent different susceptibility to ozone and late planting conditions of herbicide susceptible and resistant plants is confirmed with further studies, the existence of evolutionary tradeoffs could be suggested: plants resistant to herbicides may lose tolerance to some stressors like ozone, but on the other hand that trait may allow R plants to tolerate late planting or withstand ozone under herbicide application.

2.2 INTRODUCTION

The relative allocation of environmental resources to survival and reproduction throughout the life cycle of an organism determines its fitness (Holt and Thill, 1994). Survivorship may be further partitioned into success

of seed, seedling, and mature plant, which in turn are functions of such processes as germination, dormancy, establishment, and growth (Harper, 1977). Similarly, reproductive success depends on pollen and ovule production throughout the life cycle of plants, and fertilization success, which will determine seed yield (Crawley, 1997).

In an environment where herbicide is used, the fitness of individuals resistant to the herbicide usually increases relative to that of susceptible plants (Harper, 1956). Herbicide resistance is widespread among agricultural weeds (Le Baron and Gressel, 1982). However if the resistance gene confers negative physiological consequences to the plant, resistant (R) plants may have lower growth or reproduction than more susceptible (S) ones when compared under herbicide-free environments, thus R plants might incur in significant fitness cost (Heil and Baldwin, 2002). Under herbicide-free conditions, the relative advantage of the R- individuals over S-individuals would disappear. For example studies of growth, physiological performance and competition of biotypes of R and S-plants to triazine herbicides, have shown that triazine resistance has negative physiological consequences (Holt, 1990). Triazine R biotypes of many plant species (e.g *Amaranthus hybridus* and *Senecio vulgaris*) relative to S plants were associated with a reduced rate of electron transfer between PS II acceptors and lower CO_2 fixation rates leading to reduced photosynthetic rates,

biomass production, fecundity and competitiveness (Ort et al., 1983; Holt and Radosevich, 1983; Holt, 1988). With other weeds, resistant to acetyl CoA carboxylase (ACCase) inhibiting herbicides, the relationship of resistance mechanism to fitness was not clear (Roush et al., 1991). A paucity of information about S and R biotype performance in different environments is given as a partial reason for this ambiguity. Any environmental conditions that affect growth and yield of susceptible and resistant biotypes differentially should alter their relative fitness. Therefore, to fully understand the effect of resistance on fitness, studies of plant phenology, germination, growth, production and reproductive output over a range of environmental conditions are necessary to evaluate the variability in fitness often expressed by resistant plants in the field (Holt, 1990).

A relatively recent environmental change that could have profound deleterious effects on both plants and animals is the accumulation of ozone in the troposphere (Sharma et al., 1996). This pollutant forms from chemical reactions involving oxides of nitrogen and volatile organic compounds in the presence of sunlight. Ozone concentrations in the troposphere are already high enough to suppress plant growth and yield in many areas of the world (USEPA, 1996). Background concentrations of O_3 related to human activity have increased from ca. 10 ppb to over 40 ppb in the last decades (Finlayson-Pitts and Pitts, 1997). Ozone episodes where

the concentration exceeds 60 ppb by volume occur in some agricultural areas during the spring and summer when crops are actively growing (PORG, 1997).

In agricultural systems, plants often experience pesticide stresses simultaneously with ozone exposure. Diclofop-methyl is a post-emergence foliage-applied herbicide used to control grass weeds in cereal crops. Like ozone, exposure to diclofop-methyl produces oxidative stress and recent evidence shows that induction of senescence in susceptible species is the mode of action of this herbicide (Shimabukuro et al., 2001).

Italian ryegrass (*Lolium multiflorum* L. Lam) is an annual plant species distributed throughout most of the temperate regions of the world. It is a major grass seed crop in the Willamette Valley of Oregon and an important weed in cereal fields west of the Cascade Mountains (Appleby et al., 1976; Burrill et al., 1988). Intensive use of diclofop-methyl, a post emergence herbicide, to eliminate Italian ryegrass seedlings from winter cereal crops has led to the development of herbicide-resistant Italian ryegrass populations (Gronwald et al., 1992).

Fitness of Italian ryegrass to agricultural ecological conditions will be determined, to some extent, by how global environmental stresses (*e.g* ozone) interact with stresses from crop management practices to affect patterns of allocation of resources for survival and reproduction (Maxwell et al, 1990; Ghersa et al, 1994a). The way stressors affect this allocation patterns is strongly dependent on their timing, since stressors can generate different plant responses depending on whether they occur during vegetative or reproductive phases of the plant cycle (Grace, 1990). The probability of survivorship of plants confronted with similar acute stress changes with ontogeny (Parrish and Bazzaz, 1985).

Compensation and sometimes over-compensation to the negative effects of stressors are frequently observed in annual plants, when they are exposed to short-lasting stresses in early stages of their life cycles (Bazzaz and Morse, 1991). In contrast, negative effects of stress are irreversible when they occur during reproductive stages, or when they are chronic instead of acute (Larcher, 1995).

In this study, the effects of exposure to ozone on response to diclofop-methyl application were examined for herbicide-resistant and herbicide-susceptible biotypes of *Lolium multiflorum*. The experiments considered the following questions: (1) what is the effect of ozone on growth and

reproductive effort of Italian ryegrass plants?, (2) are herbicide tolerant plants more tolerant than herbicide susceptible plants to increasing levels of ozone?, (3) is response to ozone modified by herbicide application?, (4) does Italian ryegrass biotype or phenological stage of the plant influence herbicide response in the presence of ozone?, and (5) does duration of exposure to ozone change plant response?

2.3 MATERIALS AND METHODS

2.3.1 Plant material

Near-isogenic diclofop-methyl susceptible and resistant *L. multiflorum* lines (ORARHR-M93, Marshall cv.) developed by the USDA-ARS National Forage Seed Production Research Center were used in our experiments (Barker et al. 1997).

2.3.2 General procedures

The experiments were conducted during the growing seasons of 2000 and 2001 using the Field Ecological Research Facility (FERF) of the US Environmental Protection Agency National Health and Environmental

Effects Laboratory, Western Ecology Division in Corvallis, OR, (Hogsett et al., 1985). Seeds of both Italian ryegrass biotypes, herbicide susceptible (S) and herbicide resistant (R), were germinated in Petri dishes. Individuals of uniform size were then transplanted (one seedling per pot) to plastic pots 30 cm in height by 10 cm in diameter and containing regular potting soil. Pots were irrigated as needed throughout the experiment. A timetable of key events during the growing season is shown in Table 2.1.

Plants were grown and exposed to ozone in 12 FERF open-top chambers (Hogsett et al., 1985). Each chamber was 3.0 m diameter x 2.4 m high with a truncated cone and rain hood at the top. The experiments were arranged as a split-split plot design with three factors: ozone, Italian ryegrass biotype and herbicide treatment. At the plot level, there were two ozone concentrations: low ozone charcoal-filtered air (control) and charcoal-filtered air with added ozone (ozone), with six chambers per ozone level. Ozone was generated from oxygen and added to the chambers during the periods shown in Table 2.1 to match an episodic pattern of daily varying concentrations from 0 to 150 μL L^{-1} representative of ambient air monitoring sites (Tingey et al, 1986; Hogsett et al, 1985). Ozone was monitored in the chambers using Model 400A Advanced Pollution Instrumentation Inc., photometric analyzers. Ozone treatments were expressed as a seasonal exposure, SUM06, by accumulation over the growing season of all hourly

averages >0.060 μL L^{-1} (Lee et al., 1998). The SUM06 in 2000 was 29.8 μL L^{-1} hr for ozone chambers and 0.011 μL L^{-1} hr for control chambers. In 2001, the SUM06 in ozone chambers was 35.6 μL L^{-1} hr to the time of anthesis, and 68.3 μL L^{-1}hr to the time of final harvest. For the control treatment, the SUM06 was 0 μL L^{-1} hr.

Within each chamber there were 36 plants each of the S and R Italian ryegrass biotypes (Fig. 2.1). Nine plants from each biotype were randomly assigned to one of four herbicide treatments. When the plants were at the two-three leaf stage of growth, pots were sprayed with different herbicide doses and returned to the ozone exposure chambers. A commercial formulation (Hoelon3EC, Aventis USA) of diclofop-methyl (methyl 2-(4-(2',4'-dichlorophenoxy)-phenoxy) propanoate))) was used. Plants were sprayed at 1x the field application rate (f.a.r.) of 1120 g active ingredient (ai) ha^{-1}), 1/8 x f.a.r. (140 g ai ha^{-1}), 1/16 x f.a.r. (70 g ai ha^{-1}) and 0 x f.a.r (no herbicide) respectively.

In Oregon, Italian ryegrass seeds germinate in the field over a wide range of temperatures from early fall until late spring and the growth cycle usually ends by mid-summer when plants are dry and seeds are mature (Ghersa et al 1994). In order to assess Italian ryegrass responses to the ozone and herbicide treatments at different phenological stages, experiments in 2000

and 2001 were conducted on different dates (Table 2.1). This resulted in a

shorter growth period in 2001 and also ozone exposure at different

phenological stages. In 2000, exposure occurred during vegetative growth,

which included tillering but finished well before anthesis. In 2001, exposure

finished when plants had already flowered and had some spikes with

immature seeds.

9 individual pots to be harvested (3 each time)
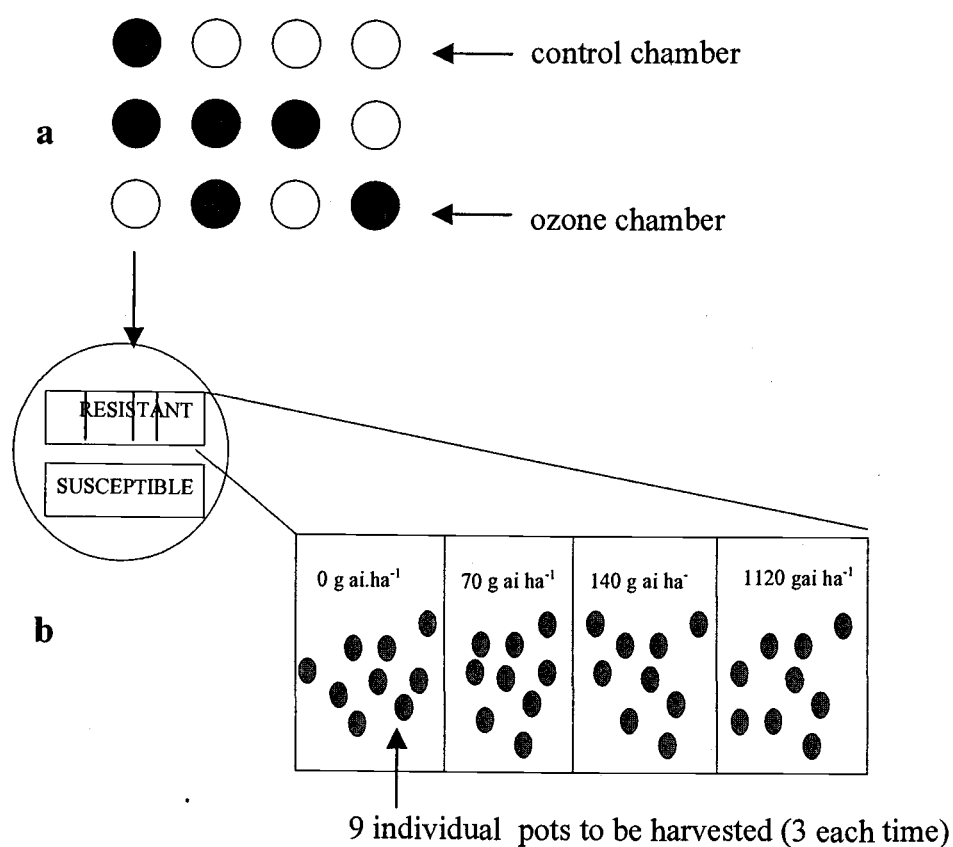
Figure 2.1: Representation of experimental layout. Air in open top chambers
was ozone-free (control) or supplied with ozone (ozone). Inside each
chamber pots sown with herbicide resistant or susceptible Italian ryegrass
and assigned to one of four herbicide doses.
 a) Position of open top chambers in the research facility. b) Detail of
arrangement of one group of pots (resistant) inside one chamber.

Table 2.1: Timetable of experimental events occurring during the 2000 and 2001 growing seasons

Event	Date		Phenological stage	
	2000	2001	2000	2001
Planting	30 August	3 May	seedling	seedling
Start of ozone exposure	8 September	16 May	1 leaf	1 leaf
Herbicide application	26 September	29 May	2-4 leaves	2-4 leaves
End of ozone exposure[a]	23 October	8 July	tillering	anthesis
Final harvest	2 May	29 August	mature seed	mature seed

[a] in 2001 a set of plants remained under continued ozone exposure (ozone-chronic)

Plants assigned to ozone treatment remained in the exposure chamber for 45 days in 2000 and 52 days in 2001. In the year 2000 experiment, when ozone exposure ended, all pots (both ozone and no-ozone, control, treatments) were moved to a greenhouse until flowering, seed production, and finally harvest. In 2001, the experiment also included season-long ozone exposure treatments with all biotypes and herbicide dose combinations. Hence, in 2001, some plants were grown in ozone free chambers the whole season (control), some were exposed to ozone and then moved to control chambers (ozone), and some remained in ozone exposure chambers for the entire growing season (ozone-chronic).

2.3.3 Measurements

Growth and yield responses to different treatments were assessed by Individual plant harvests during both years. In 2000, harvests were performed 21, 30, 50, 86, 145 and 274 days after planting and in 2001 plants were harvested 21, 35, 46, 74 and 123 days after planting. Because there was not enough room in each chamber for plants to be harvested on all days, plants to be harvested on days 21, 50 and 145 in 2000 and on days 21, and 46 in 2001 were grown in one set of three control and three ozone chambers, while plants to be harvested on days 30, 86 and 274 in 2000 and on days 35, 74 and 123 in 2001 were grown in a second set of three control and three ozone chambers. For each harvest day, three plants from a particular biotype x herbicide treatment were harvested from each of the three ozone or control chambers, for a total of nine plants. The roots were carefully washed. Length of the longest leaf, total number of leaves, and number of tillers were noted. Each plant was then divided into root, stems, leaves, and reproductive parts. When present, number of reproductive spikes was recorded. Total leaf area was measured with a LICOR 3100 leaf area meter (LICOR Inc, Nebraska). Stems, leaves, roots, and reproductive parts were dried to constant weight at 70 $^{\circ}$C and weighed. Full caryopses were separated from empty ones by blowing, and weight of full seeds was recorded.

2.3.4. Data analysis

The experiment was a split-split plot with three replicate blocks (individual chambers) per ozone or control treatment. The experimental unit was the chamber, thus data from the three plants per biotype x herbicide treatment combination within a chamber were averaged prior to statistical analysis. Data from the different years and harvests were analyzed separately. The 0.05 level of significance was used for all tests of significance. As we were mainly interested in interactions between stress factors influencing the performance of each biotype, we included in the statistical model only the main effect of ozone (O), two way interactions (ozone x biotype (OxB) and ozone x herbicide dose (OxD)) and three way interaction, OxBxD. To assess the gross performance of whole plants of each Italian ryegrass biotype we used functional growth analysis (Hunt, 1982). This method is based on fitting a polynomial curve to the growth data. The dependent variable, Y, is the log-transformed data (e.g. weight of the plant) and the independent variable x is the number of days of growth. Instantaneous relative growth rate (RGR) was calculated for each biotype and treatment as RGR= $d(\ln W)/dt$, where W is dry biomass per plant and t, period of time. This rate provides an integration of the combined performances of the various parts of the plant. It represents the efficiency of a plant as a producer of new material over time, and has been widely used to compare

species, varieties, or treatment differences on a uniform basis (Hunt, 1982).
In order to evaluate the relative contribution of the leafiness of the plant and
the photosynthesizing capacity to growth rate, two more indexes were
calculated: instantaneous values of leaf area ratio (LAR) and unit leaf rate
(or net assimilation rate) (ULR). LAR=exp[f L(t) − f W(t)] and
ULR=(1/L)(dW/dt), where L is leaf area, and W and t are as described for
RGR

Wickens and Cheeseman (1988) argue that instantaneous values of these
parameters can be weak if plants are subjected to short-term environmental
changes. Thereafter, to evaluate the influence of plant's ontogenic drift
against a background of changing environment, we also calculated interval
relative growth rate (RGR_{int}) for each biotype from data collected in 2000
and 2001 for each harvest period, using the formula:
$RGR_{int} = (\ln W_2 − \ln W_1) / (T_2 - T_1)$, where W_2 is the dry weight in grams at
the end of the observation period, W_1 is the dry weight in grams at the
beginning of the observation period, T_2 is time in days at the end of the
observation period, and T_1 is time in days at the beginning of the
observation period. The allocation to reproduction was assessed by
analyzing reproductive components (number of spikes/plant and seed
biomass/plant). Reproductive effort, (RE) was calculated RE=RB/(RB+VB)

where RB is biomass of flowers and fruits and VB is maximum plant

biomass.

SAS software (SAS Institute, 1990) was used for analysis of variance

(ANOVA) of plot means for each sub- and sub-sub-plot treatment for all

dependent variables at all harvest times. For the purpose of data

presentation, individual treatment means for all the variables considered are

presented in figures. Statements made in this paper concerning differences

among treatments, however are based on a factorial (2 ozone x 2 biotypes

X 4 herbicide dose) analysis of variance, and as such, may involve means

averaged across one or more factors. When the multi-way ANOVA showed

a statistical significant effect of ozone, a one-way (vegetative measures) or

two way (reproductive measures) analysis of variance was performed to

reveal level of significance.

2.4 RESULTS

2.4.1 Overall growth

Total biomass attained by Italian ryegrass plants was larger in 2000 than in

2001 (Figs. 2.2 and 2.3). In year 2000, average biomass per plant 50 days

after planting was similar to the value that Italian ryegrass plants had at seed maturity in year 2001, which resulted from 125 days of growth. Maximum biomass accumulation in non-herbicide treated plants was similar between the plants under ozone exposure and the respective control (not exposed to ozone). However, plants exposed to ozone, and without herbicide application, were smaller than control plants (not exposed to ozone) during the first half of the growing period (Figs. 2.2a-b and 2.3a). At the end of the experiment the ozone stressed plants (except for R in 2001) were larger than plants grown under control conditions. Plants not subjected to ozone (control) ended their growing cycle earlier than those exposed to ozone.

Figure 2.2: Dynamics of biomass production of diclofop-methyl susceptible and resistant Italian ryegrass plants in ozone and control chambers during 2000. Plants were sprayed with different diclofop-methyl doses (c-h) or received no herbicide (a-b). Horizontal arrow indicates period of ozone exposure. Vertical arrow indicates day of herbicide application. Values are means of 3 replicate chambers $\pm$ SE, with observations from 3 plants averaged per chamber. * Indicates significant difference between ozone and control plants.

SUSCEPTIBLE

RESISTANT

0 g ai.ha⁻¹

0 g ai.ha⁻¹

70 g ai.ha⁻¹

70 g ai.ha⁻¹

140 g ai.ha⁻¹

140 g ai.ha⁻¹

1120 g ai.ha⁻¹

1120 g ai.ha⁻¹

Days after Planting
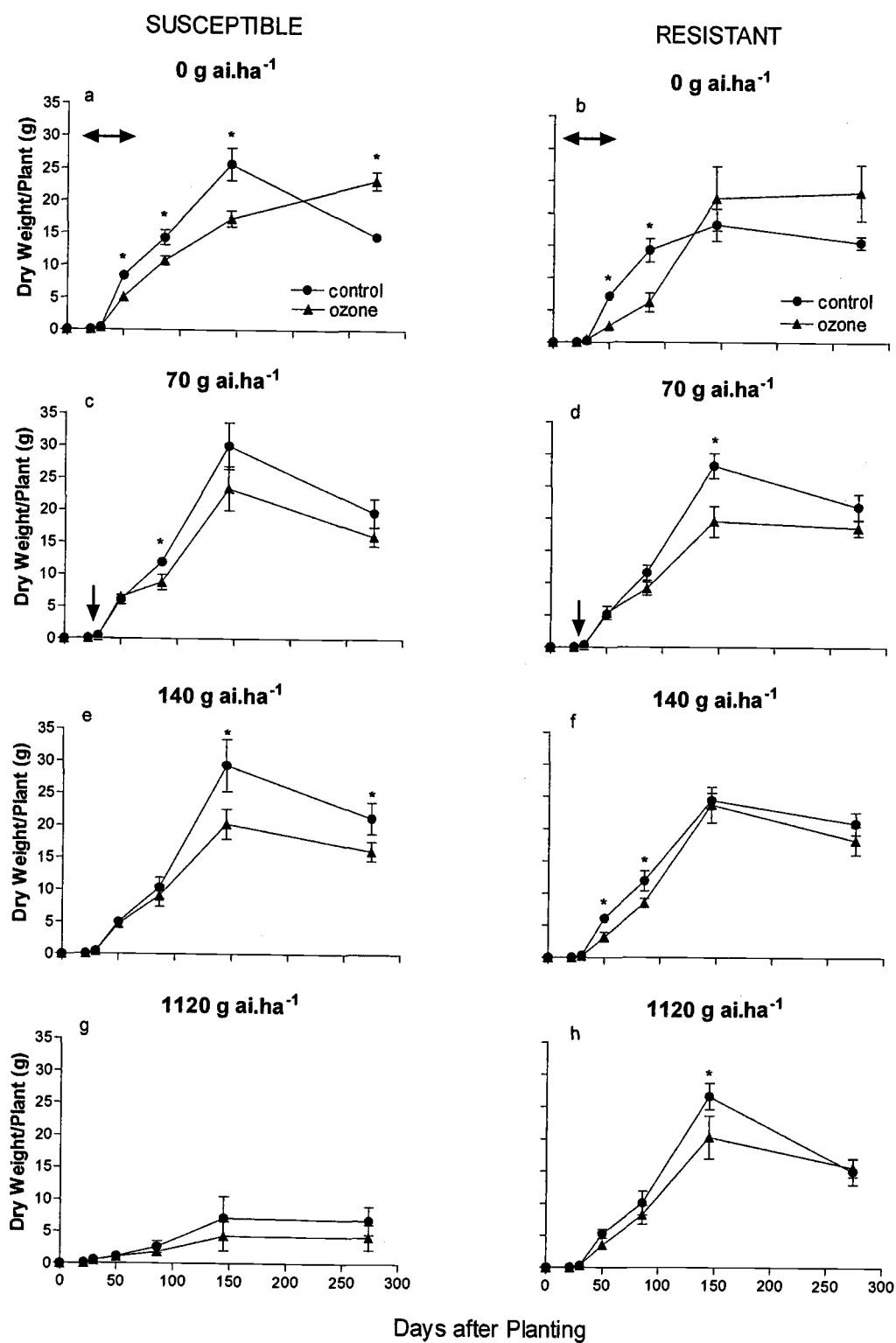
Figure 2.3: Dynamics of biomass production of diclofop-methyl susceptible and resistant Italian ryegrass plants in ozone and control chambers during 2001. In ozone chambers plants were exposed to ozone for part of the growing season (ozone) or during the whole season (chronic). Plants were sprayed with different diclofop-methyl doses (c-h) or received no herbicide (a-b). Horizontal arrow indicates period of ozone exposure, except for chronic ozone treatment where exposure lasted until final harvest. Vertical arrow indicates day of herbicide application. Values are means of 3 replicate chambers ± SE, with observations from 3 plants averaged per chamber. * Indicates significant difference between ozone and control plants.

SUSCEPTIBLE

RESISTANT

0 g ai.ha⁻¹

0 g ai.ha⁻¹

70 g ai.ha⁻¹

70 g ai.ha⁻¹

140 g ai.ha⁻¹

140 g ai.ha⁻¹

1120 g ai.ha⁻¹

1120 g ai.ha⁻¹

Days after Planting
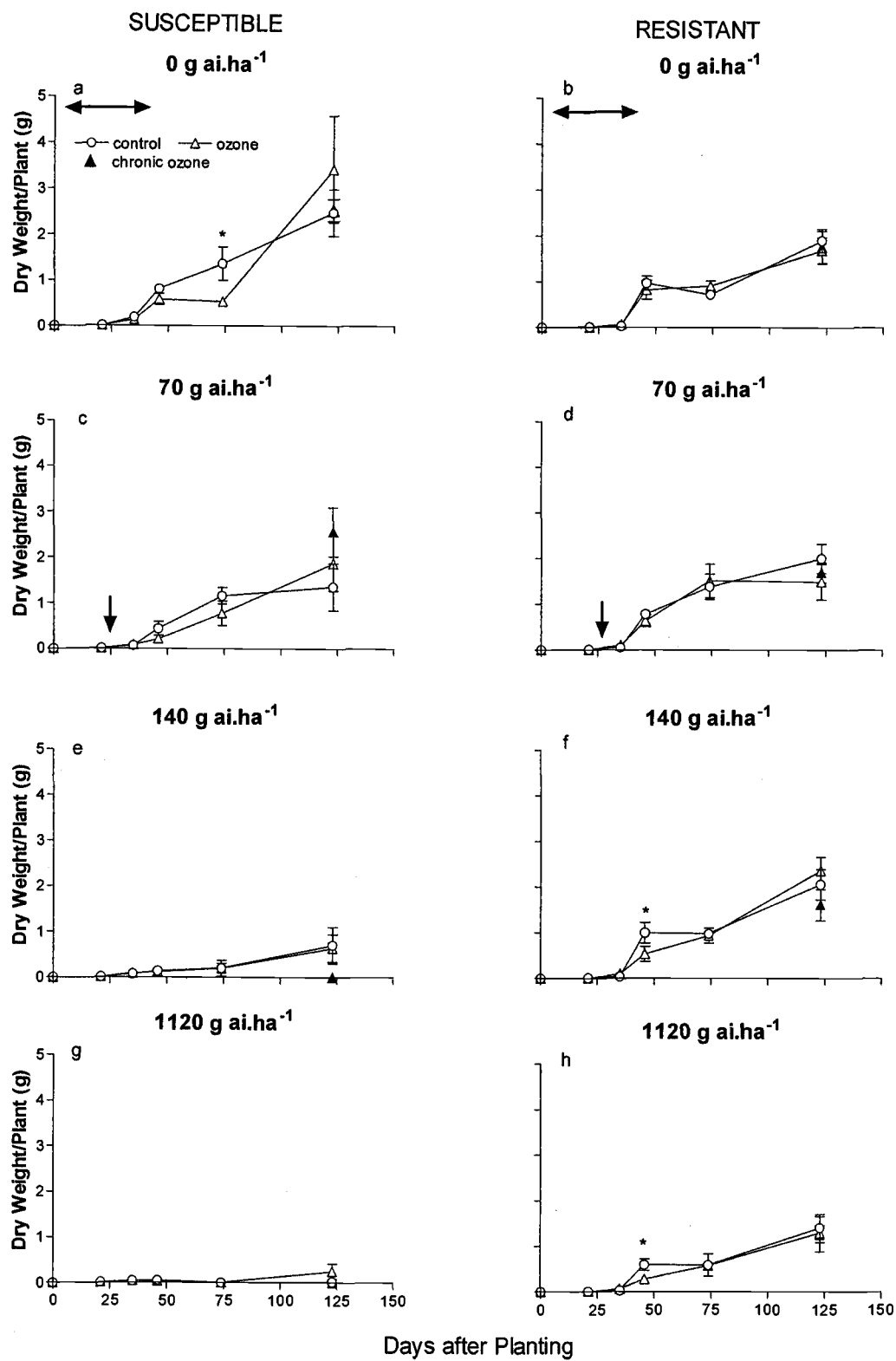
The effect of ozone on biomass production of plants exposed to herbicide depended on herbicide dose and harvest date, regardless of Italian ryegrass biotype (Tables 2.2 and 2.3). In year 2000, application of sublethal doses of herbicide (70 and 140 gai.ha^{-1}) to susceptible plants resulted in initially lower biomass than non-herbicide treated plants, followed by a rapid increase in biomass production, so that by 150 days after planting (DAP) the biomass of plants exposed to sub-lethal herbicide application was similar to that of plants not exposed to herbicide (Fig. 2.2a,c,e).

The normal use dose of the herbicide (1120 gai.ha$^{-1)}$ produced a significant decrease in biomass of the susceptible plants under both ozone and no ozone treatments that lasted for the whole growth period in 2000 (Fig. 2.2g). On the other hand, herbicide application produced an increase in maximum biomass of resistant plants, even under the normal use dose in 2000 (Fig. 2.2b,d,f,h). In 2001 the increase in biomass caused by low herbicide dosage was not observed (Fig. 2.3).

Table 2.2 : Analysis of variance for vegetative growth responses at different harvest times of two Italian ryegrass biotypes exposed to different levels of ozone and herbicide in 2000.O: ozone; B: biotype; D: herbicide dose. *P<0.05; ** P<0.01; ***P<0.001

Harvest (Days after planting)	Source	df	Number of leaves	Number of tillers	Leaf area	Plant height	Shoot/root ratio	Total biomass
21	O	1	ns	ns	ns	*	ns	*
	O x B	2	ns	*	**	**	ns	**
	O x D	6	ns	ns	ns	*	ns	ns
	O x B x D	6	ns	ns	ns	ns	ns	ns
30	O	1	ns	ns	ns	*	ns	ns
	O x B	2	ns	ns	ns	ns	ns	ns
	O x D	6	ns	ns	ns	ns	ns	ns
	O x B x D	6	ns	ns	ns	ns	ns	ns
50	O	1	ns	ns	**	*	ns	**
	O x B	2	**	**	ns	ns	ns	*
	O x D	6	**	**	**	**	*	***
	O x B x D	6	**	**	**	**	ns	***
86	O	1	ns	ns	**	*	ns	*
	O x B	2	**	**	ns	ns	ns	ns
	O x D	6	**	**	**	**	ns	***
	O x B x D	6	**	**	**	**	ns	***
145	O	1	ns	ns	ns	ns	ns	*
	O x B	2	*	**	**	ns	*	ns
	O x D	6	**	**	**	**	*	***
	O x B x D	6	**	**	**	**	ns	***

Table 2.3: Analysis of variance for vegetative growth responses at different harvest times of two Italian ryegrass biotypes exposed to different levels of ozone and herbicide in year 2001.O: ozone; B: biotype; D: herbicide dose.

