An investigation of herbicide resistance in black-grass using safeners and synergists.

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Declaration

The author has not been a registered candidate nor an enrolled student for another award of the CNAA or other academic or professional institution during this research programme. Material contained in this thesis has not been used in any other submission for an academic award and is entirely the author's individual contribution. The author has attended appropriate lectures, seminars and conferences in partial fulfilment of the requirements of the degree.

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Abstract

With the expanding world population and the decreasing amount of land available for agriculture it is not possible to grow sufficient food without the use of agrochemicals. However, the advent of herbicide resistant weeds may jeopardise our ability to feed the world in the future. Black-grass (*Alopecurus myosuroides*) resistant to the herbicide chlorotoluron was first reported in the UK in 1982 and has since become wide spread in Autumn-sown crops. The aim of this study was to characterise chlorotoluron susceptible and resistant black-grass populations in terms of whole plant growth and photosynthesis, and to investigate the activity of glutathione S-transferases (GST) using herbicide safeners and synergists. The possible role for GSTs in chlorotoluron resistance was also considered.

Susceptible and resistant black-grass plants were grown in compost under glasshouse conditions and chlorotoluron was applied to the foliage at a range of concentrations. The ED_{50} values were calculated to be 0.93 and 39.3 kg ai ha^{-1} for susceptible and resistant black-grass, respectively, giving a Resistance Factor of 42 under defined growth conditions. The morphology and development of the plants over 14 days was further investigated by assessing several growth parameters. Susceptible plants treated with field-rate chlorotoluron ceased to grow 5-8 days after treatment, whereas treated resistant plants were unaffected. The number of tillers (6), leaf area (3000 mm^2) and mean dry weight (160 mg) were the same in both biotypes (untreated) 5 weeks after sowing. Infra-red gas analysis was used to measure gaseous exchange by whole plants in specially designed chambers. Susceptible and resistant plants were equally able to perform photosynthesis at the flux densities examined, and the estimated quantum yield was 0.028-0.030 mol CO_2 mol^{-1} absorbed photons. Resistant plants treated with field-rate chlorotoluron showed no reduction in photosynthesis over this period, whereas photosynthesis by susceptible plants had ceased after 10 hours and did not recover after a dark period.

Crude cell-free extracts were made from susceptible and resistant black-grass shoots and GST activity was measured *in vitro* using CDNB as substrate. Endogenous GST activity in resistant plants was twice that in susceptible plants, 83 and 154 nmol min^{-1} mg^{-1} protein, respectively. The GST activity could be further elevated in both biotypes by pre-treating plants grown in a hydroponic system with various herbicide safeners. Benoxacor was the most effective safener, elevating GST activity by 1.5 and 3 times in resistant and susceptible plants respectively. Plants grown in hydroponics were pre-treated with 100 μM benoxacor for 24 hours and then supplied with 2.4 μM chlorotoluron for 24 hours, after which the photosynthetic rate was measured. Chlorotoluron alone reduced photosynthesis in susceptible and resistant plants by 65% and 25% respectively, but pre-treatment with benoxacor reduced those values to 47% and 0%. Thus, the increase in GST activity due to benoxacor could be correlated with a reduction in chlorotoluron injury to the plants. However, the nature of this relationship remains to be proved. It was hypothesised that elevated GST activity in resistant black-grass could protect plants from chlorotoluron by conjugating it with glutathione or by acting as a peroxidase to protect plants from oxidative stress. Alternatively, it may play no part in chlorotoluron resistance, but provide a mechanism whereby plants could be cross resistant to the herbicide fenoxaprop.

Abbreviations

Accase	Acetyl CoA carboxylase
ALS	Acetolactate synthase
AOPPs	Aryloxyphenoxypropionates
BSA	Bovine serum albumin
CDNB	1-Chloro-2,4 dinitrobenzene
CHDs	Cyclohexanediones
Dicot	Dicotyledonous plant
DMSO	Dimethyl sulfoxide
DTNB	5, 5'-Dithiobis (2-nitrobenzoic) acid
DTPA	Diethylenetriaminepentaacetic acid
DTT	Dithiothreitol
ED_{50}	Dose required to reduce fresh weight by 50%
EDTA	Ethylenediaminetetraacetic acid
EPSPS	5-enoyl-pyruvyl shikimic acid 3-phosphate synthase
GSH	Glutathione in its reduced form
GSSG	Glutathione in its oxidised form
GST	Glutathione S-transferase
GTR	Glutathione reductase
HPLC	High performance liquid chromatography
Monocot	Monocotyledonous plant
NA	Naphthalic anhydride
NADPH	Nicotinamide adenosine dinucleotide phosphate reduced
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PVPP	Polyvinylpolypyrrolidone
SDS	Sodium dodecyl sulfate
TLC	Thin layer chromatography

Contents

Chapter One

An introduction to herbicide resistance and metabolism

1.1 The biology and control of weeds

The word "weed" was originally the Anglo-Saxon name for all herbs and small plants (Weod). Many plants which are scorned as "just weeds" today were used at that time to cure illness or provide food, which demonstrates that the classification of crops and weeds is only a subjective one which has changed over the years. For the purposes of this thesis a weed is a plant growing where it is not wanted, or, as the European Weed Research Society defines it, "any plant or vegetation interfering with the objects of people" (Mortimer 1990). The world relies on 12-15 major crop species, of which 8 belong to the graminaceae family, as do half of the world's weeds. It is thought that just 75 weed species are responsible for 90% of crop loss due to weeds, and 250 species world wide are regarded as important weeds. All weeds have the common characteristic of the ability to persist repeated disturbance and periodic near total destruction of above ground biomass (Mortimer 1990, Cobb 1992).

Severe weed infestations will reduce crop quantity and quality, and it is estimated that 10-20% of global food loss can be attributed to weeds (Auld *et al* 1987). Weeds reduce the crop productivity by competing for nutrients, light, water and space. They are usually well adapted to their environment which puts crop species at a disadvantage, since they are bred for their yield and not for their competitive ability. Complete crop failure is possible if it is prevented from setting seed (Cobb 1992). If the quality of the crop is impaired then it has a lower market value. This can be due to smaller size of the grain, poor appearance or contamination by weed seed. Contamination is particularly a problem if the weed is poisonous, for example black

nightshade (*Solanum nigrum* L.) in pea crops. Problems in cleaning the grain arise when the weed and crop are closely related, such as wild oat (*Avena fatua* L.) in wheat and barley (Mortimer 1990). Weeds can also increase the time and labour required both in preparing clean seedbeds and in harvesting by interfering with machinery. Examples of this include *Polygonum aviculare* L. and *Chenopodium album* L. which have tough, wiry stems. Pests and diseases may also be harboured in weeds (Cobb 1992).

Plant species that become prolific weeds have certain biological characteristics in common which allow them to survive in the crop environment. The ideal weed is able to combine rapid growth with highly abundant seed production and a long seed dormancy for surviving adverse conditions. The seeds periodically germinate over the growing season from a persistent seed bank, and can germinate in a wide range of circumstances. Rapid colonisation depends a fast switch from vegetative to reproductive growth, investing most resources in copious seed production. Seed production can vary from an average 250 seeds per plant (*Avena fatua* L.) to 250, 000 per plant (*Chenopodium album* L.). The extent of this competitive advantage is clear when the number of seeds in a given area is considered. For example, spring barley is sown at a rate of 400 m^2 but viable weeds seeds may be present at an estimated rate of 75, 000 m^2. Most weeds are self-pollinated so only one individual is required to start a whole population. Survival sometimes depends on responding speedily to a new situation, for example, *Chenopodium album* L. can increase its internode length in response to low light exposure (Cobb 1992, Mortimer 1990).

The distribution of plants is largely determined by edaphic and climatic factors which causes some species of weeds to be prevalent in certain areas. In addition, such factors as the historical use of the land and farming practices can determine the dominant weed species (Auld *et al* 1987). Weeds can invade a new area and proliferate either because they have characteristics which pre-adapt them to the area or by evolving them over a period of time. For example, the clearing of land encourages weeds which are able to colonise bare earth such as *Chenopodium album* L. If agricultural practices remain constant over a long period of time then local races will evolve. This was first noted a long time ago when dwarf forms of fool's parsley *(Aethusa cyanapium* L.) developed after the introduction of the reaper. Gene exchanges between related weeds and crops (introgression) allow crop mimicry to occur, to the extent that they are virtually indistinguishable, a good example being the prevalence of *Zea mexicana* in maize in central America. The most extreme form of evolution results in the creation of a new species. *Ageratum conyzoides* L. is a self-compatible annual weed which has evolved as a polyploid of the perennial *A. microcarpum* L. (Mortimer 1990). Thus, the prevalence of a certain weed species in an agricultural area can be attributed to an interaction between environmental conditions, man's intervention and the innate characteristics of the weed. The continual changes in these parameters results in a constant flux in the weed populations.

1.2 The history and development of chemical weed control

It was in the 18th century that significant agricultural improvements were made with the development of new tools and methods, and it was recognised that good weed control was essential for optimal crop production. The rotation of crops was a standard practice, thus cultivation and harvest dates varied from year to year, and no single weed could gain dominance. Cereal crops were rotated with ones grown in rows such as turnips and potatoes, which could be weeded mechanically with a hoe (hence known as a cleaning crop). Leys of one or more years were also included, when the land was temporarily not sown to prevent it becoming devoid of nutrients (Lockhart *et al* 1990). Ploughing was an effective control measure, burying weed seeds and exposing weed roots to the soil surface where they desiccated. This situation continued until chemical fertilisers were developed in the early 1900s. Fertilisers removed the need to include leys in the rotations which became limited to cereals and row crops. Also at this time, the expansion into sugar beet allowed farmers in the East of England to have a rotation based entirely on cash crops. Farmers who used fertilisers and continued with crop rotations were able to achieve high levels of weed control, and this was still common in the late 1940s. However, the increasing economic pressure and reduced labour force at that time led to crop specialisation and a declining use of the cleaning crop. With the introduction of the combine harvester and selective herbicides, crop rotation was no longer necessary and whole areas were given over to constant cereal production. However, this monoculture has provided weeds with the stable conditions required to become well established and abundant (Lockhart *et al* 1990).

Copper sulphate and sulphuric acid were used as herbicides to control dicot weeds in cereals in the 1930s. The first selective herbicides to be marketed after World War II were 2,4-D and MCPA, after it was found that the salts of chlorinated phenoxyacetic acids were highly active against dicots. Between 1950 and 1970 over 50 new herbicides were developed, but nearly all were produced to control dicot weeds. The extensive use of herbicides and cereal monoculture allowed grass weeds to fill the gap left by dicots and they proliferated.

Many of the new herbicides in the 1970s were introduced specifically to control grass weeds such as wild oat, black-grass and barren brome and were mostly either substituted ureas, which inhibit photosynthesis, or graminicides, which inhibit lipid biosynthesis. Substituted ureas like chlorotoluron and isoproturon became widely used in cereals, either on their own or in mixtures to control both monocot and dicot weeds, especially in winter wheat, barley and rye (Lockhart *et al* 1990). They have soil and foliar activity and can be used pre- or post-emergence. The graminicides are a large group of herbicides which are usually subdivided into five chemical classes. Those introduced in the mid 1970s were either aryloxyphenoxypropionates (AOPPs) such as diclofop and fluazifop (the "fops") or cyclohexanediones (CHDs or "the dims") such as sethoxydim and tralkoxydim (Cobb 1992). Both chemical classes inhibit acetyl CoA carboxylase (ACCase) which is the first committed step for fatty acid biosynthesis in plants. They are applied post-emergence to control annual grasses and volunteer maize in a range of mainly broad-leafed crops such as oilseed rape and soyabeans. Another successful introduction at that time was the non-selective herbicide glyphosate, which is absorbed through the foliage and translocated to the rest of the plant (Worthing and

Hance 1991). The target site is the enzyme 5-enoyl-pyruvyl shikimic acid 3-phosphate synthase (EPSPS), which is involved in the biosynthesis of aromatic amino acids (Cobb 1992).

During the 1980s two major groups of herbicides were developed, which were far more potent and selective at low doses, and could be used at rates of grams rather than kilograms per hectare. These were the sulphonylureas such as chlorsulfuron and the imidazolinones like imazethapyr, which both act by inhibiting acetolactate synthase (ALS), a key enzyme in the biosynthesis of branched chain amino acids (Cobb 1992). These ALS inhibitors all have a wide spectrum of activity, controlling grass and broad-leafed annual and perennial weeds in cereals, with both soil and foliar activity.

The search for novel modes of action has lead to recent interest in compounds which inhibit protoporphyrinogen oxidase (PPGO), blocking chlorophyll and haeme synthesis. Two such new herbicides, F8426 and thiazimin were reported in 1993 (Saun *et al* 1993, Weiler *et al* 1993). These have good post-emergence activity on broad-leafed weeds in cereal crops, and are active at 20-40 g ha^{-1}, an almost ten fold decrease in application rate compared with the 2.5-3 kg ha^{-1} required for the older herbicides like chlorotoluron.

1.3 The development of herbicide resistant weeds

It was predicted as early as the 1950s that herbicide resistance would develop in weeds, but it was not recorded in the literature until 1968. The first reported weed with a resistant biotype was *Senecio vulgaris* L. which was discovered to be resistant to simazine, an inhibitor of photosynthesis at photosystem II. At least 57 species are now known to have biotypes resistant to the triazine group of herbicide, more than for any other group. Most are found in areas where the repeated and persistent use of triazines over several years is coupled with continuous maize monoculture (Gronwald 1994).

The information given in Table 1.1 shows the 8 classes of herbicides to which resistant biotypes have evolved from at least 5 species. There are several other groups for which just 1 or 2 species have resistant biotypes. The only herbicide for which there is no recorded resistance is glyphosate, introduced in 1974 for non-selective post-emergent weed control (Holt *et al* 1993). It would appear that the average time for the majority of herbicides between introduction and the detection of resistance is 12 years. One notable exception is the ALS (acetolactate synthesis) inhibitors group, which includes the sulfonylureas and imidazolinones. These herbicides are increasingly popular due to their high activity at low rates. Ironically, it is this high potency and specificity which quickly selected resistant biotypes, in some cases after only 4 applications (Saari *et al* 1994). Resistance to the substituted urea herbicides such as chlorotoluron and isoproturon was found about 12 years after their introduction. They have been used extensively in the East of England to control grass weeds, including black-grass, in large areas of cereal monoculture.

Table 1.1 The extent of world wide herbicide resistance in weeds. (From Holt *et al* 1993 and Lockhart *et al* 1990)

Approximate date of herbicide introduction	Herbicide class	Year resistance was first detected	Reported number of species with resistant biotypes	Number of sites where a resistant biotype is thought to have evolved
1958	Triazines	1968	57	> 1000
1976	ACCase inhibitors	1982	8	> 1000
1982	ALS inhibitors	1986	8	> 1000
1958-61	Carbamates	1988	2	70
1961-62	Bipyridiliums	1976	18	> 50
1971	Substituted ureas	1983	7	> 50
1967	Dinitroanilines	1973	5	> 20
1945	Phenoxyacetic acids	1962	6	5

1.4 Chlorotoluron

Chlorotoluron is a substituted urea herbicide which inhibits photosynthesis. It is characterised as a colourless powder, melting point of 148 ^{0}C, solubility of 70 mg l^{-1}, log K_{ow} 2.3 and half-life in the soil of 30-40 days. The structure (Figure 1.5) is such that it has a lipophilic group at one end and a positive charge, which is common to all urea herbicides and essential for activity. It is effective at 1.5-3 kg ha^{-1} both as a residual soil-acting herbicide and as a contact foliar spray against many broad-leafed and grass weeds in wheat, barley and rye, and is very effective on black-grass in winter sown cereals (Cobb 1992, Worthing & Hance 1991).

The mode of action is inhibition of electron transport in photosystem II (PS II). photosynthesis occurs on the thylakoid membrane in the chloroplast. Light is captured at the reaction centre, and this energy promotes an electron from the reaction centre to the primary electron acceptor (phaeophytin). The electron then passes to quinone, Q_A, and is transferred to plastoquinone Q_B. This Q_B is located on the D1 protein which spans the thylakoid membrane, but is not firmly bound to it. Chlorotoluron displaces plastoquinone from the Q_B site on the D1 protein, thus preventing electron flow from Q_A to Q_B. These excited electrons are free to react with oxygen and peroxide to form free radicals which attack unsaturated fatty acids in the thylakoid membrane, initiating a chain reaction of lipid peroxidation. Eventually the thylakoids disintegrate and other cell membranes are attacked, resulting in tissue disintegration (Cobb 1992).

1.5 Chlorotoluron resistance in black-grass

Black-grass (*Alopecurus myosuroides* Huds.) is an annual grass weed which produces an abundance of tillers and can reach a height of 90 cm (Elliot *et al* 1979). The flowers are protogynous and fertilised by out-crossing, although self-pollination can occur if cross pollination fails (Moss 1983). The dispersal unit is a spiklet containing a single caryopsis which becomes a characteristic purple/black colour when ripe. Propagation is solely by seed which is shed in vast quantities from late June to late August. Since black-grass produces an average of 100 seeds per head, and there can be up to 1000 heads m^2 in severe infestations, it is not surprising that populations can increase by an estimated 10 fold per annum (Moss 1981). The seeds have a short lived natural dormancy, with more than 80% of viable seed shed in July germinating in the same autumn (Gwynne & Murray 1985), which results in a small seed bank in the soil. Thus, since most seed is germinating when a winter crop seed is sown, and the seed is shed before harvest, a close association with winter cereals is not surprising.

The current dominance of grass weeds over broad-leafed ones has already been discussed, but this does not explain why black-grass has become one of the most troublesome weeds in winter cereal production in England (Flint 1985). A combination of factors have caused black-grass to become concentrated on farms with certain soil characteristics and farming methods, and to develop resistance to chemical control. A random survey of weeds in the UK in 1979 found infestations of black-grass on 50% of farms in England, but it was virtually absent from the rest of the UK (Elliot *et al* 1979). It is traditionally associated with heavy, wet soils, which are often used for autumn-

sown crops, and black-grass is reported to cause the most problems in the East, South East and East Midlands areas, in which there are more autumn-sown than spring-sown cereal crops. Herbicides were used to control black-grass on 15% of the cereal area in 1979, which was an increase from 1972 (Elliot *et al* 1979). These were mainly chlorotoluron, isoproturon and paraquat, although some metoxuron was also used. The predominance of autumn-sown crops is significant because these are harvested in mid August after most black-grass seed has been shed. Consequently, the seeds are not removed by harvesting procedures, and are left to germinate a few months later. Thus, black-grass is more prevalent in the South East on heavy clay soils.

Changes in farming practises over the last 20-25 years towards reduced ploughing, intensive cereal monoculture, earlier drilling and increased herbicide use have helped to exacerbate the problem of reduced weed control. The "minimum tillage" system of cultivation has replaced ploughing on many farms, especially on those with a heavy clay soil where it is hard to achieve a fine seed bed and an even establishment of plants by ploughing (Orson & Livingstone 1987). It has been clearly demonstrated, however, that ploughing is actually an effective way to control black-grass (Moss 1979, 1987). The majority of black-grass grows from seed which germinates in the top 2.5 cm of soil, and it has a small seed bank, which together means that ploughing leaves few viable seeds near enough to the surface to germinate. However, despite these many factors in favour of large black-grass infestations, the weed was successfully kept under control by the use of selective herbicides. Chlorotoluron and isoproturon were introduced in 1976 for black-grass control (Lockhart *et al* 1990), and approximately 50% of the winter cereal area in the UK was sprayed with these 2 chemicals (Moss &

Cussans 1985). In some areas applications of chlorotoluron were made continuously for over 10 years (Moss & Cussans 1991). On clay soils with minimum cultivation, ash residues from straw burning could build up, causing the soil to be highly absorptive (Flint 1985). Since chlorotoluron is mainly soil applied and taken up by the plant roots, this meant much less herbicide was available to the weeds. However, the situation remained under control until 1984 when populations of black-grass began to show herbicide resistance.

Black-grass was predisposed to develop resistance due to its high reproductive capacity and the absence of a large seed bank, especially where minimal tillage was practised. The first confirmed report of a resistant biotype was from the Lower 16 Acre field on Peldon Hall farm, Essex, UK (Moss & Cussans 1985). Seed was collected by these authors from several areas, including a field at Rothamsted which had never been sprayed with chemicals. This "Rothamsted biotype" was used as a control for comparison in all subsequent work. A biotype from Oxforshire was found to have partial resistance to chlorotoluron at the same time. The spread of resistant black-grass has been well documented and is shown in Table 1.2, however resistant populations are still concentrated in the South East of England, and none of the resistant populations tested to date have reverted back to being susceptible. Resistance has been increasing in Peldon and other populations since 1978 but this appears to be quite slow compared with some of the previously quoted examples. The differences between populations were noted to be due to an increased resistance in all plants, not more resistant plants in the population (Moss & Cussans 1985).

The resistance mechanism employed by black-grass has been studied by various groups. Resistance was not shown to be due to the herbicide failing to reach the target site in the chloroplast. There were no differences found in the amount of chlorotoluron either taken up by the roots or translocated into the leaves (Kemp *et al* 1990). Photosynthesis and chlorophyll fluorescence techniques proved that target site resistance was not involved (DePrado *et al* 1991, Van Oorschot & Van Leeuwen 1992). However, resistant black-grass plants metabolised chlorotoluron 2-3 times faster than susceptible plants, indicating that an enhanced ability to detoxify the herbicide forms the basis of resistance (Kemp *et al* 1990).

Table 1.2. The distribution and resistance of black-grass biotypes in England. (From Moss & Cussans 1985, Moss 1987, Clarke & Moss 1989, Clarke & Moss 1991 and Clarke *et al* 1994).

Year of seed collection or report	Number of counties with resistant biotypes	Number of farms with resistant biotypes	Percentage reduction in fresh weight resulting from field-rate chlorotoluron	
			Rothamsted	Peldon
1984	1	1	not available	
1986	1	5	98	30
1988	7	20	93	33
1989-90	19	46	99	4
1994	22	70	not available	

1.6 Herbicide resistance

Resistance is defined by the Herbicide Resistance Action Committee (HRAC) as the naturally-occurring inheritable ability of some weed biotypes within a given population to survive a herbicide treatment that should, under normal use conditions, effectively control that weed population. The selection pressure of the herbicide causes some weeds to evolve biotypes with resistance mechanisms so they can survive herbicide applications made at the recommended rate. This resistance is usually the result of an altered target site, or enhanced metabolism, or enhanced compartmentalisation. Altered target site means that there is a change in structure of that site at the molecular level so that the herbicide no longer binds to its normal site of action. Enhanced metabolism means the resistant plant is able to efficiently degrade the herbicide to non-toxic products much faster than the sensitive plant. In enhanced compartmentalisation the herbicide is removed from its site of action to a different part of the plant, usually the vacuole. Herbicides are selective because the crops are inherently resistant due to one of these mechanisms, and in terms of plant physiology, crops and weeds which are resistant to herbicides can be considered together. Table 1.3 gives an overview of the resistance mechanisms employed to combat the main groups of herbicides.

A good example of target site resistance is found in weeds resistant to triazine herbicides such as atrazine. They inhibit PS II by displacing plastoquinone from the Q_B site in the D1 protein, the same mechanism as chlorotoluron (Sigematsu *et al* 1989, Cobb 1992). Nearly all of the reported 57 resistant weed species have a mutation in the

Table 1.3. A summary of the mechanisms used by plants to avoid the toxic effects of herbicides. (From Holt *et al* 1993).

Herbicide class	Resistance mechanism
Phenoxyacetic acids eg 2,4 D	Crops: not known Weeds: enhanced metabolism
Dinitroanilines eg trifluralin	Crops: 1 incident of altered target site Weeds: partial enhanced metabolism
Bypyridiliuns eg paraquat	Resistance mechanism is not known
ALS inhibitors eg chlorsulfuron	Crops: enhanced metabolism Weeds compartmentalisation/other
Triazines eg atrazine	Crops: altered target site Weeds: enhanced metabolism
Substituted ureas eg chlorotoluron	Crops: enhanced metabolism Weeds: enhanced metabolism
AOPPs eg diclofop	Crops: altered target site Weeds altered target site
CHDs eg sethoxydim	Crops: altered target site and enhanced metabolism Weeds altered target site

*psb*A gene, which encodes the D1 protein, resulting in the substitution of glycine for serine at residue 264 (Trebst 1991). This point mutation greatly reduces the affinity of atrazine to the Q_B binding site, resulting in a 100 fold or more increase in whole plant resistance (Gronwald 1994). In contrast, the selectivity of atrazine in maize and sorghum is due to enhanced metabolism by the crop species (Cobb 1992), and there is one resistant weed species, *Abutilon theophrasti* Medic., which also has the mechanism of enhanced metabolism. This demonstrates that generalisations about resistance mechanisms should not be made, nor should it be assumed that weeds will evolve the same mechanisms as those present in the crop species.

The AOPP and CHD herbicides such as diclofop-methyl and sethoxydim act by inhibiting the enzyme ACCase which catalyses the first committed step in fatty acid biosynthesis (Babczinski & Fischer 1991, Cobb 1992). The reason for these being selective grass weed killers in broad-leaved crops is that the ACCase in dicot plants is insensitive to these herbicide, although not all grass plant are equally sensitive (Walker *et al* 1988, Devine *et al* 1993). In Australia, diclofop-methyl was used to control *Lolium rigidum* Gaud. (annual ryegrass) for just four years before resistance was detected (Heap & Knight 1982). In some populations resistance is due to a less sensitive target site (Tardif *et al* 1993) but in other biotypes there is no difference in target site sensitivity, herbicide uptake or metabolism. A resistance mechanism based on membrane depolarisation has also been proposed (Hausler *et al* 1991, Ditomaso 1993), but this may be a secondary effect of the herbicide.

1.6.1 Cross and multiple resistance

Black-grass and annual ryegrass (*Lolium rigidum*) are both major out-crossing weeds which have evolved resistance to more than one herbicide. Cross resistance is defined as the expression of a resistance mechanism that endows the ability to withstand herbicides from different chemical classes (Hall *et al* 1994a). Annual ryegrass provides a good example of target site cross resistance where a change at the biochemical site of action of one herbicide also confers resistance to other herbicides from different chemical classes which inhibit the same site of action (Powles & Preston 1995). For example, a biotype of annual ryegrass became resistant to sethoxydim after repeated exposure to the herbicide. This population was also resistant to eight other herbicides

including clethodim, tralkoxydim, diclofop-methyl and fluazifop-butyl which all share ACCase as the target site (Tardif *et al* 1993). Resistance levels varied from 300 fold for fluazifop-butyl to three fold for clethodim, indicating that cross resistance does not mean an equal level of resistance to each herbicide. There is also considerable variation between populations in the spectrum of herbicides they are resistant to and the degree of resistance to each one (Heap & Knight 1990). These differences may be due to mutations in at least five different alleles of the ACCase structural gene, which were identified using maize mutants with different patterns of cross resistance (Marshall *et al* 1991).

Black-grass cross resistance is due to enhanced metabolism which provides resistance to dissimilar herbicide classes, which often includes ones which they have never been exposed to. A list of 19 herbicides for which resistance has been demonstrated in Peldon black-grass was given by Moss & Cussans (1991). These include other phenylureas, chlorsulfuron, diclofop-methyl and imazamethabenz, and fenoxaprop-ethyl has been added to the list more recently (Hall *et al* 1994). These herbicides are from different chemical classes with different modes of action, but all appear to be detoxified by the cytochrome P450 monooxygenases, with the exception of fenoxaprop-ethyl for which a different resistance mechanism involving GST is implicated (Moss & Clarke 1994). The degree of resistance to each chemical varies and, although most populations show the same pattern of cross resistance as the Peldon population, there are some exceptions (Moss & Cussans 1991).

Multiple resistance is a relatively new phenomenon in plants, defined as the existence of more than one resistance mechanism in an individual plant or population (Powles & Preston 1995). This can be two resistance mechanisms to a single herbicide as in a chlorsulfuron-resistant biotype of annual ryegrass which has a resistant ALS enzyme and enhanced chlorsulfuron metabolism (Christopher *et al* 1992). A much more complicated case is seen in the annual ryegrass biotype designated VLR69, which has a resistant form of the ACCase enzyme (Powles & Preston 1995), a two fold enhanced metabolism of chlorsulfuron (Burnet *et al* 1994), a 1.5 fold enhanced metabolism of diclofop-methyl (Powles & Preston 1995), enhanced metabolism of simazine and chlorotoluron (Burnet *et al* 1993a, b) and 5% of the population have a resistant ALS enzyme (Burnet *et al* 1994). It is unlikely that all the herbicide enhanced metabolism is the result of one enzyme and several cytochrome P450s are thought to be responsible for this cross resistance (Powles & Preston 1995). In black-grass just one biotype (Lincs E1) has been reported to have multiple resistance from a combination of enhanced metabolism of a range of herbicides and resistant ACCase, although this only occurs in about 15% of the population (Powles & Preston 1995). These examples indicate that weed control may be severely hampered in the future if more species evolve cross and multiple resistance.

The selection pressure of a herbicide will select for any differences in plant morphology which confer an advantage. Although altered target site and enhanced metabolism are the main resistance mechanisms, reduced uptake and translocation, and herbicide placement can also have a role. Morphological changes such as reduced leaf area or a more waxy leaf surface may decrease the uptake of foliar applied herbicides.

The position of the roots in relation to the herbicide in the soil will also influence herbicide uptake (Devine *et al* 1993). These morphological features may be selected for alongside other mechanisms of resistance, depending on the natural variation within the population and the intensity of the selection pressure. It follows that since no two populations are the same each population has the ability to evolve different characteristics and resistance mechanisms. Each herbicide resistant population must be characterised individually, and resistance may be due to the combination of several mechanisms and morphological adaptations. The fitness of the resistant biotype is also important as this will contribute to its rate of evolution. For example, atrazine resistant biotypes are less fit and have a reduced ability to photosynthesis. This lack of fitness explains why resistant populations are relatively easy to control, and there are few problems with cross and multiple resistance (LeBaron 1991).

1.7 Herbicide metabolism

Since plants cannot avoid toxic chemicals by movement or active secretion, they rely on the metabolic detoxification of foreign compounds. The process occurs in 3 stages, oxidation, conjugation and compartmentalisation. The initial oxidation or hydroxylation of herbicides is mediated by the cytochrome P450 monooxygenase family of enzymes, and renders the lipophilic molecules more polar, thus increasing reactivity. The molecule can then be conjugated, usually either to a sugar moiety, amino acid or glutathione, producing an inert water-soluble compound. Much less is known about the subsequent sequestration, into the plant vacuole or the cellular matrix. In some cases, the herbicide is non-toxic after the initial reactions, but in others it requires conjugation before deactivation. There are also cases where conjugation is the initial reaction (Cole 1994).

1.7.1 Primary metabolism

The cytochrome P450 family of enzymes is well known in mammals to catalyse many monooxygenase reactions, but far less is known about them in green plants. They catalyse the following reaction using molecular oxygen and NADPH. One atom of oxygen is incorporated into the substrate and the other atom used to produce water, hence the term mixed function oxidase.

$$NADPH + H^+ + O_2 + RH \rightarrow ROH + H_2O + NADP^+$$

In mammals, these reactions are associated with the formation of steroids, bile acids and vitamin D in the smooth endoplasmic reticulum (Jones & Caseley 1989). They have

proved much harder to study in plants because they only exist in tiny amounts, are highly labile, have low activity and may only be expressed in certain conditions or cell types (Donaldson & Luster 1991). However, they have been reported to exist in at least 27 species, and are known to catalyse many reactions, including aromatic ring and alkyl chain hydroxylation, N-oxidation, S-oxidation, aromatic ring epoxidation, N-dealkylation and O-demethylation (Jones 1991). In plants they are involved or implicated in the hydroxylation of trans-cinnamic acid (which leads to the synthesis of coumarins, tannins, lignins and flavenoids), the hydroxylation of some fatty acids, the hydroxylation of geraniol and nerol (alkaloid formation), the hydroxylation of kaurene (involved in gibberellin synthesis) and general N-demethylations and N-hydroxylations (Hendry 1986, Jones & Caseley 1989).

Cytochrome P450s are membrane-bound proteins with a molecular weight of approximately 50, 000 daltons. They contain a haem group which binds to molecular oxygen and carbon monoxide, and the binding to CO is photoreversible. When bound to CO it has a characteristic intense absorption maximum at 450 nm and further redox activity is blocked. These enzymes are further characterised by a requirement for NADPH and molecular oxygen. NADPH donates electrons to cytochrome P450 via a membrane bound flavoprotein, NADPH:cytochrome P450 (also referred to as NADPH: cytochrome-C reductase because it also donates electrons to cytochrome C), as shown in Figure 1.1 (Donaldson & Luster 1991). Although NADPH is the preferred electron donor, NADH can also support monooxygenase activity by donating electrons via NADPH: cytochrome b_5 reductase. The NADPH:cytochrome P450 reductase has been purified and its functional role confirmed using antibodies. It has a molecular weight of

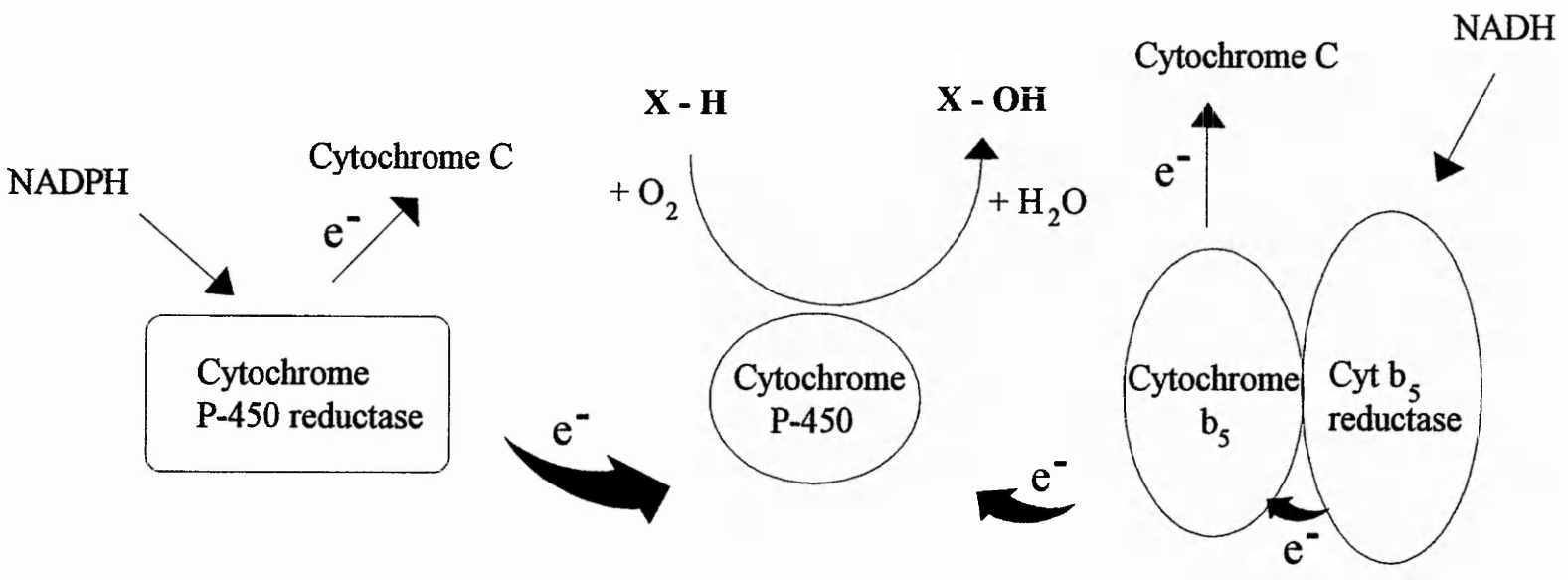


Figure 1.1 A schematic diagram to show electron flow to cytochrome P-450 and the subsequent oxidation of a substrate. (From Donaldson & Luster 1991)

82, 000 daltons and contains equimolar amounts of FAD and FMN (Benveniste *et al* 1986). It is associated with the endoplasmic reticulum, is less abundant than cytochrome P450 and appears able to donate electrons to more than one cytochrome P450 (Jones 1991). The location of these enzymes is difficult to pinpoint because they are isolated from microsomal fractions containing a mixture of membrane fragments. There have been reports locating them on the endoplasmic reticulum, the plasma membrane and in the mitochondria (Donaldson & Luster 1991).

Cytochrome P450 activity in mammalian liver is not absolute, but depends on factors such as age, sex and stress, and can be induced by a range of chemicals. Cytochrome P450s also exist in multiple forms in plants, and several chemicals and stresses are known to induce activity (Jones 1991). Two well known reactions mediated by the cytochrome P450s are cinnamic acid 4-hydroxylase (CA4H, Potts *et al* 1974) and lauric acid hydroxylase (LAH, Salaun *et al* 1978). The effect of pre-treatment with various chemicals on cytochrome P450 can be assessed by measuring the rate of these reactions. Using microsomes prepared from Jerusalem artichoke tubers (*Helianthus tuberosus* L.) and etiolated wheat (*Triticum aestivum* L.), ethanol, manganese, 2,4D, phenobarbitol, monuron, and mecoprop were shown to increase activity, as did wounding and ageing of tubers. Inhibiting chemicals included piperonyl butoxide, tetcyclasis and ABT (1-aminobenzotriazole) (Reichart *et al* 1980, Reichart *et al* 1982, Mougin *et al* 1991). However, some chemicals preferentially affected one or other reactions, and several altered the amount of cytochrome P450 detected. Paclobutrazol also decreased cytochrome P450 activity, but it acts by specifically inhibiting the oxidation of *ent*-kaurene to *ent*-kaurenoic acid in gibberellin biosynthesis (Hedden &

Graebe 1985). The requirement for NADPH and molecular oxygen, and the photoreversibility to carbon monoxide are used to characterise reactions involving cytochrome P450. Metabolism of primisulfuron in maize (*Zea mays* L.) (Fonne-Pfister *et al* 1990), chlorotoluron in wheat (Mougin *et al* 1990) and diclofop-methyl in wheat (McFadden *et al* 1989) have been shown to be dependent on cytochrome P450s in this way.

The metabolism of chlorotoluron by crop plants has been well documented. Wheat, barley and cotton metabolise chlorotoluron faster than susceptible weeds (Ryan *et al* 1981). However, the proportions of metabolites produced varied between the species. The 2 main pathways of chlorotoluron degradation are shown in Figure 1.2. In wheat, barley and maize metabolism proceeds via the ring-methyl hydroxylated metabolite (II), which is quickly conjugated, whereas metabolism in susceptible weed species such as *Avena fatua* L., *Lolium perenne* L., *Alopecurus myosuroides* Huds., *Galium aparine* L. and *Bromus sterilis* L. proceeds little beyond the mono N-demethylated derivative (I) (Ryan *et al* 1981, Gonneau *et al* 1987, Fonne-Pfister & Kreuz 1990). It is this differential metabolism which determines the susceptibility of species to chlorotoluron, although increased uptake may play a part in the susceptibility of *Avena fatua* L. (Ryan & Owen 1982). The importance of the type of metabolite is reflected in toxicity studies showing that whilst metabolites II and III do not inhibit photosynthesis, the mono N-demethylated derivative is almost as effective as unmodified chlorotoluron (Ryan & Owen 1982). However, there are some exceptions to this rule. Resistant cotton and *Veronica persica* Poir. are able to quickly metabolise chlorotoluron by step-wise N-demethylations resulting in substantial amounts of the di-

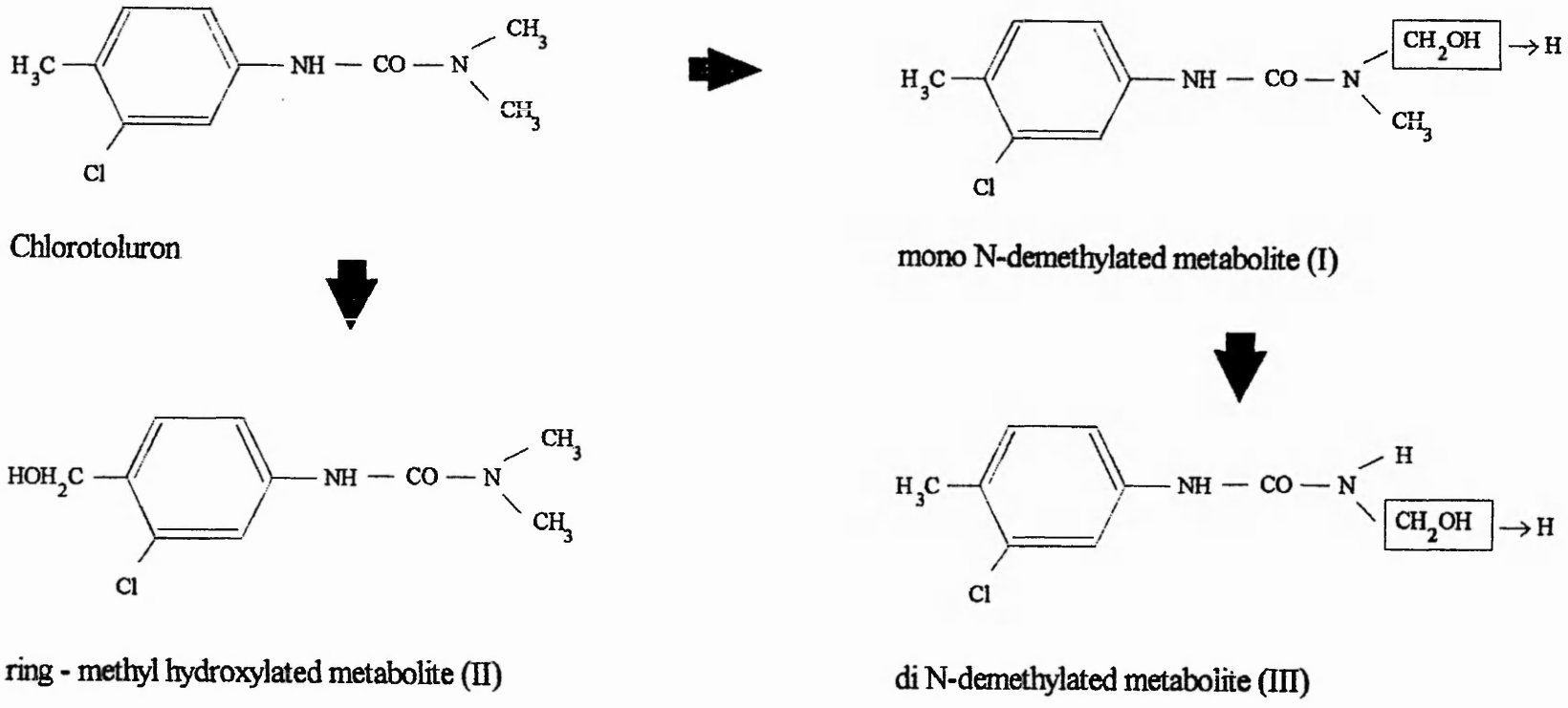


Figure 1.2 The initial reactions in chlorotoluron metabolism. The chemical groups shown in boxes inticate the intermediate stage in the reaction. (Modified from Devine et al 1993)

N-demethylated derivative(III) (Owen & Donzel 1986, Gonneau *et al* 1987). The ability to quickly conjugate metabolites is also important, and *Veronica persica* Poir. has the added advantage in conjugating both metabolites I and III. Another exception is a resistant biotype of the Australian ryegrass, *Lolium rigidum* Gaud. which predominantly metabolises chlorotoluron via the N-demethylation route (Burnet *et al* 1993). Thus, the degradation pathway, proportion of metabolites and speed of metabolism is species specific. The role of cytochrome P450 in oxidative metabolism of chlorotoluron *in vitro* was first proved in wheat microsomes (Mougin *et al* 1990) and then in maize (Fonne-Pfister & Kreuz 1990).