Harvest (Days after planting)	Source	df	Number of leaves	Number of tillers	Leaf area	Plant height	Shoot/root ratio	Total biomass
21	O	1	ns	ns	ns	ns	ns	ns
	O x B	2	ns	ns	ns	ns	ns	ns
	O x D	6	ns	ns	ns	ns	ns	ns
	O x B x D	6	ns	ns	ns	ns	ns	ns
35	O	1	ns	ns	****	ns	ns	****
	O x B	2	ns	ns	ns	ns	ns	ns
	O x D	6	***	***	***	*		***
	O x B x D	6	**	*	***	*		***
46	O	1	ns	ns	*	*	ns	*
	O x B	2	**	*	*	ns	ns	*
	O x D	6	***	****	****	**	ns	****
	O x B x D	6	***	****	*	**	ns	**
74	O	1	ns	ns	ns	ns	ns	ns
	O x B	2	***	***	ns	***	ns	***
	O x D	6	****	***	***	***	*	****
	O x B x D	6	****	***	ns	***	ns	ns
123	O	1		ns			ns	ns
	O x B	2		**			ns	ns
	O x D	6		***			*	ns
	O x B x D	6		***			ns	ns

* $P<0.05$; ** $P<0.01$; ***$P<0.001$; ****$P<0.0001$

2.4.2. Rate of biomass accumulation in each harvest period

Ozone affected the timing at which maximum biomass was reached, but did not affect the overall growth efficiency of the plants to produce new biomass as shown by the instantaneous RGR for the growing season (Table 2.4).

Table 2.4: Contribution of leaf area ratio (LAR) and unit leaf rate (ULR) to instantaneous relative growth rates (RGR) for a herbicide susceptible and a herbicide resistant Lolium *multiflorum* biotype in 2000 as determined by dry weight accumulation over 145 days under ozone (OZ+) and ozone free (OZ-) environments and different doses of diclofop-methyl applications

Biotype	Ozone	Herbicide Dose	RGR (week^{-1})	LAR (cm^{2}g^{-1})	ULR (gm^{-2}week^{-1})
Susceptible	OZ -	0 gai ha^{-1}	0.15	21.63	69.00
		70 gai ha^{-1}	0.15	23.76	63.13
		140 gai ha^{-1}	0.15	24.57	61.06
		1120 gai ha^{-1}	0.08	---	---
	OZ +	0 gai ha^{-1}	0.15	21.33	70.32
		70 gai ha^{-1}	0.15	18.36	81.68
		140 gai ha^{-1}	0.15	20.80	72.11
		1120 gai ha^{-1}	0.01	1.23	89.96
Resistant	OZ -	0 gai ha^{-1}	0.15	24.81	60.45
		70 gai ha^{-1}	0.16	26.10	61.30
		140 gai ha^{-1}	0.16	25.89	61.80
		1120 gai ha^{-1}	0.16	22.16	70.60
	OZ +	0 gai ha^{-1}	0.15	23.29	64.40
		70 gai ha^{-1}	0.15	23.53	63.73
		140 gai ha^{-1}	0.16	19.70	81.22
		1120 gai ha^{-1}	0.16	23.74	67.67

[a]RGR= d(ln W) / (dt), LAR=exp[fL(t) − fW(t)], ULR=fW'(t) exp[fW(t) - fL(t)], where W is dry weight, L is leaf area and t is time.

Italian ryegrass plants were similar in growth pattern for both years, i.e. an increase in the interval RGR (RGR_{int}) for the first 2 or 3 harvests with a subsequent continuous fall for the remaining harvests (Tables 2.5 and 2.6). The effect of ozone and herbicide application on the biomass of Italian ryegrass plants is reflected in the different growth attained in each harvest period (Table 2.5). In 2000, RGR_{int} of unsprayed plants of both biotypes was affected by ozone in some intervals for S plants and most intervals for R plants (Table 2.5). Often when a significant difference in RGR_{int} between ozone and control plants was observed in a certain period, the difference was reversed in the following growing interval.

Regardless of ozone exposure, the pattern of change in RGR_{int} of the susceptible biotype plants sprayed with herbicide was very similar to those observed for the unsprayed controls (Table 2.5). The highest herbicide dose reduced the RGR_{int} of the susceptible plants after the second interval, reaching negative values (biomass decay) in the ozone chambers during the last two intervals, and 86 DAP in control chambers. Herbicide also produced a strong response in the RGR_{int} of the resistant biotype plants, which became evident at different intervals depending if they were exposed to ozone or not.

Table 2.5 : Interval Relative growth rates (RGR_{int}) for a herbicide susceptible and a herbicide resistant *Lolium multiflorum* biotype in 2000 as determined by dry weight accumulation over time[a]

Biotype	Ozone	Herbicide Dose	RGR[b]				
			0-21 DAP[c]	21-30 DAP	30-50 DAP	50-86 DAP	86-145 DAP
			------------------- mg g^{-1} d^{-1} -------------------				
Susceptible	OZ -	0 gai ha^{-1}	148 a	205 bc	149 a	16 bc	14 c
		70 gai ha^{-1}	150 a	214 b	133 b	20 b	15 c
		140 gai ha^{-1}	140 a	236 ab	121 b	21 b	19 a
		1120 gai ha^{-1}	141 a	255 ab	39 d	11 c	-10 e
	OZ +	0 gai ha^{-1}	135 ab	242 ab	126 b	20 b	13 c
		70 gai ha^{-1}	140 a	224 b	144 ab	7 c	17 a
		140 gai ha^{-1}	138 a	243 ab	122 b	16 bc	15 c
		1120 gai ha^{-1}	136 a	251 ab	44 d	-11 d	-31 e
Resistant	OZ -	0 gai ha^{-1}	142 a	185 c	151 a	23 b	11 cd
		70 gai ha^{-1}	120 b	266 a	134 b	23 b	16 ab
		140 gai ha^{-1}	118 b	279 a	140 ab	18 b	13 c
		1120 gai ha^{-1}	119 b	267 a	132 b	19 b	18 a
	OZ +	0 gai ha^{-1}	118 b	287 a	88 c	26 a	19 a
		70 gai ha^{-1}	126 ab	224 b	140 ab	20 b	13 c
		140 gai ha^{-1}	125 ab	221 b	114 bc	34 a	17 a
		1120 gai ha^{-1}	125 ab	263 a	110 bc	27 a	13 c

[a]Means represent the averages of values taken from a total of 9 plants. Means followed by the same letter within a column are not significantly different.
[b]RGR_{int} = (ln W_2 – ln W_1) / (T_2 – T_1) where W_2 is the dry weight (g) at the end of the observation period, W_1 is the dry weight (g) at the beginning of the observation period, T_2 the time (d) at the end of the observation period and T_1 the time (d) at the beginning of the observation period.
[c]DAP: days after planting

In the second experiment (year 2001), despite the fact that the experiment lasted less time and the RGR_{int} values were generally lower, responses to

all treatments followed the same trends as in the first experiment (Table

2.6).

Table 2.6:   Interval Relative growth rates (RGR$_{int}$) for a herbicide susceptible and a herbicide resistant *Lolium multiflorum* biotype in 2001 as determined by dry weight accumulation over time[a]

Biotype	Ozone	Herbicide Dose	RGR[b]					
			0-21 DAP	21-35 DAP	35-46 DAP	46-74 DAP	74-123 DAP	74-123 DAP ozone all season
			--------------------- mg g^{-1} d^{-1}-----------------------					
Susceptible	OZ -	0 g aiha^{-1}	70 a	208 a	74 d	31 b	4 c	
		70 g aiha^{-1}	69 a	107 c	126 c	66 a	30 a	
		140 g aiha^{-1}	70 a	123 bc	42 d	6 c	--	
		1120g aiha^{-1}	76 a	90 c	--	--	--	
	OZ +	0 gaiha^{-1}	64 a	184 ab	156 b	0.5	29 ab	*27 ab*
		70 gaiha^{-1}	66 a	140 b	64 d	28 b	21 ab	*44 a*
		140 gaiha^{-1}	67 a	133 bc	51 d	0.0	25 ab	--
		1120 gaiha^{-1}	72 a	92 c	--	--	--	--
Resistant	OZ -	0 gai ha^{-1}	80 a	94 c	282 a	--	16 b	
		70 gai ha^{-1}	72 a	99 c	258 ab	17 c	16 b	
		140 gai ha^{-1}	53 a	115 c	290 a	10 c	21 ab	
		1120 gai ha^{-1}	63 a	149 b	184 b	9	36 a	
	OZ +	0 gai ha^{-1}	59 ab	165 b	178 b	11	23 ab	*18 b*
		70 gai ha^{-1}	59 ab	162 b	176 b	29 b	20 ab	*10 b*
		140 gai ha^{-1}	56 b	150 b	138 c	37 b	25 ab	*16 b*
		1120 gai ha^{-1}	58 a	132 bc	131 c	38 b	18 b	*22 ab*

[a]Means represent the averages of values taken from a total of 9 plants
[b]RGR$_{int}$ = (ln W$_2$ – ln W$_1$) / (T$_2$ – T$_1$) where W$_2$ is the dry weight (g) at the end of the observation period, W$_1$ is the dry weight (g) at the beginning of the observation period, T$_2$ the time (d) at the end of the observation period and T$_1$ the time (d) at the beginning of the observation period

2.4.3 Allocation patterns

2.4.3.1 Vegetative structures

Ozone affected most vegetative measures of both Italian ryegrass biotypes. However, the magnitude of responses was variable across harvest dates and biotypes resulting in significant interactions (Tables 2.2 and 2.3). As with biomass, in 2000 the maximum number of leaves and tillers, and maximum plant length and leaf area did not differ between ozone and control treatments (Figs 2.4a-b, c-d, 2.6a-b and 2.8a-b). Nevertheless plants in ozone chambers reached a plateau in leaf area and tiller production later than control plants. By 150 DAP number of tillers and leaf area in control (no ozone and no herbicide) plants were decreasing while the rate of production of new photosynthetic tissue in ozone plants was still rising (Figs. 2.6a-b and 2.8b). Shoot/root ratio was not modified by ozone for both biotypes throughout the growing season (Fig. 2.4e-f). In year 2001, when plants were exposed to ozone until late in the growing cycle, we recorded differences in vegetative parameters between ozone and control plants only on certain dates (Fig. 2.5). In some cases the response to ozone was opposite to that observed the previous year: on day 75, ozone S plants were higher than control plants (Fig. 2.5c) and on day 50 ozone R plants had more tillers than control plants (Fig. 2.9b-d).
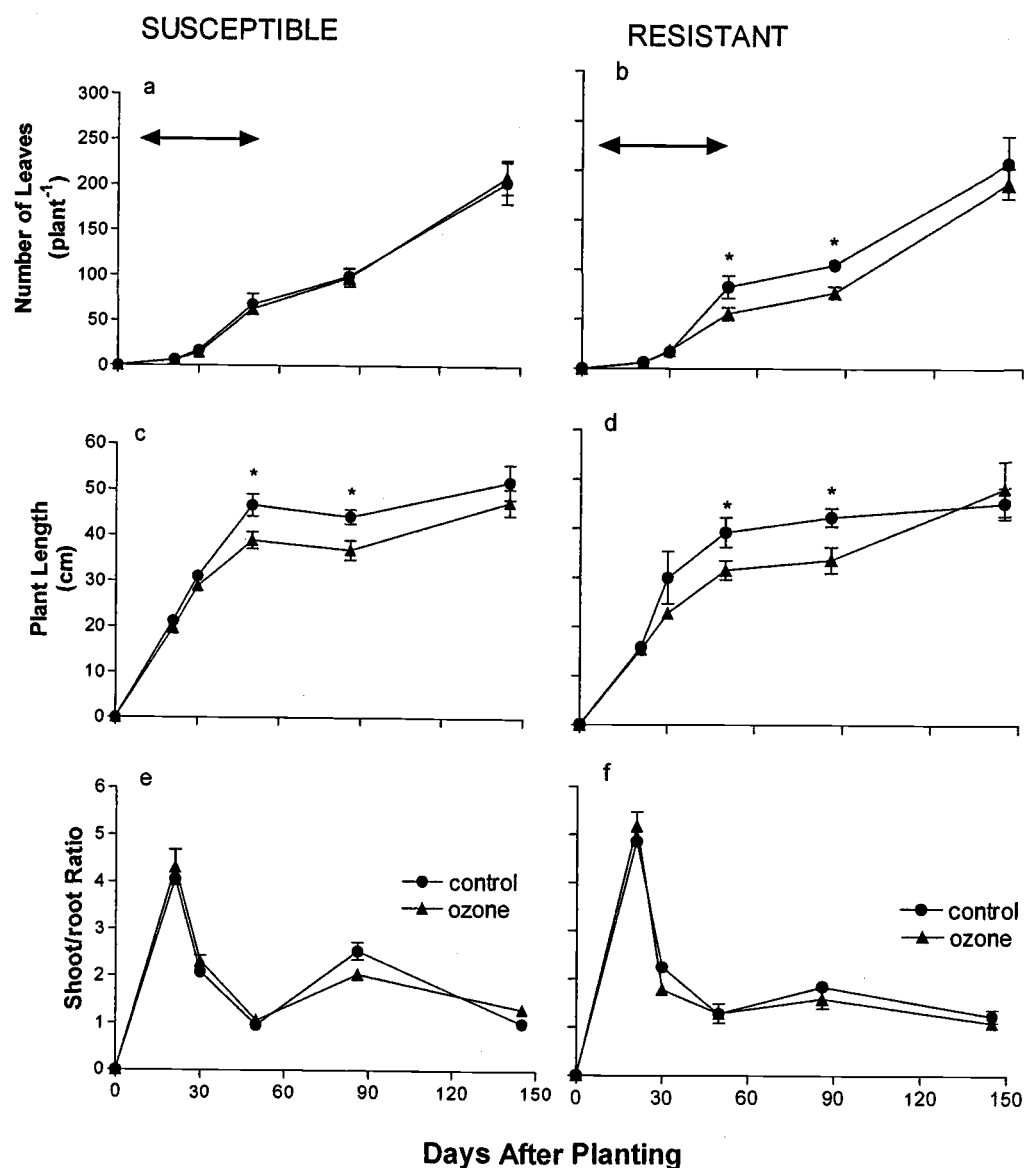
Figure 2.4: Number of leaves (a-b), plant length (c-d, measured as length of the longest leaf) and shoot/root ratio (e-f) of diclofop-methyl susceptible and resistant Italian ryegrass plants grown in control and ozone chambers during 2000. Horizontal arrows represent period of ozone exposure. Values are means of 3 replicate chambers ± SE, with observations from 3 plants averaged per chamber. * Indicates significant difference between ozone and control plants.

Figure 2.5: Number of leaves (a-b), and plant length (c-d, measured as length of the longest leaf) of diclofop-methyl susceptible and resistant Italian ryegrass plants grown in control and ozone chambers during 2001. Horizontal arrows represent period of ozone exposure. Values are means of 3 replicate chambers ± SE, with observations from 3 plants averaged per chamber. * Indicates significant difference between ozone and control plants.

Moreover, a continuous exposure to ozone (chronic) under no herbicide or low herbicide doses produced a slight increase in the number of tillers of both biotypes relative to plants exposed to ozone for only a short period of time (ozone) (Fig. 2.9a,b,c,d).

The dynamics of tiller and leaf area production of plants exposed to herbicide in 2000 paralleled biomass accumulation for both biotypes (Figs. 2.6, and 2.8). Except for normal-use dose in susceptible plants, the herbicide produced little or no decrease in initial leaf area and number of tillers of control plants with a subsequent increase in the rate of new tillers and leaf area production after herbicide application. Interestingly, in the ozone plants herbicide also produced an increase in leaf area and number of tillers, decreasing the difference between control and ozone plants. In 2001, the increase in tiller number and leaf area after herbicide application was only observed for R plants (Figs. 2.7c-d and 2.9d-f-h). As a result of these treatment interactions on the production of vegetative tissues, the average leaf area per unit of biomass contributing to growth (LAR) was different among herbicide and ozone treatments (Table 2.4).

Figure 2.6: Dynamics of leaf area production of diclofop-methyl susceptible and resistant Italian ryegrass plants in ozone and control chambers during 2000. Plants were sprayed with different diclofop-methyl doses (c-h) or received no herbicide (a-b). Horizontal arrow indicates period of ozone exposure. Vertical arrow indicates day of herbicide application. Values are means of 3 replicate chambers ± SE, with observations from 3 plants averaged per chamber. * Indicates significant difference between ozone and control plants.

SUSCEPTIBLE

RESISTANT

0 g ai.ha^{-1}

0 g ai.ha^{-1}

70 g ai.ha^{-1}

70 g ai.ha^{-1}

140 g ai.ha^{-1}

140 g ai.ha^{-1}

1120 g ai.ha^{-1}

1120 g ai.ha^{-1}

Leaf Area (cm^2 plant^{-1})

control
ozone

Days after Planting
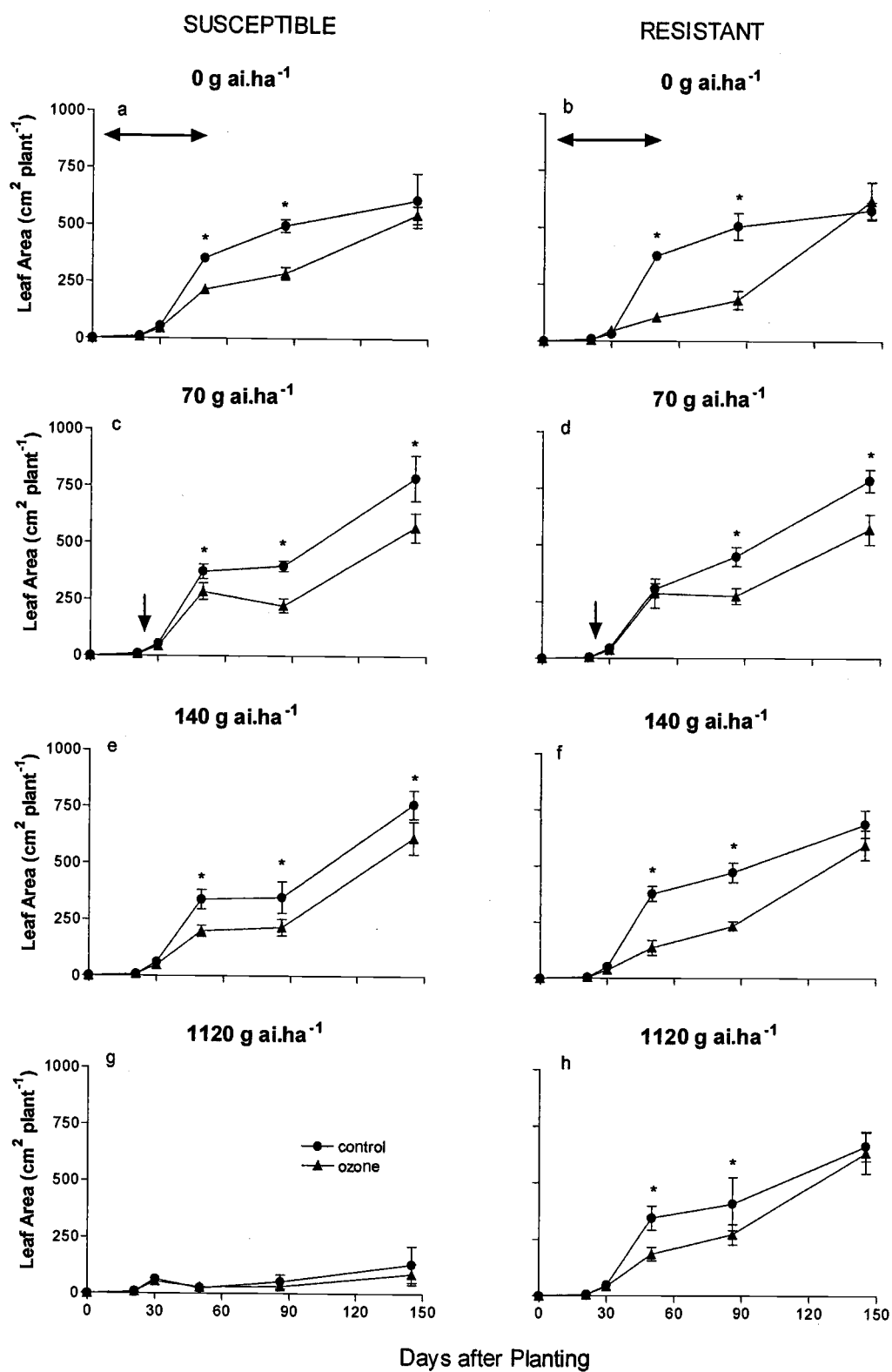
Figure 2.7: Dynamics of leaf area production of diclofop-methyl susceptible and resistant Italian ryegrass plants in ozone and control chambers during 2001. Plants were sprayed with different diclofop-methyl doses (c-h) or received no herbicide (a-b). Horizontal arrow indicates period of ozone exposure. Vertical arrow indicates day of herbicide application. Values are means of 3 replicate chambers ± SE, with observations from 3 plants averaged per chamber. * Indicates significant difference between ozone and control plants.

Days After Planting
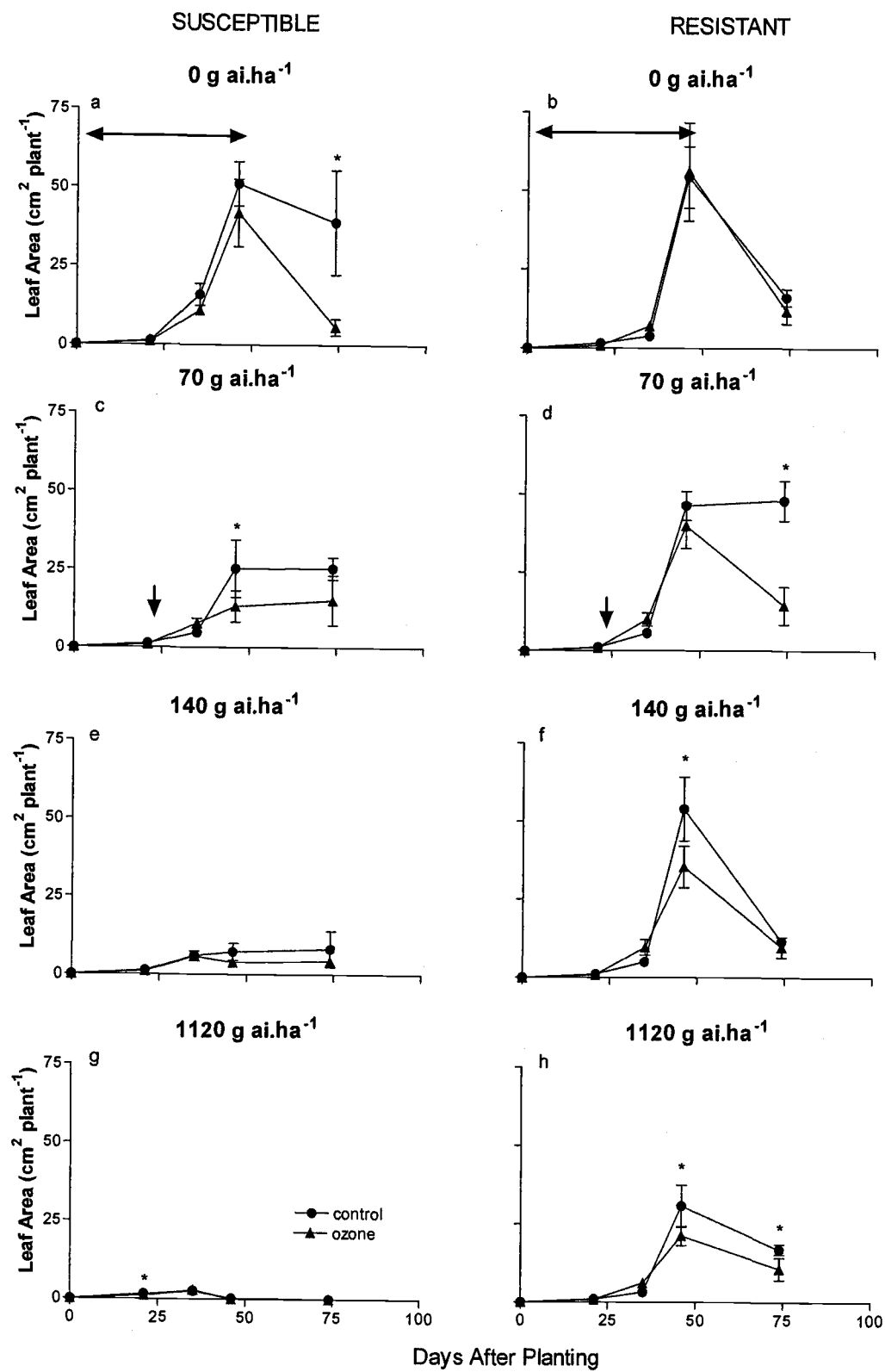
Figure 2.8: Dynamics of tiller production of diclofop-methyl susceptible and resistant Italian ryegrass plants in ozone and control chambers during 2000. Plants were sprayed with different diclofop-methyl doses (c-h) or received no herbicide (a-b). Horizontal arrow indicates period of ozone exposure. Vertical arrow indicates day of herbicide application. Values are means of 3 replicate chambers $\pm$ SE, with observations from 3 plants averaged per chamber. * Indicates significant difference between ozone and control plants

SUSCEPTIBLE

RESISTANT

Days after Planting
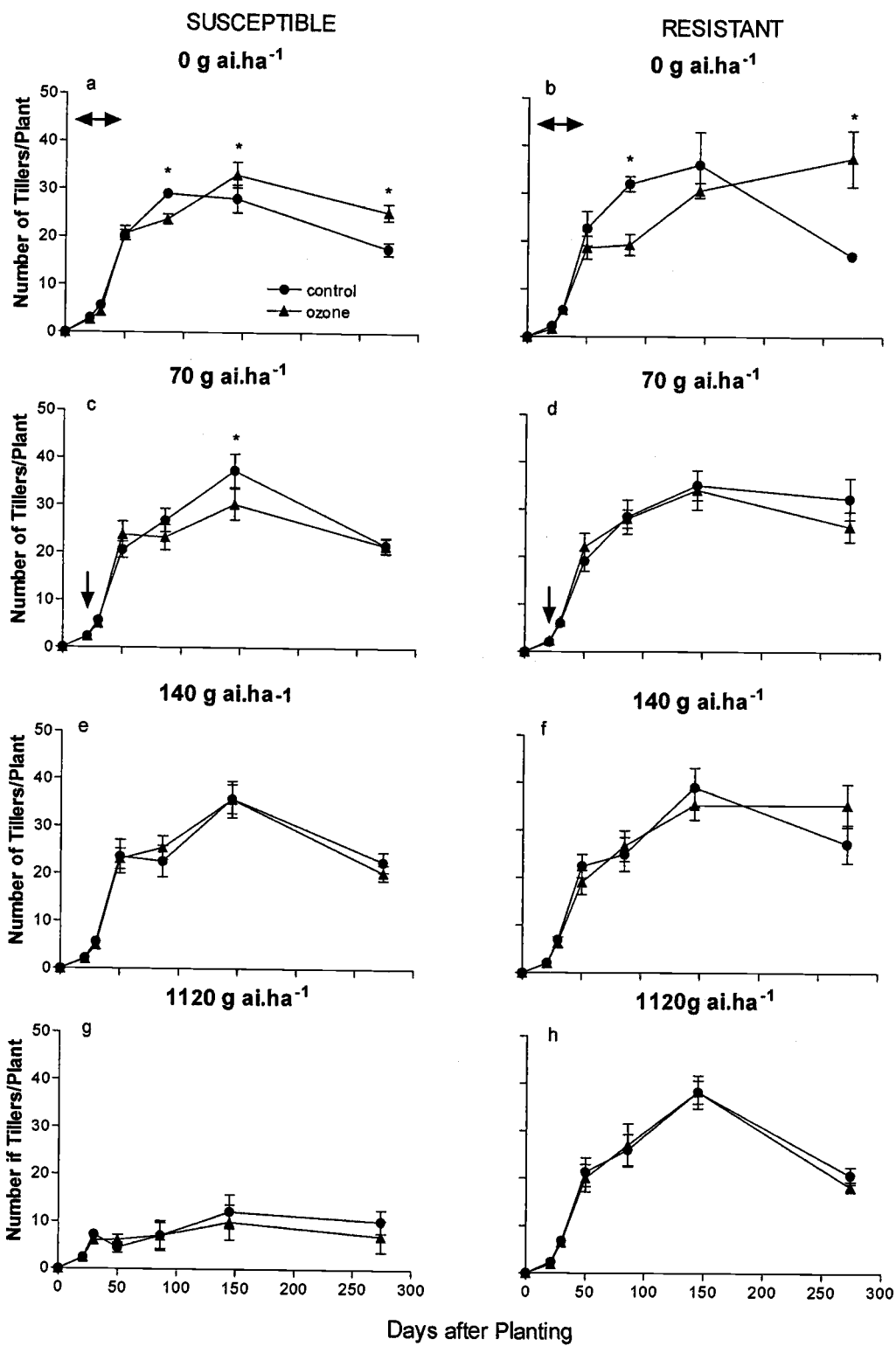
Figure 2.9: Dynamics of tiller production of diclofop-methyl susceptible and resistant Italian ryegrass plants in ozone and control chambers during 2001. Plants were sprayed with different diclofop-methyl doses (c-h) or received no herbicide (a-b). Horizontal arrow indicates period of ozone exposure, except for chronic ozone treatment where exposure lasted until final sampling date. Vertical arrow indicates day of herbicide application. Values are means of 3 replicate chambers $\pm$ SE, with observations from 3 plants averaged per chamber. * Indicates significant difference between ozone and control plants.
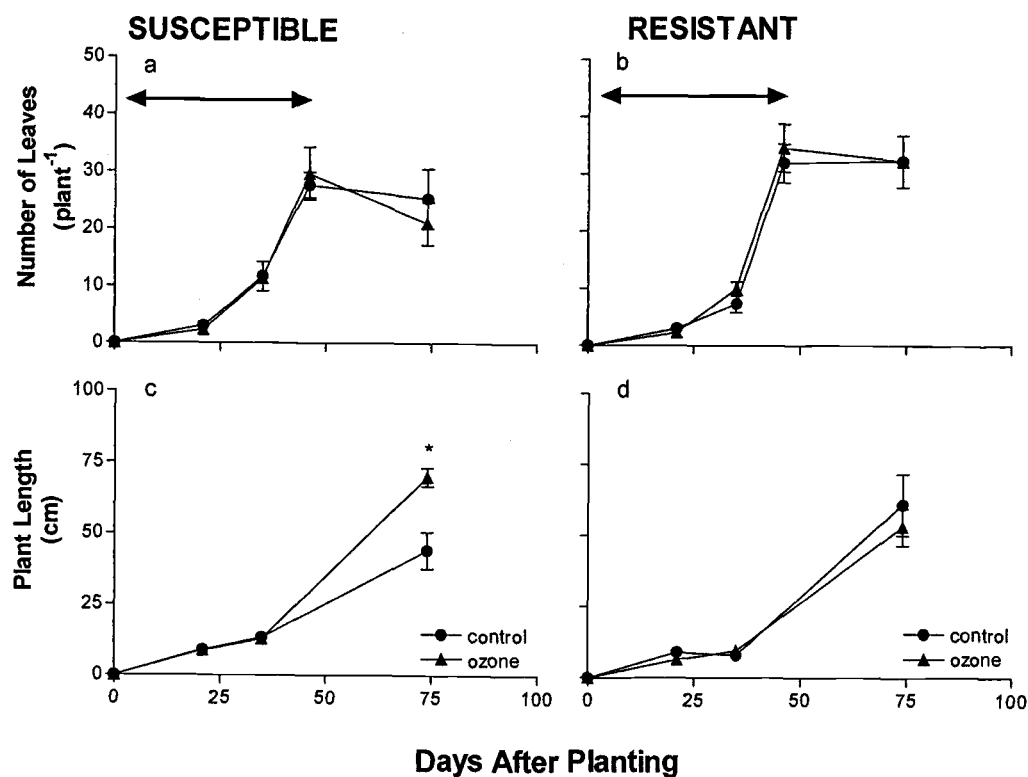
SUSCEPTIBLE