Evidence for the monooxygenase-dependant metabolism of chlorotoluron in black-grass first came from observations that ABT (1-aminobenzotriazole) and other known inhibitors of herbicide metabolism increased chlorotoluron toxicity, particularly in resistant plants (Kemp & Caseley 1987, Kemp *et al* 1988). A study using an *A. myosuroides* cell suspension culture showed that the resistant biotype metabolised chlorotoluron twice as fast as the susceptible and that more ring-methyl hydoxylated products were formed. The resistant plants also had elevated levels of cytochrome P450 (Caseley *et al* 1990). Thus, resistance in black-grass appears to be due to the differential metabolism of chlorotoluron.

However, there is still much discussion over the specificity of the cytochrome P450s studied. There are many examples where ABT was found not only to generally decrease chlorotoluron metabolism, but to preferentially inhibit the ring-methyl hydroxylase (Cabanne *et al* 1987, Gonneau *et al* 1987, Canivenic *et al* 1989). The two

enzymes also differ in their response to other stimulants or inhibitors, for example diclofop stimulated N-demethylase activity but not ring-methyl hydroxylase (Mougin *et al* 1991). Whilst ring-methyl hydroxylase was characteristically inhibited by carbon monoxide, N-demethylase was unaffected, leading to the speculations that the two reactions could be catalysed by different cytochrome P450 isozymes or that the N-demethylase could be either dependent on a peroxygenase enzyme, or a cytochrome P450 which used hydroperoxide as an oxygen donor (Mougin *et al* 1990).

The debate over cytochrome P450 specificity is not limited to chlorotoluron metabolism. There is evidence that the metabolism of the herbicides diclofop, chlorsulfuron, triasulfuron, linuron and chlorotoluron is catalysed by distinct cytochrome P450s in wheat which have different substrate specificities and kinetics (Frear *et al* 1991, Mougin *et al* 1991). The cytochrome P450 which mediates cinnamic acid 4-hydroxylase does not support chlorotoluron metabolism (Mougin *et al* 1992) or in-chain lauric acid hydroxylase (Zimmerlin *et al* 1992). However, data has also been presented that indicates that one isozyme can catalyse several reactions (Hallahan *et al* 1992, Kelly *et al* 1992, Zimmerlin & Durst 1992). It is also interesting to note that there are cases where one isoform requiring a specific substrate produces more than one product. For example, one cytochrome P450 mediates the aryl hydroxylation of diclofop in wheat, but three different isomeric products are possible (Zimmerlin & Durst 1992). There is strong homology between an isozyme in avocado (*Persea americana* Mill.) and catmint (*Nepeta racemosa* L.) which hydroxylates nerol, but whereas epoxides are formed in avocado, the main product in catmint is 10-hydroxylated (Hallahan *et al*

1994). This differential metabolism by one isozyme in two different species could also form the basis of herbicide selectivity.

Our understanding of these enzymes and their biosynthetic role(s) has been limited by recurrent problems in their isolation and purification, and especially in obtaining sufficient quantities of them for further study. Recent advances in molecular biology have enabled the purification of cytochrome P450 from nine species, and activity is attributed to cinnamic acid 4-hydroxylase in four of them (Hyde *et al* 1993). Another technique has been to use transgenic tobacco plants created using a fused enzyme of rat and yeast cytochrome P450 reductase. These were chlorotoluron resistant and produced a predominance of ring-methyl hydroxylated products (Shiota & Ohkawa 1995).

In an attempt to establish the natural *in vivo* role of cytochrome P450s, P450 cDNA clones from *Catheranthus roseus* Don were isolated using a PCR (polymerase chain reaction) method. Although the genes were expressed in the yeast system they failed to detect any activity (Vetter *et al* 1992, Meijer *et al* 1993,). Purified cytochrome P450 from avocado mesocarp (*Persea americana* Mill.) appeared to naturally use nerol and geraniol, however, their role in ripening avocado has not been identified, and there is no evidence of these monoterpenoids present in the fruit (Hallahan *et al* 1992). Enzyme binding data from tulip bulbs (*Tulipa gesneriana* L.) also indicated geraniol to be the natural substrate and not cinnamic acid. However, these isozymes also bound to 2,4D and benzo[*a*]pyrene, and so may be part of the plant defence mechanism (Kelly *et al* 1992).

The ability to detoxify xenobiotics may well be a minor role, as metabolism of herbicides has been found to be carried out by only a fraction of the total cytochrome P450 content (Zimmerlin *et al* 1992). This may also explain why increases in activity are not always matched with the same increase in cytochrome P450 content. For example, the safener benoxacor stimulated a 15 fold increase in chlorotoluron metabolism but only doubled the P450 content (Fonne-Pfister & Kreuz 1990). Either only a small amount of constitutive enzyme was involved or a small amount of specific enzyme was induced. Hydroxylation of triasulfuron activity was estimated to be 6% of total induced activity (Thalacker *et al* 1994). Thus, xenobiotic detoxification may not be the main function of these enzymes. It has been proposed that the detoxification role may be important in emerging plants and young seedlings, but that once the vacuole is fully formed secondary metabolites and xenobiotics are deposited in it (where they play a role in plant defence). This would explain why activity is not induced in mature tissue (Hendry 1986) and the observation that cytochrome P450 levels decrease with age (Thalacker *et al* 1994). It appears that the main function of P450s in mature tissue is biosynthetic (Hendry 1986). The current situation is that no P450s responsible for herbicide detoxification have been isolated, although about 50 physiological substrates have been characterised which are all plant-specific secondary metabolites (Werck-Reichhart 1995). Since all the cytochrome P450s isolated so far have been from achlorophyllous material and storage organs, then our understanding of these enzymes is based on the biosynthetic P450s and not the detoxification P450s.

1.7.2 Secondary metabolism

The second phase of metabolism usually results in the formation of herbicide conjugates. Various conjugates can be formed from an initial combination with sugars, amino acids or peptides. However, it is conjugation with glutathione, catalysed by the glutathione S-transferases (GST) which is the most common in herbicide detoxification (Devine *et al* 1993). The tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine) is the major form of free thiol in most higher plants, although some legumes for example soybean (*Glycine max* Merr.) and white clover (*Trifolium repens* L.) contain the alternative peptide homoglutathione. Concentrations of between 0.18 and 0.59 μmol glutathione g^{-1} fresh weight in plants have been reported, but there are distinct intracellular variations (Lamoureux & Rusness 1989). In tobacco cells, a concentration gradient was found from 20 μM in the vacuole, to 60 μM in the cytoplasm, through to 2000 μM in the chloroplast (Rennenberg 1982). Glutathione is at least partially synthesised in the chloroplast, and the biosynthetic pathway resembles that found in animals. The ATP-dependent, 2-step process is shown in Figure 1.3. Regulation of the enzyme γ-glutamylcysteine synthetase is by feedback inhibition of glutathione, and synthesis may also be influenced by the concentration of L-cysteine in the cells (Rennenberg 1982).

Glutathione S-transferases have been extensively studied in animals and are known to exist in all vertebrates. Several soluble isozymes have been isolated from the cytosol and classified into groups which correspond to multigene families (Armstrong 1993). In comparison, much less is known about these enzymes in higher plants,

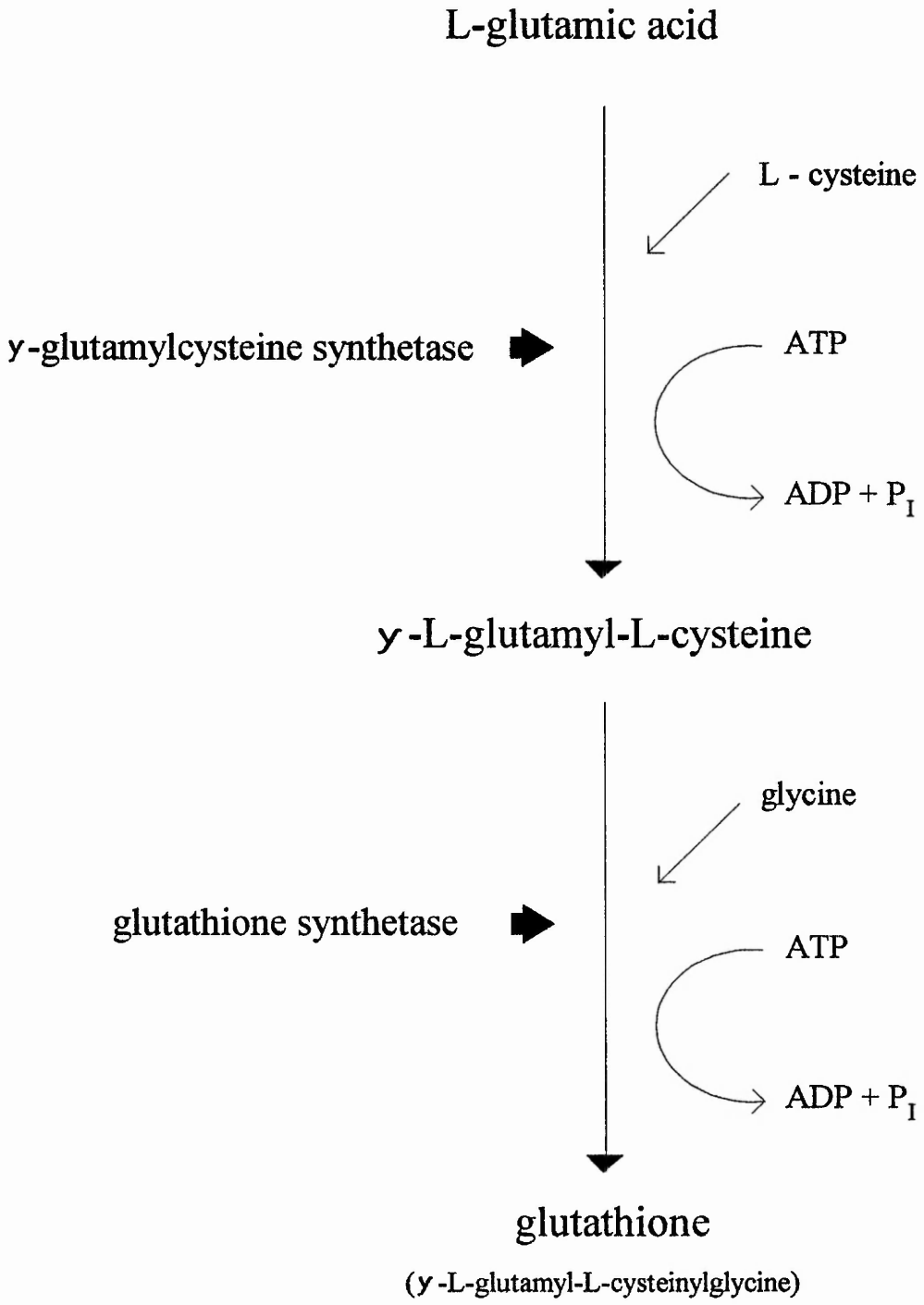
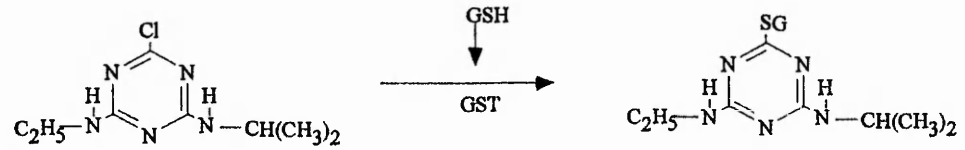
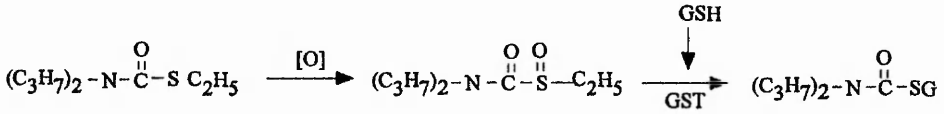


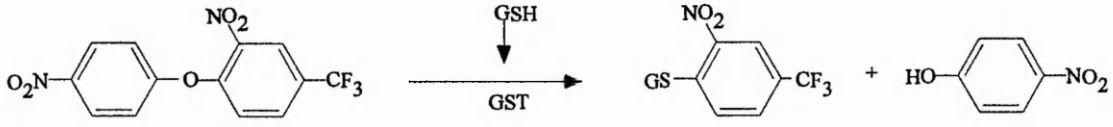
Figure 1.3 Biosynthesis pathway of glutathione, modified from Rennenberg 1982.



Atrazine



EPTC



Flurodifen

Figure 1.4 The conjugation reactions of atrazine, EPTC and fluorodifen with glutathione.

although their role in herbicide metabolism is well established and has been recently reviewed (Lamoureux & Rusness 1989, Cole 1994). Glutathione (GSH) conjugation occurs with herbicides that contain an electrophilic functional group such as chloroacetanilides, triazines, thiocarbamates, diphenylethers and sulfonylureas (Cole 1994), according to the generic equation:

$$GSH + RX \rightarrow GSR + XH \qquad \text{(Armstrong 1993)}$$

This reaction is catalysed by the glutathione S-transferases, although it can occur spontaneously, depending on the degree of electrophilicity of the herbicide (Han & Hatzios 1991a). It is an irreversible reaction resulting in a covalent linkage of GSH to another substrate, and the resulting conjugate is usually less toxic than the parent herbicide and less reactive (Lamoureux & Rusness 1989). The involvement of GST in herbicide detoxification was first identified for atrazine metabolism in maize, in which the chlorine anion is substituted with a thiol group, as shown in Figure 1.4 (Lamoureux *et al* 1970). This is an example where glutathione conjugation is the initial detoxification reaction, whereas other herbicides such as EPTC are activated by primary phase reactions, such as oxidations first (Figure 1.3). Another variation was discovered in the conjugation diphenylether herbicides which includes cleavage of the ether bond (shown in Figure 1.4).

Unlike the cytochrome P450s, these enzymes are relatively easy to isolate and purify, and the complete amino acid sequence of at least one isozyme is known (Wiegland *et al* 1986). Most of the isozyme characterisation has been carried out in maize which detoxifies the herbicides metolachlor, alachlor (both chloroacetanilide herbicides) and atrazine by glutathione conjugation (Cole 1994). Two GST isozymes

were identified in 1983 (Mozer *et al* 1983), a constitutively present isozyme (GST I) and one which was only present when maize was treated with a chloroacetanilide safener (GST II). A third isozyme was found to account for most of the alachlor conjugation activity (O'Connell *et al* 1988), and a more recently discovered GST IV was characterised by a different substrate specificity to the other three (Fuerst & Irzyk 1992). This isozyme was not discovered earlier because GST activity was regularly assayed using the model substrate CDNB (1,chloro 2,4 dinitrobenzene). However, this is often misleading, as a wide range of activities can be measured for one isozyme using different substrates (Irzyk & Fuerst 1993). GST activity is often much higher when herbicides are used as substrates compared with CDNB. GST IV shows no activity with CDNB but readily conjugates GSH with metolachlor. Thus, GST I and GST III are both constitutive, with GST III having a high affinity for chloroacetanilides. GST II is only present when induced by safeners and GST IV is constitutive and induced. These enzymes have been characterised as dimers with subunits of 25-29 kDa. Antiserum for GST I is cross reactive with many GST peptides, thus these subunits may combine in different ways to give isozymes with different and overlapping substrate affinities (Fuerst *et al* 1993). None of these four isozymes have been attributed with atrazine-glutathione conjugation, and recent research suggests that the multiple GSTs are controlled by at least five genes (Edwards 1995).

Investigations using other crop plants also indicate the presence of several isozymes. In sorghum, two constitutive isozymes with different substrate specificities were identified with an additional four or five inducible GSTs (Dean *et al* 1990). The characterisation of GSTs in mesocarp tissue and callus from pumpkin (*Cucurbita*

maxima Duch) identified two isozymes in the tissue and seven in the callus (Fujita *et al* 1994). The substrate specificities, induction mechanism and role in herbicide metabolism of these isozymes has yet to be established. Resistance to alachlor in wheat is attributed to endogenous GSTs, although pre-treatment with the herbicide increased activity (Jablonkai & Hatzios 1991).

However, comparatively little is known about GST activity in weeds species. A comparison was made between maize and giant foxtail (*Setaria faberi* Herrm.) in which GST activity was found to be 14 fold higher in maize (Boydston and Slife 1986). A recent study comparing GST activity in six weed species and maize, revealed a wide range of activities (Hatton *et al* 1995). Although GST activity in maize was generally higher, there were exceptions when certain substrates were used.

The natural role of these enzymes is poorly understood. It is generally accepted that glutathione maintains proteins in their metabolically active (reduced) form (Rennenberg 1982). High levels of reduced glutathione may also be required for optimal protein synthesis (Lamoureux & Rusness 1989). Another role for glutathione is thought to be in providing a means of long distance transport for sulphur in its reduced from. Glutathione is translocated in the phloem from mature leaves to roots and other sinks where it is catabolised (Rennenberg 1982). The increase in GST activity noted when plants are under stress has implicated these enzymes in responses to pathogens, air pollution such as ozone, temperature extremes and drought (Price *et al* 1990, Farago & Brunold 1994). These theories are consistent with findings that measurable GST activity can be altered by the environmental conditions plants are grown in, particularly

temperature (Boydston & Slife 1986, Kunkel *et al* 1991). Another role for GST may be detoxification of hydrogen peroxide in chloroplasts, acting as a glutathione peroxidase (Rennenberg 1982, Price *et al* 1990), or limiting limit lipid peroxidation (Price *et al* 1990). Although not proved in plants, a protein isolated from rat liver microsomes exhibited both glutathione peroxidase and GST activity, could protect cellular membranes from lipid peroxidation (Reddy *et al* 1981).

1.8 Safeners and synergists

With any herbicide, the degree of selectivity between the crop and the weeds is not absolute, but depends on various interactions between the chemical, the plants and the environment. Problems are frequently encountered when the crop species and the weed are closely related, and some herbicides like glyphosate have no selectivity. Thus, there is a continual need to develop herbicides with improved selectivity, despite the increasing costs of developing new herbicides and the need to comply with stringent rules for registration. One solution to this problem is to add a safener, a chemical which can be used in small amounts to increase the effectiveness of an existing herbicide, and can be developed at a much reduced cost. A good safener has no phytotoxicity, consistently protects crops without affecting the weeds and is effective at low doses (Hatzios 1989).

1.8.1 History and commercial use of safeners

The potential of safeners was first discovered in the late 1940s by Otto Hoffman who noticed that the tomato plants treated with 2,4,6-T were not injured when accidentally exposed to vapours of the herbicide 2,4D. Numerous greenhouse tests were conducted and eventually the first commercial safener, naphthalic anhydride, was patented in 1971 for herbicides used in maize. It was used as a seed dressing to protect monocot crops such as maize, sorghum, wheat and rice from several herbicides, mainly thiocarbamates, chloroacetamides, imidazolinones and sulfonylureas. Although naphthalic anhydride could provide moderate protection for several crop-herbicide combinations, it had little selectivity and was never a commercial success (Hatzios 1989, Stephenson & Yaacoby 1991). The first selective safener, dichlormid (R25788) was developed a few years later, with the added advantage that it could be tank mixed with the herbicide. However, its use was restricted to protecting maize from thiocarbamate injury because it had little safening ability for other monocot crops. The most successful safeners since then have been developed for chloroacetamide herbicides, and are applied as a seed dressing or soil treatment (Stephenson & Yaacoby 1991).