RESISTANT

Days After Planting
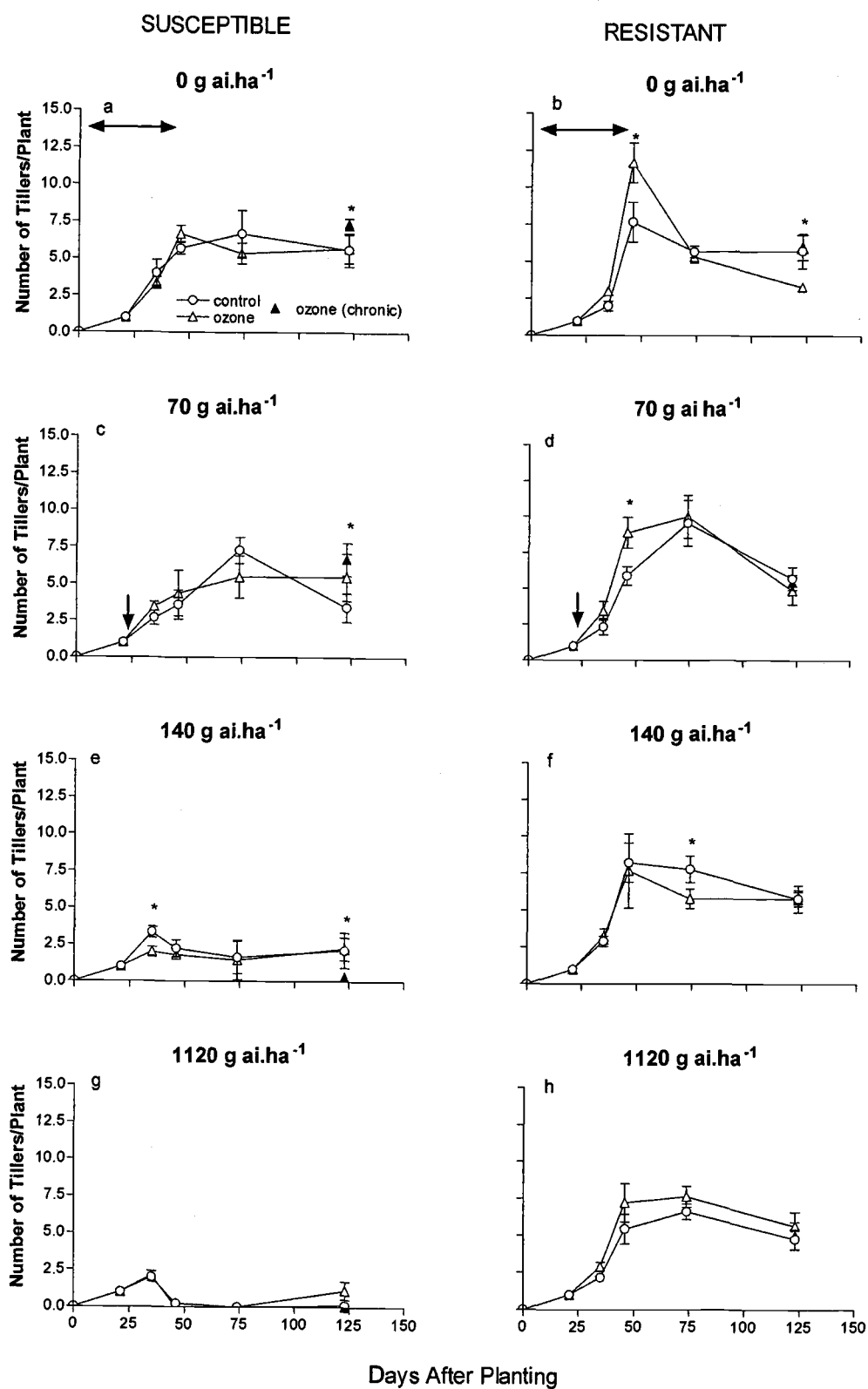
2.4.3.2 Seed production and reproductive effort

In year 2000, ozone curtailed seed production, in both S and R plants (Table 2.7 and Fig. 2.10). Like for parameters of plant growth, the response of seed production to ozone was different depending on biotype and herbicide dose. In the absence of herbicide, ozone reduced seed production of both biotypes, but the effect was particularly important in the resistant biotype, which produced less than 50 % of seed biomass when exposed to ozone as when not exposed (Fig. 2.10). As expected, herbicide application reduced seed production of the susceptible biotype plants; this effect was larger when plants grew in the chambers with ozone (compare difference between herbicide and no herbicide plants in control and ozone chambers, Fig. 2.10a). Herbicide application in the first year of experiment decreased seed biomass in R plants in control chambers, and increased it under ozone exposure (Fig. 2.10b).

Table 2. 7 : Significance levels derived from analysis of variance for yield components of two Italian ryegrass biotypes exposed to different levels of ozone and herbicide in years 2000 and 2001.O: ozone; B: biotype; D: herbicide dose

Source	df	2000		2001	
		Number of spikes	Seed biomass	Number of spikes	Seed biomass
O	1	ns	**	ns	ns
O x B	2	ns	ns	**	*
O x D	6	**	**	***	*
O x B x D	6	*	ns	ns	ns

* $P<0.05$; ** $P<0.01$; ***$P<0.001$; ****$P<0.0001$

In year 2001, there was a great variability in seed production. Contrary to the first experiment where both biotypes produced similar amount of seeds, in 2001, regardless of the treatment, the resistant biotype produced an order of magnitude more seeds than susceptible plants. Nevertheless as in the first experiment, seed production was different depending on biotype and herbicide dose (Fig. 2.11).

A- SUSCEPTIBLE
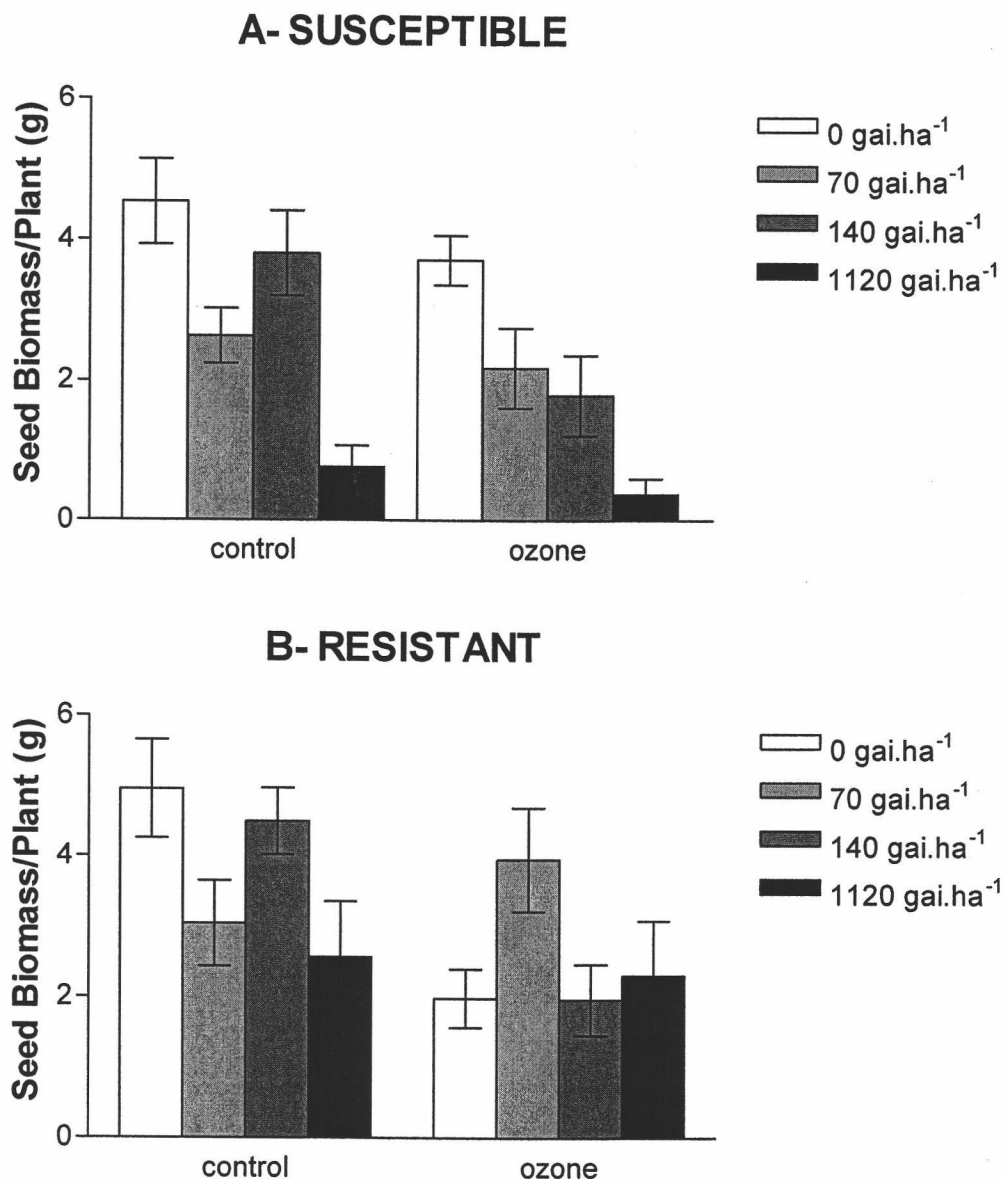
B- RESISTANT

Figure 2.10: Seed production of diclofop-methyl susceptible (A) and resistant (B) Italian ryegrass plants exposed to different doses of the herbicide in control and ozone chambers during 2000. Bars are means of 3 chambers $\pm$ SE with 3 plants averaged per chamber. ANOVA for A-Susceptible: herbicide $P<0.0001$, ozone $P=0.0079$, herbicide x ozone $P=0.29$; B-Resistant: herbicide $P=0.31$, ozone $P=0.009$, herbicide x ozone $P=0.01$
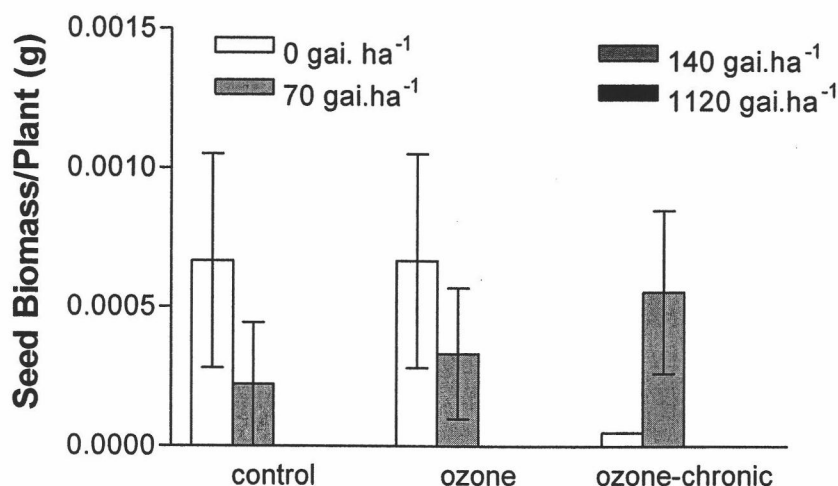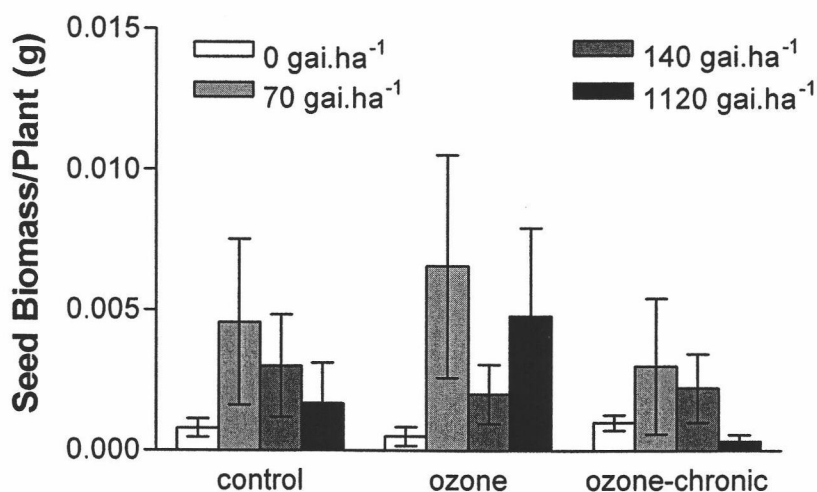
Figure 2.11: Seed production of diclofop-methyl susceptible (A) and resistant (B) Italian ryegrass plants exposed to different doses of the herbicide in control and ozone chambers during 2001. Control: plants in control chambers the whole growing season, ozone: plants exposed to ozone for 54 days and then taken to control chambers, ozone-chronic: plants in ozone chambers the whole growing season. Bars are means of 9 plants ± SE. ANOVA for A- susceptible: herbicide $P=0.006$, ozone $P=0.77$, herbicide x ozone $P=0.31$; B-resistant: herbicide $P=0.12$, ozone $P=0.44$, herbicide x ozone $P=0.84$.

In the absence of herbicide, seed production by plants of both biotypes was not affected by exposure to ozone early in the cycle. But when the plants were exposed to ozone for the full growing season (chronic), seed production by the susceptible plants was reduced to almost zero. Herbicide application strongly reduced seed production of susceptible plants, which became zero when the dose was greater than 70 gai/ha. Interestingly, when susceptible plants received the lowest dose of herbicide and were under ozone for the entire growing cycle the plants produced a similar amount of seed as the ozone-free controls. Thus this sub-lethal herbicide dose eliminated the chronic ozone treatment effect on seed production (Fig. 2.11). Seed production by resistant biotype plants was strongly augmented by herbicide application regardless of whether they were or were not exposed to ozone (except for highest herbicide dose and ozone-chronic) (Fig. 2.11).

The number of spikes per plant did not always explain the changes in seed biomass produced from exposure to ozone or herbicide. In 2000, plants in ozone chambers produced on average numbers of spikes similar to plants in control chambers (Fig. 2.12). Moreover, the increase in number of tillers observed for ozone plants in 2001 did not translate into more spikes per plant. As expected, production of spikes was decreased by herbicide dose in susceptible plants during both years (Figs. 2.12 and 2.13).
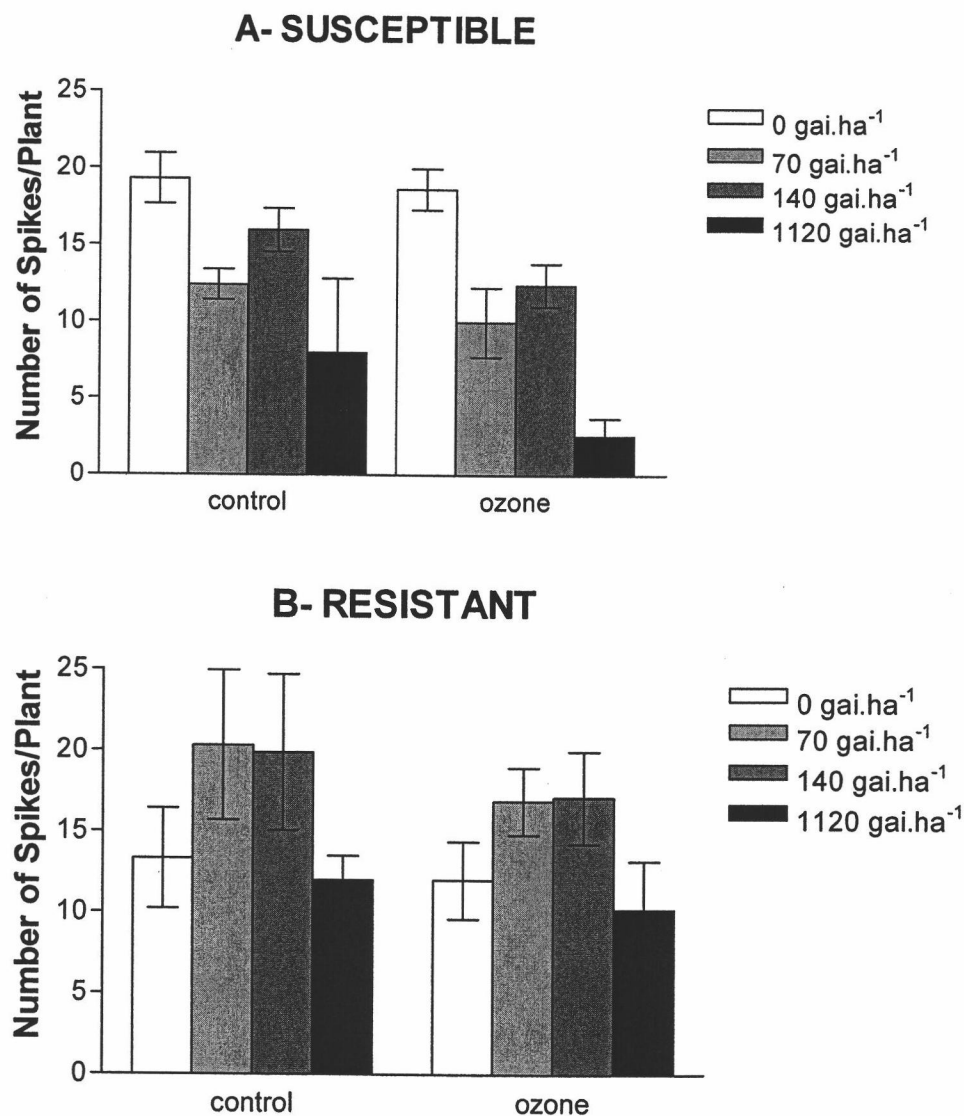
Figure 2.12: Number of spikes produced by diclofop-methyl susceptible (A) and resistant (B) Italian ryegrass plants exposed to different doses of the herbicide in control and ozone chambers during 2000. Bars are means of 9 plants $\pm$ SE (3 plants averaged in each of 3 chambers). ANOVA for A-susceptible: herbicide (H) $P<0.0001$, ozone (O) $P<0.05$, HxO $P=0.75$; B-resistant: herbicide (H) $P=0.04$, ozone (O) $P=0.31$, HxO $P=0.98$

A- SUSCEPTIBLE
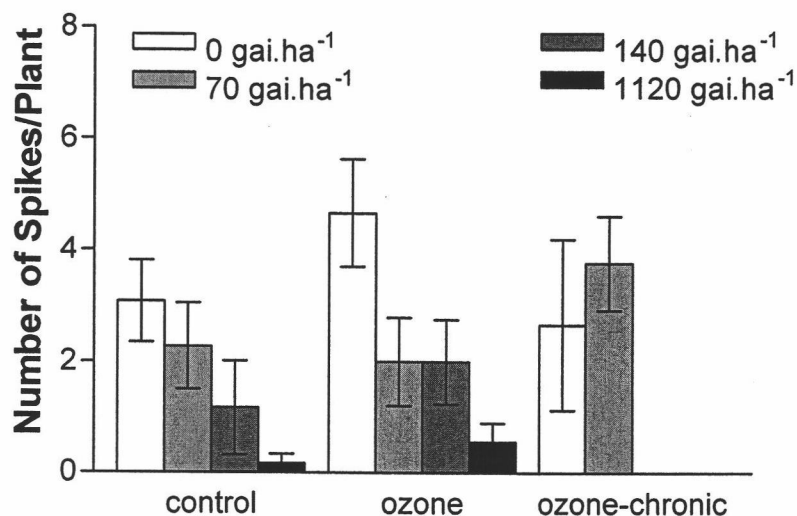
B- RESISTANT
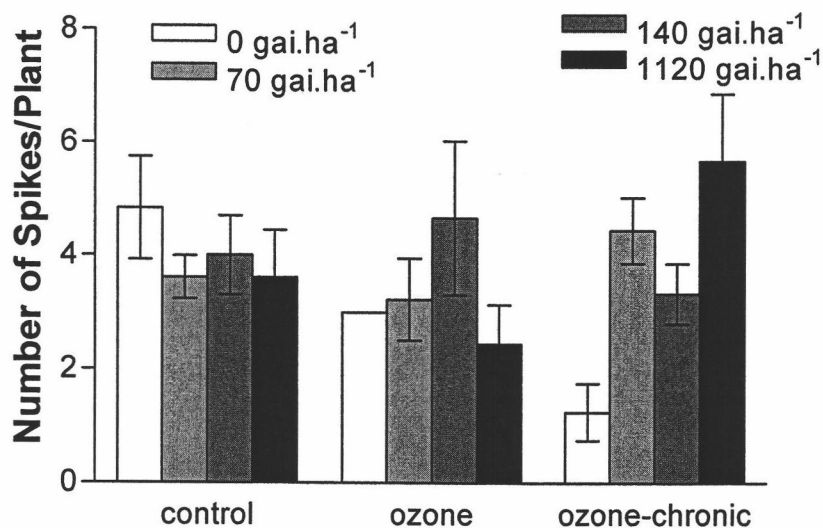
Figure 2.13: Number of spikes produced by diclofop-methyl susceptible (A) and resistant (B) Italian ryegrass plants under herbicide in control and ozone chambers during 2001. Ozone-chronic: plants in ozone chambers the whole growing season. Bars are means of 9 plants $\pm$ SE. ANOVA for A-susceptible: herbicide (H) $P=0.006$, ozone (O) $P=0.77$, HxO $P=0.32$; B-resistant: herbicide (H) $P=0.12$, ozone (O) $P=0.44$, HxO $P=0.84$

In year 2000 the reproductive effort (reproductive biomass as a percent of maximum biomass achieved) of susceptible plants growing in ozone free chambers (control) and no herbicide application was 18 % and lower than the 26 % calculated for control resistant plants (Table 2.8). Ozone did not affect this ratio in the susceptible plants but strongly reduced it in the resistant biotype to about 12 %. On the contrary, the effect of herbicide application on the reproductive effort was similar for both biotypes, lowering the ratio depending on the dose, to a minimum of 10 % in the susceptible plants that were subjected to highest herbicide level (Table 2.8).

In 2001, the late planting date reduced the reproductive effort of the control susceptible biotypes plants to only 5 % but did not affect that of the resistant plants. However, the stress imposed by herbicide and ozone together generally increased reproductive effort of both biotypes reaching values as high as 89 % in resistant plants (Table 2.8).

Table 2.8: Reproductive effort (RE) of a herbicide susceptible and a herbicide resistant *Lolium multiflorum* biotype in 2000 and 2001 under ozone (OZ+) and ozone free (OZ-) environments and different doses of diclofop-methyl applications. RE for control plants is highlighted.

Biotype	Ozone	Herbicide Dose	RE[a]	
			2000	2001
Susceptible	OZ -	0 gai ha^{-1}	**0.18**	**0.05**
		70 gai ha^{-1}	0.16	0.25
		140 gai ha^{-1}	0.13	0.01
		1120 gai ha^{-1}	0.10	0.00
	OZ +	0 gai ha^{-1}	0.17	0.18
		70 gai ha^{-1}	0.13	0.11
		140 gai ha^{-1}	0.13	0.24
		1120 gai ha^{-1}	0.16	0.05
Resistant	OZ -	0 gai ha^{-1}	**0.26**	**0.25**
		70 gai ha^{-1}	0.13	0.18
		140 gai ha^{-1}	0.22	0.16
		1120 gai ha^{-1}	0.12	0.22
	OZ +	0 gai ha^{-1}	0.12	0.22
		70 gai ha^{-1}	0.20	0.89
		140 gai ha^{-1}	0.11	0.76
		1120 gai ha^{-1}	0.13	0.28

[a] RE=RB/(RB+VB) where RB= total reproductive biomass per plant, and VB=maximum total vegetative biomass accumulated per plant.

2.5 DISCUSSION

Our experiments show that ozone exposure can affect growth of *Lolium multiflorum* , modulating biomass production and allocation patterns as well as interactions with another abiotic stress factors such as herbicide. Moreover, our data show that the magnitude and direction of responses might be quite different depending on the plant biotype, phenological stage and environmental conditions.

The degree of ozone stress response of plants is determined by their relative sensitivity to ozone, by O_3 concentrations and by duration of exposure (Heagle et al., 2000). The impact of ozone on growth and reproduction of both Italian ryegrass biotypes was more important in the first year of the experiment, carried out during the normal growing conditions for this species (Figs. 2.2, 2.3, 2.10 and 2.11). This result was surprising because ozone exposure in other cereal species decreases yield more after anthesis than before (Pleijel et al., 1998). Ozone levels in the exposure chambers were similar both experimental years but plants were exposed to the stress at different times in their life cycle. In 2001, high temperatures and longer days shortened the growing cycle. Plants were significantly smaller than plants from the previous year experiment as a consequence of early flowering induced by the photo-thermal conditions.

Thus overall environmental conditions were probably the main stress factor for the plants in 2001. For other atmospheric pollutants such as sulfur dioxide, the amount that actually enters leaf cells is dependant on stomata conductance at the time that atmospheric SO_2 is high (McLaughlin and Taylor, 1981). Therefore, internal pollutant dose is due to the interaction between factors that regulate stomata conductance (and growth) and factors that regulate atmospheric concentrations of the pollutant. Even when acute ozone exposures occurred during and after anthesis in 2001, its effect on growth and seed production was only evident when plants were under ozone for the whole season.

Both biotypes were equally efficient in producing new biomass (Table 2.4). However, plant exposure to ozone affected growth of each Italian ryegrass biotypes changing leaf area ratio, without changing total biomass accumulation. Compensation mechanisms were especially evident in year 2000. Given enough time vegetative growth parameters of ozone-stressed plants eventually approached that of the controls, not exposed to ozone. Injury caused by ozone resulted in significant reductions in photosynthetic tissues. This is in accordance with existing literature showing foliar injury caused by ozone exposure in a number of crops (Heggestad et al., 1984; Deveaou et al., 1987; Krupa and Kickert, 1997). However, other responses

to ozone such as changes in shoot/root ratio (Polle et al., 2000) were not observed in our experiments (Fig. 2.4).

We observed typical signs of ozone damage early in the cycle, around 10 days after beginning exposure. Symptoms of O_3 injury included chlorosis, bronzing, and early senescence of mid-aged and older leaves. However, damage was not permanent, but rather generated a change in plant phenology. By the last harvest in year 2000, the rate of production of new tillers and leaf area in control plants was declining (Figs. 2.6 and 2.8). This is an expected pattern for an annual semelparous plant, ie. translocation of assimilate to flowers and respiration, which usually accompanies senescence. On the other hand, rate of leaf area production of ozone-stressed plants increased soon after exposure ended. The delay in the time of leaf area accumulation of the plants under ozone stress was accompanied by higher unit leaf rates (ULR) than control plants, resulting in equal RGR and similar maximum biomass. ULR is the net gain of assimilates per unit of leaf area and time (Gardner et al., 1985). ULR provides an estimate of long-term photosynthetic efficiency by measuring how well a plant uses the available leaf area to produce biomass. Increased photosynthetic efficiency was probably an important compensation mechanism for Italian ryegrass plants under ozone or herbicide stress conditions.