It is difficult to make many generalisations about safeners because they belong to chemically diverse groups which can be seen by comparing their structures (Figure 1.5), and only specific combinations of safener, herbicide and crop are effective. Flurazole (a substituted thiazole) and oxabetrinil (an oxime ether) are used to protect sorghum from alachlor and metolachlor injury (Brooks *et al* 1987, Yenne *et al* 1990). Dichlormid and benoxacor are dichloroacetamides which protect maize from

thiocarbamates and chloroacetamides (Hatzios 1991). The most frequently used safener for rice is fenclorim (a phenylpyrimidine) which protects against pretilachlor (Han & Hatzios 1991b). Most of the commercially available safeners are used as seed dressings to protect monocot crops against chloroacetamide or thiocarbamate herbicides. Another development has been the safener fenchlorazole-ethyl which is applied as a mixture with the herbicide fenoxaprop-ethyl (Bieringer *et al* 1989). This mixture is more active on weeds and causes less crop injury than fenoxaprop-ethyl alone (Stephenson *et al* 1993).

The earliest safeners, naphthalic anhydride and dichlormid were shown to enhance chloroacetanilide metabolism, which was known to proceed via glutathione conjugation. However, the mode of action of safeners is not exclusive to the elevation of GST activity and GSH content. Increases in the formation of herbicide-glutathione conjugates, glutathione reductase, herbicide uptake, lipid synthesis from acetate, herbicide monooxygenation and glucosylation have all been observed (Devine *et al* 1993).

1.8.2 The effect of safeners on herbicide metabolism

Many safeners are known to increase herbicide-glutathione conjugation in maize, sorghum and rice. Pre-treatment of maize with dichlormid elevated GST activity and increased the conjugation of EPTC sulfoxide with glutathione (Lay & Casida 1976). Also in maize, benoxacor increased metolachlor conjugation (Kreuz *et al* 1989, Cottingham & Hatzios 1991, Fuerst *et al* 1993), dichlormid and MG-191 elevated acetachlor conjugation (Jablonkai 1991, Ekler *et al* 1993), and MG-191 promoted EPTC

sulfoxide conjugation (Jablonkai 1991). In sorghum, oxabetrinil increased the amount of glutathione-metolachlor conjugates (Yenne *et al* 1990) and the rice safener fenclorim increased pretilachlor conjugation with glutathione (Han & Hatzios 1991c).

In several cases where elevated levels of glutathione-herbicide conjugates are measured, they can be attributed to an elevated GST activity and correlated to the induction of specific GST isozymes. This may be a specificity towards a particular herbicide, for instance, dichlormid increased GST activity towards metolachlor in maize whilst having no effect on atrazine conjugation (Edwards and Owen 1988). Benoxacor induced a new isozyme in maize with activity towards metolachlor and enhanced the activity of 4 constitutive isozymes, 2 with activity towards metolachlor and 2 which conjugated EPTC-sulfoxide (Dean *et al* 1991, Fuerst *et al* 1993). Flurazole can increase the activity of constitutive isozymes in maize and sorghum. In addition, an isoenzyme with specific activity towards chloroacetamide herbicides was induced in maize, and 5 such isozymes in sorghum (Mozer *et al* 1983, Dean *et al* 1990). Oxabetrinil, NA and dichlormid could induce 4 or 5 different isozymes with activity towards metolachlor (Dean *et al* 1990). The safeners do not act directly on the GST enzymes (Gronwald *et al* 1987), and recent evidence suggest that benoxacor acts by inducing the *de novo* synthesis of mRNA transcripts required for GST synthesis (Miller *et al* 1994).

There are several examples where elevated GST activity has been associated with an increase in glutathione content, although this does not always happen (Cottingham & Hatzios 1991). Dichlormid and benoxacor doubled the glutathione content in maize (Farago & Brunold 1990) and AD-67, MG-191 and fenclorim also

increased GSH concentrations (Han & Hatzios 1991b, Ekler *et al* 1993,). In certain cases the elevated glutathione content is due to the stimulation of enzymes in the glutathione biosynthesis pathway. For example, dichlormid increased adenosine 5'-phosphosulfate sulfotransferase (APSSTase) activity and benoxacor elevated APSSTase and ATP-sulfurylase activity, with a significant increase in sulfate uptake (Farago & Brunold 1990). Dichlormid has also been shown to increase GSH synthetase (Rennenberg *et al* 1982) and glutathione reductase (Komives *et al* 1985). The main mechanism of fenclorim safening is by direct or indirect stimulation of glutathione synthesis, elevated GST activity being a secondary consequence of this action (Han & Hatzios 1991b).

Most safeners can elevate GST activity, but their ability to protect crops from injury can vary enormously, even when considering the protection of one species against one herbicide. For example, in sorghum, the safeners inducing GST activity towards metolachlor could be ranked, according to the measured GST activity as, flurazole > oxabetrinil > naphthalic anydride > dichlormid (Gronwald *et al* 1987). Although there is generally a good correlation between the ability of safeners to reduce injury and GST activity, there are some exceptions. The safeners BA 145138 and AD 67 increased GST activity to a similar level, but only BA145138 was effective at protecting maize from alachlor injury (Ekler & Stephenson 1991). It may be that only certain safeners induce the activity of the specific GST isozyme required to detoxify a given herbicide.

A good example of a species-specific response is found in the interaction of fenchlorazole-ethyl with the graminicide fenoxaprop-ethyl. The interaction is synergistic on some weeds species, for example, increasing herbicide toxicity to crab-grass, but protects wheat and barley from injury. Metabolism is increased in all species, resulting in rapid de-esterification of fenoxaprop to fenoxaprop acid, which is the active herbicide. Further metabolism to non-toxic, water soluble metabolites, however, only occurs in wheat and barley. When fenoxaprop is hydrolysed the products may be conjugated with glucose or glutathione. The safener may increase hydrolysis, or elevate either of the conjugation enzymes or act on any given combination of these enzymes (Yaacoby *et al* 1991).

It is interesting to note that herbicide metabolism and safener efficacy can be localised in certain plant parts. In maize, metolachlor is conjugated 4-5 times faster in the roots than the shoots. This is reflected by observations that metolachlor is much less phytotoxic when taken up by the roots than when uptake is via the coleoptile. Benoxacor increases metolachlor conjugation in both the roots and shoots, but it is twice as active in the shoots (Kreuz *et al* 1989). The safeners themselves may be conjugated by glutathione (Yenne *et al* 1990) and some herbicides can induce their own metabolism (Jablonkai & Hatzios 1994).

There are several incidences recorded of safeners providing protection from herbicides which are metabolised by the cytochrome P450 dependent monooxygenases. For example, the tolerance of maize to imazethapyr is increased by naphthalic anhydride and this is associated with an elevated cytochrome P450 content (Barrett & Maxon

1991). Naphthalic anhydride also increased the P450 content of wheat microsomes, further stimulating the aryl hydroxylation of diclofop and triasulfuron hydroxylation (Frear *et al* 1991, Zimmerlin & Durst 1992). It is difficult to fully understand herbicide-safener interactions when the mode of action of thiocarbamates and chloroacetamides is not understood. Although their metabolism proceeds via glutathione conjugation there is indirect evidence that cytochrome P450s may also be involved in acetochlor metabolism (Jablonkai and Hatzios 1994). Flurazole increased alachlor metabolism and induced mixed function oxidase activity in sorghum, but alachlor failed to be metabolised by isolated P450s *in vitro*, with or without flurazole, although flurazole itself was rapidly degraded (Brooks *et al* 1987).

1.8.3 Effects of safeners on other metabolic processes

Safeners are reported to affect other plant processes, particularly biosynthetic pathways, but these are usually regarded as secondary effects. Flurazole and oxabetrinil at 100 μM inhibited carbon dioxide fixation and lipid, protein and DNA synthesis (Zama & Hatzios 1986). The interaction of fenclorim with pretilachlor may be antagonistic at the site of protein synthesis, and may cause the redistribution of carbon in the lipid fraction (Han & Hatzios 1991b). The herbicide norflurazon prevents carotenogenesis in plants by inhibiting phytoene desaturase. The safening of norflurazon by NA is not fully understood, but does not appear to involve altered metabolism of the herbicide (Wilkinson 1993). There are also indications that pre-treatment with NA and dichlormid can increase the extractable ALS activity from maize (Rubin & Casida 1985, Milhomme *et al* 1991).

1.8.4 Herbicide synergists

Synergists are compounds which differentially increase herbicide toxicity in weeds, usually by inhibiting herbicide metabolism. Cytochrome P450 inhibitors such as 1-aminobenzotriazole (ABT, Table 1.4), piperonyl butoxide and various triazoles increase the toxicity of any herbicides metabolised by these enzymes. For example, ABT increases the toxicity of chlorotoluron to herbicide susceptible and resistant black-grass to the extent that it overcomes resistance (Kemp & Caseley 1987).

Tridiphane is a herbicide which can act synergistically with atrazine. The herbicidal action of atrazine on the panicoid grasses such as *Setaria faberi* Herrm. is very poor, but when used in conjunction with tridiphane, which has only a limited effect itself, a synergistic interaction gives selective control of such weeds in maize (Devine *et al* 1993). GST enzymes from maize, giant foxtail (*S. faberi*), onion and pea were all shown to catalyse the conjugation of tridiphane with glutathione in *in vitro* studies (Lamoureux & Rusness 1986). Thus, the synergism of tridiphane and atrazine is based on tridiphane acting as a competitive inhibitor for atrazine-glutathione conjugation, the initial step in atrazine metabolism. The GS-tridiphane conjugate also acts as an inhibitor, and was 4 times more effective in giant foxtail than maize, thus providing a method of selectivity. Tridiphane has been shown to inhibit mouse hepatic cytochrome P450s (Moreland *et al* 1989), and there is indirect evidence that it can inhibit cytochrome P450s in plants (Caseley *et al* 1991b).

1.9 The aims of the investigation

Black-grass is a major weed problem in Britain and chlorotoluron-resistant populations are very difficult to control, especially as most are cross resistant to several herbicides. Chlorotoluron metabolism has been shown to involve the action of cytochrome P450s, but the possible role of other detoxification enzymes has yet to be established. Thus, there is a need to further investigate the resistance mechanism(s) in black-grass.

1. The first objective was to characterise a chlorotoluron-resistant black-grass population in terms of whole plant growth and photosynthesis, and to compare this with a susceptible population. This was necessary because herbicide selectivity is not an absolute characteristic but a dynamic condition, dependant on factors such as herbicide interception, uptake and translocation; the metabolic pathway and speed of detoxification; the resistance mechanism, and any differential ability to tolerate toxic affects. Since the selection pressure of a herbicide will favour any plants with a characteristic which confers a margin of increased survival, each herbicide-resistant population may have different mechanisms and has to be considered as an individual case. The aim of this characterisation was to establish the level of resistance using ED_{50} values based on the reduction in fresh weight under defined conditions. A quantitative assessment of plant growth, development, and photosynthesis was made to assess variations within and between populations, particularly to investigate a possible link between resistance and changes in growth or photosynthesis.

2. The second objective was to investigate glutathione conjugation in black-grass, and consider a possible role for glutathione S-transferases in chlorotoluron resistance. This is an important area of herbicide chemistry which so far has received little attention in weeds. The aim was to optimise existing *in vitro* assays using CDNB (1-chloro-2,4 dinitrobenzene) as the model substrate, and to investigate the effect of several herbicide safeners and synergists on GST activity in black-grass. The interaction of these safeners and synergists with chlorotoluron and the possible role of GST in herbicide resistance was also considered. The synergists used were ABT and tridiphane (Table 1.4), and the 7 safeners are given with their structures in Table 1.5.

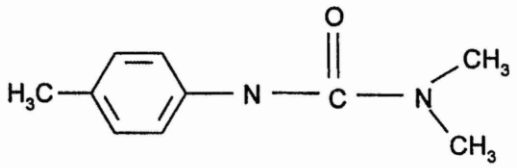


Figure 1.5 Schematic drawing of the chemical structure of chlorotoluron.

Table 1.4 The chemical structures of ABT and tridiphane

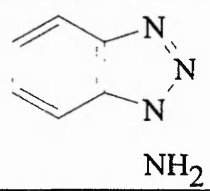
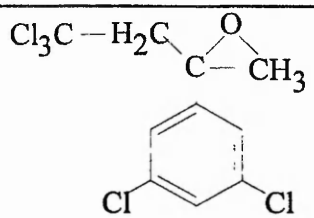
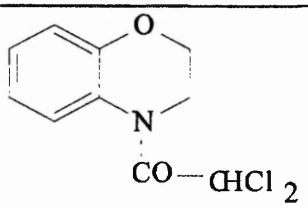
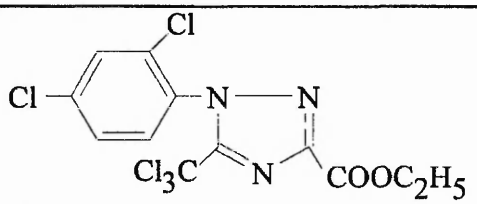
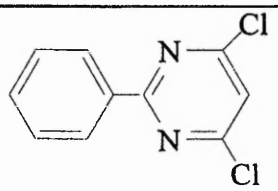
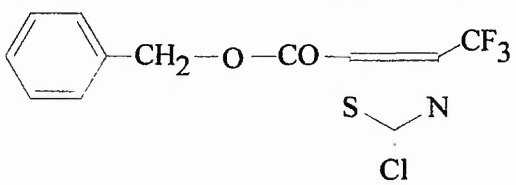
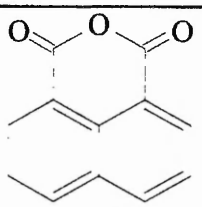
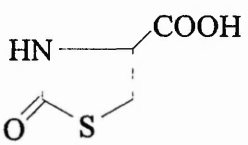
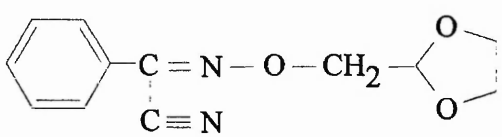
1-Aminobenzotriazole (ABT)	
Tridiphane	

Table 1.5 The chemical structures of the herbicide safeners used in this study.

Benoxacor	
Fenchlorazole-ethyl	
Fenclorim	
Flurazole	
Naphthalic anydride	
OTC	
Oxabetrinil	

Chapter Two

The characterisation of two black-grass populations susceptible and resistant to chlorotoluron

2.1 Introduction

Before conducting a biochemical study of a herbicide it is appropriate to first characterise the growth of the experimental species under defined conditions to ascertain the nature of the interaction between the weed and the herbicide at the whole plant level. This is of particular importance when comparing susceptible and resistant biotypes because resistance may be associated with certain morphological features or a particular growth stage. The aim of this study was to establish the ED_{50} value for the Peldon population of resistant black-grass, under our glasshouse conditions, and to make a quantitative assessment of susceptible and resistant black-grass using the parameters of growth and photosynthesis.

2.2 Materials and methods

2.2.1 Plant material

Two seed stocks of *Alopecurus myosuroides* Huds. were used in this study. The susceptible seed was bought from Herbiseed Ltd, Berkshire, UK, in large quantities. The company obtained the original seed stock from an area which had not been previously treated with substituted phenyl ureas, and had been cultivated for the last 12 years with exposure only to 2,4 D and ioxynil to select out dicotyledonous weeds (personal communication). The resistant seed was collected from Peldon, Essex, UK,

with the help of Dr SR Moss in 1993, which is the farm where resistance was first detected. This is the most chlorotoluron-resistant population currently known.

The seeds were sown in J Arthur Bowers multipurpose compost (in pots or trays as described below) and initially watered from above. The germination rate was 50-60 % for both biotypes. All plants were raised under glasshouse conditions, which allowed moderate control of environmental conditions. The temperature was nominally 20 ^{0}C during the day and 15 ^{0}C at night (± 5 ^{0}C), although it regularly reached 30-35 ^{0}C during the summer months and dipped as low as 5 ^{0}C on some winter nights. Supplementary lighting from high pressure sodium lamps in the winter ensured that photosynthetic photon flux density did not drop below 120 μmol m^{-2} s^{-1}; the maximum in summer being approximately 1100 μmol m^{-2} s^{-1}. Plants received a minimum of 14 hours of light per day and watering was on a daily basis (twice daily in summer) via automatic sub-irrigation. Although this gave some environmental variations, resistant and susceptible plants were always grown at the same time, so that they were subjected to exactly the same growth conditions.

2.2.2 Chlorotoluron application to whole plants grown in compost

Chlorotoluron, as the formulation Dicurane 500SC, was sprayed using a Mardrive Evaluation Unit set to deliver 200 l ha^{-1} (fixed nozzle, 80^0 flat fan tip, 3 bar pressure, spray height 450 mm) in accordance with the manufacturers instructions. Chlorotoluron was applied at a range of concentrations (0, 0.44, 0.88, 1.75, 3.5, 7, 14, 28, 35, 42 and 49 kg ai ha^{-1}) for the dose-response experiments. For all other

experiments plants were sprayed with either field-rate chlorotoluron (3.5 kg ai ha^{-1}) or tap water.

2.2.3 Characterisation of herbicide-resistant black-grass

To establish the dose-response of black-grass to chlorotoluron, seed was sown at a rate of 12 seeds per 9 cm pot and the plants thinned to six per pot where necessary. The dose-response was determined using five pots of each biotype for each treatment, including pots which were sprayed with tap water only (ie control). Plants were sprayed at the 2 leaf stage, approximately 3 weeks after sowing, and placed in a randomised block in the glasshouse. Fresh weight of the aerial portions of the plants was assessed two weeks later and the experiment was carried out on three separate occasions

For the sequential growth analysis, seed was sown in seed trays and the plants transplanted at the coleoptile stage, about 10 days after sowing, into individual cells (36.5 x 36.5 x 56 mm) in plastic multicell trays (Fordingbridge Ltd, West Sussex, UK). This allowed each plant an equal amount of space and light, and avoided root-root competition. At the 2 leaf stage, half the plants of each biotype were sprayed with field rate chlorotoluron and half with tap water as above. On the day of spraying, six susceptible and six resistant plants were harvested, and harvests of six control and six treated plants from both biotypes were made five times in the following 14 days after treatment. This experiment was also repeated on three separate occasions. The plants (minus the roots) were assessed for fresh and dry weight (and total leaf weight for each plant), and the number of leaves and tillers. Total leaf area for each plant was measured

using a camera mounted leaf area meter (Delta T Devices Ltd, Cambridge, UK). Total leaf water content was calculated by drying the leaves in an oven at 80 ^{0}C for 2 days and expressing the difference in fresh and dry weight as a percentage of fresh weight. Specific leaf area (leaf area/leaf dry weight), leaf-weight ratio (leaf dry weight/ whole plant dry weight) and relative growth rates were determined as described in Hunt (1990).

2.2.4 Photosynthesis by susceptible and resistant black-grass

Whole plant photosynthesis and transpiration were determined using an infra-red gas analyser (IRGA, model LCA4, ADC Ltd, Hoddesdon, UK).

2.2.4.1 The plant chambers: The chambers were designed and purpose-built to enclose the whole plant, as shown in Figure 2.1. A tall, narrow design was chosen (160 x 40 x 40 mm) so that the plant could be accommodated without undue restriction and with a maximum tissue to volume ratio. This was important since the larger the volume of air, the longer it takes for the system to equilibrate or to register small changes in CO_2 concentration. Chambers were constructed in two halves out of 6 mm acrylic (Perspex) held together by four brass screw threads and wing nuts. The base of the chamber was enlarged so that it could rest firmly on a section of multicell plastic tray, and a 2 mm groove in one side accommodated the plant stem. It was imperative that the whole system remained airtight, as even tiny leaks could result in big errors and so a

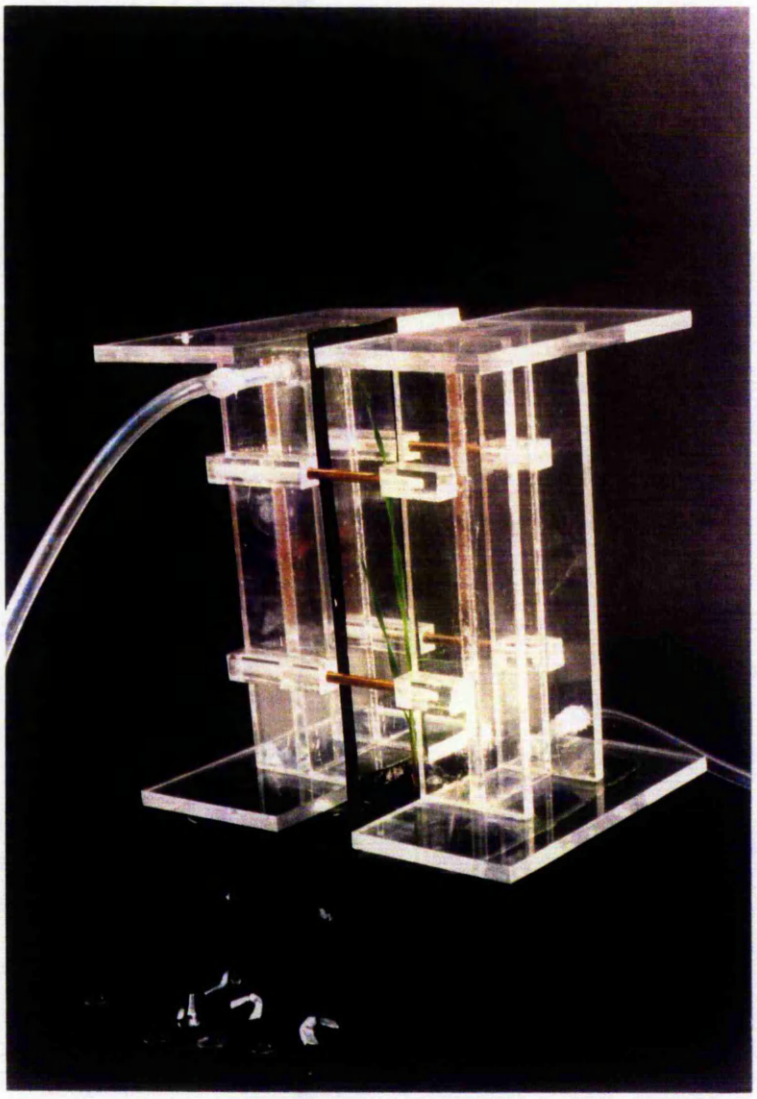


Figure 2.1 Experimental chamber for the measurement of whole plant gaseous exchange. The plant enclosed is at the 2-3 leaf stage. The chamber is shown at approximately half its actual height.

gasket of plastic sealant was placed between the two chamber halves, and a ring of plasticine was placed around the stem base of each plant. The air inlet was positioned at the bottom of the chamber and the outlet at the top on the opposite side, to ensure an air flow across the plant. All joints in the pipework were sealed using household silicone sealant or araldite. The consistency of air flow through the chambers was monitored using CO_2 vapour because it was visible as a white gas. The CO_2 released by a brick of dry ice was pumped through a chamber and was seen to be evenly distributed. One chamber was modified using an ADC transducer kit to contain a thermistor and a small fan. Measuring the temperature inside the chamber enabled the measurement of transpiration rates.