The increase of tiller number after exposure to stress had ceased was a consistent response to ozone and sublethal doses of herbicide in both years (Figs. 2.8 and 2.9). It was apparent in plants grown under both acute and chronic exposure to ozone. The stimulation of tillering may be due to the effects of the stresses on hormonal balance controlling apical dominance, similar to that reported in early works by Engle and Gabelman (1967) and Bennet and Runeckles (1977) for ozone, and by de Prado et al. (1999) for the effect of diclofop-methyl. The injuries caused by the stress might have reduced the hormonal effect of the main tiller and hence allowed the growth of axillary meristems resulting in more tillers per plant. Only a proportion of those tillers reached reproductive stage and as a result, plants growing in ozone and control chambers had similar numbers of spikes (Figs. 2.12 and 2.13). Nevertheless, seed biomass per plant was significantly affected, mainly from differences in number of seeds per spike. Even when maximum biomass attained per plant was not affected by ozone, a decrease in grain yield suggests that partitioning to vegetative structures was at the expense of reproductive tissues, *ie* not only did ozone reduce the biomass of reproductive tissue produced, but also the proportion of total biomass that was allocated to make seeds.

There is a large body of information showing that exposure to chronically elevated O_3 may cause changes in carbohydrate allocation patterns,

decreases in photosynthesis, reductions of biomass, changes in growth patterns, and premature senescence (Chameides et al., 1994; Heath and Taylor, 1997), as well as alterations in biochemical defence systems (Foyer et al., 1994, Polle, 1998). However, consistent O_3 responses have not always been found. A reason for this paucity of responses may be that O_3 sensitivity is affected by the interaction of internal plant-specific factors on the one hand, and external, environmental factors on the other (Polle et al., 2000). To date little attention has been paid to the question of how defence mechanisms against O_3 may be affected by interaction with nutrition or other environmental factors. Our data demonstrates that herbicide application had effects on both the susceptible and resistant plants responses to ozone, which depended on herbicide dose and the moment and length of exposure to ozone.

As a consequence of the interaction of both stressors we observed different responses for vegetative and reproductive plant structures. The result of the interaction of stress was also different for each biotype. In general, the decrease in vegetative parameter produced by each stress factor did not determine the effect of the stresses together (Figs. 2.6, 2.7, 2.8 and 2.9). In contrast, ozone and herbicide together produced synergistic (Fig. 2.10a) or antagonist (Fig. 2.10b) effects on reproductive structures. The interaction of ozone and herbicide had an effect on seed production and reproductive

effort of both biotypes that could not be predicted from plant responses to the individual stresses. In 2000, ozone and herbicide alone had a negative effect on the reproductive effort of both biotypes (Table 2.8). In 2001, while ozone alone did not modify reproductive ratio of resistant plants, herbicide and ozone together produced a significant increase in the ratio.

Ozone mainly enters plants mainly via the stomata and it subsequently reacts with cell wall and membrane components resulting in the formation of reduced oxygen species (ROS), such as, hydroxyl and superoxide radicals and hydrogen peroxide, which are highly reactive with biological molecules. Consequently membrane integrity is disrupted, leading to cell death (Benton et al. 2000). Like ozone, exposure to diclofop-methyl produces oxidative stress that results in induction of senescence in susceptible individuals (Shimabukuro et al., 2001). Two mechanisms of action are recognized for diclofop-methyl herbicide that affects membrane integrity. One mechanism is the inhibition of de novo fatty acid biosynthesis in plastids through inhibition of actyl-Coa carboxylase (ACCase) (Gronwald, 1991; Tardif et al., 1996). A second mechanism is a catabolic reaction in which membrane disassembly and production of free radicals results in plant death due to numerous damaging reactions resulting in oxidative stress (Shimabukuro et al., 1999). Despite formation of ROS seems to be a common underlying element in the damage caused by ozone and diclofop-

methyl herbicide, their effect on growth and reproduction of Italian ryegrass plants was different.

The biotypes behaved differently when exposed to stress. While the effect of herbicide was greater on the susceptible plants, ozone seemed to affect more the herbicide resistant than the susceptible plants. The tissues of both biotypes were injured by ozone. Both herbicide susceptible and resistant plants initially decreased in biomass, which was compensated later in the growth cycle when exposure to ozone ended (Fig. 2.2). However, the magnitude of decrease in leaf area and biomass due to ozone, was greater in resistant than on susceptible plants (Figs. 2.2 and 2.6). On the other hand, the effect of herbicide on seed production was significantly larger in S-plants than in R-plants, but seed production was more affected by ozone in the resistant plants (Fig. 2.10). Late planting of Italian ryegrass plants (second year experiment) reduced seed production significantly more in the in the susceptible than in the resistant plants (Fig. 2.11).

Resistant individuals have a modified ACCase that is insensitive to the herbicide. Both S and R-plants cell membranes are depolarized by the herbicide, but only R plants have the ability to repolarise them. Because of this physiological difference between the Italian ryegrass biotypes, it is reasonable to expect different responses not only to herbicide application

but also to ozone, another oxidizing stress and perhaps to the combination of both. Our study demonstrated that sensitivity to one oxidizing stress is not necessarily controlled by relative sensitivity to the other. The idea that tolerance mechanisms to several kinds of stresses are interconnected and partially overlapping has long been proposed. Because of the multiple roles that oxygen radicals play in plant metabolism different studies have pointed to shared rather than separate protective pathways in the plant (Leshem and Kuiper, 1996; Smirnoff, 1998). Moreover, it has been suggested that because of ROS involved in signalling, resistance could be selected against for one stress, resulting in resistance against other oxidative stresses (Perl-Treves and Perl, 2002).

While the metabolic mechanisms behind our observed responses are unknown, it is clear that the physiological changes in R plants that enable herbicide tolerance, did not enable plants to tolerate ozone as well. One explanation could be the physical location of the defense systems in the cell. Plants respond to ROS levels by activating a number of antioxidative defense mechanisms. The primary event in diclofop-methyl induced phytotoxicity is a catabolic process which leads to membrane disassembly, lipid peroxidation, ethylene evolution and the metabolic induction of ROS with resulting oxidative stress (Shimabukuro et al, 2001). In R plants, resistant ACCases, auxinic compounds, and antioxidants including

enzymes protecting from ROS and constituents of the ascorbate pathway are located in the cytoplasm. On the other hand, ozone responses, such as the accumulation of ascorbate and different defense proteins are typically directed to the cell wall and apoplastic fluid (Sandermann et al., 1998). It is possible that R individuals with modified ACCase have some impediment in producing antioxidants, enzymatic and non-enzymatic scavengers of free radicals in the apoplast. The ability of R plants to tolerate ozone exposure could be modified by sublethal doses of herbicide application. Auxin levels increase resulting in membrane repolarisation in R plants, which may become over expressed as a consequence of the acute stress imposed by a sub lethal herbicide dose (Vila Aiub et al., 2003). This could then be involved in reinforcing the repair system in the plan, *ie* the membrane repolarisation ability of R-plants could be involved in the antagonist interaction between ozone and herbicide plant exposures. We observed some antagonistic effects of both stresses on the resistant plants (e.g. Fig. 2.10). Herbicide resistant and susceptible plants should be examined for levels of constitutive and induced antioxidants enzymes to start testing these hypotheses.

If the apparent different susceptibility to ozone of herbicide susceptible and resistant plants is confirmed with further studies, evolutionary tradeoffs could be suggested. When Italian ryegrass plants evolve to be resistant to

herbicides they may lose tolerance to other stressors. Current theory of adaptive evolution suggests occurrence of fitness tradeoffs when populations undergo adaptive change forced by high selective pressures (Bazzaz et al., 1987). Previous work shows that susceptible and resistant Italian ryegrass biotypes do not exhibit large difference in fitness under herbicide free conditions (Roush et al., 1994). However if, as was shown in these experiments, the pollutant differentially affects susceptible and resistant plants, fitness cost of resistance might be seen in polluted environments without herbicide spraying. In this context, our findings provide new and important information on the potential for multiple stress interactions.

This information is important because, the intensity of each individual stress may be low but in combination with other stresses, capable of exerting significant selection pressure on alleles that determine sensitivity (Winner, 1994). On the other hand, late planting had a greater effect on susceptible than on resistant plants, which produced a significant greater amount of seeds than susceptible plants in the second year. In agro ecosystems where herbicides and ozone pollution may occur simultaneously, our results suggest that the resistant biotype should have a strong advantage over the susceptible biotype. This advantage is particularly evident for late emerging

plants, which may be most frequent in commercial farms because early emerging would be efficiently controlled by soil tilling.

The development of research in plant stress physiology has had a long and varied history. However the number of studies on single stressors far exceeds the number of studies on multiple-stressor interaction. This study shows that not only do we need more information on multiple stressors, but also a closer link between stress physiology and ecophysiology experiments, looking at evolutionary processes associated with adaptation to a wide variety of environmental conditions. Only in this way will we understand the complex interactions among stresses such as ozone and other environmental factors that determine fitness in the agricultural systems.

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CHAPTER 3

ITALIAN RYEGRASS DENSITY RESPONSES UNDER OZONE AND HERBICIDE STRESS

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3.1 ABSTRACT

Adaptations to overcrowding of individuals have probably been evolving since the beginning of life on the earth, resulting in density dependant controls of growth and development. There is little information on how new anthropogenic stress factors could modify responses to density. Level of pollutant in the atmosphere is known to affect not only general plant growth but also allocation patterns. It is reasonable to think that atmospheric stressors could affect differentially the dominant and subordinate individuals in a population. We investigated whether a combination of diclofop-methyl herbicide and ozone could erase the bimodal pattern for average individual weight typically resulting from intraspecific competition, and also whether the interaction of the stresses curtailed the expected growth compensation of individuals when density declines. Seeds of Italian ryegrass were sown at a density of 500 seeds/pot and plants were grown in ozone or control chambers in 2000 and 2001, early and late in the normal growing period for the species respectively. Half of the pots were sprayed with diclofop-methyl herbicide, and one week later all pots were thinned to densities of 1, 10, 20 or 50 plants per pot. Individual plant vegetative parameters and total seed production per pot were analyzed. Both ozone and herbicide modified competition relationships among Italian ryegrass plants, but only herbicide

had an impact on seedling mortality. The biomass density relationship followed an hyperbolic model, which reached saturation at lower densities in 2000 than in 2001. Total biomass per pot as affected by density was modified by stress only in 2001. Ozone did not modify the effect of density on individual average plant biomass, leaf area or number of leaves. Herbicide significantly intensified density effects in ozone and control chambers. Ozone and herbicide changed the expected frequency distribution of individual plant weights as density increased. In 2000 all pots (control, herbicide and ozone-treated) had a similar seed biomass-density relationship. Compensation was achieved even though, in the lower densities, pots under stress had lower numbers of spikes per pot and larger proportions of flowering tillers. In 2001 the number of spikes per pot increased with density. Seed production was significantly lower than in 2000 and there was a tendency for an inverse relationship with density in control and ozone-treated pots. The present study suggests that information obtained about individual plant responses to human induced stress is useful to understand how populations will perform. Yet, because compensatory mechanisms occur at the population level, predictions derived only from individual studies may be inconsistent or incorrect.

3.2 INTRODUCTION

Competition among neighboring plants for limiting resources is a central

process in plant ecology, both in wild and managed situations. Plant

intraspecific competition due to increasing density, *i.e* number of plants on a

unit area, has been widely studied and reviewed, leading to a significant

amount of theory (deWit, 1960; Harper, 1977; Cussens, 1985; Radosevich,

1988; Grace and Tilman, 1990).

Experiments have repeatedly addressed the relationships among plant

yield, number of individuals and resources available in a specific area. It is

well known that as biomass production in a plant population increases, it

becomes independent of density, and is controlled by the carrying capacity

of the environment. This relationship is known as constant final yield

(Harper, 1977; Radosevich, 1987). Density produces stress, increasing

mortality risks not only to whole plants, but also to parts (leaves, roots, etc).

The rate of death becomes a function of the growth rate of the survivors-a

process known as self-thinning (Harper, 1977). Density stressed

populations form a hierarchy of dominant and subordinate individuals. The emergent pattern of this hierarchy is a bimodal or log-normal frequency distribution of plant weights, with the highest death risk present within the classes of suppressed individuals. Likewise, effects of density are not equal for all parts of a plant. Branches are very plastic, whereas leaves and seeds are relatively less so. These differences between plant parts in response to density are controlled by physiological mechanisms that detect changes in environmental signals. For example, changes in light quality related to plant density can induce differences in branching, leaf location within the canopy, and internode elongation (Ballare et al., 1990) and roots can change their elongation rate in response to the proximity of other roots (Mahall and Callaway, 1992).

Goldberg (1990) proposed a framework to understand competition effects between plants, based on her observation that most interactions between individuals occur through an intermediary. The intermediary can be a soil resource, light, an herbivore, or toxins. If the individuals deplete soil resources causing a negative effect on the environment, the net result on plant growth, as a consequence of increased density, would be negative, because plants respond positively to increasing levels of soil resources. If density results in soil accumulation of a toxin, increased density would also

have a negative impact on plant growth due to the negative response of plants to the toxin.

Aphalo and Ballare (1995) presented another important framework to represent interaction between neighboring plants. They view plants as having resource acquiring systems (*ie*. roots and leaves) that are "fed" by the resources in the environment. Plant responses also are, in part, determined by the amount and rates at which the resources accumulate in the plant (Aphalo and Ballare,1995). These authors also view plants as having an information acquiring system, which could gain information from the external and internal pools of resources (*e.g* nitrogen, phosphorus, water). Such information acquiring systems also could receive flows of information from non-resource environmental factors, such as temperature, light quality or pH. In this way plant-plant interactions at a mechanistic level are driven by information controlling plastic responses significant for space capture and resource acquisition by individual plants (Aphalo and Ballare, 1995).

Adaptations to overcrowding of individuals have probably been evolving since the beginning of life on the earth, resulting in density dependant controls of growth and development that are especially important to reduce the population's risks for extinction. In this way, specific plant populations

are formed by large numbers of metameric organisms with great plasticity, which thus, appear with a variety of phenotypes. Molding of these phenotypes depends on when, which, and at what rate mechanism/s are set to function as a consequence of genetic and environment signals interactions. In the human intensively managed ecosystem, herbicides and air pollutants are novel factors in the environment to which plants were not exposed during their evolutionary history (Larcher, 1995). There is little information on how anthropogenic stress factors could modify responses to density. Air pollution is known to affect not only general plant growth but also allocation patterns (*e.g* Chameides et al., 1994; Matyssek et al., 1995; Rennenberg et al., 1996). It is reasonable to think that atmospheric stressors could affect differentially the dominant and subordinate individuals in a population changing the expected bimodal/log distribution of individual weights. Allometric relationships may change, directly as some organs in the plant are injured more than others, and also indirectly as organ damage changes hormonal balance. Ozone typically decreases above-ground allocation more than below-ground, but the magnitude of the decreases varies widely (Pell et al., 1994). Also, these stressors induce physiological responses to overcome the oxidative reactions they cause (Sandermann et al., 1998), which could improve the performance of individuals when exposed to other stress conditions such as those generated by depletion of soil resources.

Italian ryegrass (*Lolium multiflorum* Lam) is a major grass seed crop in the Willamette Valley of Oregon and an important weed in cereal fields west of the Cascade Mountains (Appleby et al., 1976; Burrill et al., 1988). Intensive use of diclofop-methyl, a post emergence herbicide, to eliminate Italian ryegrass seedlings from winter cereal crops has led to the development of herbicide-resistant Italian ryegrass populations (Gronwald et al., 1992). Italian ryegrass is planted for turf and grazing purposes and has naturalized in many of the worlds' temperate regions (Appleby et al., 1976). For this reason it has attracted the attention of many researchers who have studied eco-physiological processes of this species, including individual plant response to intra- and inter- specific competition (Liebl and Worsham, 1987; Hashem et al., 1998, 2000). Diclofop and haloxyfop herbicides cause rapid inhibition of growth in susceptible Italian ryegrass (Shimabukuro, 1990). Plants show a biomass decrease dose response to the herbicide, and acclimation has been observed after recurrent sub-lethal dose application (Vila Aiub et al., 2004).

Growth responses to ozone and diclofop methyl herbicide on individual plants have been investigated (Martinez-Ghersa et al., in prep). While herbicide reduced biomass of susceptible Italian ryegrass plants, ozone often reduced growth rates without necessarily modifying maximum

biomass. Both stresses, alone and in combination, affected production of leaves and tillers, which in turn had an effect on seed production. Work at Nottingham (Sanders et al., 1990) showed that although a mixture of air pollutants had no significant effect on the number of leaves of field beans (*Vicia faba* L.) or on their total area, the younger leaves tended to have larger areas in charcoal filtered treatments than in non-filtered treatments. Such nearly imperceptible differences probably have implications for the effects of pollutants in combination with other stresses, but the significance of this possibility has not yet been quantified.

These experiments give information on the effect of pollutants and combination of abiotic stresses on individual plants. In general, they show that air pollutants can affect many aspects of plant development and reproduction, that some of these effects are short-term, and that the integrated consequences for the plant cannot easily be predicted due to multiple compensation mechanisms. However, in natural and agricultural systems plants usually grow in association with other plants of either the same or different species. In order to understand how novel stressors introduced by human activities may affect mechanisms controlling plant response to competition, it is important to investigate their effects on plant populations grown both as monocultures and mixtures of species.

In this paper we investigate the effects of ozone and a sub-lethal dose of diclofop-methyl on Italian ryegrass plants growing at various densities. Since ozone sometimes affected biomass accumulation rate over the growing period more than the maximum biomass (Martinez-Ghersa et al, in prep, Chapter 2) and oxidative stressors like ozone and diclofop produce greater effects on plants growing at high rates (McLaughlin and Taylor, 1981), we hypothesized that the impact of these stressors should be greatest on dominant individuals of the population. These stressors could erase the bimodal pattern for average individual weight resulting from competition at high densities. It is further hypothesized that the interaction of the pollutants will curtail the expected growth compensation of individuals that allows for constant per unit area biomass when density declines.

3.3 MATERIALS AND METHODS

3.3.1 Plant material

A diclofop-methyl susceptible *L. multiflorum* line (ORARHR-M93, Marshall cv.) (Barker et al. 1997) was used in our experiments.

3.3.2 General procedures

The experiments were conducted during the growing seasons of 2000 and 2001 using the Field Ecological Research Facility (FERF) of the US Environmental Protection Agency National Health and Environmental Effects Laboratory, Western Ecology Division in Corvallis, OR, (Hogsett et al., 1985). Around 500 seeds per pot were sown in plastic pots 30 cm in height by 10 cm in diameter and containing regular potting soil. Pots were irrigated as needed throughout the experiment. A timetable of key events during the growing season is shown in Table 3.1.

Plants were grown and exposed to ozone in 12 FERF open-top chambers (Hogsett et al., 1985). Each chamber was 3.0 m diameter x 2.4 m high with a truncated cone and rain hood at the top. The experiments were arranged in a split-split plot design with three factors: ozone, herbicide treatment and plant density. At the whole plot level, there were two ozone treatments: low ozone charcoal-filtered air (control) and charcoal-filtered air with added ozone (ozone), with six chambers per ozone level. Ozone was generated from oxygen and added to the chambers during the periods shown in Table 3.1 to match an episodic pattern of daily varying concentrations from 0 to 150 μL L^{-1} representative of ambient air monitoring sites (Tingey et al, 1986; Hogsett et al, 1985). Ozone was monitored in the chambers using Model 400A Advanced Pollution Instrumentation Inc., photometric analyzers. Ozone treatments were expressed as a seasonal exposure, SUM06, by

accumulation over the growing season of all hourly averages >0.060 μL L^{-1} (Lee et al., 1998). The SUM06 in 2000 was 29.8 μL L^{-1} hr for the ozone treatment and 0.011 μL L^{-1} hr for the control. In 2001, the SUM06 was 35.6 μL L^{-1} hr and 0 μL L^{-1}hr for the ozone and control treatments respectively.

Within each chamber there were 40 pots (Fig. 3.1). Half of the pots were randomly assigned to one of two herbicide treatments: ¼ x the field application rate (far) (280 g ai ha^{-1}), and 0 x far (no herbicide) respectively. Plants were sprayed at the two-to three leaf stage of growth in a track sprayer and returned to the ozone exposure chambers. A commercial formulation (Hoelon3EC, Aventis USA) of diclofop-methyl (methyl 2-(4-(2',4'-dichlorophenoxy)-phenoxy) propanoate))) was used.

All herbicide treated and untreated pots were randomly assigned to four seedling densities: 1, 10, 20 and 50 plants per pot. Approximately one week after herbicide application plants were thinned to the desired densities by randomly pulling out seedlings from each pot. Minimal disturbance occurred to the remaining plants during thinning. Pots were visited one week later and any re-growth from remaining crowns of previously thinned seedlings was eliminated. Any newly emerged seedlings were also pulled to maintain the desired densities. Some pots were left unthinned to evaluate the effect of thinning in each treatment.

In Oregon, Italian ryegrass seed germinates in the field over a wide range of temperatures from early fall until late spring and the growth cycle usually ends by mid-summer when plants are dry and seeds are mature (Ghersa et al., 1994). In order to assess Italian ryegrass responses to the ozone and herbicide treatments at different phenological stages, experiments in 2000 and 2001 were conducted over different time periods (Table 3.1). This resulted in a shorter growth period and ozone exposure at different phenological stages in 2001 than 2000. In 2000, exposure occurred during vegetative growth, in late summer and early fall which included tillering but finished well before anthesis. In 2001, exposure occurred in late spring and early summer and finished when plants had already flowered and had some spikes with immature seeds.
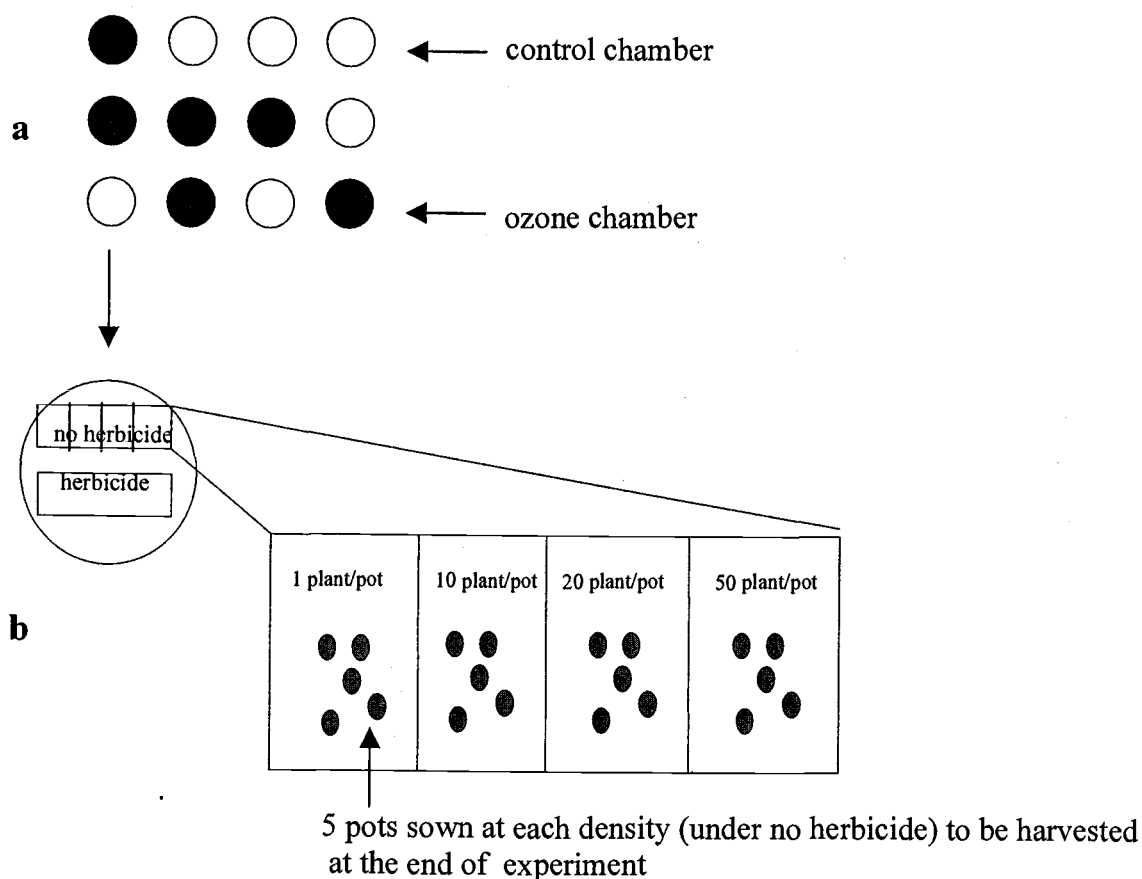
Figure 3.1. Representation of experimental layout. Air in open top chambers was ozone-free (control) or supplied with ozone (ozone). Inside each chamber pots sown with Italian ryegrass were assigned to herbicide application or no herbicide and plant density thinned to 1, 10, 20 or 50 plants per pot. a) Position of open top chambers in the research facility. b) Detail of arrangement of one group of pots (e.g. no herbicide treatment) inside a chamber.

Table 3.1 Timetable of experimental events occurring during the 2000 and 2001 growing seasons

Event	Date		Phenological stage	
	2000	2001	2000	2001
Planting	30 August	3 May	seed	seed
Start of ozone exposure	5 September	16 May	1 leave	1 leave
Herbicide application	27 September	30 May	2-4 leaves	2-4 leaves
Plant thinning	10 October	5 June	2-4 leaves	2-4 leaves
End of ozone exposure[a]	23 October	8 July	tillering	anthesis
Harvest of individual plants	28 February	-------	anthesis	--------
Final harvest	2 May	29 August	mature seed	mature seed

Plants assigned to ozone treatment remained in the exposure chamber for 45 days in 2000 and 52 days in 2001. In the year 2000 experiment, when ozone exposure ended, all pots (both ozone and control treatments) were moved to a greenhouse until flowering, seed production, and finally harvest.

The effects of density on growth of individual plants were studied only in 2000. Fifteen individual plants were randomly harvested from each

treatment combination. Number of tillers, number of leaves, leaf area and dry weight per plant was recorded. Also, one pot per density and herbicide treatment from each of three of the ozone and three of the control chambers was evaluated for biomass production. Total density and dry biomass per pot were measured after 72 h at 60 °C.

In both years, when spikes with mature seeds were observed, all pots were evaluated weekly, until no more new spikes appeared. Number of spikes per pot was recorded, harvested and seed biomass was determined.

2.3.4. Data analysis

Regression analysis was used to relate final and initial densities, and vegetative and reproductive yield to density. Linear or non-linear models were fitted by means least square using the regression utility in GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, 2003). A function of the form: $Y = Y_{max}.N/(K_n + N)$, where Y_{max}= maximum yield, N=density and K_n=density at ½ Y_{max} was fitted to data following a non-linear model.

3.3 RESULTS

In both years, there was a linear positive relationship between final density of control (neither ozone or herbicide) Italian ryegrass plants at harvest time and density to which pots were hand-thinned (Fig. 3.2). However, the model slope differed between years. While in year 2000 final densities were twice as large as initial densities due to regrowth of some of the original 500 plants (Fig. 3.2a), in year 2001 final densities remained around the same as initial densities (Fig. 3.2b). Ozone did not modify the relationship between initial and final density, while herbicide application both in control and ozone chambers significantly lowered it. In year 2001 final density in pots under herbicide application, with or without ozone, was reduced to only a few plants/pot independent of initial density.