2.2.4.2 The experimental design: The apparatus was set up as shown in Figure 2.2 to give an open system for gas analysis. PVC tubing (7 cm external, 4 cm internal diameter) was used throughout and a flow rate of 0.3 l min^{-1} was maintained. Early attempts to heat the air to 20 ^{0}C before it entered the chambers proved unnecessary because it always heated up to room temperature (20-24 ^{0}C) beforehand. Ambient air was drawn from outside the building via an inlet 1.5 m above ground and then split 7 ways. Six tubes went to the inlet ports on the 6 plant chambers and one went into the reference port on the gas handling unit (ADC WA161). The six outlet pipes from the chambers went into the sample ports on the WA161. This gas handling device controlled which sample went through to the IRGA for analysis. It was set to switch between chambers every 5 min and so the IRGA received sample gas from a different chamber every 5 min. The readings from each chamber were automatically logged, thus providing a record of photosynthesis and transpiration for each plant at 30 min intervals. A photosynthetic flux density of 350 μmol m^{-2} s^{-1} was chosen after preliminary

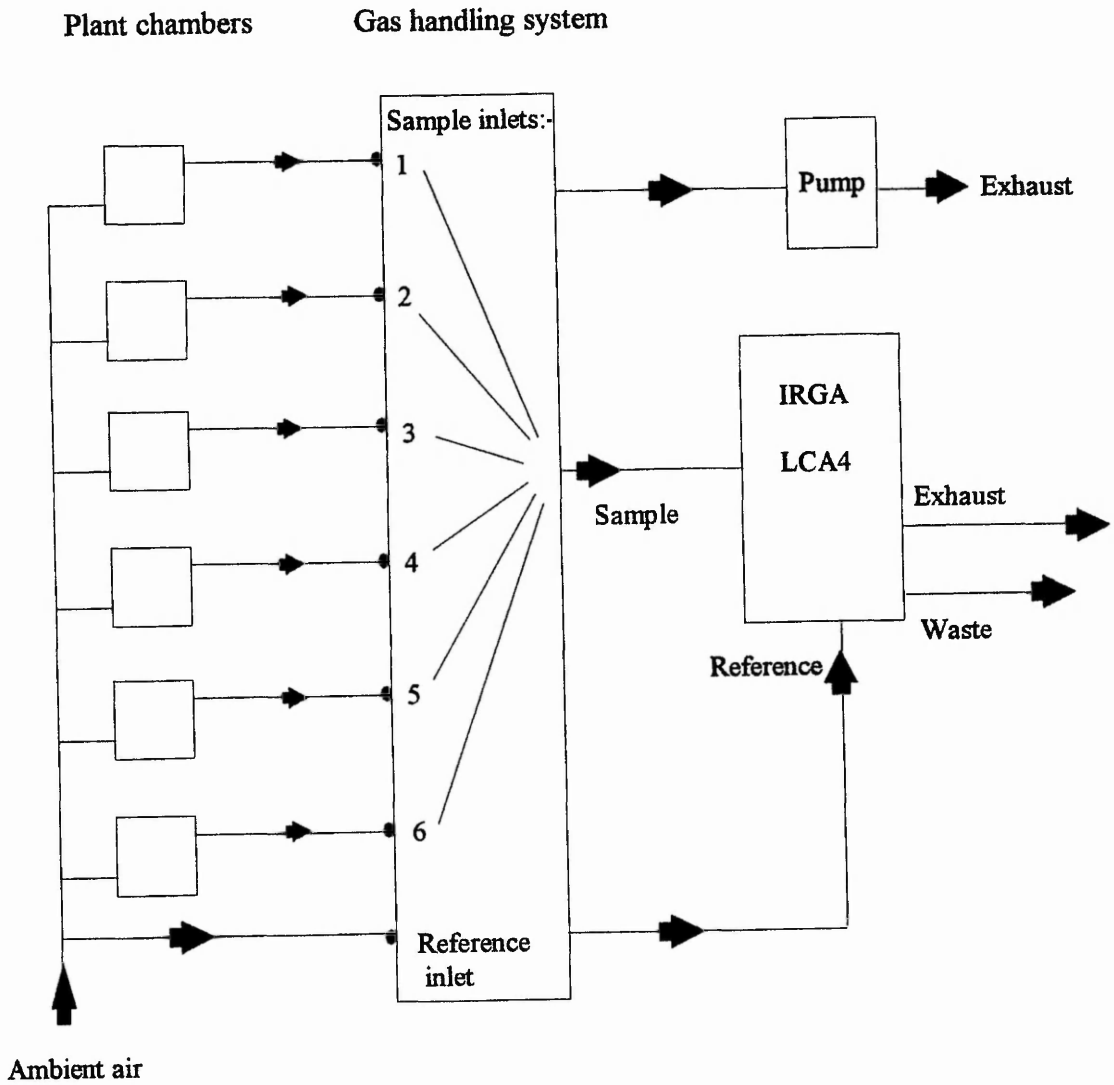
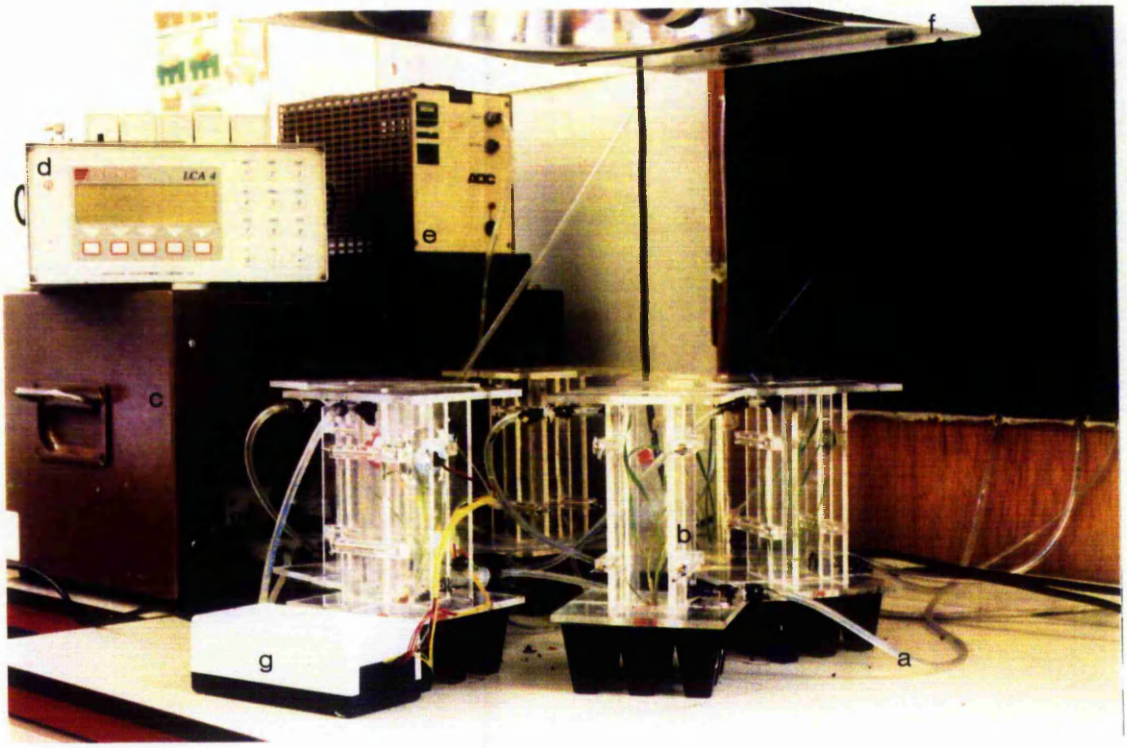


Figure 2.2 Experimental design for the measurement of gaseous exchange by infra-red gas analysis



Key

a. Ambient air is drawn from outside
b. Experimental plant chamber
c. Gas handling device
d. Infra-red gas analyser
e. External pump
f. Overhead sodium lamp
g Transducer box to control fan and thermistor in one chamber

Figure 2.3. The equipment in use for the measurement of gaseous exchange.

experiments demonstrated this to be optimum for these experiments. Lighting was supplied by an overhead high pressure sodium lamp, which caused the temperature inside the chambers to reach between 24 and 28 ^{0} C. It was not possible to provide cooling with the resources available, but an external fan was used to prevent overheating. The chambers were arranged under the lamp such that they all received the same amount of light and heat. As only one chamber had a thermistor fitted, transpiration measurements rely on the assumption that the temperature in all chambers was equal. The photograph in Figure 2.3 shows the equipment in use.

2.2.4.3 The effect of flux density on photosynthesis: Plants were grown in separate cells, as described above, until they had reached the 3-4 leaf stage. Initially, just three susceptible plants were chosen at random and placed in the experimental chambers. The three chambers not in use were closed securely to prevent air from the laboratory being drawn into the system. Measurements of photosynthesis were taken from each plant by switching the gas handling device manually at a range of flux densities from 0 to 750 μmol photons m^{-2} s^{-1}. The plants were given 30 mins to adapt to each flux density before the measurements were taken. This was repeated once more with susceptible and twice with resistant plants, to determine the optimum flux density for further experiments.

2.2.4.4 The effect of chlorotoluron on gaseous exchange: Six susceptible plants were taken, three were sprayed with field rate chlorotoluron and three with tap water as

described above. These were placed in the chambers at random, and 0.5 - 1, hour after spraying CO_2 uptake was monitored and recorded automatically over a 24 hour period with constant illumination. This was repeated using six resistant plants, again taken at random from the greenhouse. A further two repeats of the full experiment were performed for each biotype. Another set of experiments were conducted with the sodium lamp on a timer switch to give an 8 hour period of darkness to simulate night. Transpiration rates were also measured for each of these plants.

2.2.5 Analysis of data

The initial dose-response data were based on observations from at least 30 plants per treatment and were expressed as the percentage reduction in fresh weight compared with the controls because plant growth varied between tests. The dose-response curves were computed using the mathematics functions in SlideWrite (Advanced graphics software Inc, Carlsbad, USA), which uses the Levenberg-Marquardt alogarithm in an iterative process to find the line of best fit. Results from the sequential growth analysis were expressed as means from six plants per treatment and the standard error was calculated. Means from 3, 6 or 9 plants and the standard error were calculated from the gaseous exchange experiments and regression analysis was used to estimate the quantum yields.

2.3 Results

2.3.1 Characterisation of herbicide-resistance by growth analysis

Figure 2.4 shows the typical appearance of susceptible and resistant black-grass plants 2 weeks after they were sprayed with various doses of chlorotoluron. Differences in plant size were visible after 7 days, but no other symptoms were seen until 8-10 days after treatment. The limp and yellowing leaves shown in Figure 2.5 were common on susceptible plants given field rate or higher doses of chlorotoluron. The first leaves to become chlorotic were those which were fully expanded at the time of herbicide application and symptoms first appeared at the leaf tips or margins. At the time of harvest some plants had lost all pigmentation and were clearly necrotic, as illustrated by the plant on the left in Figure 2.5. In other plants, the emerging leaf was still green and in a few cases appeared to be growing. This illustrates the observed variation in response within black-grass populations used in this study.

In the three experiments carried out to determine the dose-response, the mean fresh weight of the control plants was 0.82, 0.48 and 1.33 g respectively, for susceptible and 1.39, 0.82, and 1.49 g for resistant plants. Overall values ranged from 3.18 g for a resistant control plant to 0.01 g for a susceptible plant treated with twice field rate. The marked difference in susceptibility to chlorotoluron between the two biotypes is clearly shown in Figure 2.6. Percentage of control values were used so that results from the different experiments were comparable. Under the growth conditions described above field rate chlorotoluron (3.5 kg ai ha^{-1}) reduced the fresh weight of susceptible plants by

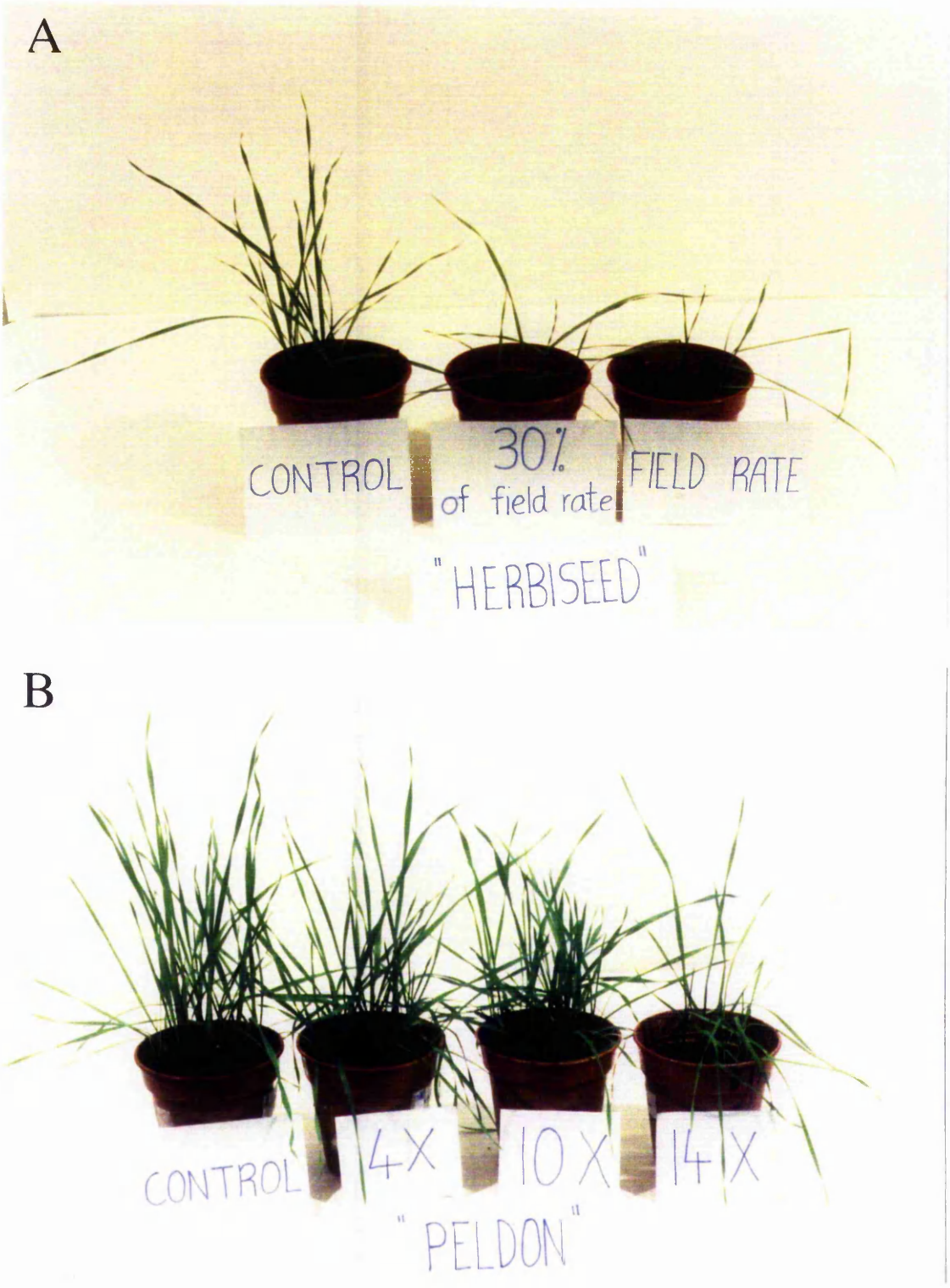


Figure 2.4 The appearance of (A) susceptible and (B) resistant black-grass plants 2 weeks after treatment with chlorotoluron.

Rates shown as multiples of the field rate (3.5 kg ai ha^{-1}).

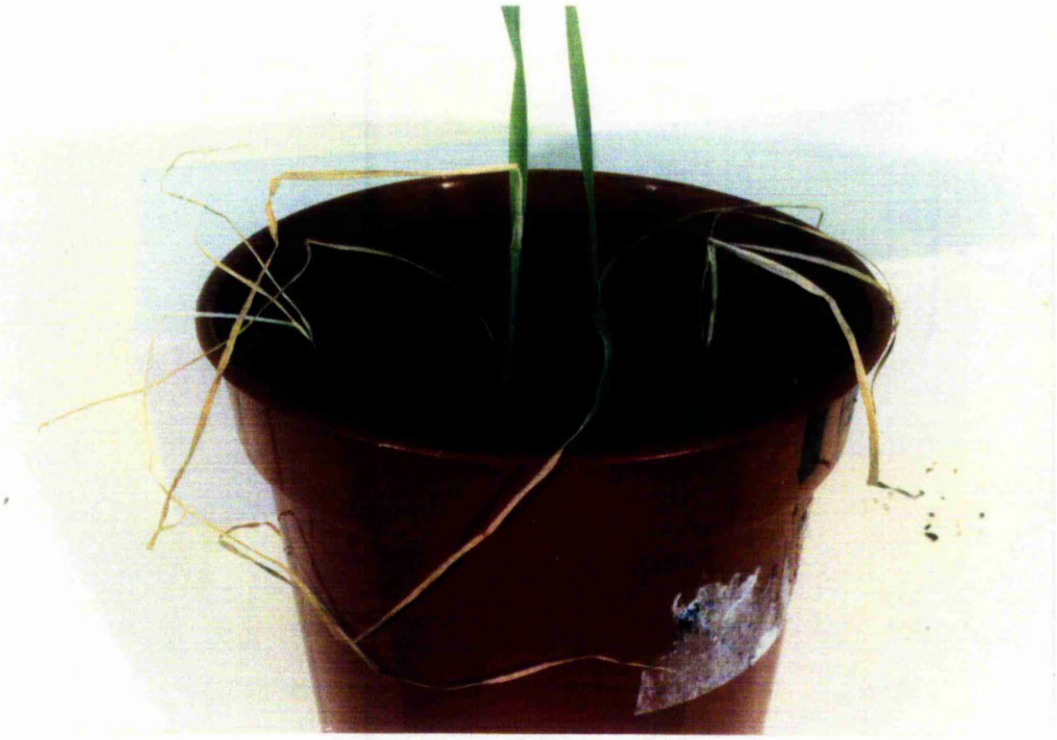


Figure 2.5 Susceptible plants 2 weeks after treatment with field-rate chlorotoluron showing chlorosis and necrosis.

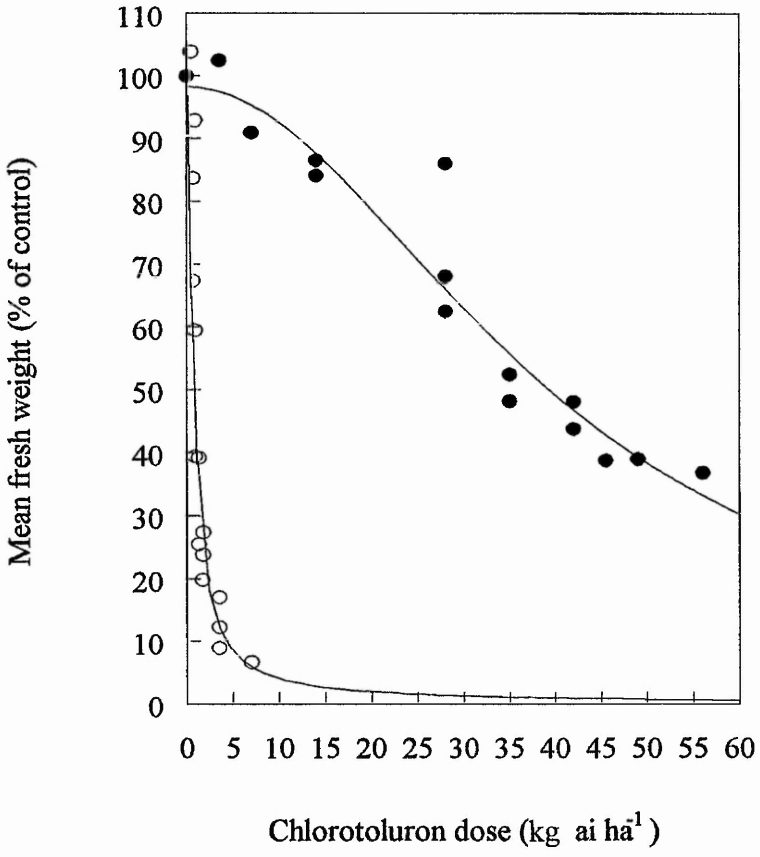


Figure 2.6 The effect of increasing herbicide dose on the fresh weight of susceptible (○) and resistant (●) black-grass 2 weeks after herbicide application. This graph shows combined data from 3 experiments, where each value is a mean of at least 25 plants. Field rate = 3.5 kg ai ha^{-1} .

83-91%, but had no effect on resistant plants (2% increase). This corresponds well to the reported values of 99% for Rothamsted and minus 3.6% for Peldon under simulated field conditions (Clarke & Moss 1991). The maximum reduction in fresh weight obtained for resistant plants was 65% when treated with 56 kg ai ha^{-1}. At this rate the herbicide solution contained over 50% of the thick, white formulation, hence it was not practical to use higher doses. Using the fitted curves the ED_{50} values (dose required to reduce fresh weight by 50%) were calculated to be 0.93 kg ai ha^{-1} (r^2 0.91) for susceptible and 39.3 kg ai ha^{-1} (r^2 0.94) for resistant black-grass, giving a Resistance Factor (ratio of ED_{50} values) of 42 under the growth conditions described.

Figure 2.7 shows the changes in dry weight, tiller number and leaf area of susceptible and resistant plants over 14 days after treatment with chlorotoluron at field rate or water. The comparison of treated and untreated susceptible plants revealed that whilst the dry weight of control plants increased exponentially over the 14 days, from an average 10 to 160 mg, the treated plants gained only 12 mg on average over the first 8 days and then ceased to grow. Control and treated susceptible plants both had an average of 1 tiller and 3 leaves after 5 days. However, by day 14 control plants had 6 and 10, respectively, whereas treated plants had not produced any more tillers or leaves. This indicated that when field rate chlorotoluron was applied to susceptible plants they had stopped growing after 5-8 days. In contrast, the resistant plants appeared to grow equally well, regardless of treatment. The dry weight, tiller number and leaf number

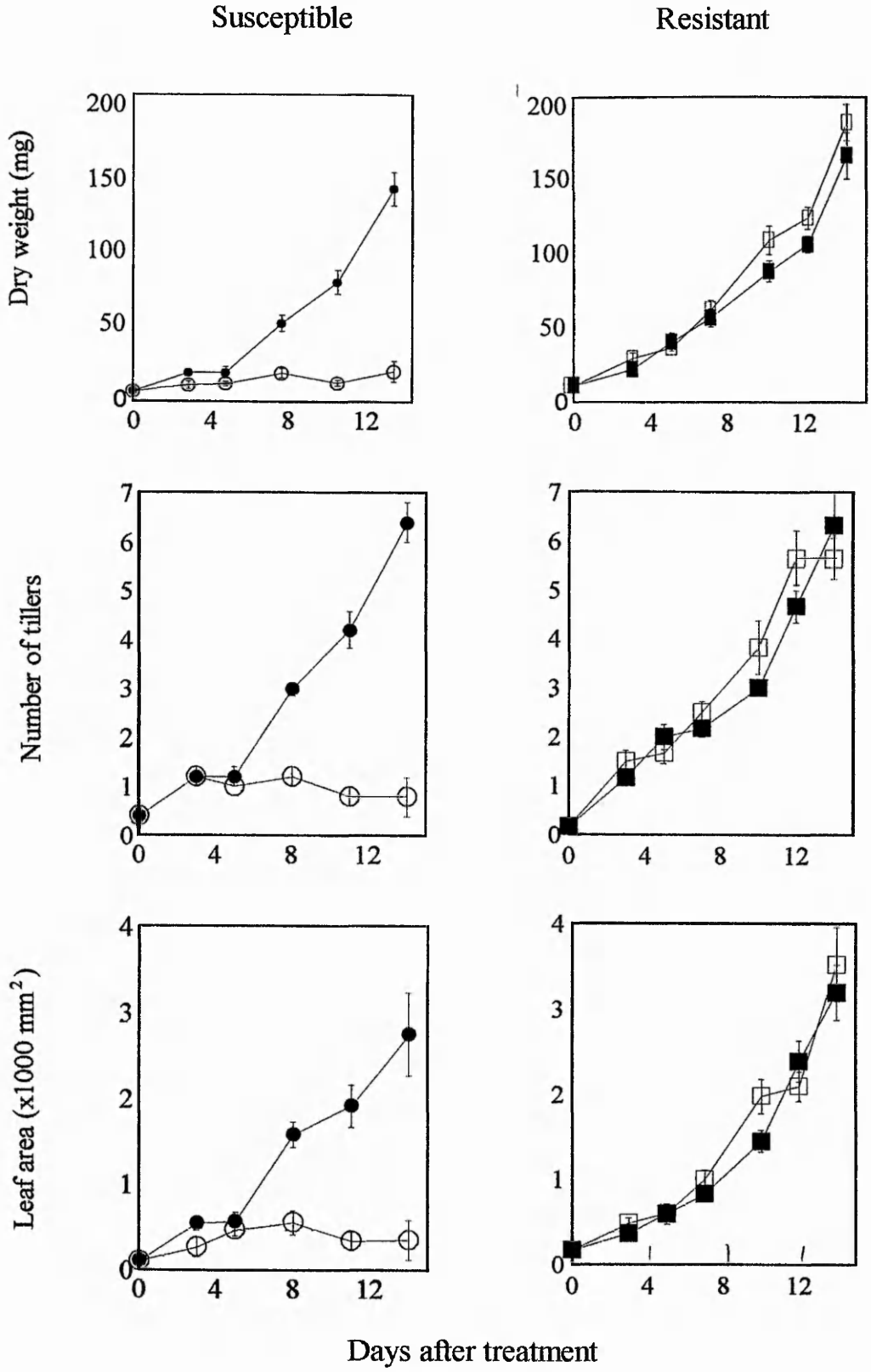


Figure 2.7 Growth analysis of susceptible (-●-control, -⊖-treated) and resistant (-⊟- control, -■- treated) blackgrass plants,using the parameters of dry weight, tillering and leaf area. Treated plants were sprayed with chlorotoluron at field rate. Values represent means ± SE where n = 6 plants.

Table 2.1 The effect of chlorotoluron on susceptible and resistant black-grass plants using specific leaf area, leaf-weight ratio and leaf water content. Values represent means from 3 experiments ± SE, where n = 6 plants.

	Biotype	Initial value	After 14 days - control	After 14 days - treated
Specific leaf area ($mm^2\ mg^{-1}$)	Susceptible	29.3 ± 2.40	34.9 ± 4.34	28.2 ± 3.40
	Resistant	28.1 ± 0.89	34.1 ± 4.36	34.0 ± 1.60
Leaf-weight ratio	Susceptible	0.569 ± 0.038	0.544 ± 0.026	0.576 ± 0.037
	Resistant	0.636 ± 0.047	0.590 ± 0.028	0.582 ± 0.035
Leaf water content (%)	Susceptible	83.5 ± 1.20	84.5 ± 1.13	68.4 ± 12.5
	Resistant	80.5 ± 1.69	83.6 ± 1.14	83.5 ± 0.85

Table 2.2 The relative growth rates of susceptible and resistant black-grass plants with and without chlorotoluron treatment at field rate (3.5 kg ai ha^{-1}). Values expressed as mg g^{-1} dry weight day^{-1} ± 95% confidence intervals, n = 6 plants for each experiment.

Experiment	Susceptible		Resistant	
	Control	Treated	Control	Treated
1	180 ± 90	73 ± 160	180 ± 56	190 ± 48
2	200 ± 32	45 ± 20	190 ± 53	200 ± 48
3	220 ± 48	78 ± 84	150 ± 32	170 ± 25

Experiment 3		Time interval (Days)					
		0-3	3-5	5-7	7-10	10-12	12-14
Control	Susceptible	344	180	173	187	54	199
	Resistant	226	316	156	145	94	222
Treated	Susceptible	64	80	0	146	43	0
	Resistant	320	112	264	187	64	209

of control and treated resistant plants were not significantly different on any of the days measurements were made. There was no evidence to suggest that field rate chlorotoluron had any effect on the growth or development of resistant plants. Differences between susceptible and resistant black-grass plants grown without herbicide could be determined by comparing the control plants of each biotype. Five weeks after sowing the mean number of tillers on susceptible and resistant plants was 6.40 and 6.33 respectively, final mean leaf area was 2751 and 3200 mm^2, and final mean fresh weight was 928 and 1169 mg. None of the small differences between the biotypes were significant, indicating that there was no difference in the growth and development of susceptible and resistant plants up to this growth stage under these glasshouse conditions.

The parameters of specific leaf area, leaf-weight ratio and leaf percentage water content were calculated from fresh and dry weights and leaf area, and the results are given in Table 2.1. Specific leaf area was calculated by dividing leaf area (mm^2) by leaf dry weight (mg) to obtain an indication of leaf thickness (Hunt 1990). The average specific leaf area for control susceptible and resistant plants was 32 and 31 $mm^2\ mg^{-1}$ respectively, over the 14 days, and any differences between the biotypes were not significant. Treated resistant and susceptible plants produced leaves with a similar thickness to the control plants and again, differences were not significant. The leaf weight ratio indicates the amount of resources invested in photosynthesis compared with investment in structural components. Although this is difficult to determine for monocots, the values do confirm that susceptible and resistant plants equally invested in leaf production. The water content of the leaves remained at 81-85% in both biotypes. Fourteen days after treatment, treated susceptible plants showed a 10% reduction in

water content, but this was not significant at the $p<0.05$ level. Three separate growth analysis experiments were carried out in which the trends for each growth parameter were very similar, but the results could not be combined to give an overall mean due to differences in initial size of the plants. The relative growth rates were calculated for each experiment (Table 2.2) based on the increase in dry weight per gram per day, according to Hunt (1990). The rates determined for each experiment were not significantly different at the $p<0.05$ level. Thus, although the plants were different sizes initially they had the same relative growth rates. The average growth rates for susceptible and resistant control plants were 200 and 180 mg g^{-1} day^{-1} respectively, under the conditions described.

2.3.2 Photosynthesis by susceptible and resistant black-grass

Photosynthesis by susceptible and resistant black-grass was measured at various photosynthetic photon flux densities (Figure 2.8). The maximum rates obtained for susceptible and resistant plants were 8.94 ± 1.5 and 6.78 ± 1.4 μmol m^{-2} s^{-1} respectively. The difference in rates of photosynthesis between the two biotypes was not significant, due to the amount of variation between individual plants. Using the linear portion of the graph (0-350 μmol photons s^{-1}) the quantum yield was estimated to be 0.028 mol CO_2 mol^{-1} absorbed photons for susceptible and 0.03 mol CO_2 mol^{-1} absorbed photons for resistant black-grass. These values were only estimates because they were based on the amount of light supplied and not the number of photons absorbed. Elevating the light intensity above 400 μmol photons m^{-2} s^{-1} did not further increase the rate of

photosynthesis by either biotype, nor was photoinhibition evident at the range of flux densities studied.

Figure 2.9A clearly demonstrates the effect of field-rate chlorotoluron on photosynthesis by susceptible and resistant plants illuminated with 350 μmol photons m^{-2} s^{-1} over a 24 hour period. Photosynthesis by susceptible plants sprayed with chlorotoluron ceased 4 hours after treatment and did not recover over the time period studied. Although no value was obtained for time 0, the results indicated that photosynthesis had already declined within the first 2 hours after spraying. Photosynthesis by control plants increased over the 24 hours from 2.65 to 3.45 μmol m^{-2} s^{-1}, which is a typical observation when plants are placed in a stable enclosed environment with no limitations and high hunidity. Thus, any reduction in photosynthesis cannot be attributed to enclosing the plants in chambers. In contrast, the treated resistant plants showed no decline in photosynthesis, and rates were very similar to those of the control plants over the 24 hour period. Thus, field rate chlorotoluron had no affect on photosynthesis by resistant plants. Under these conditions the maximum rates of photosynthesis measured were 4.61 ± 0.7 μmol CO_2 m^{-2} s^{-1} for susceptible and 3.45 ± 0.5 μmol CO_2 m^{-2} s^{-1} for resistant plants. There was considerable variation in rates of photosynthesis within treatments, which may have been due to the wide-ranging size of the plants. The plants used had a final, total leaf area of between 590 and 2597 mm^2.

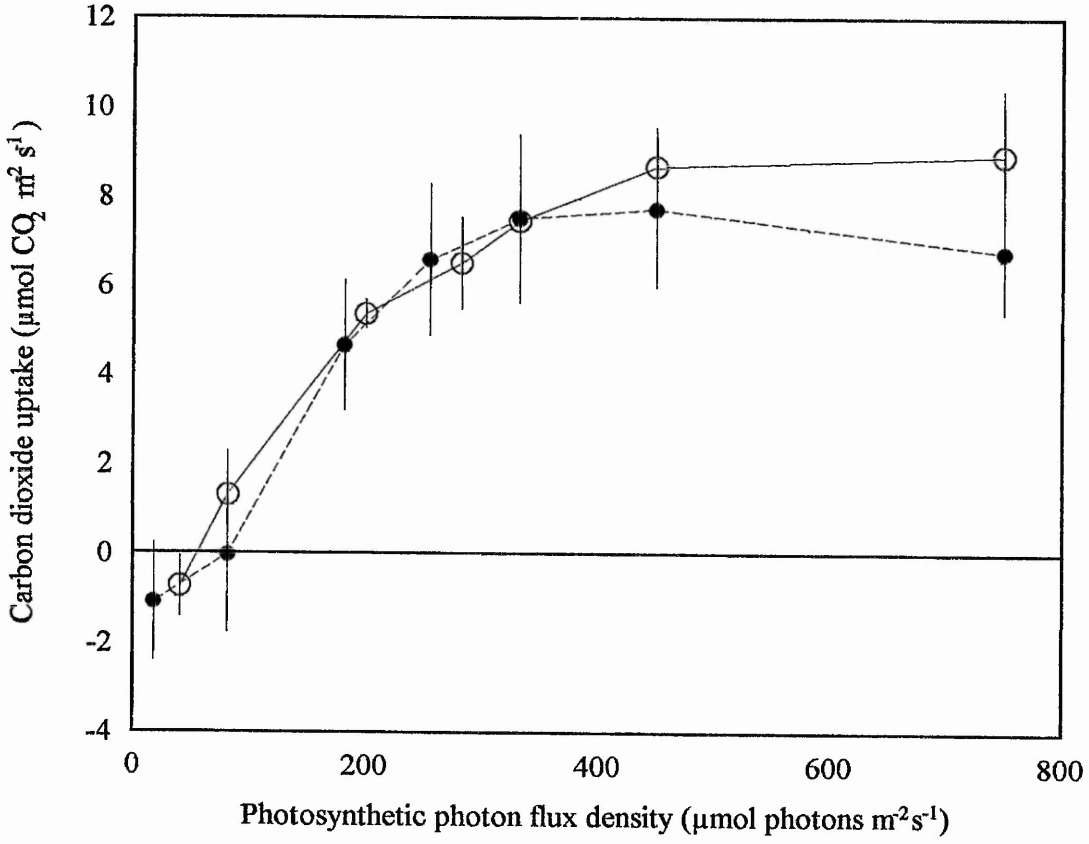


Figure 2.8 The effect of increasing light intensity on photosynthesis by susceptible (-○-) and resistant (-●-) black-grass. Values represent means ± SE where n = 3 plants.

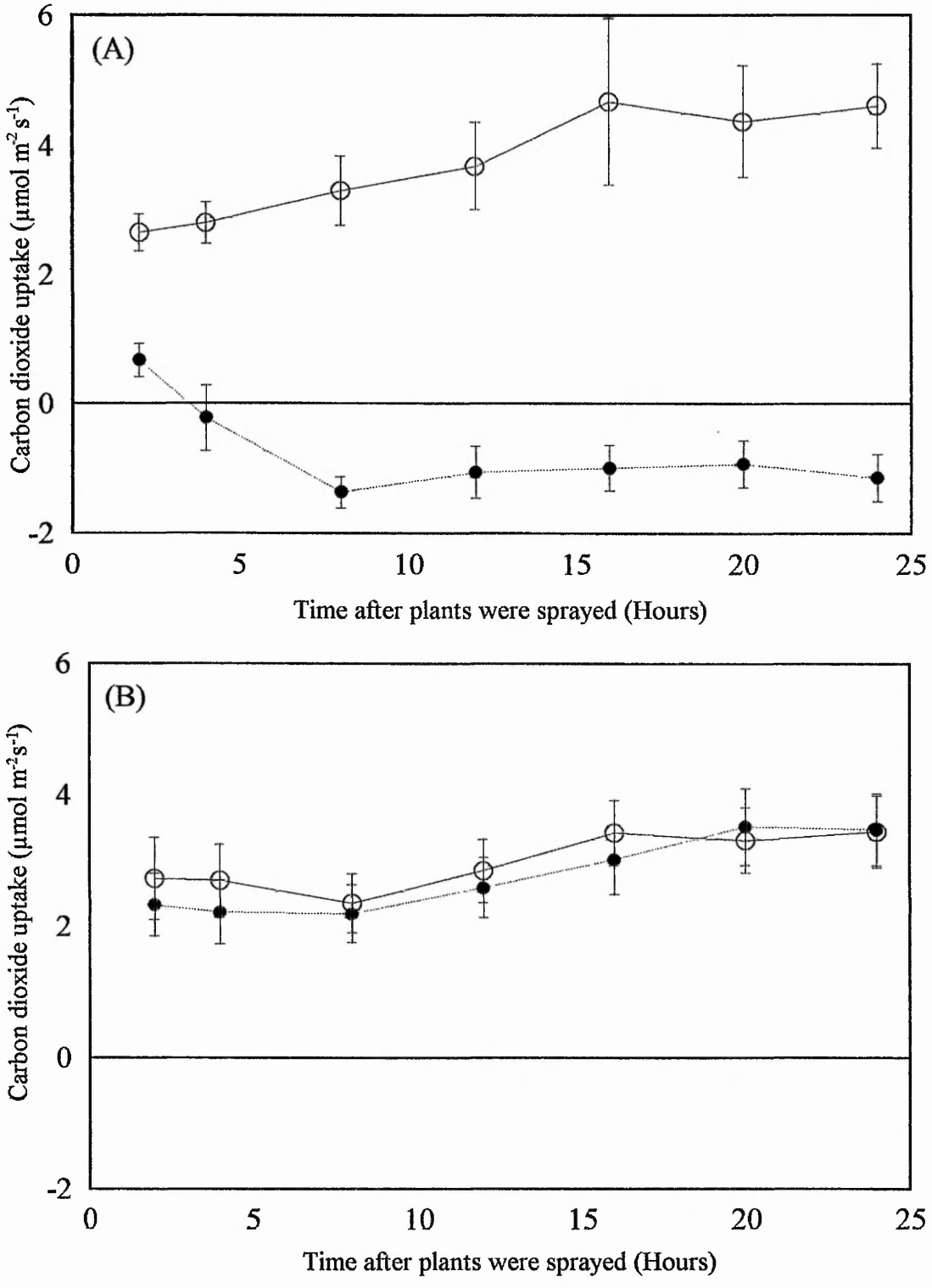


Figure 2.9 Photosynthesis by susceptible (A) and resistant (B) plants over a 24 hour period with constant illumination. Plants were sprayed with either distilled water (⊖) or field rate chlorotoluron (●). Values represent means ± SE of 3 experiments, each containing 3 replicates, (n = 9 plants).

These experiments were repeated, but with a dark period of 7 or 8 hours introduced into the 24 hour time period, to simulate night. Figure 2.10 shows rates of gaseous exchange for susceptible plants provided with a period of darkness 12 hours after chlorotoluron application, as would be the case if plants were sprayed early in the morning in the field. Total inhibition of photosynthesis by treated plants occurred after approximately 10 hours and no recovery was evident after the 8 hour period of darkness. Photosynthesis by the control plants remained constant at 3.75 ± 0.06 μmol CO_2 m^{-2} s^{-1} for the first 12 hours, and quickly returned to this rate after the 8 hours of darkness. Transpiration by control plants also remained constant in the light periods at an average 1.2 mmol H_2O m^{-2} s^{-1}. Initial transpiration rates for treated plants were much higher than those of the control plants, but they rapidly declined. Treated plants continued to transpire after the period of darkness, but only at half the rate of control plants.

Figure 2.11 shows how an earlier dark period occurring 6 hours after treatment affects photosynthesis and transpiration by susceptible plants. The control plants maintained an average photosynthetic rate in the light of 3.45 ± 0.08 μmol CO_2 m^{-2} s^{-1} both before and after the period of darkness. Photosynthesis by treated plants declined over the first 6 hours but had not completely ceased before the dark period. When lighting resumed, there was a minimal rate of photosynthesis, but this finally stopped about 18 hours after treatment. This suggests that the earlier dark period delayed the inhibition of photosynthesis by about 8 hours, which was the length of the darkness. Transpiration by control plants increased after the dark period from 1.24 ± 0.04 to 1.52 ± 0.06 mmol H_2O m^{-2} s^{-1}. Transpiration by treated plants continued after the dark period, but again the rate was only half that observed by control plants.