In 2000, 173 days after planting, production of biomass per pot in control pots increased with density, following a rectangular hyperbolic model, and reached a plateau at a density of about 10 plants/pot (Fig. 3.3a). Stress imposed by herbicide and ozone on Italian ryegrass plants modified the biomass-density relationship. Maximum yield was attained at higher densities; around 150 plants/pot for plants in ozone chambers no herbicide, and 100 plants/pot for plants with herbicide application in control or ozone (Fig 3.3a, Table 3.2).
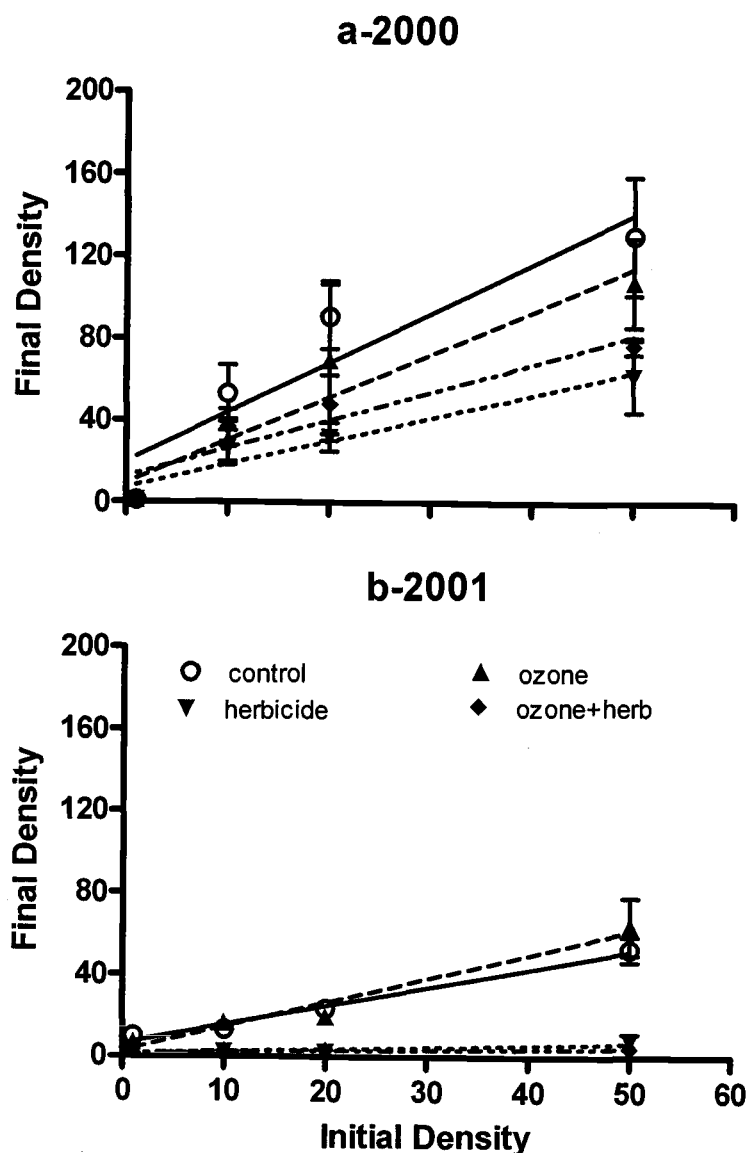
Figure 3.2. The relationship between initial Italian ryegrass plant density, at the moment of hand-thinning and final density at harvest time. Plants were grown in control or ozone chambers with or without herbicide application. Regression model fitted is final density=a+b*initial density. Model parameters are: a-year 2000: control a=19.9, b=2.4, r^2=0.68; ozone a=7.9, b=2.3, r^2=0.63; herbicide a=7.4, b=1.1, r^2=0.64; ozone+herbicide a=12.6, b=1.4, r^2=0.73. b-year 2001: control a=6.7, b=0.9, r^2=0.74; ozone a=2.6, b=1.2, r^2=0.51; herbicide a=2.4, b=0.09, r^2=0.07; ozone+herbicide a=2.6, b=0.04, r^2=0.11

Figure 3.3. Relationship between total biomass produced per pot by Italian ryegrass plants harvested at 173 days after planting in 2000 (a) and 135 days after planting in 2001 (b). Plants grew in control chambers without herbicide application (control) or sprayed with herbicide (herbicide), and in ozone chambers without herbicide application (ozone) or sprayed with herbicide (ozone+herb.). Model fitted is $Y=Y_{max}.N/(K_n + N)$. Inset in b) shows relationship for all density ranges.

a- 2000
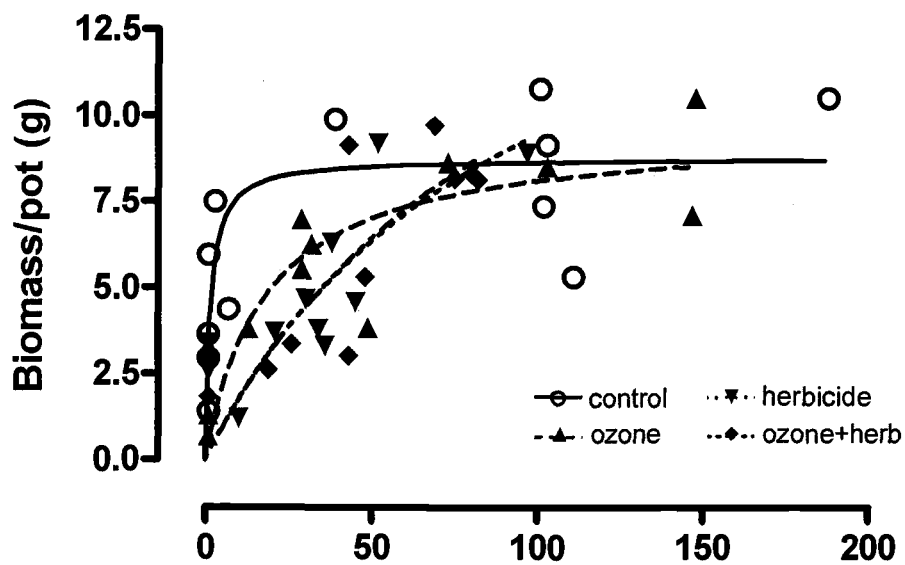
b-2001
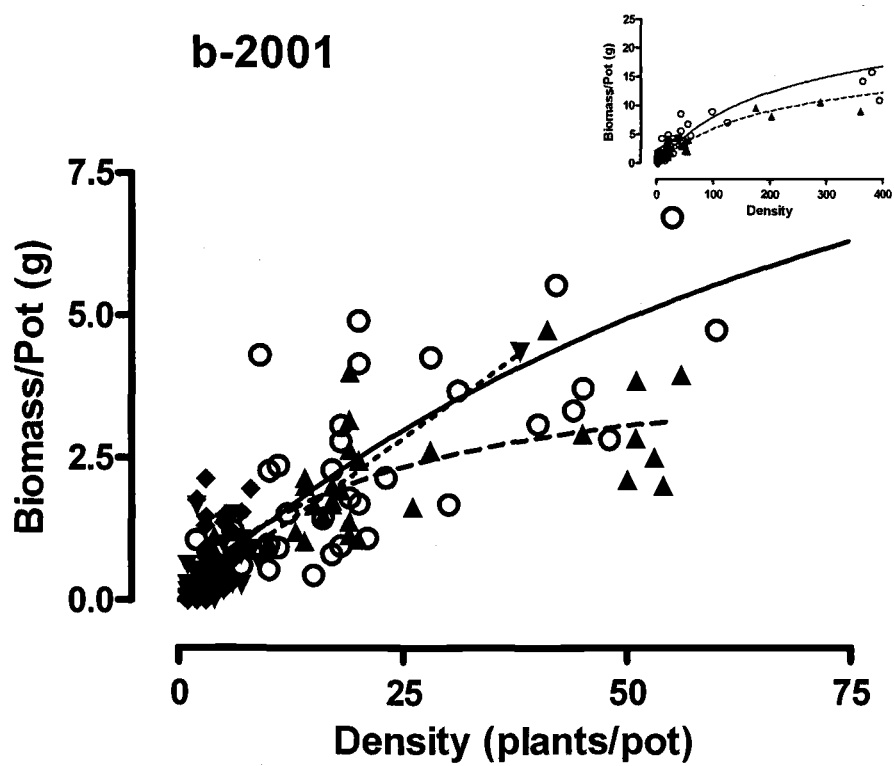
In year 2001 the relationship between average biomass per unit area and density for the control pots sampled 135 days after planting, also fit a hyperbolic model. However, density saturation occurred at about 400 plants/pot (Fig. 3.3b). Ozone imposed a limitation on maximum average biomass per unit area. Thus, the plant production became independent of density at about 50 plants per pot resulting in a lower plateau than that of model fitted for the control treatment (Fig 3.3b and Table 3.2). As in the first experiment, herbicide applied singly or in combination with ozone, reduced density and average biomass per unit area (Fig.3.3b).

The inverse of the average individual plant biomass, number of leaves and leaf area for 2000, was regressed against density to represent the loss in each parameter's average value in relation to density increase ("reciprocal yield law", Shinozaki and Kira, 1956) (Fig. 3.4).

Table 3.2. Parameter estimates for Italian ryegrass vegetative and reproductive biomass production as influenced by exposure to ozone and herbicide, for responses shown in Figs. 3.3, 3.4b and 3.6a and c. SE standard error of parameter estimates.

Response variable	Treatment	Year	Parameter estimates			SE	
			Y_{max}	K_n	R^2	Y_{max}	K_n
Biomass/ pot	control	2000	8.79	1.52	0.59	0.89	0.76
	ozone		9.61	18.45	0.73	1.51	11.14
	herbicide		18.47	95.13	0.50	12.30	9.71
	herb+ozone		20.29	107.5	0.58	15.51	10.64
	control	2001	26.42	228.9	0.89	2.78	55.9
	ozone		18.78	214.2	0.86	2.21	59.1
	herbicide		4.5×10^{10}	3.9×10^{11}	0.74	3.9×10^{11}	3.44×10^{12}
	herb+ozone		1.91	7.29	0.16	1.60	10.2
Number of Spikes/ pot	control	2000	82.78	10.10	0.99	0.40	0.24
	ozone		80.00	20.89	0.99	0.23	0.19
	herbicide		73.55	23.48	0.91	2.70	2.11
	herb+ozone		77.38	20.65	0.98	1.16	0.82
Seed Biomass/ pot	control	2000	2.84	1.00	0.45	0.04	0.14
	ozone		3.15	2.63	0.70	0.06	0.29
	herbicide		2.37	0.66	0.82	0.02	0.04
	herb+ozone		2.85	1.68	0.94	0.02	0.08
Number Leaves/ plant	control	2000	0.17	10.56	0.98	0.00	0.66
	ozone		0.19	12.03	0.99	0.00	0.63
	herbicide		0.14	3.38	0.84	0.00	0.76
	herb+ozone		0.14	2.28	0.77	0.00	0.63

In general, ozone did not alter the effects of density on individual plant weight and leaf area, while herbicide increased density effect and herbicide and ozone combined erased density effects altogether (Fig. 3.4a and c and Table 3.3). Control and ozone treatments showed similar weight reductions in relation to unit density increase (b=0.2 and 0.3, Table 3.3).

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Figure 3.4. Relationship between the inverse of individual weight (a), number of leaves (b), leaf area (c) and plant density in year 2000. .Plants were grown in control or ozone chambers with or without herbicide application. Regression model fitted is for (a) and (c) 1/y=a+b*initial density, for (b) $Y=Y_{max}.N/(K_n + N)$.
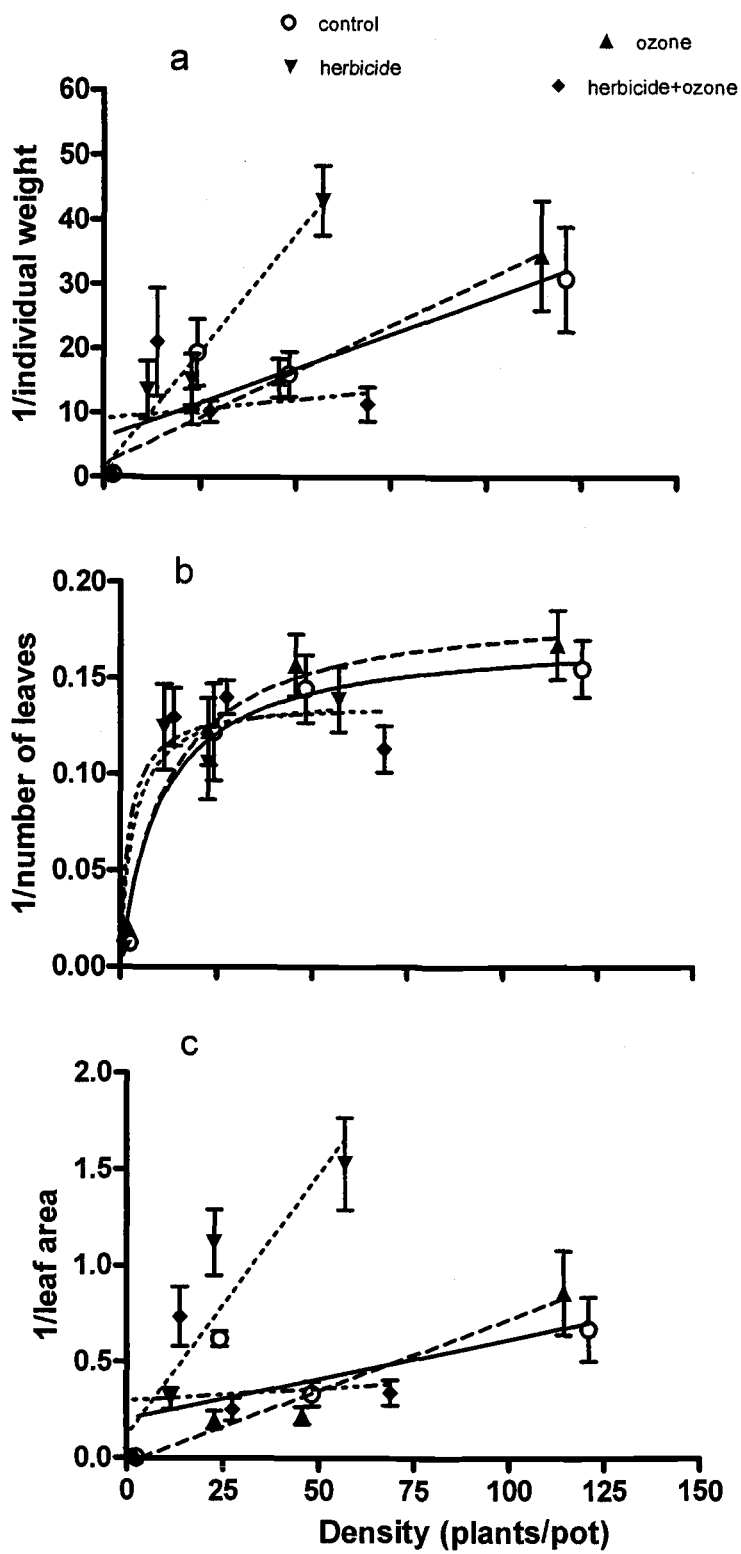
Table 3.3. Parameter estimates for Italian ryegrass vegetative and reproductive biomass production as influenced by exposure to ozone and herbicide, for responses shown in Figs. 3.4a and c and 3.7a.
SE standard error of parameter estimates.

Response variable	Treatment	Year	Parameter estimates			SE	
			a	b	r^2	a	b
Biomass/ plant (1/W)	control	2000	6.1	0.2	0.78	5.3	0.08
	ozone		2.1	0.3	0.98	1.5	0.02
	herbicide		1.2	0.7	0.97	2.7	0.08
	herb+ozone		9.0	0.1	0.04	7.5	0.10
Leaf area/ plant (1/LA)	control	2000	0.2	0.004	0.48	0.2	0.00
	ozone		- 0.0	0.007	0.97	0.06	0.00
	herbicide		0.1	0.03	0.87	0.2	0.00
	herb+ozone		0.3	0.001	0.01	0.2	0.00
Number of Spikes/ pot	control	2001	0.9	0.9	0.76	2.8	0.09
	ozone		0.9	1.1	0.94	0.6	0.05
	herbicide		-2.5	1.1	0.87	0.6	0.07
	herb+ozone		-0.2	0.8	0.29	0.9	0.20

However, this slope's value was more than doubled by the herbicide treatment (0.70), indicating higher loss of individual weight per unit of density increase. When plants were treated with ozone and herbicide combined, average individual weight was insensitive to density variations within the studied density range (Fig. 3.4a and Table 3.3).

At the lowest densities, herbicide treated and control plants had similar leaf areas (ca. 200 cm^2) and around twice that of plants from the other

treatments (Fig. 3.4c). However density strongly reduced individual average leaf area of the herbicide treated plants (Fig 3.4c). Density affected number of leaves in plants of all treatments in a similar way, by a reduction in the number of leaves with density until a certain point when number of leaves per plant became independent of plant density (Fig. 3.4b).

The frequency distribution of individual plant average weights of the control treatment plants varied as density increased (Fig. 3.5a-d). At the lowest density class (0 –10 plants per pot) plant sizes greater than 2.2 g were the most frequent, and as density increased these size classes became less represented until in the 100-120 plant/pot density the maximum plant size sampled corresponded to the 0.4-0.6 g class (Fig.3.5e).

The effect of the stressors on plant weight differed at the lower and higher densities, was but similar at medium densities. At the lowest plant densities (0-10 plants/pot), ozone increased the frequency of lower size classes compared to control pots, but herbicide and herbicide with ozone combined produced an opposite effect (Fig. 3.5 f-k-p). However, these larger size individuals disappeared in pots under herbicide application when density increased.

At medium densities (20 to 50 plants/pot), ozone and herbicide had a

similar effect on plant weight distribution by reducing the frequency of the highest and the lowest size classes compared to controls. This resulted in a more even distribution of sizes than for plants in the control at the corresponding densities. At the higher densities (>100 plants/pot density), there were no pots with surviving plants after herbicide application (Fig. 3.5 o-t).

Figure 3.5 The frequency distribution of individual plant weights of Italian ryegrass growing in pots with different plant densities in 2000. Plants grew in control or ozone chambers and with or without herbicide application.

CONTROL

a 0-10 plants b 10-20 plants c 20-30 plants d 40-50 plants e 100-120 plants

OZONE

HERBICIDE

OZONE+HERB

Weight/plant (g)
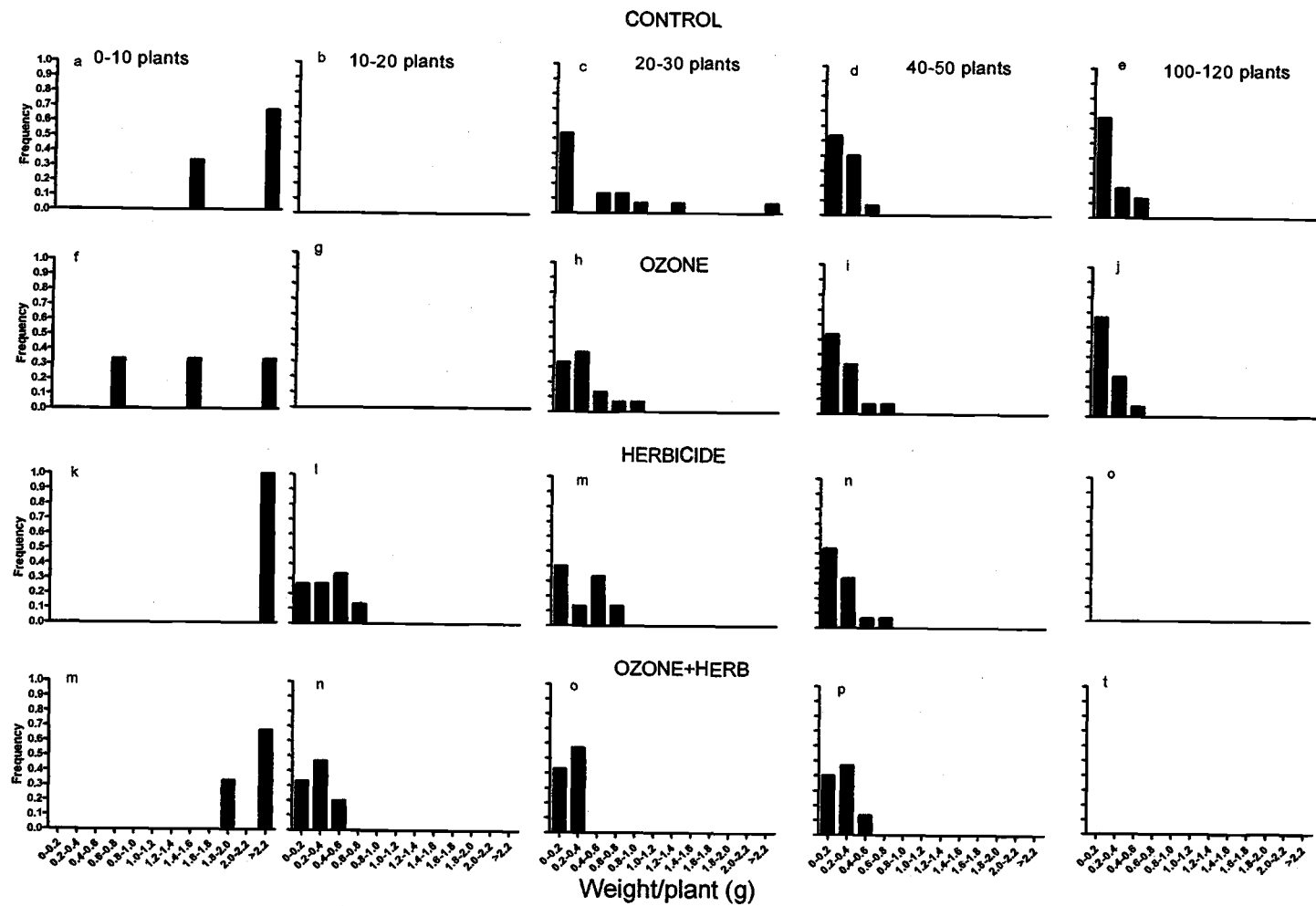
In year 2000, the number of spikes per pot increased with density following a hyperbolic pattern (Fig 3.6). All pots reached a similar maximum number of spikes per pot of ca. 80 spikes. However control plants reached this plateau at lower densities than any stressed plants (i.e. all other treatments) (Table 3.2), indicating that fewer tillers were added per unit of plant density increase. The proportion of total tillers bearing spikes (spike to tiller ratio) for all treatments followed an "optimum response model" with a maximum value (ca 0.90) and minimum values (ca 0.50) at the lowest and highest plant densities (Fig 3.6b). Control and ozone treatments had similar patterns of change in proportion of flowering tillers with density. Herbicide, regardless if combined with ozone or not, pushed the ratio peak to a lower density value (around 10 plants/pot). At the lowest plant density the combination of herbicide and ozone produced an increase in the spike/tiller ratio to 0.80, while the ratio for the remaining treatments was around 0.5 (Fig. 3.6b). Seed biomass/pot increased with density following a similar hyperbolic model in all treatments (Fig. 3.6c).

Figure 3.6. Relationship between number of spikes per pot (a), proportion of flowering tillers (b), seed biomass per pot (c) and plant density in year 2000. .Plants were grown in control or ozone chambers with or without herbicide application. Regression model fitted for (a) and (c) 1/y=a+b*initial density.
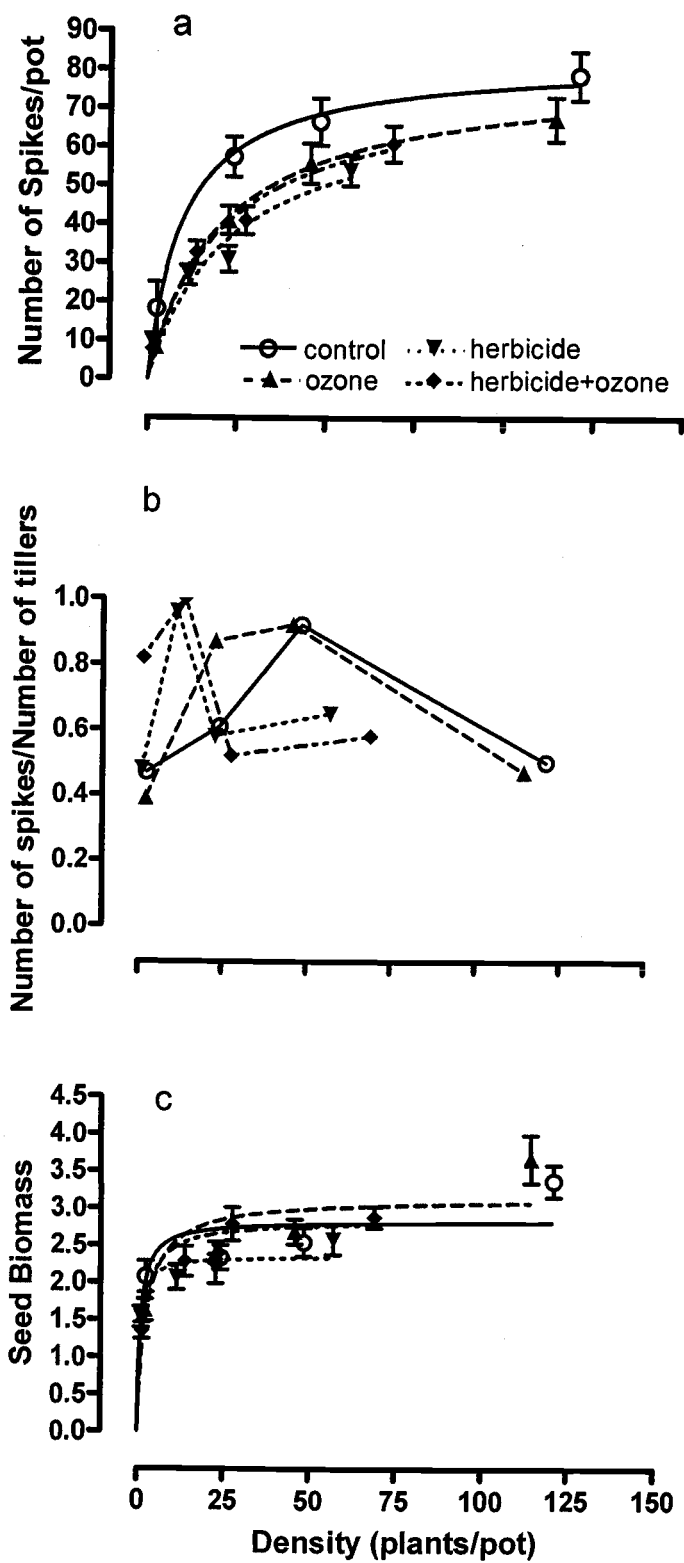
In 2001 the relationship between spike density and plant density was similar for all treatments and followed a linear model (Fig. 3.7a). In contrast to the experiment conducted in 2000, seed production in 2001 was variable, and tended to be higher at the lowest densities especially with ozone treated plants (Fig. 3.7b).
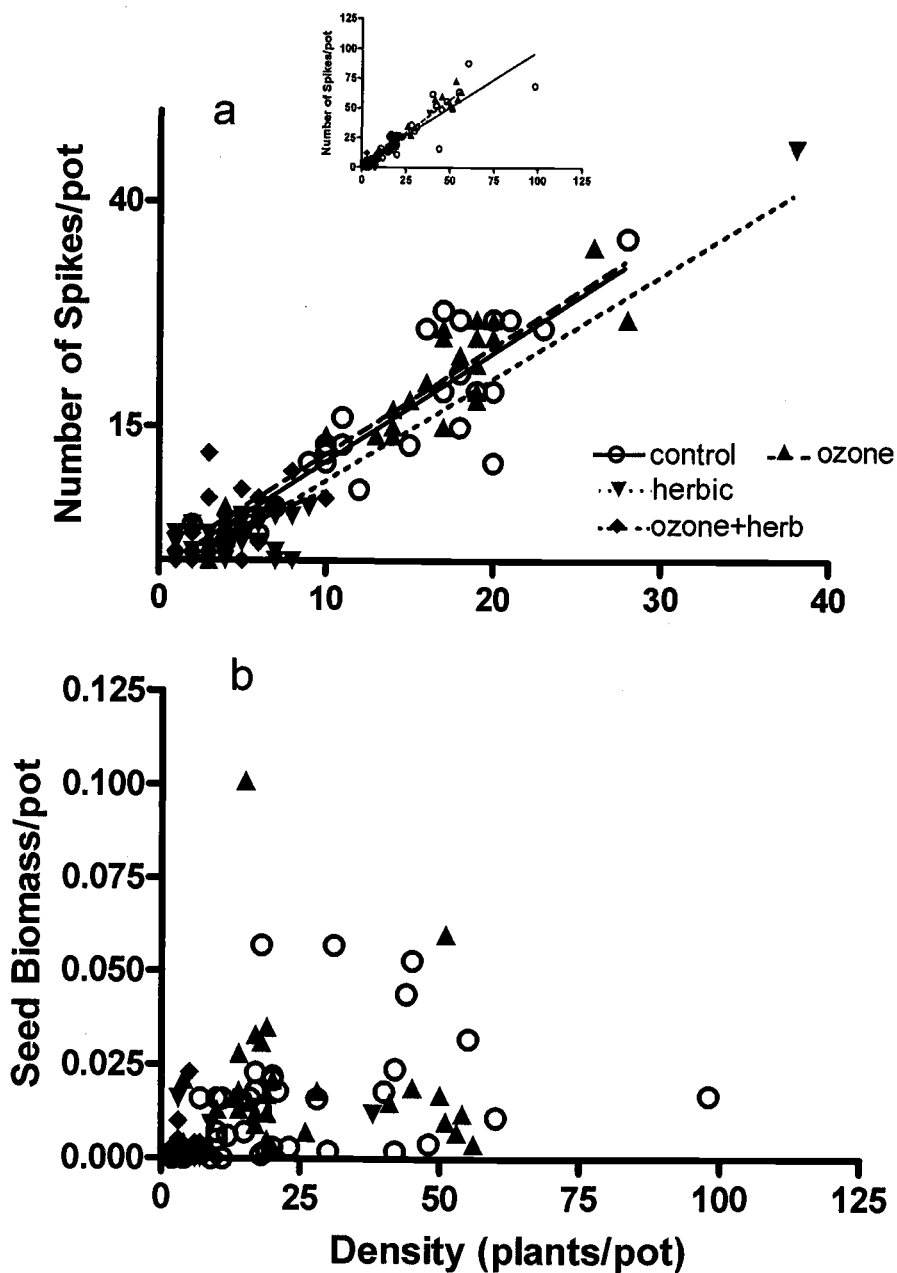
Figure 3.7. Relationship between number of spikes per pot (a), seed biomass per pot (b) and plant density in year 2001. .Plants were grown in control or ozone chambers with or without herbicide application. Regression model fitted for (a) Y=a+b*density.