The corresponding results for resistant plants given a 7 hour dark period 10 hours after treatment are shown in figure 2.12. There was no significant difference between control and treated plants in either their photosynthesis or transpiration. Photosynthetic rates increased by 25% over the time course from 2.34 ± 3.14 μmol CO_2 m^{-2} s^{-1} for control and 2.07 ± 0.3 to 2.78 ± 0.1μmol CO_2 m^{-2} s^{-1} for treated plants. This increase was similar to that previously observed, although the actual rates were lower. In contrast transpiration decreased from 1.47 ± 0.2 to 0.79 ± 0.2 μmol CO_2 m^{-2} s^{-1} for control and 1.39 ± 0.1 to 0.93 ± 0.1 μmol CO_2 m^{-2} s^{-1} for treated plants. Thus, field rate chlorotoluron had no effect on either the photosynthesis or transpiration in resistant black-grass plants.

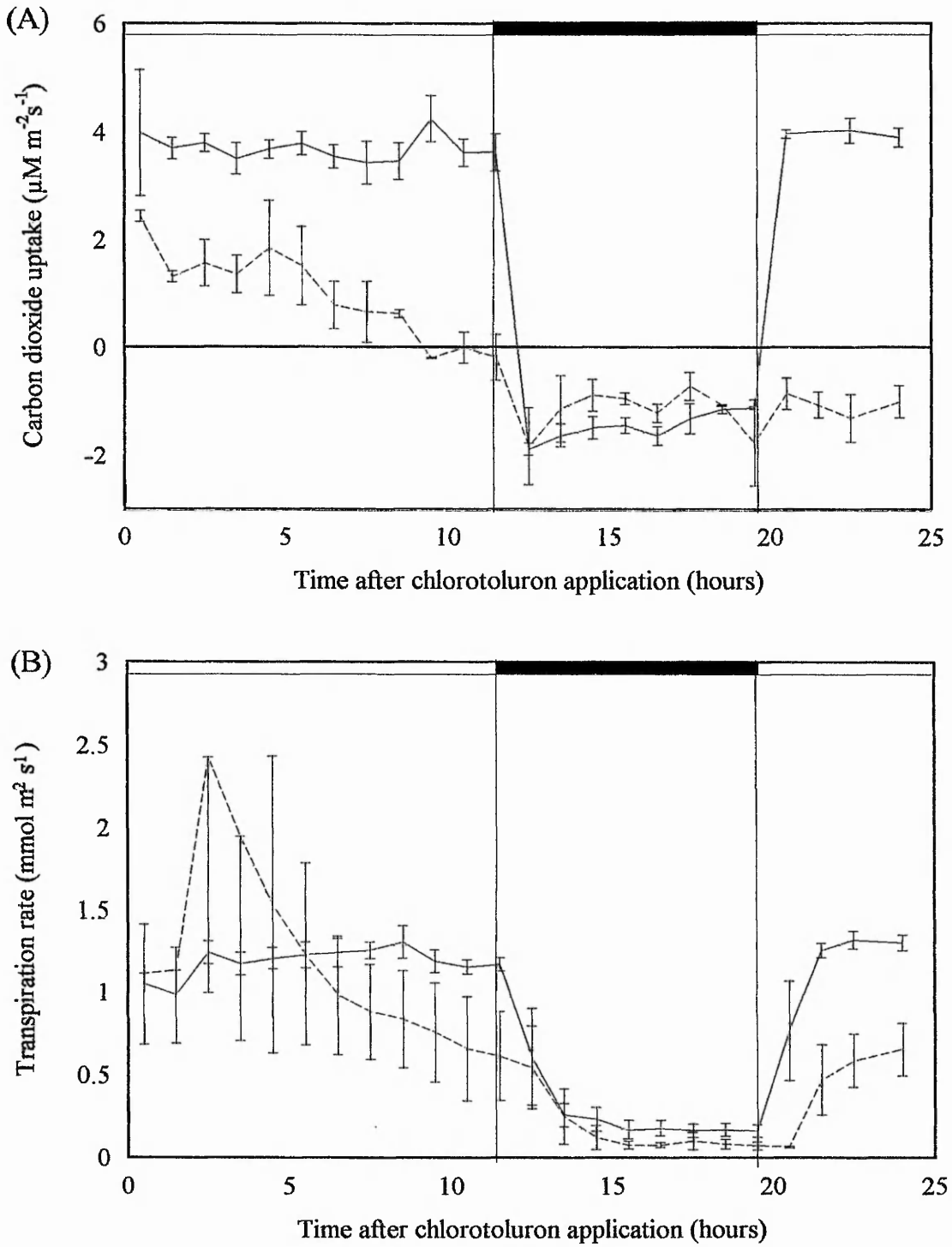


Figure 2.10 Photosynthesis (A) and transpiration (B) by susceptible black-grass over a 24 hour time period. Plants were initially sprayed with either distilled water (—) or field-rate chlorotoluron (----). Overhead illumination was fixed at 350 µmol photons $m^{-2}s^{-1}$, except for the 8 hour period of darkness. Values represent means ± SE where n = 3 plants.

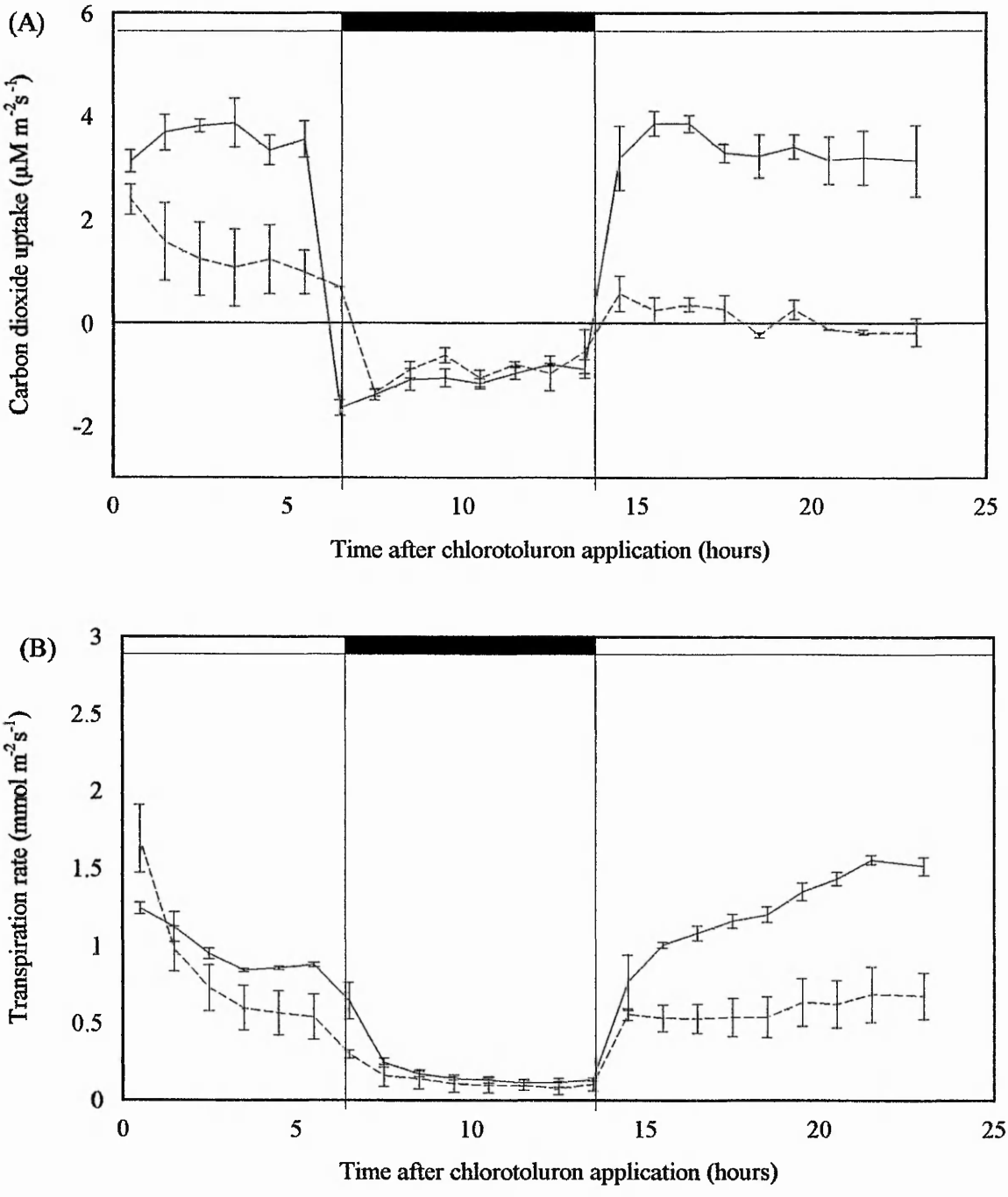


Figure 2.11 Photosynthesis (A) and transpiration (B) by susceptible black-grass over a 24 hour time period. Plants were initially sprayed with either distilled water (—) or field-rate chlorotoluron (---). Overhead illumination was fixed at 350 µmol photons $m^{-2}s^{-1}$, except for the 8 hour period of darkness. Values represent means ± SE where n = 3 plants.

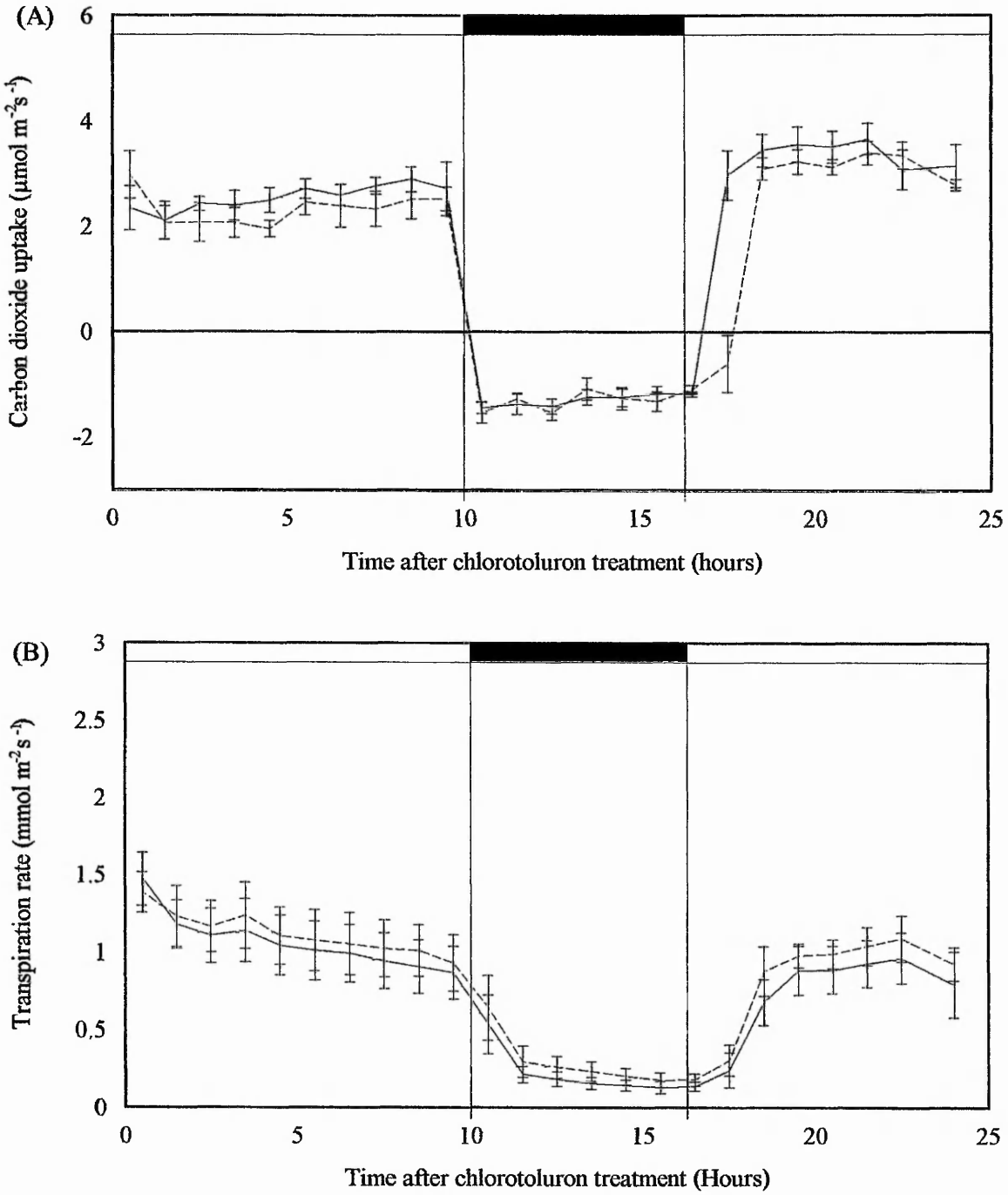


Figure 2.12 Photosynthesis (A) and transpiration (B) by resistant black-grass after an initial spray with either distilled water (—) or field rate chlorotoluron (---). Illumination was interupted after 10 hours by a 7 hour period of darkness. Values represent means ± SE where n = plants.

2.4 Discussion

The principal aim of the initial experiments was to characterise resistance by establishing the ED_{50} values for susceptible and resistant plants. The ED_{50} values will vary depending on growth conditions and experimental procedures, although if susceptible and resistant biotypes are grown at the same time the Resistance Factors should be constant. The wide variation obtained in final fresh weight values may be due to the experiments being carried out at different times of year, such that light and temperature were not consistent for each experiment. The night temperature regularly dropped to 4 - 8 ^{0}C during the second experiment which may explain the low fresh weight values. There are few published Resistance Factors for resistant black-grass, probably due to the high dose rates required to establish the ED_{50} value. However, Clarke & Moss (1991) reported that field-rate chlorotoluron did not reduce the growth of black-grass from Peldon, and that a dose of 10.5 kg ai ha^{-1} reduced growth by 12% which is similar to the results from this study. In 1985 a Resistance Factor of 16 was obtained using ED_{80} values for Peldon and Rothamsted biotypes in glasshouse trials (Moss & Cussans 1985). This is less than half of the value determined in these experiments, which is either indicative of an increase in resistance levels at this site or of differences in methodology, particularly in the growing medium used. The resistance factor for a Spanish resistant biotype was given as 29.3 (Menendez *et al* 1994), although the ED_{50} values were only 0.08 (susceptible) and 2.43 kg ai ha^{-1}, (resistant) which is below field-rate. This may be due to the method of herbicide application, which was a

soil drench, or maybe because the susceptible biotype used was much more susceptible than the ones used in England.

The ED_{50} value is based on the amount of herbicide applied, however herbicide efficacy is determined by the amount reaching the target site. This in turn is regulated by complex interactions between the soil, herbicide, weed species and environmental conditions. Chlorotoluron is mostly taken up by the roots (Blair 1978, Schmidt & Pestemer 1980,) so soil moisture content and absorptive capacity will largely determine the amount available to the plant. Generally, herbicide injury increases with soil water content (Blair 1985). The pattern of seedling root development can also be important. Most of the herbicide applied will remain in the top layer of the soil. Plants which quickly develop lateral roots in this area will take up more herbicide than those which concentrate on growing a long tap root. It is the total amount of herbicide available to the plant which determines injury, not necessarily the concentration at which it was applied (Blair 1978). The environmental factors of light and temperature, and rainfall in the field, will exert an influence on growth and herbicide toxicity. The rate of photosynthesis is linked to temperature and light availability, and the phytotoxicity of substituted ureas is known to increase with light intensity (Pallett & Dodge 1980, Dodge 1983). Temperature also influences metabolic processes including the rate of herbicide metabolism.

Herbicide absorption is maximal at the root tip (Strang & Rogers 1971) and typically occurs rapidly for 5-30 minutes and then more slowly over a longer period (Balke & Price 1983a). Chlorotoluron, as a lipophilic molecule, will enter root cells

rapidly (Briggs *et al* 1982) and cross the plasma membrane by simple diffusion. It may also accumulate slightly against the concentration gradient by partitioning into lipid fractions in the tissue (Price & Balke 1983a). Once inside the plant herbicides are translocated in the xylem, phloem, or both, which is determined by the physiochemical properties, principally the pK_a and log K_{ow} (1-octanol/water partition coefficient) of the molecule. Bromilow *et al* (1990) proposed a model based on this which indicated that the substituted ureas would only be xylem mobile. Thus, chlorotoluron translocation will be dependent on water potential gradients within the plant, affected by soil water content and relative humidity and not on metabolically active processes. The herbicide must finally enter the chloroplasts in the leaf cells to exert its phytotoxic action. Herbicides tend to accumulate at the end point of water flow, which are the leaf tips and margins (Devine *et al* 1993) and this explains why chlorosis was observed to start in these areas of the leaf.

Since herbicide toxicity is determined by the amount of herbicide reaching the target site, and this is dependent on many variables, it is obvious that ED_{50} values can be of limited value unless the conditions of the experiments are clearly defined. For example, watering plants from above results in more herbicidal damage than sub-irrigation (Blair 1985). This is especially true of ED_{50} values established in the field. An interesting study was made of the effect of the absorptive capacity of the soil (Moss 1987). The ED_{50} for Peldon was 3.56 in soil with a low absorptive capacity (kD 4.7), whereas in a more absorptive soil (kD 11.1) the ED_{50} value was 32.5. The absorptive capacity of the compost used in this study is not known, but presumed to be high as this

would explain why the ED_{50} value was higher than that reported by other workers, but is close to the value given on highly absorptive soil.

This study was carried out using whole plants under glasshouse conditions. Whole plants have the advantage that all the normal plant processes for regulating the amount of herbicide at the target site are in place. This gives a good indication of herbicide action in the field and good correlations between field and glasshouse results can be obtained (Clarke & Moss 1991). However, the lack of control over environmental conditions, particularly light and temperature was a disadvantage and caused wide test-to-test variations. The ED_{50} values also depend on the time between spraying and harvesting and the control biotype used. Resistance Factors can also be determined using other parameters such as the inhibition of electron flow at photosystem II or chlorophyll fluorescence in detached leaves, although these often result in values far higher than those obtained in whole plants. For instance, the Resistance Factor for atrazine resistant *Amaranthus cruentus* was 500 using chloroplasts but only 69-87 with whole plants (De Prado *et al* 1991c).

The survival of resistant biotypes in the field is determined by their competitive ability and the efficacy of the resistance mechanism. It has been suggested that any small changes in plant morphology which confer a degree of resistance will be selected for (Devine *et al* 1993). For example, resistance to foliage applied herbicides may be attributed to thicker cuticles or narrower leaves (Gasquez & Darmency 1991). In other cases changes in morphology are a consequence of the resistance mechanism, for example triazine resistant *Senecio vulgaris* L. has a reduced growth rate compared to the

susceptible biotype (Holt 1988). An increased rate of tiller production would establish the plants faster and thus may confer a competitive advantage, and an increase in leaf area could reduce the amount of herbicide reaching the soil. Although the two populations of black-grass used in this study displayed morphological variations, there were no significant differences between them in terms of tillering, leaf production and growth rate. These results confirm previous reports that resistant black-grass does not show any reduction in growth compared with susceptible plants (Kemp *et al* 1990).

Resistant and susceptible black-grass plants appeared equally efficient at assimilating carbon dioxide, and performed photosynthesis at similar rates under low and high flux densities, with an optimum rate at 300-350 μmol photons m^{-2} s^{-1}. There was an indication that resistant black-grass may have a reduced capacity to photosynthesise at high flux density compared with susceptible plants, but the values used were not high enough to confirm this. Consequently, no difference in photosynthesis could be correlated with the acquisition of the chlorotoluron resistance mechanism. Photosynthesis by untreated susceptible and resistant plants increased by 40% and 20% respectively over 24 hours under constant illumination which could be due to several factors. It may be that the maintenance of a constant environment with optimal conditions for photosynthesis allows maximum rates to be obtained. It could also be explained by an increase in leaf area since these plants were actively growing. The leaf area of black-grass plants at the 2-3 leaf stage was noted to increase by 10% over 24 hours (which was also seen by Van Oorschot 1970), but this is not enough to account fully for the increase in photosynthesis. When the chambers were under constant illumination the temperature rose by 2-5^{0}C, but when given a period of darkness the increase was not so great, and photosynthesis by control plants was

constant. Thus, the difference in photosynthesis by control plants between the two sets of experiments may be due to differences in temperature which could not be controlled.

Photosynthesis by susceptible plants was inhibited by chlorotoluron between 4 and 10 hours after herbicide application. Monuron inhibited photosynthesis by flax cotyledons after 2 hours of floating on a solution of the herbicide and no chlorophyll remained after 96 hours (Pallett & Dodge 1980, Dodge 1982). Metamitron inhibited photosynthesis by black-grass after 4 hours, although no visible symptoms were observed until several days later (Van Oorschot & Van Leeuwen 1979). The introduction of a dark period soon after herbicide application appeared to delay the inhibition of photosynthesis by chlorotoluron as could be predicted. After the inhibition of electron flow at photosystem II, light energy is dissipated by reacting with oxygen to produce oxygen free radicals, which results in the breakdown of the thylakoid membranes and cellular disruption (Pallett & Dodge 1980). The period of darkness predictably delays the build up of oxidative damage and the inhibition of photosynthesis.