3.4 DISCUSSION

This study presents evidence that abiotic stressors can produce significant changes in the size structure of an annual plant species, modifying the hierarchies of individuals within the population by changing density-dependence responses. In both years of study, herbicide and density were the two most important factors controlling seedling mortality. Plant density was mostly determined by thinning, aiming to achieve a gradient from 1 to 50 plants per pot (130 to 6600 plants per m^2), and by further regulation of plant density that differed between treatments and years (Fig. 3.2). Thinning was accomplished using hand-held tweezers to completely eliminate the plants basal crown. However crown removal was not always achieved and some thinned plants sprouted again increasing the target pot densities from two to almost five times in some pots. On the other hand, sprouting did not occur in the pots with herbicide application. The ratio of final to initial density in pots that received herbicide application was always lower than that of control pots (Fig 3.2). The plants already stressed by herbicide application did not withstand additional stress produced by clipping, and died without sprouting. Thereafter, target density remained unchanged until the last harvest. It is difficult to determine whether mortality in each treatment was density-dependent, density-independent or both. However, the constant relationship between final and initial density in year 2000

suggests that mortality risk was constant at all densities (density-independent), and all of the variation in density was absorbed in plastic responses in plant weight (Harper, 1977). However, in 2001, when unfavorable temperatures for growth occurred, mortality became density-dependent as suggested by a low and constant final density in herbicide pots.

The biomass to density relationship was different between years and treatments (Figs. 3.3a and b). Stress treatments in the year 2000 experiment were applied during early developmental stages of the Italian ryegrass populations and plants grew under low radiation and temperature conditions during a great part of their growing cycle (Table 3.1). Biomass production by the control plants growing during the first six months of the first experiment became insensitive to density at about 50 plants/pot, and all the stress treatments lowered biomass production within the 1-50 densities range in (Fig 3.3a). Independently or in combination, ozone and herbicide treatments reduced individual growth resulting in smaller average size per plant. Therefore the smaller individuals increased the total biomass per unit area less than in the control treatment, as density increased. Interestingly, in the 2000 experiment, the stress treatments imposed on Italian ryegrass did not change the maximum biomass production attained per pot, ie. the population's ability to capture resources (Fig 3.3a). If a reduction in

resource capture or transformation abilities had occurred due to exposure to any of the stress treatments, the maximum biomass achieved by the population would have fallen. This latter response has been repeatedly demonstrated where resource availability for plants is experimentally manipulated (Harper, 1977). The inability to capture resources under environmental conditions that were far from optimal in 2001, because of late planting, may have decreased maximum yield per pot attained by plants when they were subjected to further stress of ozone or herbicide exposure (Fig. 3.3b).

It has been argued that any influence that slows the rate of plant growth can be expected to delay and reduce the intensity of density stress among plants (Clatworthy and Harper, 1962; Harper, 1977). Taking into account that herbicide and ozone independently or in combination, reduce the rate of plant growth (Martinez-Ghersa et al., in prep, Chapter 2 this thesis), the differences in biomass-density relationship between any of the stress treatments and the control in this study (Fig. 3.3a) support this argument. However, sensitivity to plant crowding was different among stress treatments (Fig. 3.4). While herbicide-treated plants had the greatest reductions in plant weight per unit of density, ozone-exposed plants followed the same response as the control. Only when both stressors were applied in combination did individual plant biomass become independent of

density. Therefore, these experiments do not support fully the generalization first presented by Clatworthy and Harper (1962). Herbicide stress even when reducing plant growth, intensified, rather than delayed or reduced the onset of density effects, while ozone stress had no effect. These results better support the model for interference presented by Aphalo and Ballare (1995), in which negative interactions are not only based on depletion of resources but also on environmental signals (changes in light quality, accumulation of toxins, etc) received by plants sometimes well before competition actually begins.

The frequency distribution of individual plant weights was affected by plant crowding in the control treatment (Fig.3.5a-d). It followed the expected skewing of the plant size frequency distribution, where the frequency of small subordinate individuals increases rapidly as a few large individuals dominate over time or increase in density (Firbank and Watkinson, 1990). When we considered changes in mean plant weight with density, only one of the stress factors, herbicide, affected growth differently than the control plants (Fig. 3.4a). However, the average value of some individual parameter might not be the commonest in the population, and the average value may be obscuring plant-to-plant variation. Our analysis of the frequency distribution of individual weights, in turn, revealed that ozone also had an effect on the weight/ density relationship by changing plant weight

distributions. The pattern presented in Fig. 3.5 of these histograms was changed by the stress treatments depending upon density level, and the stress factor (Fig. 3.5e-p). These changes were the result of a combination of individual plant death especially occurring among the lower size class plants and a reduction in growth of dominant individuals. Subordinated individuals have the highest death risk (Harper, 1977), and any additional reduction in their ability to use or capture resources will increase death rate. The dominant individuals can tolerate more stress than smaller plants, but because of the greater growth rate of larger plants, herbicide and ozone effects may be intensified (Reinert, 1984; Reich, 1987).

It has been suggested that novel or extreme stresses, such as soil contaminants and gaseous pollutants, may unmask variation within the populations, on which natural selection acts .The increased variability may result from differential resistance to pollutants among genotypes or the differential disruption of developmental homeostasis among genotypes (Coleman et al., 1990). In this study, it is uncertain whether changes in size hierarchies are linked to heritable traits. However our data indicate that strong competitive interactions that occur at high densities, when combined with abiotic stress, may have demographic consequences for an outbreeding species such as Italian ryegrass. These consequences may result from changes in the relative contribution of the individuals

(genotypes) to the next generation. The pattern of change in number of spikes per pot with increasing density followed a similar saturation pattern in all treatments, with the value of stressed plants always slightly below that of control ones (Fig. 3.6a). But this similarity in the pattern of spike-density change among the treatments was a result of important variation in the spike to tiller ratio (Fig 3.6b). The change in proportion of fertile tillers with density followed the expected optimum model for Italian ryegrass growing in monoculture (Hashem et al., 1998). Proportion of fertile tillers was low at very low and very high densities probably due to apical dominance of the main tiller and intense competition respectively. At medium densities almost all tillers produced a spike. On the other hand, all stressors alone or in combination decreased the density at which the ratio spike to tillers was maximum. Plants receiving herbicide application had high proportion of flowering tillers only at low densities; but ozone expanded the range of densities where most of the tillers had spikes. We have previously showed that the damage caused by herbicide or ozone can reduce the dominance of the primary tiller and induced plant tillering (Martinez-Ghersa et al., in prep, chapter 2). This observation may explain why the number of spikes to tiller production at lower densities was higher respect to the control. At higher plant densities, competition among plants and branches would increase tiller mortality and reduce spike development (Donald, 1951; Hashem et al., 1998).

Seed production in our experiment became insensitive to increases in density at low density values, indicating that differential spike size compensated for the lack of spikes at the lowest densities (Fig. 3.6c).

High temperatures and radiation during 2001 made the plants grow faster and induced flowering early in the growing cycle. Under these environmental conditions plant competition and the stress treatments did not determine individual growth responses. Therefore, plant spike density per pot increased proportionally to plant density in a similar way regardless of treatments (Fig. 3.7a). Only in the last phase of the plants' life cycle, during seed production, did interactions between density and stress factors become apparent (Fig. 3.7b). Control and especially, the ozone treatment pots tended to produce more seeds at lower values of the experimental density range. The two treatments with herbicide applied produced small amounts of seed and were insensitive to density change. Similar to the previous year, at the lower values of the density range (<30 plants per pot) seed production increases overcompensated for reductions in spike density, *ie*. a positive relation of spikes to density was found as opposed to a negative tendency relating of seed biomass to density (Fig. 3.7a and b).

In conclusion, the stressors changed the frequency distribution for average individual plant weight resulting from increasing densities (Fig. 3.4). In

2000, interactions between density and abiotic stress reduced biomass and number of spikes. However, contrary to our predictions, plants were able to compensate during grain filling maintaining similar seed production–density relationships in all treatments (Fig. 3.6b). In 2001, because plants grew at higher radiation and temperature than in 2000, they had a shorter life cycle and negative regulation of density on biomass and spike production was less important (Fig. 3.3b and 3.7a).

This study suggests that information obtained from individual plant responses to human induced stress is useful to understand how populations will perform. Yet, because compensatory mechanisms occur at the population level, predictions derived only from individual studies may be inconsistent or incorrect. The different population responses caused by each of the assayed stress agents as they interacted with plant density indicates that empirical information is still lacking to build a strong framework to understand how novel stress agents may affect plant populations.

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CHAPTER 4

HERBICIDE SELECTION OF ITALIAN RYEGRASS UNDER DIFFERENT LEVELS OF UVB RADIATION

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4.1 ABSTRACT

Ultraviolet- B radiation is an environmental stress for plants that will become aggravated in the next decades by stratospheric ozone depletion. In this study we used Lolium multiflorum as a model system to test whether an environmental stress derived from global change, UVB, can influence the efficacy of weed control and evolution towards herbicide resistance. We grew three generations of L. multiflorum plants with and without UVB light and subjected each generation to a series of diclofop-methyl doses (from 0 to 1120 gai.ha^{-1}). The effect of selection history was tested with herbicide screenings. The effect of herbicide application on plant survival and biomass varied significantly among herbicide doses and with presence or absence of UVB light. UVB light regimes and herbicide levels also interacted modifying the relationship between vegetative and reproductive biomass in both parental and first-generation plants. In the absence of herbicide, a decrease in individual fecundity with increasing plant density was similar under both no-UVB and UVB light treatments. High herbicide pressure resulted in increased production of reproductive structures in response to the decrease in density when plants were grown without UVB. When herbicide selection pressure was high, UVB light did not significantly affect the evolution toward herbicide tolerance. When selection pressure on the parental plants was lower, UVB stress was synergistic, and augmented

the selection response to herbicide dose. Our study shows that UVB light was a weak stress factor for the ryegrass plants. However, with low selection pressure caused by herbicide application, the two stress factors had a synergistic effect, causing changes in herbicide efficacy that in turn had demographic and evolutionary consequences. In the field, such interactions between stress factors might be of significance for success of annual weed species in which seed output is a major determinant in fitness.

4.2 INTRODUCTION

The impact on biological systems of multiple stresses caused by human activities is far from understood (Mooney and Winner, 1991). Nevertheless, there is some consensus among scientists that broad scale changes in the environment- including soil water, vegetation, and the atmosphere- will alter the productivity of agroecosystems, influence the selection of crop varieties, and affect the distribution of agricultural pests and their response to control (Vitousek, 1994; Patterson, 1995; Ziska et al., 1999; Sala et al., 2000). One of the most striking environmental global changes is the decrease in thickness of column ozone of tropical regions (Bojkov et al., 1990; Niu et al., 1992), which varies with month, latitude and longitude. In some regions, scientists have reported a concomitant increase in surface ultraviolet-B radiation (UVB 280-320 nm) due to loss of ozone and its function in absorption of ultraviolet light (Lubin et al., 1989). UVB radiation is believed to be an environmental stress for plants (Cybulski and Peterjohn, 1999; Hunt and McNeil, 1999). Even with the control of ozone-depleting substances such as chlorofluorocarbons, significant ecological impacts from UVB are likely to continue for several decades (Day et al., 1999; Paul and Gwynn-Jones, 2003). Although the direct effects of UVB light on plants have been extensively reported, it is critical to determine the effects of

increasing UVB radiation on the response of biological systems to
management practices such as herbicide application.

Weed control, most commonly using herbicides, has become one of the
most important activities in crop production because weeds compete with
crops and decrease yield (Radosevich et al., 1997). The response of a plant
to an applied chemical is influenced by other stresses to which it is
subjected both prior to or following treatment (Muzik, 1976; Stanton et al.,
2000). Plants are rarely exposed to only a single stress, and adjustments to
several concurrent stresses are usually required for attaining high yields
(Mooney and Winner, 1991). However, studying the effects of individual
stress components has been the most common approach.

Italian ryegrass (Lolium multiflorum Lam.) is a major grass seed crop and a
highly competitive weed in cereal fields (Appleby et al., 1976; Burrill et al.,
1988), leading to as much as 60% loss in grain yield (Appleby et al., 1976).
Diclofop-methyl has been extensively used to control ryegrass in cereal
production in the United States and throughout the world, which has lead to
the development of resistant populations (Brewster and Appleby, 1988;
Stanger and Appleby, 1989). Genetic, physiological, and ecological
research on processes regulating herbicide resistance in ryegrass has been
conducted (Ghersa et al., 1994a; Ghersa et al., 1994b; Powles and

Holtum, 1994). Studies on resistance to diclofop-methyl in Italian ryegrass have demonstrated that a single partially dominant gene encodes resistance to this herbicide, conferring to heterozygous individuals an intermediate tolerance at field-applied rates (Betts et al., 1992). More recently, glyphosate resistance also has been reported in Italian ryegrass (Powles et al., 1998).

Acetyl-coenzyme A carboxylase (ACCase), a key enzyme involved in the biosynthesis of lipids and UVB-absorbing pigments (flavonoids), is the target site of several selective herbicides such as diclofop-methyl (Kobek et al., 1988). We speculate that the combined effects of diclofop-methyl selection and UVB radiation may cause demographic and evolutionary plant responses different from that caused by each stress separately. Diclofop-methyl and UVB light have opposite effects on ACCase: while the enzyme is inhibited in susceptible individuals by diclofop-methyl (Gronwald et al., 1992), previous reports have indicated that total, citosolic and plastidic activity of ACCase is inducible under UVB exposure (Ebel and Hahlbrock, 1977; Konishi et al., 1996). Moreover, efficacy of ACCase-inhibiting herbicides under field conditions can be improved in the absence of UVB radiation, suggesting that herbicide applications should be carried out at late evenings or nights (McMullan, 1996).

There is no published information on the response of annual ryegrass to the combined effects of herbicide application and UVB light. In this study we used *L. multiflorum* as a model system to test whether an environmental stress derived from global changes, such as UVB light, can influence the success of herbicides in controlling weeds. We considered the effects of dose on both the efficacy of the control procedures and the plant's evolution toward herbicide resistance. We hypothesize that the efficacy of the herbicide in controlling ryegrass changes with the level of environmental UVB. We further hypothesize that under more stressful conditions (higher levels of UVB radiation), the rate herbicide resistance evolution will decrease due to changes in mortality and fecundity of surviving individuals.

4.3 MATERIALS AND METHODS

All experiments were carried out during winter and spring of 1996, 1997, and 1998 at the experimental field of Facultad de Agronomía, University of Buenos Aires (34º35' S, 58º29' W).

4.3.1 Response to herbicide under UVB light environments: parental population and progeny (F$_1$)

A factorial experiment (Fig. 4.1) in a randomized block design was carried out twice, in 1996 and replicated in 1997. The purpose of the study was to examine the response of a ryegrass population that was grown under different UVB light levels and then subjected to a series of herbicide doses. Thirty 0.25-m^2 15-cm-high wooden boxes filled with organic soil were sown with a commercial accession of endophyte-free Italian ryegrass from Oregon. The accession was never exposed to herbicide application, and each box was planted with at 1024 seeds/m^2 and placed in the field. Seeds from the same parental population were used in both years. We chose an endophyte-free population to avoid plant–fungal interactions, which are known to be affected by UVB (Newsham et al., 1998).

Half of the boxes (plots) were randomly assigned to each of the UVB light treatments: (1) ambient light with exclusion of UVB light (UVB-) and (2) ambient solar light (UVB+). All plots were covered immediately after sowing with plastic film that transmitted more than 90% of the photosynthetically active radiation (400–700 nm). The film over the UVB- plots was Mylar-D film (0.1 mm thick; DuPont), which blocks all radiation below 320 nm (see spectrum in Ballare et al., 1996). The film over the UVB-transparent control plots (UVB+) was Aclar film (0.04 mm thick; Allied Signal Plastics, Morristown, NJ), which has very high transmittance over the whole UV waveband. The two types of film allow over 90 % penetration of PAR (400-

700 nm). Sheets of film were draped over 1 x 1 m metal frames that were maintained about 3 cm above the plant canopy during the course of the experiments. Each plot had an individual frame above it, and the sides were left open to allow air circulation. Air temperature under the filters was recorded in the morning and afternoon on three different days using a model 21X Micrologger (Campbell Scientific, Logan, UT), and no differences between plots were detected. All plots were watered as needed to maintain the soil near field capacity. Experiments started on June 13 in 1996 and July 10 in 1997.

Three weeks after sowing, when the plants were at the two-leaf stage, seedling density was measured in a 78.5 cm^2 area placed at random in each plot. Plots under UVB- and UVB+ were randomly assigned to a series of herbicide doses: 0, 140, 280, 560 (label dose), and 1120 g ai ha^{-1} of diclofop-methyl {($\pm$)-2-[4-(2,4-dichlorophenoxy) phenoxy] propanoic acid, methyl ester} (commercial formulation 284 g ai l^{-1}, Iloxan, Hoechst-Aventis, Argentina). Each treatment combination (UVB light x herbicide dose) was replicated three times in both years. Herbicide was applied with a constant-pressure hand sprayer over the entire plot 1 day after density was measured. Seedling survival in the sampling areas was recorded 1 month after herbicide application, and surviving individuals were harvested. Total dry weight was obtained after oven drying for 72 h at 72°C.
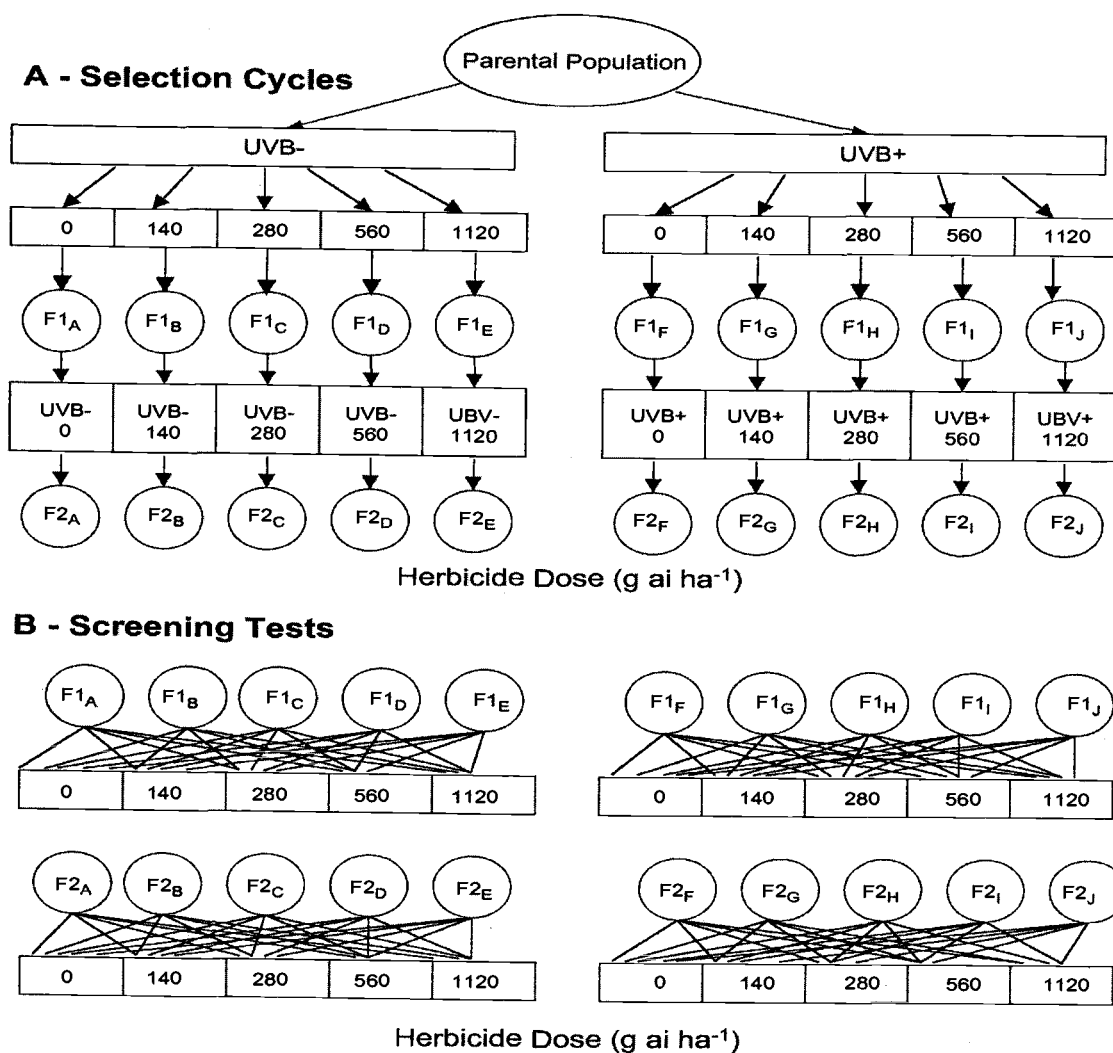
Figure 4.1: Overview of the selection protocol and experimental design. (A) Selection cycles were carried out during 1996 and 1997; (B) herbicide screening tests were carried out in 1998. (A) Plants grew under filtered solar light without UVB radiation (UVB-) or ambient solar light (UVB+) and were then subjected to a series of herbicide doses: 0, 140, 280, 560 or 1120 gai.ha^{-1} during three generations: parental plants, second generation (F$_1$) and third generation (F$_2$). (B) F$_1$ and F$_2$ seedlings were evaluated for herbicide resistance by a dose response screening test.

Surviving plants were isolated when they began to flower, to prevent pollen transfer among plots. The metal filter frames also supported a fiberglass fabric that covered the openings around each plot. This material did not prevent air circulation or light penetration, but did prevent pollen flow among plots.

Another 78.5-cm^2 area was sampled in each plot to evaluate seed production. Plots were visited every 10 days from early November until the end of December in both years, and all newly produced spikes were harvested and seed weight recorded. Seeds produced outside of the sampling area were also harvested and stored under laboratory conditions for further use.

Mature seeds from each treatment were harvested at the end of 1996 and kept separate. We sampled a subset of the seeds from each treatment to produce the next generation of seedlings (progeny). These seeds were again sown in 1997 as above. Each group of second-generation seedlings was subjected to the same UVB light x herbicide dose treatments as in the corresponding parental plants. Seedling survival, biomass, and seed production of surviving individuals were recorded as already described.

4.3.2 Selection Response to Stress in F_1 and F_2 Populations

To allow examination of selection responses to the different herbicide and UVB light environmental conditions (selection histories), seed produced under each treatment by the parental plants in 1996 and 1997 (F_1) and those produced by the progeny plants in 1997 (F_2) were exposed to a common stress environment. One hundred seeds from each selection history were sown in organic soil in plastic pots (1.5 L.). Initial seedling density was recorded after emergence. Three pots from each selection history were assigned to each of the five diclofop-methyl doses (0, 140, 280, 560, and 1120 g ai ha^{-1}). Herbicide was applied at the two- to four-leaf stages, as described earlier. Seedling survival and biomass of the surviving plants were determined.

4.3.3 Data analysis

All data were analyzed using SAS Version 6.12 statistical package (SAS Institute, Cary, NC). Two- and three-way ANOVA tests were performed on survival and biomass data. We tested the main effects (year, selection history, and herbicide dose) and the interactions using the General Linear

Model procedure. Arcsine transformations were used on all survival data to meet the assumptions of ANOVA. A probability level of P < 0.05 was used to delineate main and interaction treatment differences.

Data from herbicide screening of F_1 and F_2 populations were evaluated with least square non-linear regression . Regression analysis was further used to fit linear or nonlinear models to fecundity data as appropriate. Nonlinear models were used if ANOVA indicated that higher order polynomial effects were more significant than linear effects.

4.4 RESULTS

4.4.1 Herbicide efficacy

4.4.1.1 <u>Survivorship in the first selection cycle (parent plants)</u>

Average temperature and UVB radiation during the months that experiments were carried out are presented in Table 4.1. Average temperatures in June and July 1996 were lower, and temperatures in December were higher, than temperatures for the corresponding months in the other two years. UVB radiation in the October–December 1996 period was higher than in 1997. In both years of the experiments with parent plants (1996 and 1997), the average efficacy for the herbicide treatments was ca.

90 percent. However, the effect of UVB and herbicide application on plant survival differed between years, as indicated by a significant three-way interaction ($P < 0.05$). To explore the nature of these effects, we examined each year separately.

In the first year, average plant survival was 92 percent for the untreated plots and 10 percent for the herbicide-treated plots, but survival of treated plants did not differ among herbicide doses or light treatments (Fig. 4.2a). In the second year, overall survival was higher and more variable than during the first year (Fig. 4.2b). The response to herbicide dose depended on the UVB light regime to which plants had been exposed ($P=0.04$).

Table 4.1 Monthly temperature and light conditions for the three years that experiments were conducted

	Temperature (°C)			UVB[a] light dose ($J\ m^{-2}day^{-1}$)		
	1996	1997	1998	1996	1997	1998
June	10.2	12.1	12.1	33.9		
July	10.0	12.9	13.0	23.6	24.4	
August	14.7	14.4	12.8	35.5	34.9	
September	14.1	14.5	13.9	58.8	65.2	
October	18.6	17.6	18.8	85.9	82.4	
November	21.8	20.1	20.6	136.7	111.3	
December	24.5	21.8	23.1	152.0	125.4	
January			23.2			151.1
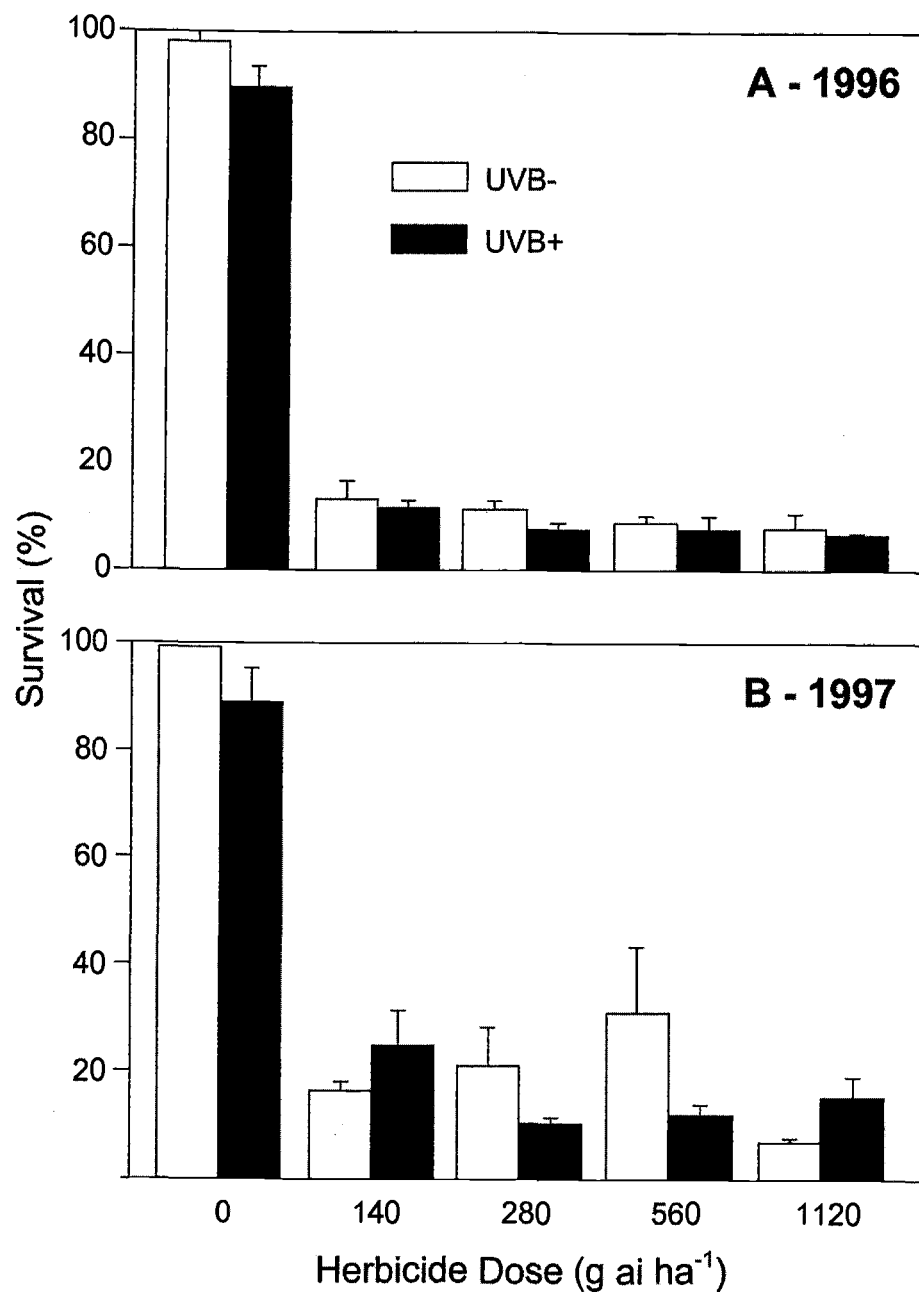
Figure 4.2. Effect of ultraviolet-B (UVB) radiation on survival of Italian ryegrass seedlings of a parental population when treated with different doses of diclofop-methyl. Bars represent means $\pm$ SE. ANOVA for (A) 1996: UVB light (L), $P = 0.18$; herbicide dose (D), $P = 0.0001$; L x D, $P = 0.95$. For (B) 1997: L, $P = 0.12$; D, $P = 0.00001$; L x D, $P = 0.04$.

4.4.1.2 Biomass of individuals in the first selection cycle (parent plants)

Like survivorship, biomass of the surviving ryegrass plants was significantly affected by the interaction among year, UVB light, and herbicide dose ($P <$ 0.05), which suggests that the consequences of treatment manipulations on the same ryegrass population differed for the two years. Italian ryegrass biomass was not significantly affected by UVB light in either untreated or herbicide-treated plants during 1996 (Fig. 4.3a). In the second year, the UVB+ treatment resulted in more variability in final plant sizes among doses than that of the UVB- treatment (Fig. 4.3b). UBV light increased the effects of herbicide, only under low-dose treatments ($P=0.03$).

4.4.1.3 Effect of herbicide and UVB light on fecundity

UVB light and herbicide interacted, modifying the relationship between vegetative and reproductive biomass in parent plants (Figs 4.4a and b). The interaction of year and vegetative biomass was not significant for individual reproductive biomass, therefore data from both years was pooled. When plants were not treated with herbicide, UVB light had little impact on fecundity of individual plants (Fig. 4.4a, difference between slopes $P =$ 0.19). For herbicide-treated plots without UVB light, there was a correlation between reproductive and vegetative biomass ($P=0.038$) (Fig. 4.4b). When treated plots included UVB+, on the other hand, production of reproductive

biomass was relatively low and was independent of vegetative biomass (P = 0.17). Consequently, UVB light and diclofop-methyl herbicide interacted to affect the relationship between reproductive biomass per plant and plant density (Figs. 4.5a and b). In the absence of herbicide, individual fecundity decreased with the increase in density of plants under both UVB- and UVB+ treatments (Fig. 4.5a). The mortality caused by the herbicide caused a decrease in plant density, but only plants growing under UVB- responded to this decrease in density by increasing their production of reproductive structures (Fig. 4.5b).

4.4.1.4. Survivorship in the second selection cycle (F_1 plants)

After the first selection cycle, herbicide efficacy was reduced significantly; survival values increased more than 50% (Fig. 4.6). The effect of herbicide dose on plant survival depended on the UVB light environment, as shown by the strong interaction found (P=0.02). There was no definite pattern in the relationship between herbicide dose and survival rate of the second generation (F_1) plants (Fig. 4.6).
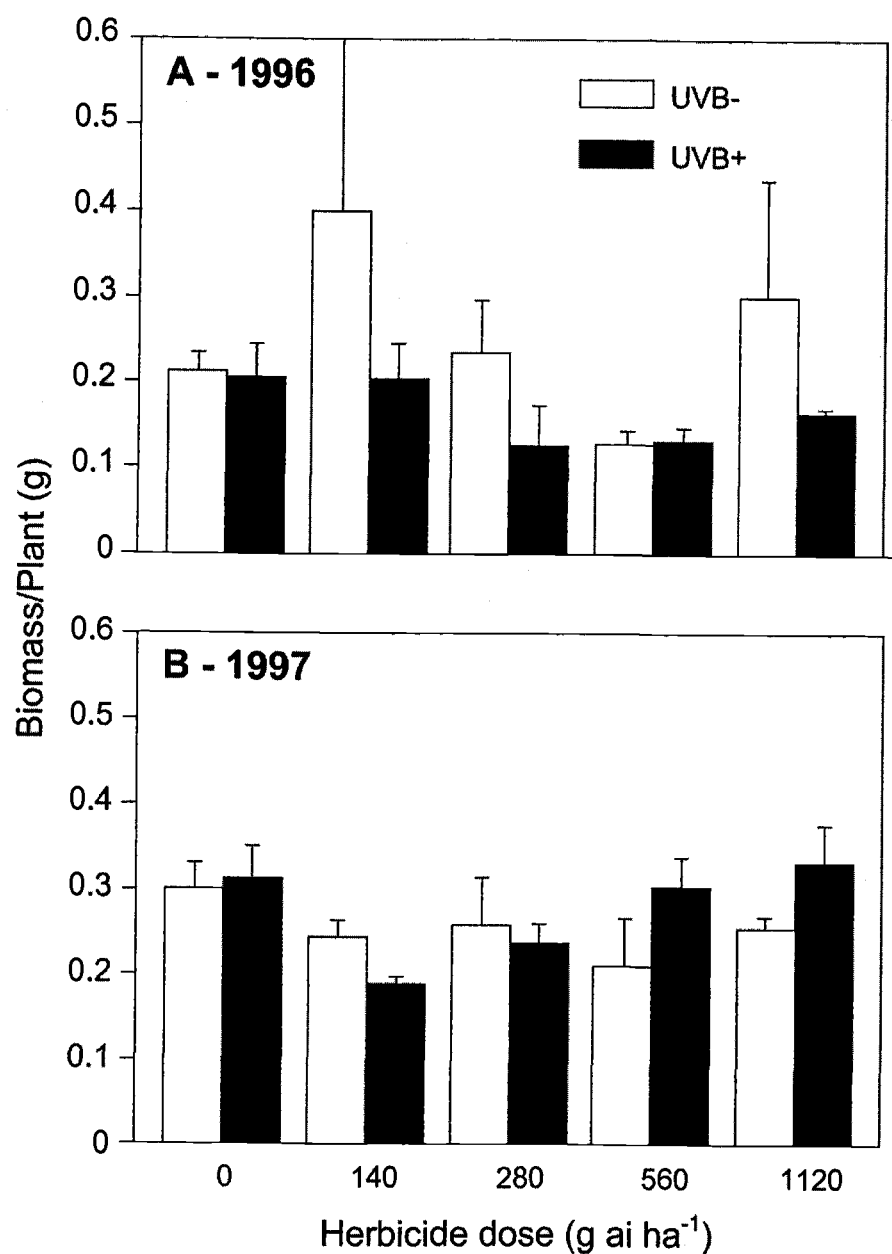
Figure 4.3 Effect of ultraviolet-B (UVB) radiation on biomass of seedlings of a parental population of Italian ryegrass surviving treatment with different doses of diclofop-methyl. Bars represent means $\pm$ SE. ANOVA (acronyms as in Fig. 2) for (A) 1996: L, $P = 0.13$; D, $P = 0.43$; L x D, $P = 0.76$. For (B) 1997: L, $P = 0.39$; D, $P = 0.10$; L x D, $P = 0.03$.
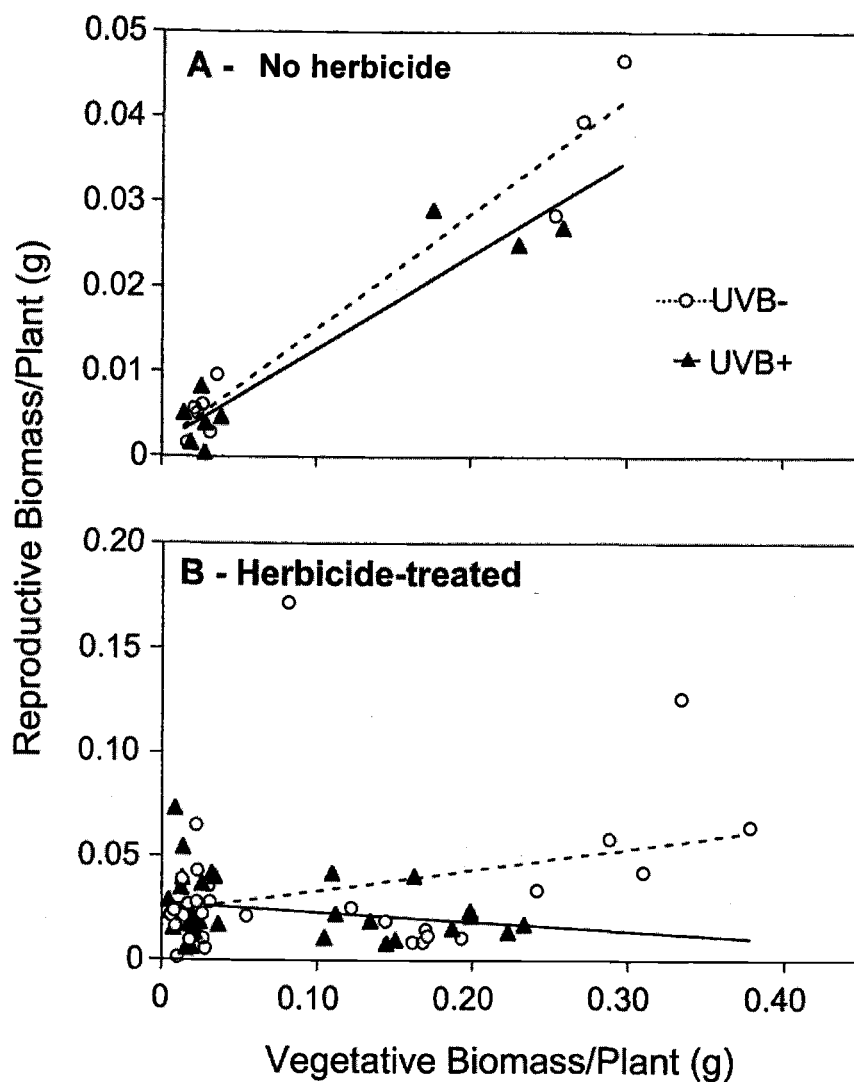
Figure 4.4 The effect of ultraviolet-B (UVB) light on the relationship between reproductive and vegetative biomass per individual Italian ryegrass plant growing in (A) non-treated (control) plots, and (B) herbicide-treated plots. Both parent populations for experiments conducted in 1996 and 1997 were pooled for analysis. Regression equations: (A) UVB-: y = 0.1354x + 0.0013, R^2 = 0.95, P = 0.001, SE slope 0.01; UVB+: y = 0.1102x + 0.0015, R^2 = 0.89, P = 0.001, SE slope 0.01 ; (B) UVB-: y = 0.1011x + 0.0227, R^2 = 0.31, P < 0.05, SE slope 0.05; UVB+: y = -0.0461x + 0.0276, R^2 = 0.06, P > 0.05.
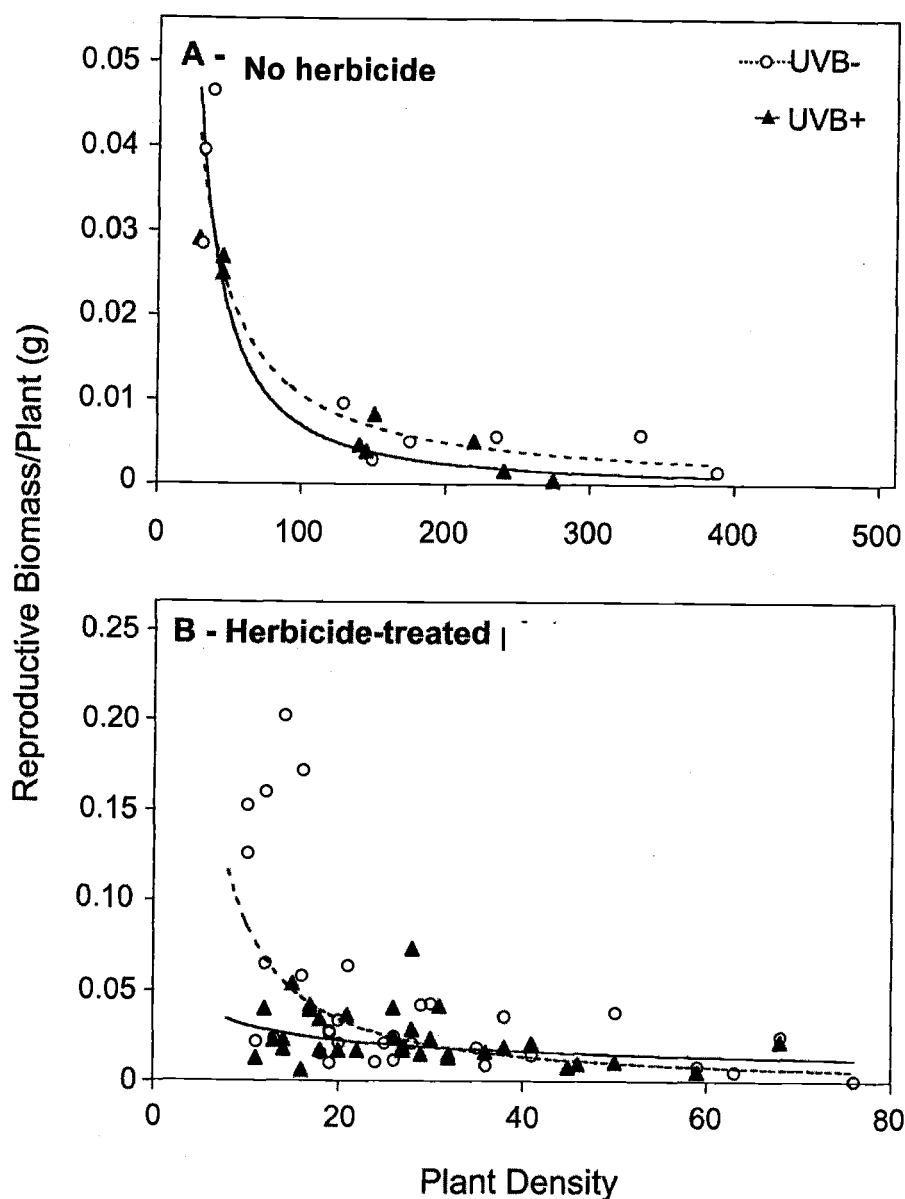
Figure 4.5 The effect of ultraviolet-B (UVB) light on the density-dependent production of reproductive biomass in (A) non-treated (control) plots, and (B) herbicide-treated plots. Parental and F_1 populations were pooled for analysis.

Regression model fitted : $y = a\ exp\ (-K^*x)$ (A) UVB-: a=0.061, K=0.017 (SE 0.01), R^2 = 0.88; UVB+ : a=0.045, K=0.014 (SE 0.004), R^2 = 0.97; (B) UVB- : a=0.18, K=0.12 (SE 0.055), R^2 = 0.47; UVB+: ns
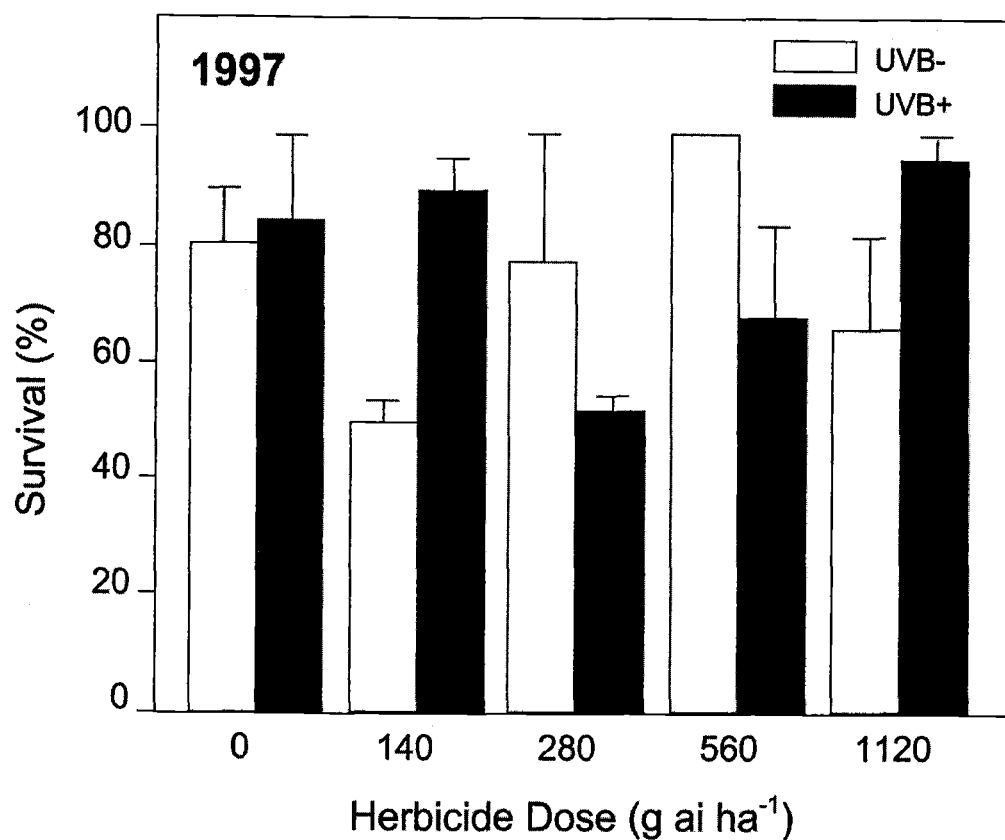
Figure 4.6 Effect of ultraviolet-B (UVB) radiation on survival of Italian ryegrass seedlings of an F_1 population when treated with different doses of diclofop-methyl. Bars represent means $\pm$ SE. ANOVA (acronyms as in Fig. 2): L, $P = 0.17$; D, $P = 0.39$; L x D, $P = 0.02$.

4.4.1.5 <u>Biomass of individuals in the second selection cycle (F1 plants)</u>

The light environment had a significant effect on the average weight of the individuals surviving herbicide application (Fig. 4.7). Plant biomass was almost 25% greater without UVB light than with it. However, the effect of UVB light on final plant size was not consistent across all herbicide treatments; it was especially evident at the two lowest herbicide doses (Fig. 4.7).

4.4.2 Selection responses to stress

4.4.2.1 <u>F_1 population</u>

There was a significant interaction between parental herbicide doses and herbicide dose response, showing that one generation of diversifying selection was enough to alter the response to herbicide dose in ryegrass plants. A strong year effect on survival resulting from herbicide dose response of plants coming from different selection histories ($P<0.001$) was found. Separate year analysis revealed a highly significant main effect of UVB light history, suggesting that over all parent and screening herbicide doses, the UVB light environment affected the probability of surviving herbicide application (Fig. 4.8 and Table 4.2). Moreover, during the second year of the experiment, the effect of selection history (parental dose) on plant survival varied significantly with light history, as indicated by the

interaction term UVB x dose in Table 4.2 ($P = 0.0487$). This response was not affected by screening herbicide dose, as suggested by the lack of significance for the three-way interaction for light environment, parental dose, and screening dose ($P= 0.5727$). We also found evidence of variation in survival to herbicide application associated with the selection history in both years ($P = 0.0001$) (Table 4.2).

Equations fitted to describe herbicide dose response of different selected populations (Table 4.3) are useful to explain the above interactions. The values for the slope and model fit (R^2) are estimators of the plant response to herbicide dose. A decrease in the value of either of the estimators can be interpreted as decreased sensitivity in the assayed population to the herbicide dose response, and thereafter, an increased selection response. In the dose response carried out with the 1996 F_1 population, the steepest slope (-9.60) and the best model fit (0.96) were for pots without previous herbicide selection under UVB+. As herbicide selection pressure increased (herbicide dose applied to parent plants), the slope and the model fit decreased, regardless of light treatment. However, it is important that plants selected in the absence of UVB light had lower herbicide response slopes than those selected under UVB+ (Table 4.3). This difference was especially evident in plants selected by the 280 g ai ha^{-1} herbicide dose.

The lower selection pressure caused by the herbicide treatments in the 1997 parental generation was reflected in the herbicide dose response of the F_1 generation. In this case, the decrease in slope of the response with an increase in selection pressure (parental dose) was much smaller than the decrease found during the first year, regardless of light environment. Again, the UVB environment affected the response of the different ryegrass populations to herbicide, as shown by the differences in model slope. However, it is interesting that in this case, slopes for plants under UVB+ were less steep than those for UVB- (Table 4.3).
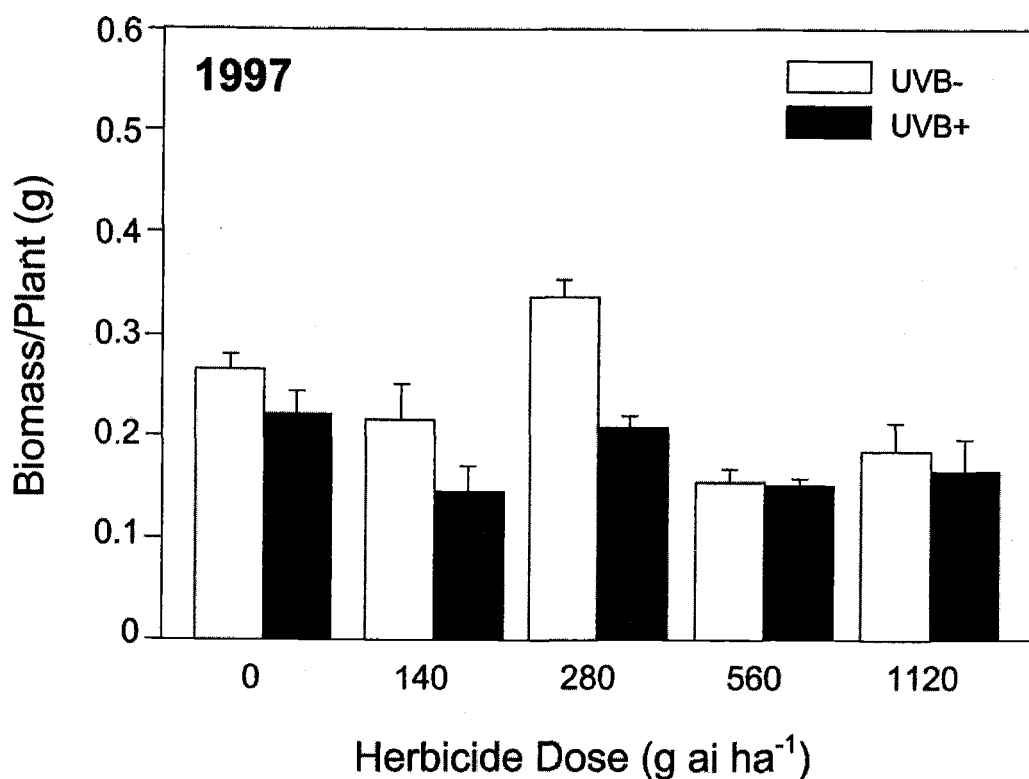
Figure 4.7 Effect of ultraviolet-B (UVB) radiation on biomass of seedlings of an F_1 population of Italian ryegrass surviving treatment with different doses of diclofop-methyl. Parental and F_1 populations were exposed to the same UVB light and herbicide environments. Bars represent means ± SE ANOVA (acronyms as in Fig. 2): L, $P = 0.0034$; D, $P = 0.0007$; L x D, $P = 0.31$.
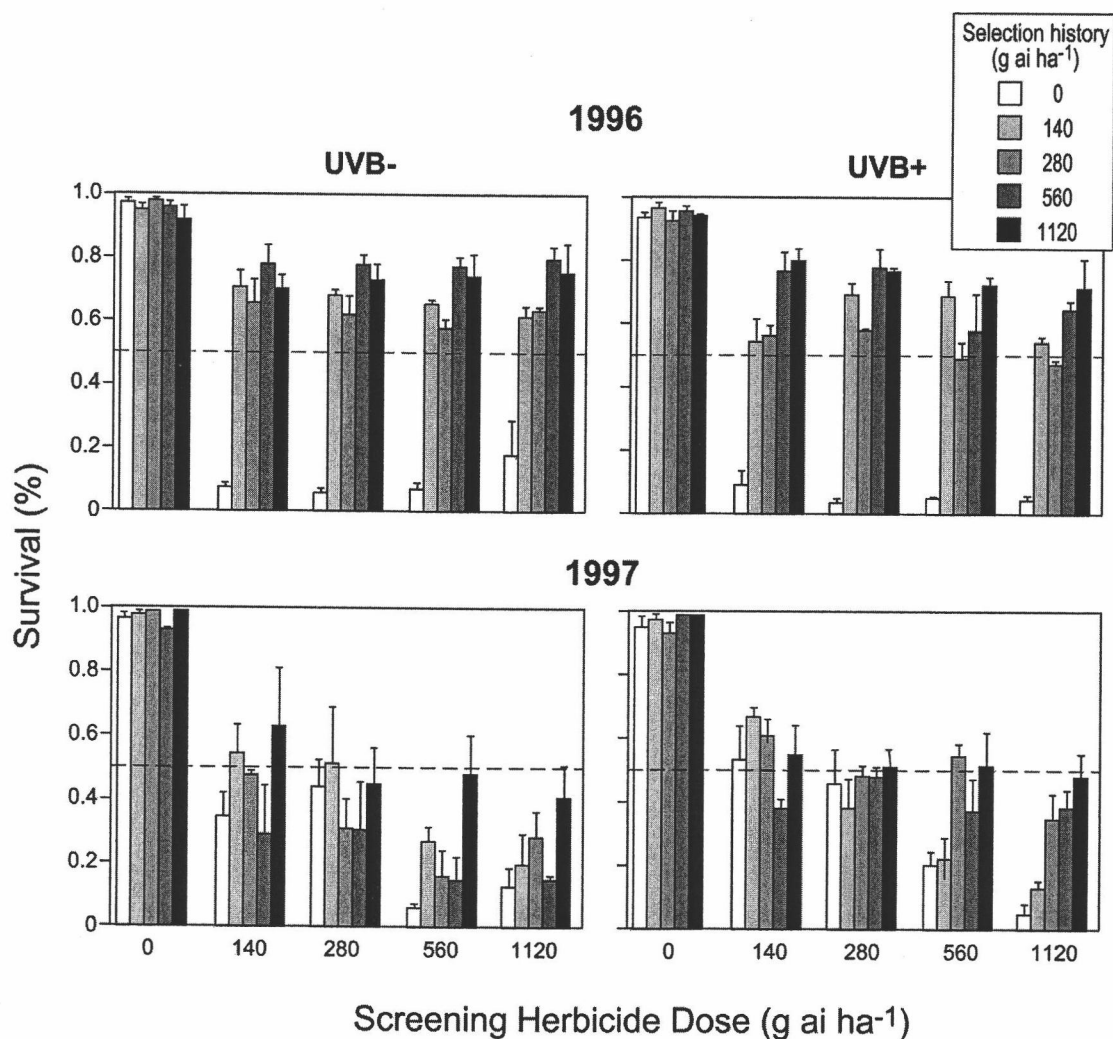
Figure 4.8 Selection of ryegrass populations in different UVB light and herbicide environments as determined by plant survival to herbicide application of F1 seedlings arising from seeds produced in 1996 and 1997 (bars represent mean and SE). The figure illustrates the significant interactions between current environment (herbicide screening dose) and selection history shown inTable 4.2

Table 4.2 The effect of current environment (herbicide screening dose) and ultraviolet-B (UVB) light and herbicide exposure history (dose) on survival of F_1 ryegrass seedlings from seeds produced in 1996 and 1997 and of F_2 ryegrass seedlings from seeds produced in 1997 (herbicide screened in 1998)

		F_1 seedlings		F_2 seedlings
		1996	1997	1997
Source of variation	Df^a	P^b	P	P
UVB	1	0.0157	0.0048	0.4887
dose	4	0.0001	0.0001	0.0001
UVB x dose	4	0.1518	0.0487	0.0563
screen	4	0.0001	0.0001	0.0001
UVB x screen	4	0.4112	0.2692	0.8190
dose x screen	16	0.0001	0.0422	0.0016
UVB x dose x screen	16	0.5538	0.5727	0.6810

Table 4.3. The effect of herbicide and UVB light history (treatments applied to parental generation) on the survivorship of the F_1 population of Italian ryegrass seedlings to diclofop-methyl dose screening. Seedlings grew from seeds produced in 1996 and 1997, and bioassays were carried out under the same environmental conditions in 1998. Equation parameters and coefficient of determination (R^2) describe the response of plants treated with increasing doses of the herbicide. Model fitted: survival=b ln (screening dose)+ a SE standard error of the slope

Parental herbicide dose (g ai ha^{-1})	1996								1997							
	UVB-				UVB+				UVB-				UVB+			
	a	b	SE	R^2	a	b	SE	R^2	a	b	SE	R^2	a	b	SE	R^2
0	72.1	-9.3	1.1	0.87 *	69.9	-9.6	0.6	0.96 *	85.4	-6.9	1.3	0.72 *	81.4	-5.8	1.2	0.75 *
140	87.5	-3.2	0.3	0.86 *	87.4	-3.8	1.2	0.67 *	84.45	-4.9	1.4	0.44 *	84.7	-5.6	1.2	0.76 *
280	88.3	-3.9	0.2	0.81 *	83.2	-4.5	0.4	0.91 *	87.1	-5.5	0.8	0.85 *	83.1	-4.0	0.1	0.80 *
560	90.5	-1.9	0.4	0.56 *	90.0	-2.4	1.0	0.35 *	75.2	-5.6	1.4	0.79 *	82.9	-4.4	1.2	0.86 *
1120	86.0	-1.9	0.8	0.34	90.3	-2.2	0.4	0.69 *	86.8	-4.8	1.0	0.58 *	86.3	-4.4	0.7	0.75 *

* probability of a slope significantly non-zero ($P < 0.01$)

4.4.2.2   F2 population

A second year of selection under the same herbicide and UVB light conditions further increased the degree of herbicide tolerance in the ryegrass populations (Fig. 4.9 and Table 4.2). Again, the effect of the selection history on plant survival changed with the light environment (p=0.05), independently of the herbicide dose used for the screening test (herbicide dose response) (p=0.681) (Table 4.2). When the selection was carried out twice in the absence of UVB light (UVB-), both the slopes and the R^2 values obtained from the herbicide dose response followed the expected parental dose selection pressures; i.e., the highest sensitivity values (slopes) were for the control (no herbicide application made to parent plants), intermediate values were for the mid-leve herbicide doses to parent plants (140 and 280 g ai ha^{-1}), and lowest values were for the highest herbicide doses to parent plants (560 and 1120 g ai ha^{-1}) (Table 4.4). In contrast, when selection was carried out twice in presence of UVB light (UVB+), sensitivity to herbicide was lost at all parental herbicide doses except the highest (1120 g ai ha^{-1}), which still responded to the increase in herbicide screening dose with a slope of -3.8 (Table 4.4).

Table 4.4. The effect of herbicide and UVB light history (treatments applied to parental and F_1 populations) on survival to diclofop-methyl dose response of the F_2 population of Italian ryegrass seedlings. Seedlings grew from seeds produced in 1997, and bioassays were carried out during the next growing cycle. Equation parameters and coefficient of determination (R^2) describe the response of plants treated with increasing doses of the herbicide. Model fitted: survival=b ln (screening dose)+ a. SE standard error of the slope

Parental herbicide dose (g ai ha^{-1})	UVB-				UVB+			
	a	b	SE	R^2	a	b	SE	R^2
0	76.2	-8.1	0.9	0.85 *	76.2	-7.63	1.5	0.71 *
140	91.3	-2.6	0.9	0.32 *	81.7	-0.8	0.2	0.01
280	80.9	-2.7	1.0	0.23	83.4	0.0	1.2	0.00
560	56.6	0.17	0.9	0.00	81.9	-0.3	1.3	0.00
1120	84.2	-0.73	0.9	0.17	91.0	-3.8	1.1	0.26 *

* probability of a slope significantly non-zero ($P < 0.01$)
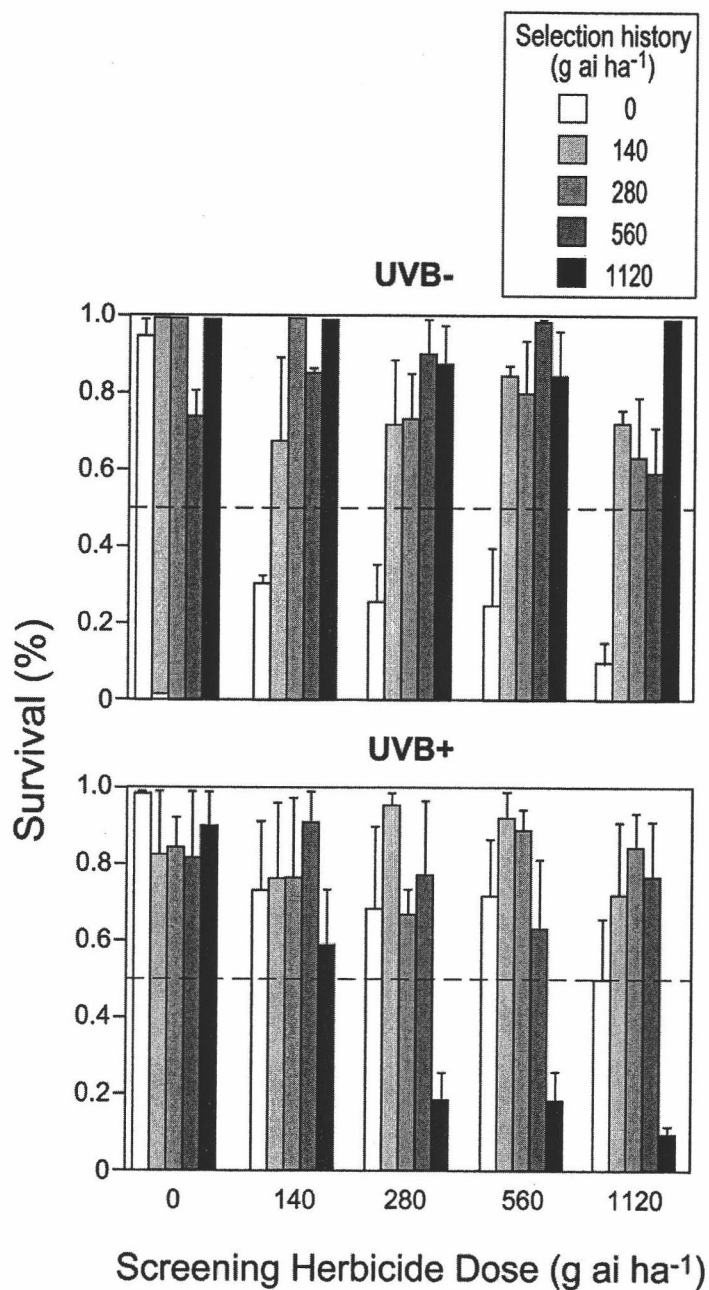
Figure 4.9 Selection of ryegrass populations in different UVB light and herbicide environments as determined by plant survival to herbicide application made to F_2 seedlings from seeds produced in 1997. The figure illustrates the significant interactions between current environment (herbicide screening dose) and two cycles of the same selection history from Table 4.2. Bars represent means $\pm$ SE.

4.5 DISCUSSION

These results indicate that environmental stress caused by UVB light interact with diclofop-methyl to affect the efficacy of the herbicide annual ryegrass control, as well as plant growth and reproduction of the plants surviving the combined UVB-herbicide treatment. Therefore, UVB light affects the evolutionary response to diclofop-methyl selection pressure.

The effect of UVB light combined with increasing herbicide doses varied between years as well as among doses. Variability of plant response to the combined effects of UVB light and other stress factors is to be expected (Teramura and Sullivan, 1994). In order for UVB radiation to be effective it must first penetrate the leaf, reach sensitive targets and be absorbed by the chromophores present. A variety of plant responses may alter the penetration of UVB into mesophyll. Anatomical changes in the leaf epidermal layer, quantitative or qualitative changes in epicuticular waxes or an increase in leaf thickness or specific leaf weight may reduce penetration of UVB radiation to sensitive targets (Tevini and Steinmuller, 1987). In 1997, UVB light increased the efficacy of intermediate herbicide doses (Fig. 4.2B). In that year, the percent survival of the UVB+ seedlings was statistically equal in the three highest herbicide doses, but lower for the

lowest (140 g ai ha^{-1}) dose, whereas for the UVB- seedlings, the percent of survival decreased only with the highest dose.

A UVB effect on average individual aerial biomass was not observed until second generation (F$_1$) plants (Figs 4.3a, b & 4.7). Previous studies show that UVB may affect the rate of seedling emergence, as well as affecting plant canopy structure (Ballare et al., 1996; Barnes et al., 1996). The importance of these factors in determining the outcome of intraspecific competition has not yet been determined. However, it maybe that a delay in the emergence of seedlings and a reduction in growth rate could increase the asymmetry in individual size that occurs as a response to density, which in turn could lead to a greater mortality of smaller individuals under a UVB+ environment than in the absence of this stress factor.

The effect of the herbicide–UVB light interaction on individual aerial biomass varied between years and among doses. In part, the different responses might be explained by the environmental conditions prevailing during each experiment year, for the periods when seedlings were emerging and herbicide was most lethal for plants. Herbicide was sprayed at the end of June in 1996 but not until the end of July in 1997. The average temperature during June of 1996 was 10°C, with UVB averaging 33.9 J/m^{2}day; during July the temperature remained the same, but UVB radiation

dropped to 23.6 (Table 4.1). During the July–August period of 1997, on the other hand, the average temperature was 12.9°C in the first month, and UVB was 24.4 J/m²day; in August, the average temperature increased by 1.5°C and the UVB radiation level by 10.5 J/m²day (Table 4.1). In the 1996 experiment, although no statistical differences were found among treatments, the coefficient of variation for the mean of the treatments with the lowest and the highest herbicide doses was lower with UVB light than without (Fig. 4.3a). In 1997, the presence of UVB and herbicide interacted to reduce not only the variance of the mean in some dose treatments, but also the biomass at the lower herbicide dose (140 g ai ha⁻¹). Moreover, the aerial biomass of individuals receiving the highest herbicide dose (1120 g ai ha⁻¹) was greater with UVB light than without it (Fig. 4.3b). Plant biomass accumulation in response to stress interactions is thus nonlinear. A combination of stress factors or a series of stressful events can reinforce, weaken, mask, or even reverse the response of plants to a single stress factor (Larcher 1995). Our data suggest that the combined effects of herbicide and UVB stresses will be evident when the herbicide acts as a weak stress factor. In the F_1 population subjected to herbicide and UVB treatments in 1997, as the level of one stressor (herbicide concentration, in this case) became so growth limiting, growth prediction became uncoupled from, and thus independent of the other stressor (UVB) (Fig. 4.7). The effects depended not only on the level of the stressors, but also on the

particular year. Only in 1997, when herbicide selection pressure was low, was there a significant interaction between UVB light and herbicide application that affected both the survival of plants and the biomass of surviving individuals.

The most striking effect of the UVB light–herbicide dose interaction was on the fecundity of individuals in relation to individual biomass and plant density (Figs 4.4 & 4.5). Even though examples in the literature indicate increased fecundity per g biomass in response to various environmental stresses (e.g. water and temperature, Bazzaz, 1996), it is well documented that stresses, in general, reduce fecundity of individuals (Stanton et al., 2000), as does UVB light alone (Mazza et al., 1999). Similarly, in *Avena fatua,* diclofop-methyl not only produces seedling mortality but also reduces plant fecundity (Scursoni et al., 1999). To our knowledge, however, this is the first report on the effects of UVB light–diclofop-methyl herbicide interaction on plant fecundity and density responses.

The effects on fecundity may have been related to environmental differences during tiller, flower, and seed production. Temperature and UVB radiation during the October–December period were greater in 1996 than in 1997. These differences may have caused differences in stress levels

(Mazza et al., 1999), leading to first year's low average total biomass per plant for surviving plants in the UVB+ treatment (Fig. 4.3).

The conditions created in our experiments had significant effects on fecundity of individual plants. This response strongly supports our prediction that the interactions between environmental stresses and herbicide control practices will have evolutionary consequences. The first evidence of such a consequence in this study was the change in herbicide efficacy of the on the offspring of the 1996 parental plants (Fig. 4.6). Herbicide application on the parent populations should have eliminated the individual plants susceptible to each dose, therefore complete survival to the same herbicide dose was expected in the second generation of plants (F_1). Even though the percentage of seedling survival increased for all doses in relation to that of the parents (Figs 4.2 and 4.6), variability among doses and between UVB light environments was high. This is especially true for the plants selected under low herbicide doses. The differential selection was probably because in the presence of UVB light, only the more herbicide-tolerant phenotypes produced seeds, whereas in the absence of UVB light, the individuals with a wider tolerance range contributed to the seed pool that was produced by plants surviving the herbicide doses. No matter what mechanism is responsible, the reduced fecundity of some ryegrass plants under UVB (Fig. 4.5) is likely to have a significant effect on the performance of the species in

the natural or agricultural environment. As for other annual species, seed output is a major determinant of ryegrass fitness (Newsham et al., 1998).

Selection caused by high doses of diclofop-methyl herbicide (1120 g ai ha^{-1}) should leave only individuals carrying a resistant homozygous genotype, while lower sub-lethal doses (mainly 140 g ai ha^{-1}) should allow the survival of heterozygous and probably a few susceptible homozygous individuals. Since ryegrass is an obligate outbreeder, when there is a low frequency of herbicide-resistant genes in the population, these genes will be present in individuals with heterozygous genotypes because of the swamping effect of susceptible gametes during reproduction (Mettler et al., 1988). In this case, selection by the highest herbicide dose should yield no evolved response. Because the highest dose is lethal for heterozygous individuals, all resistance genes would be eliminated. In our study, because of the dramatic rate of change in herbicide tolerance for the herbicide doses used, it appears that the ryegrass population had a high initial frequency of individuals with resistant genes. Even so, herbicide dose response revealed a clear selection related to herbicide dose, UVB light environment, and yeare(environment) history. Because seedling survival was lower for 1996 parental plants than for those in 1997, regardless of herbicide dose or light environment, the 1996 plants showed a greater selection response to all treatments. In the 1996 experiment, when selection pressure was high,

UVB stress reduced the selection response, as shown by the slopes calculated for the regression models fitted to the results of the herbicide-screening test (compare slopes for the UVB- and UVB+ results in the 1996 screening, Table 4.3). This apparent antagonistic effect of UVB on the evolution of herbicide resistance was still evident in the second generation (F_2); that is, one more selection cycle with the highest herbicide dose (1120 g ai ha^{-1}) under UVB+ did not increase herbicide tolerance (Table 4.4). In 1997, with much lower selection pressure on the parental plants, the absolute values of the slopes describing the screening dose response were smaller when there was UVB light stress, suggesting that in this case the UVB stress was synergistic with that imposed by the herbicide, thus augmenting the selection response towards herbicide tolerance (Table 4.3).

In conclusion, our study demonstrates that ambient UVB light is a weak stress factor for the ryegrass plants. However, when the selection pressure caused by herbicide application was low, the two stress factors had a synergistic effect, causing changes in herbicide efficacy that in turn had demographic and evolutionary consequences. The importance of the interactions between these light environments and herbicide doses on the evolution of herbicide-resistant populations may be greater in populations with lower initial resistant gene frequencies or in cases of tolerance conferred by polygenic systems. Research is needed to assess the

practical importance of ongoing weed evolution (Jordan and Jannink, 1997). Our findings suggest that interactions between human-made environmental stressors may not only be important for driving herbicide resistance, but also for the evolution of other phenotypic characteristics in weeds and wild vegetation.

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CHAPTER 5

GENERAL CONCLUSION

The research presented in this dissertation was developed to understand the extent that individual plant responses to multiple environmental stresses can be extrapolated to population-level responses in an annual species. I address both individual and population responses because the former reveals the plasticity of a response through the ontogeny of an individual, while population-level responses reflect the ecological and evolutionary potential of a species to respond to stress (Bazzaz and Morse, 1991).

I concentrated on one species, Italian ryegrass (*Lolim multiflorum* Lam.). This species is both an important seed crop in the Pacific Northwest, and a weed in cereal crops in many parts of the world. I analyzed the responses of ryegrass individuals of two genotypes to interactions of ozone and a herbicide during their ontogenic cycle (Chapter 2); the responses of competing individuals in a ryegrass population to ozone and the herbicide during one generation (Chapter 3); and the responses of a ryegrass population to ultraviolet light and the herbicide, for three generations (Chapter 4). Existing information on the biology of this species, the rapid generation time of one annual cycle, and the existing evidence that

ryegrass could be affected by all three stresses made Italian ryegrass a good model system.

My experiments answered questions of both economic and ecological importance. They also provided answers to agricultural questions. I wanted to study abiotic stress factors over a range of spatial scales, and that shared a common mode of action. The chosen stress factors: diclofop-methyl herbicide, UVB radiation and tropospheric ozone fulfilled those study requirements.

A herbicide is local stress factor acting mainly where it is sprayed. Ozone and UVB light are regional or global stress factors. Changes in the levels of air pollutants and of ultraviolet radiation in the UVB range (280-315 nm) at the earth's surface are components of the widely discussed global changes. Thinning of the stratospheric ozone layer is projected to increase UV radiation reaching the terrestrial surface (Kerr and McElroy, 1993; Madronich et al., 1998). On the other hand, tropospheric ozone increased over the past years because of the release of precursor substances such as nitrogen oxides (NO_x) and hydrocarbons by human activities (Stockwell et al., 1997; Kley et al., 1999). Recent models predict that ozone and UVB radiation will simultaneously increase in the troposphere in the future (Kley et al., 1999). Increased UVB radiation and air pollutants are considered

oxidative stresses since they are assumed to influence plants via elevated levels of reactive oxygen species (ROS) (Langebartels et al, 2002). Like ozone, exposure to diclofop-methyl produces oxidative stress that results in induction of senescence in susceptible individuals (Shimabukuro et al., 2001).

As described in Chapter 1, I used ecological theory as a framework to ask specific questions in each chapter, and to design experiments to answer them. This final chapter: (1) places the results of my thesis into a context for ongoing research on the subject, (2) describes the scenarios where these results have relevance, (3) indicates how these results might contribute to management of weed populations, and (4) discusses the direction that future research in stress ecophysiology might take.

The information contained in this dissertation can be critical in a scenario of global change and of stress factors resulting from human activities, especially since biological systems are faced with multiple stress factors acting at the same time. It is in this aspect where my thesis contributes to the knowledge base: *i.e* interactions rather than responses to individual stresses.

5.1 SUMMARY OF RESULTS WITHIN CURRENT RESEARCH DEVELOPMENTS

Ecology is intended to understand relationships between organisms and their environment, and at different organization levels. A continuing question has been how organisms live where they do and at times where environmental conditions are far from optimum. Darwin's main concern had to do with struggles for life, i.e. survival of the fittest organisms in an environment (Darwin, 1859). He was aware of the various responses plants express toward environmental factors. More recently, descriptive studies provided functional characterization of species, depending on their ability to withstand stress and disturbance (Grime, 1979). This notion has been used for management of weed populations (Radosevich et al, 1997). Awareness of the importance of environmental factors as stresses to agricultural systems began with early agriculture. Around 1930, it became clear that drought and soil salinity could exert a powerful influence on agricultural productivity (Nilsen and Orcutt, 1996). Since then, the literature has been filled with studies of plant stress physiology, but there is virtually no information on how adaptations to stress in plants develop.

In the later 1970s, applied research in plant stress physiology (crop stress physiology) took a slightly different path than research in natural systems

(plant ecophysiology). Ecophysiology began to examine interactions among populations and ecosystem biology, while agriculturalists, studying plant stress physiology, developed a closer affinity to molecular biology (Nilsen and Orcut, 1996). This difference is a reflection of different philosophies about how plants respond to environmental stress. Ecophysiologists are most concerned with evolutionary processes associated with adaptation for a wide variety of species (Clausen et al., 1940; Penning de Vries, 1975; Bjorkman et al, 1972). In contrast, the philosophy of agricultural research centered on the acclimation of plant varieties or lines of narrow genetic diversity to the variation in agricultural systems.

When pollutants first became an important component of plant stress physiology, scientists focused their research on toxicology. However, over time, crop physiologists have expanded into learning about detoxification mechanisms, especially as influenced by their work on herbicide mode of action and potential adaptation of herbicide tolerance in weeds and crops. Ecophysiologists, on the other hand, have tended to follow research lines concerning effects of atmospheric pollutant at larger scales. Recently, effort has been targeted toward modeling ecosystem responses to changing atmospheric factors (Norman, 1993).

The answers to the questions posed in my thesis could not be provided solely by either of those approaches. Stress physiology cannot be understood well with models that reduce botanical diversity to "the plant". Rather, a coherent theoretical framework for predicting and interpreting stress responses needed be derived from multi-scale studies. Making the link between plant and population responses to environmental changes allows us the elucidation of patterns for existing adaptations to stress, and to forecast the rate and direction of contemporary evolutionary responses to stresses of recent origin.

My dissertation looks at stress from a different perspective, at a range of scales from the ecophysiology of the organism to the population. By conducting simple experiments in controlled and field environments, I covered processes that occur in the life of an individual (Chapter 2) to population processes that must take place over ecological (Chapter 3) or evolutionary time (Chapter 4).

The experiments with *Lolium multiflorum* in Chapter 2 showed that ozone exposure can produce significant effects on the physiology of an annual species, modulating the growth rate and allocation patterns of the plants. Ozone can also interact with the effects of other abiotic stress factors, such as, diclofop-methyl herbicide. My data also show that magnitude and

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direction of responses can be quite different depending on the plant

biotype, phenological stage, and environmental conditions (Table 5.1)

Table 5.1 Summary of responses of herbicide susceptible (A) and herbicide resistant (B) plants when they grew under ozone, UVB, herbicide, or a combination of two stress factors. A negative (-), positive (+) or neutral (0) effect respect to the control plants (no stress) is indicated. Two responses for a given stress factor means that response is dependent on herbicide dose. Effects are indicated on individual (plant⁻1) or per pot (pot⁻¹) bases. NP: normal planting date, DP: delayed planting date, nm: variable not measured, RE: reproductive effort, S/R: shoot/root

(A) Herbicide susceptible plants

Response Variable	ozone		UVB		herbicide		O_3 + herb		UVB+herb	
	NP	DP	NP	DP	NP	DP	NP	DP	NP	DP
Plant⁻¹										
Biomass	-	0	-	-	- 0	-	+ -	0	-	-
Leaf area	-	-	nm	nm	-	-	+ -	+ -	nm	nm
Tillers	-	0	nm	nm	+ 0	- 0	+ -	+ -	nm	nm
Seedbiom	-	0	0	0	-	-	-	-	-	-
Spikes	0	0	nm	nm	-	-	-	-	nm	nm
Leaves	0	0	nm	nm	nm	nm	nm	nm	nm	nm
Length	-	+	nm	nm	nm	nm	nm	nm	nm	nm
RE	0	+	nm	nm	-	+	-	+	nm	nm
S/R ratio	0	nm	nm	nm	0	nm	0	nm	nm	nm
Pot⁻¹										
Biomass	-	-	nm	nm	- 0	0	- 0	0	nm	nm
Spikes	-	0	nm	nm	-	0	-	0	nm	nm
Seedbiom	0	0	0	0	0	0	0	0	-	-

Table 5.1 (continued)

(B) Herbicide resistant plants

Response Variable	ozone		UVB		herbicide		O_3 + herb		UVB+herb	
	NP	DP	NP	DP	NP	DP	NP	DP	NP	DP
	Plant^{-1}									
Biomass	-	0	nm	nm	+	0	+	0	nm	nm
Leaf area	-	0	nm	nm	0	0	+	+ -	nm	nm
Tillers	-	-	nm	nm	+	0	+	+ -	nm	nm
Seedbiom	-	0	nm	nm	0	+	+ 0	+	nm	nm
Spikes	0	-	nm	nm	+	+	+	+	nm	nm
Leaves	-	0	nm	nm	nm	nm	nm	nm	nm	nm
Length	-	0	nm	nm	nm	nm	nm	nm	nm	nm
RE	-	+	nm	nm	-	+	-	+	nm	nm
S/R ratio	0	nm	nm	nm	0	nm	0	nm	nm	nm

The impact of ozone on growth and reproduction of both Italian ryegrass biotypes used in this study was greater in the first year of the experiment, carried out during the normal growing conditions for this species (Table 5.1) Plant exposure to ozone affected growth of both ryegrass biotypes changing leaf area ratio, without changing total biomass accumulation. Given enough time vegetative growth parameters of ozone-stressed plants eventually approached that of the controls. This delay in the time of leaf area accumulation of the plants under ozone stress was accompanied by

higher average unit leaf rates (ULR) than control (non-ozone treatment) plants, and resulted in equal RGR and similar maximum biomass.

As a consequence of the interaction between stressors, very different responses for vegetative and reproductive structures were found, which also differed with biotypes (Table 5.1). While the effect of herbicide was greater on the herbicide susceptible plants, ozone affected the herbicide resistant plants more that susceptible plants. On the other hand, late planting of ryegrass plants (second year experiment) decreased seed production significantly more in the in the herbicide susceptible than in the resistant plants. Reproductive effort of herbicide-susceptible plants was about 50% smaller than that of resistant plants in that experiment. Herbicide and ozone stress decreased reproductive effort in both biotypes in the first year of the experiment but increased it in the second year when environmental (temperature) stress was greater.

In Chapter 3, I present evidence that abiotic stressors can produce significant changes in the population structure of an annual species, modifying the hierarchies of individuals within the population by changes in density-dependent responses. The stressors changed the frequency distribution for average individual weight resulting from increasing densities. Based on the experiments to stress individual plants (Chapter 2), we

expected that density effects could be delayed under stress, because stress had an effect on all the vegetative parameters measured. However, ozone did not modify the individual weight/density relationship, whereas herbicide increased it, and both stresses together made weight per plant independent of density.

Moreover, and contrary to what we had hypothesized, despite interactions between density and abiotic stress that reduced biomass and number of spikes, plants were able to compensate during grain filling and maintain similar seed production–density relationships in all treatments. Because several, compensatory mechanisms occurred at the population level (Chapter 3) predictions on the effect of stresses derived only from individual studies (Chapter 2) were sometimes incorrect.

Results in Chapter 4 indicate that the environmental stress caused by UVB light can interact with diclofop-methyl to affect the efficacy of the herbicide as well as impact plant growth and reproduction in the plants surviving the chemical treatment (Table 5.1). Therefore, UVB light interfered with the evolutionary trend to resistance determined by herbicide selective pressure. The effect of UVB light combined with increasing herbicide doses varied between years as well as among dose levels. In part, the different responses to UVB and herbicide were explained by the environmental

conditions during each experimental year. The combined effects of herbicide and UVB stresses were most evident when the herbicide acted as a weak stress factor. When the level of one stressor (herbicide concentration, in this case) became growth limiting, growth prediction became uncoupled from, and thus independent of the other stressor (UVB). Just as reported in Chapters 2 and 3, the effects observed depended not only on the level of the stressors, but also on the particular environmental conditions (year).

The conditions in our experiments had significant effects on fecundity of individual plants (Chapters 2 and 3). This response supports our prediction that the interactions between environmental stresses and herbicide application practices will have evolutionary consequences. However, the direction of those consequences was not always the same (Chapter 4). The herbicide dose response revealed a clear selection related to herbicide dose, UVB light environment, and year (environmental conditions) selection history. In the 1996 experiment, when selection pressure was high, UVB stress reduced the selection response. In 1997, with much lower selection pressure on the parent plants, the absolute values of the slopes describing the herbicide dose response were smaller when there was UVB light stress, suggesting that the UVB stress was synergistic with that imposed by the

herbicide, and thus augmenting a selection response towards herbicide tolerance.

5.2 CONTRIBUTION OF THIS RESEARCH TO GENERAL AND APPLIED KNOWLEDGE

Stress sensitivity and responses are related to the form, duration, intensity and spatial/temporal distribution of the stresses experienced. But they also depend heavily upon the ecological and evolutionary history of the species or population. A species that has evolved in a given resource matrix, will have a genetically controlled partitioning schedule that ensures maximal growth during the prevailing environmental conditions. These conditions, however, change from day to day and, hence flexibility must be built into the adjustment involving a great number of processes at the plant and population level. We are still far from understanding what mechanisms plant species use to maximize growth and reproductive output. We know however that new demands are being imposed on plants as human activities modify natural environments. I hope my thesis is a contribution to fill in that gap in knowledge.

I believe the information gathered here has significance for agriculture as well as environmental sciences, to understand how crops cope with less than optimum growing conditions. It has been repeatedly demonstrated that crop productivity is significantly inhibited by natural environmental factors and anthropogenic factors such as air pollution. Neither of these environmental stresses is expected to become less intense.

From a more applied perspective, weeds have been recognized as a major economic pest because of the losses they cause to crops. Reduction in weed competition in modern agriculture is achievable with the wide spectrum of tools and chemicals existing today, but weed management strategies are not related solely to the use of herbicides or other tools. Instead, most of the information necessary to design practices for weed management are derived from studies of weed population dynamics. These studies consist of monitoring temporal changes in the number of individuals (or amount of biomass) within a weed population. Demographic analyses helps to identify a life stage or process in a weed population which is particularly critical for its regulation, but often fails to predict the occurrence of that process in time and space. Another population approach, more mechanistic than the demographic one, is required to establish functional relationships between biological and environmental factors and rates of

critical processes. My studies represent a step towards establishment of those relationships

An important result of my thesis is that environmental human stresses have an impact not only on the individual plant level but also on populations, changing competition relationships and having potential evolutionary consequences. Those stress factors should be considered in weed experiments evaluating, for example, herbicide effects or weed-crop competition. Such experiments are usually conducted in greenhouses (which exclude most UVB radiation) or in experiment stations with different degrees of atmospheric contaminants depending on the location relative to urban areas. Usually the only environmental factors considered are soil and air humidity content, PAR, temperature and soil organic matter.

If the apparent differences in susceptibility to ozone and late planting conditions of herbicide-susceptible and -resistant plants is confirmed, the existence of evolutionary tradeoffs could be suggested, whereby plants resistant to herbicides may lose tolerance to some stressors like ozone. On the other hand that trait may also allow R-plants to better withstand the negative effects of late spring emergence or ozone when herbicide is applied.

Another important outcome of my thesis is the efficiency of seed production under stress conditions. Herbicide efficacy experiments usually consider survival rate or biomass reductions of treated plants. Differences in reproductive effort of each ryegrass biotypes used in my experiments suggest that seed production might also be a parameter of high importance. The herbicide-resistant plants originally produced twice as much seed per unit of vegetative biomass than S-type plants, the stresses lowering the reproductive effort of R-plants to that of unstressed susceptible plants. Because of the higher reproductive effort of R-plants, even with significant reductions in biomass, plants can be represented in the next generation. Moreover, when delayed planting shortened the plants' growth cycle, the already greater reproductive effort of the resistant biotype was increased by the stress treatments whereas little change occurred in susceptible plants. These results suggest why weeds of any species have not become extinct despite the continuing increase in herbicide efficiency for their control them.

Finally, increases in reproductive effort with stress observed in the warmer conditions of the second year experiment, might be important for the range expansion of this plant species to more sub-tropical areas or to areas with higher levels of ozone contamination.

5.3 CONCLUSIONS

Below is a list of the important and relevant questions derived from this thesis

1) Plants are capable of growth and reproductive compensation under novel stresses produced by human actions.

2) Stress factors with similar biochemical mechanisms can have different effects at the individual plant and population levels of organization.

3) Compensation occurs at all levels of organization: as individuals modifying growth and allocation to different organs, and as populations modifying birth, and death rates and density dependence responses.

4) The ability to compensate sometimes decreases with the number of stress factors, e.g. compensation under herbicide application is decreased in plants exposed to high levels of ozone or UVB. In other cases, compensation ability increases with the number of stress factors (e.g., ozone and herbicide).

5) The common assumption that the extinction rate will increase with the increase in number of stress factors, due to a loss of compensatory ability, does not always apply

5.4 FUTURE RESEARCH

The information presented in this thesis highlight the importance of conducting more research on the links between levels of organization; organisms, populations and ecosystems. Processes in ecosystems are, in part, determined by responses of individual organisms. To model the function of an ecosystem in a mechanistic way, the linkage between organisms and ecosystems must be better understood.

There is also much more need to learn about multiple stresses, since plants experience many interactive stresses in all habitats. However, not all stresses are abiotic. The ability of plants to respond to a biotically caused stress, such as herbivory, depends on the availability and ability to use environmental resources. There is probably a large cost to a plant's ability to acclimate to environmental stress caused by a biotic stress which it is also experiencing. On the other hand, some environmental stresses may trigger internal defense mechanism that also serves against biotic stress.

Ecological studies demonstrate the essential role of plants for sustaining life the biosphere since all life is supported by plant productivity. Perhaps the more we understand about plant responses to the environment, the greater

will be our ability to manage both natural and manmade systems to sustain the biosphere and our own future.

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CHAPTER 6

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