Treated susceptible plants showed a much greater variation in rates of transpiration compared with the control plants, producing large error bars in Figure 2.10, which may be due to herbicide action. Higher initial transpiration rates were related to a faster decline in photosynthesis, which may reflect an increase in herbicide uptake (Muzik 1976, Robinson & Dunham 1982). Transpiration continued in susceptible plants after photosynthesis was completely inhibited. It has been observed that with some urea herbicides it can take several days for transpiration to reach the levels

observed in the dark, although photosynthesis stops after a few hours (Van Oorschot 1970).

Clearly, the black-grass biotypes used in this study show a different response to the herbicide chlorotoluron on both a fresh weight basis and in whole plant photosynthesis after treatment. The first herbicide resistant weeds reported were resistant to triazine and thus most of the work on biotype comparisons has been carried out on triazine resistant species. Atrazine resistant biotypes of *Senecio vulgaris* L. and *Chenopodium album* L. have a reduced biomass (Elliot & Peirson 1983, Holt 1988), lower rates of CO_2 fixation and lower quantum yields compared to susceptible biotypes (Van Oorschot & Van Leeuwen 1984, Hobbs 1987). It appeared that modifications to the thylakoid membrane which confer resistance also impaired photosynthesis (Holt *et al* 1981). This was not the case with certain triazine resistant biotypes of *Phalaris paradoxa* L. and *Solanum nigrum* L. which had equal biomass and CO_2 uptake under non-competitive conditions (Schonfeld *et al* 1987, De Prado *et al* 1991b). Atrazine resistant velvetleaf (*Abutilon theophrasti* Medic.) has no altered target site (resistance was due to enhanced metabolism) but growth was reduced and photosynthesis was not as efficient (Gronwald *et al* 1989).

Much less is known about the growth of biotypes resistant to other herbicides. There was no difference in plant height, tiller production or dry weight between dinitroanaline susceptible and resistant goosegrass (*Eleusine indica* Gaerth., Murphy *et al* 1986). Sulfonylurea resistant prickly lettuce (*Lactuca serriola* L.) had less biomass but was equally competitive (Alococer-Ruthling *et al* 1992). Reduction in growth cannot be correlated to a particular herbicide or resistance mechanism. However, fitness

and survival needs to be considered over the whole life cycle including mortality rates, seed production, and the ability to compete successfully with the crop.

2.5 Conclusion

The Peldon population of resistant black-grass was estimated to have a Resistance Factor based on fresh weight of 42 when grown under these glasshouse conditions. There were no differences between resistant and susceptible plants in their growth and development over a period of 5 weeks, or in their ability to perform photosynthesis and transpiration at the flux densities examined. Chlorotoluron resistance in black-grass was not associated with a reduction in growth or photosynthesis, and the differences in resistance must clearly lie at a more biochemical level of organisation.

Chapter Three

A study of glutathione S-transferases in susceptible and resistant black-grass

3.1 Introduction

The metabolism of chlorotoluron by resistant and susceptible black-grass is known to involve the cytochrome P450 monooxygenases (Caseley *et al* 1990), but the possible involvement of other detoxifying enzymes has yet to be established. The glutathione S-transferases are an important group of enzymes which can catalyse the conjugation of many herbicides with glutathione, and they also have a role in plant responses to stress such as air pollution (Price *et al* 1990). The aim of this study was to measure endogenous GST activity in susceptible and resistant black-grass and to investigate the effect of safeners and synergists upon this. The interactions between these compounds and chlorotoluron was also investigated in the search for a possible role for GSTs in the chlorotoluron resistance mechanism in black-grass.

3.2 Materials and Methods

3.2.1 Optimisation of GST assay procedures

All chemicals were obtained from Sigma Chemical, Sigma-Aldrich Company Ltd, Dorset, England, unless otherwise stated.

3.2.1.1 Plant material: Tissue from plants that were susceptible to chlorotoluron was used throughout the optimisation of both the extraction method and the glutathione S-transferase (GST) assay. The plants were grown in compost as previously described, and used at the 2-3 leaf stage, except when the effect of plant age was investigated where plants with up to 5 leaves were used.

3.2.1.2 Preparation of cell free extracts: The initial method used was that published for a study of oxygen scavenging enzymes by Hull (1991), except that all the eluent from the desalting columns was collected. Approximately 1 g of whole plant tissue was ground to a fine powder in liquid nitrogen and stored at -70 ^{0}C until required. The powder was defrosted in a centrifuge tube containing 5 ml of chilled extraction medium (discussed below), homogenised for 20 s using an Ultra Turax and centrifuged (MSE 21) at 20 000 g for 10 mins at 4 ^{0}C. The supernatant was desalted on a Sephadex G-25, PD-10 column (Pharmacia) by loading 2.5 ml onto the column after equilibration with 25 ml 0.1 M potassium phosphate buffer (pH 7) containing 0.2 mM DTPA. This was eluted with 3.5 ml of equilibration buffer and the collected sample was the cell-free extract used in all further studies.

To establish the best extraction medium for black-grass four different media (given in Table 3.1) were tested. The GST activity was measured using a published assay (Price *et al* 1990) and the results are given in Table 3.2. The extraction medium did not markedly influence the final GST activity obtained, although medium no. 4 was

Table 3.1 The composition of the 4 different extraction media tested. Each was made up to a total volume of 5 ml.

Number	Extraction medium	Reference (where applicable)
1	0.1 M potassium phosphate, pH 7 5 mM DTPA 10 mM ascorbate 0.2 g PVPP	Hull 1991
2	0.1 M potassium phosphate, pH 7 5 mM DTPA 10 mM ascorbate 0.2 g PVPP 1 mM sodium metabisulphite	
3	0.1 M potassium phosphate, pH 6.8 0.25 g PVPP 1 mM sodium metabisulphite	Jablonkai & Hatzios 1991
4	0.25 M Tris-HCi, pH 7.8 1 mM DTPA 0.2 mM DTT 0.1 mM PMSF 0.2 g PVPP	Irzyrk & Fuerst 1993

Table 3.2 The specific GST activity in cell-free extracts from susceptible and resistant plants measured after extraction in 4 different media. Values are means ± SE, n = 3 experiments.

Extraction medium	Specific GST activity (nmol min^{-1} mg^{-1} protein)
1	27.7 ± 5.5
2	29.3 ± 5.5
3	27.2 ± 4.7
4	28.9 ± 5.6

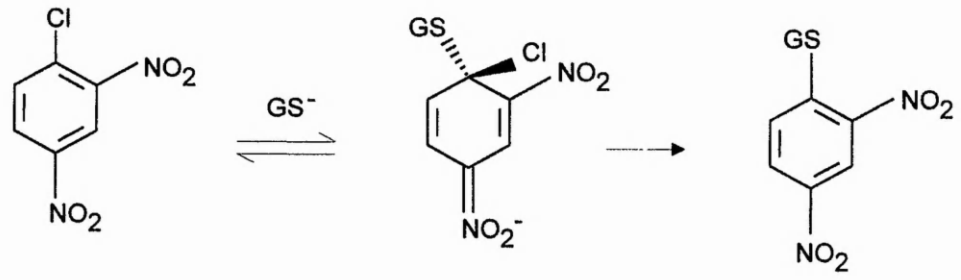


Figure 3.1 The conjugation of CDNB with glutathione

disregarded since PMSF is unstable in aqueous solution (James 1978) and so extraction medium 2 was chosen for all subsequent work.

3.2.1.3 The GST assay: Since there is no published assay for GST activity in black-grass, the spectrophotometric method of Price *et al* (1990) was used as a starting point and subsequently optimised. It is based on measuring the production of a conjugate of 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) when they are added to the extract, as shown in Figure 3.1. The concentrations of CDNB and GSH were optimised as shown in Figure 3.2. The stock solution of CDNB was made by dissolving in ethanol to give a final ethanol concentration of 9.6% (v/v) in the cuvette. An attempt to reduce the ethanol concentration was made, but this could not be reduced beyond 5.8% (v/v) due to the insolubility of CDNB. However, reducing the ethanol concentration in the reaction mixture did not appear to alter the enzyme activity. The pH of the assay buffer was optimised (Figure 3.3) within the pH range of the phosphate buffer and background conjugation was also measured at each pH. This resulted in the following optimum assay reaction mixture: 0.1M potassium phosphate pH 7.5 containing 0.2 mM DTPA, 1 mM CDNB, 2 mM GSH, and 50 µl cell free extract.

The total reaction volume was 1 ml and the temperature was maintained at 20 ^{0}C by the cuvette temperature controller (Peltier cell) in the Perkin Elmer 550S spectrophotometer. Immediately after the extract was added, the activity was measured at 340 nm using a chart recorder with the paper speed set at 10 cm min^{-1}. The rate was linear over the recording period of approximately 2-3 mins. The activity was calculated by subtracting the background level of conjugation and then using the extinction

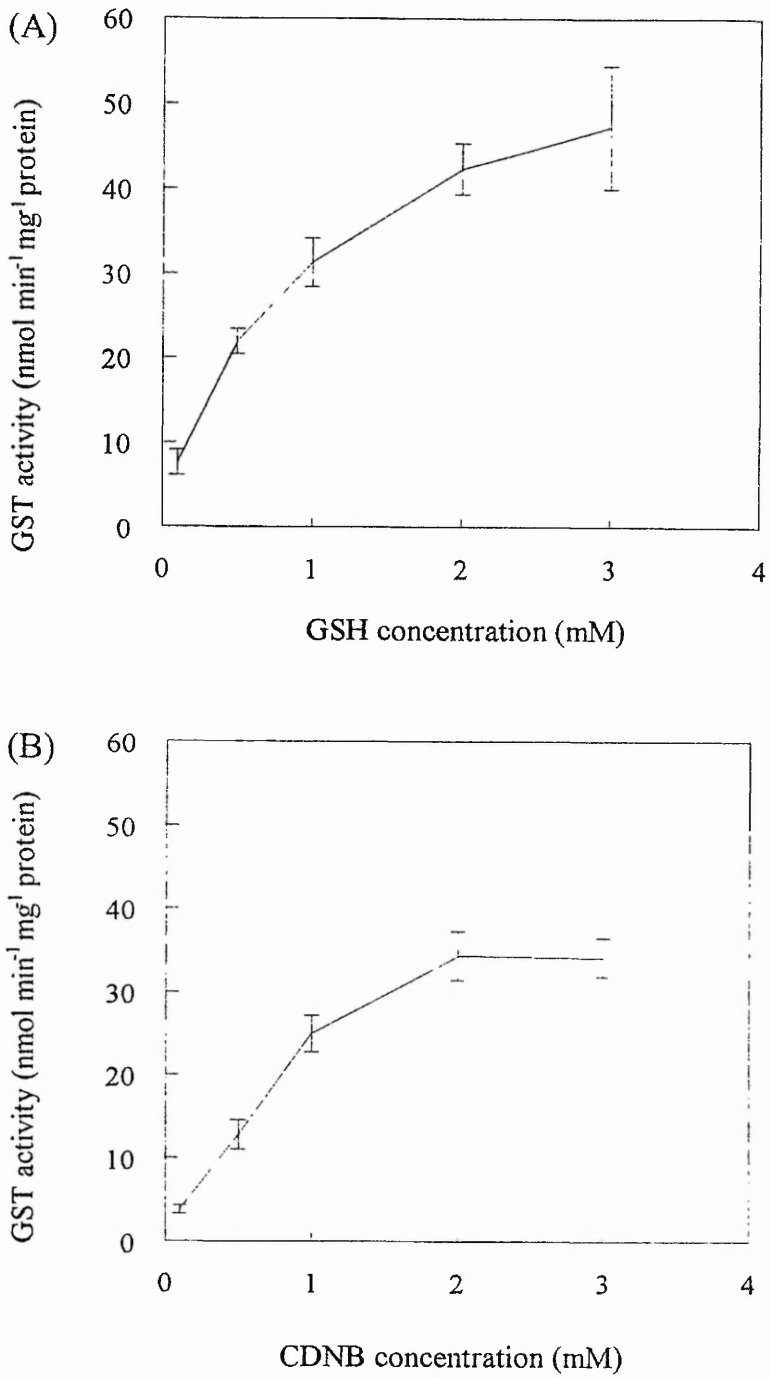


Figure 3.2 Optimisation of the concentrations of (A) GSH and (B) CDNB required for GST activity. Values represent means ± SE, where n = 3 samples of plant material.

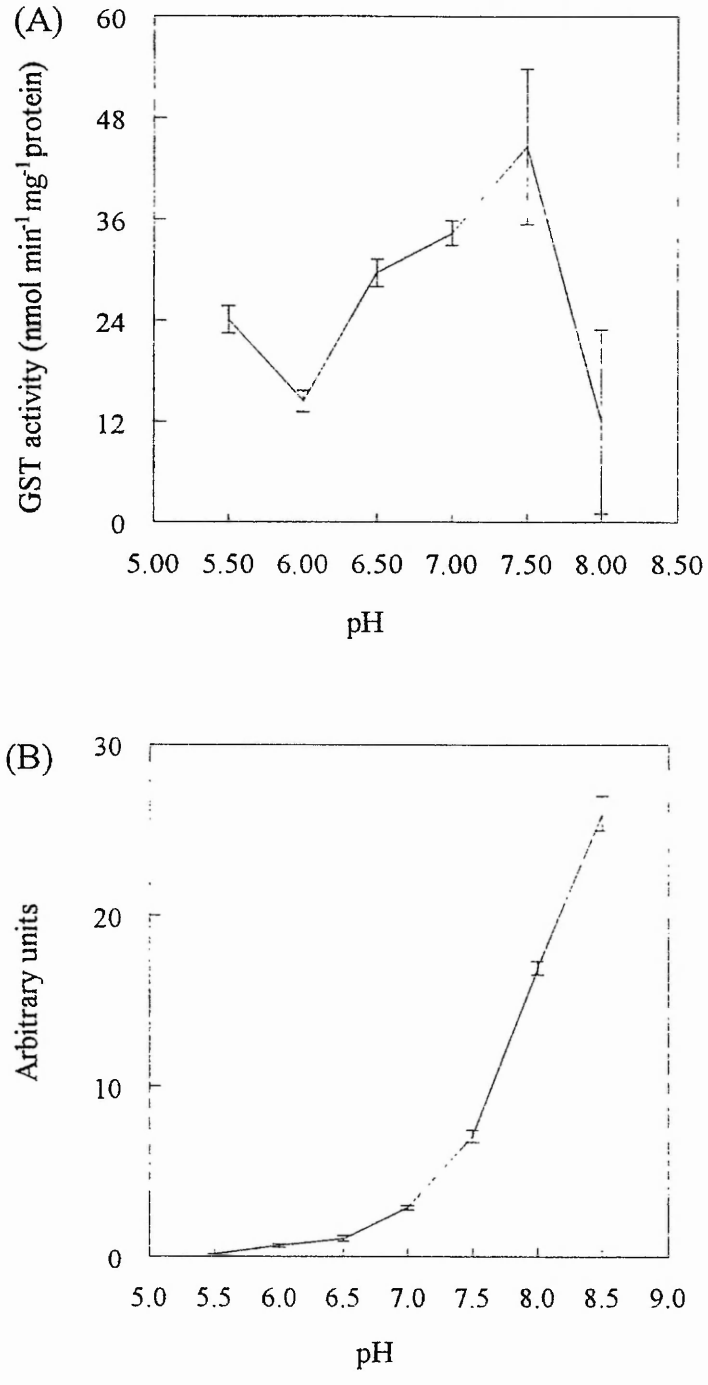


Figure 3.3 Optimisation of the assay buffer pH (A) and the background conjugation (B). Values represent means ± SE where n = 2 samples of plant material.

coefficient of the conjugate (9.6 mM cm^{-1}). Activity was expressed on a soluble protein basis.

3.2.1.4 Protein determination: The protein content of each extract was determined using the Bradford (1976) dye-binding assay. A stock solution of protein reagent was made by dissolving 200 mg of Coomassie Brilliant Blue G-250 in 50 ml of 95% (v/v) aq ethanol. To this was added 100 ml 85% (w/v) aq phosphoric acid and the solution made up to 1 litre with distilled water. The protein reagent was prepared by taking 75 ml of stock solution and 5 ml of 0.3% SDS (w/v) and adding distilled water to give a final volume of 500 ml. A standard curve was established using bovine serum albumin fraction V (BSA) at a range of concentrations (0-50 $\mu g\ ml^{-1}$). A solution of 200 µl of BSA and 1 ml of reagent was vortexed and incubated at room temperature for 3 min, after which the absorbance at 595 nm was measured. This was repeated 3 times for each BSA concentration and a linear standard curve obtained. The assay was conducted in this manner on each cell-free extract using 50 µl of extract, 150 µl of distilled water and 1 ml of reagent. The cell-free extracts were diluted first when necessary so that all absorbance readings fitted on the standard curve.

3.2.1.5 Plant age: To eliminate the possibility that differences in GST activity could be related to the age of the plants, a further experiment was undertaken. Plants were grown to the 5 leaf stage, harvested and the plant material sorted according to leaf number. The results (Figure 3.4) demonstrate that activity is not linked to leaf maturity for plants up to the 5 leaf stage and validates the use of whole plants for these experiments.

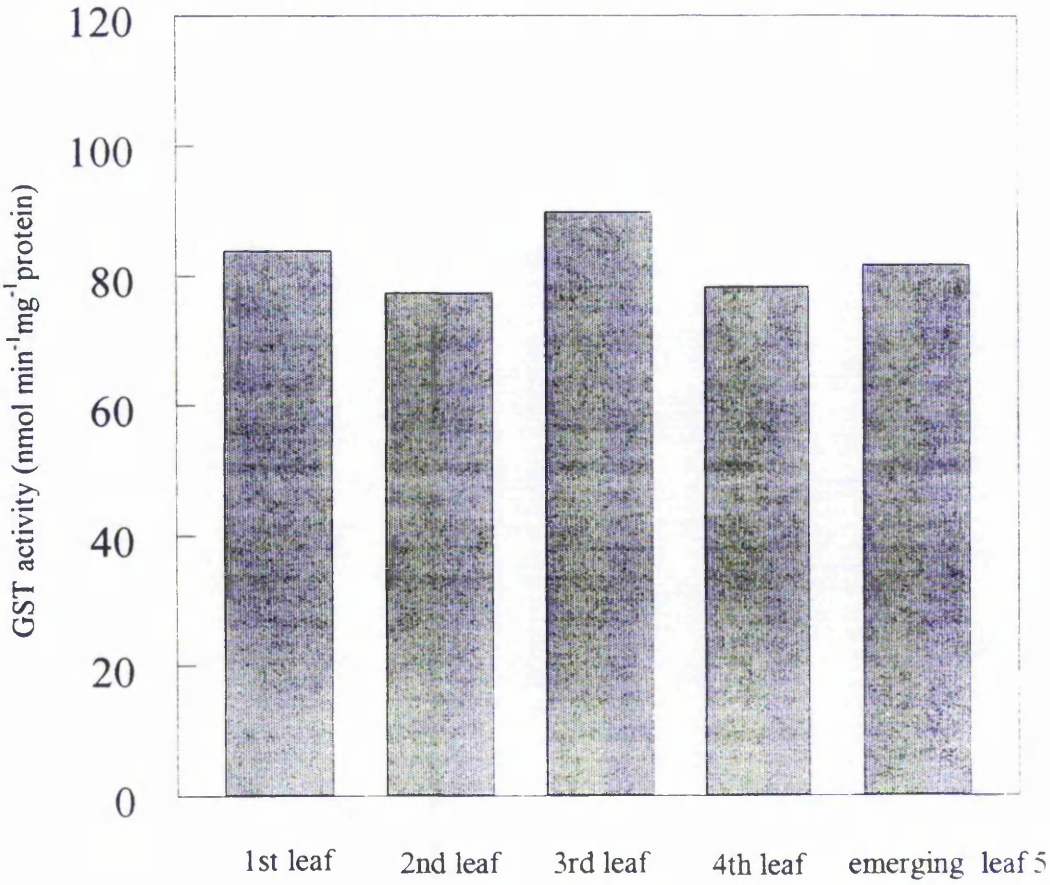


Figure 3.4 A comparison of GST activity in black-grass leaves of different ages. Values presented are from one typical experiment, repeated three times.

3.2.2 The effect of chlorotoluron on glutathione S-transferase, glutathione reductase and the glutathione pool

3.2.2.1 Plant material and treatment: Plants from susceptible and resistant populations were sprayed at the 2 leaf stage (as described in section 2.2.3) with field rate (3.5 kg ai ha^{-1}) chlorotoluron and sequential harvests were made 0, 2, 4, 8, 10 and 24 hours after treatment. Each harvest consisted of 15 susceptible and 15 resistant plants. Plants of each biotype were ground in liquid nitrogen and the resultant powder divided to give two samples at each harvest which were stored at -70 ^{0}C. One sample was used to assess GST activity (as described in section 3.2.1.3), protein content and glutathione reductase (GTR) activity, whilst the other sample was used to determine the glutathione pool.

3.2.2.2 Glutathione reductase: This assay was modified from Schaedle and Bassham (1977), as described by Hull (1991). The same extract as above was used and the 1 ml reaction mixture contained 0.7 ml 0.1 M potassium phosphate buffer (pH 7.8, containing 0.2 mM DTPA), 100 µl 3 mM GSSG, 100 µl of cell free extract and 100 µl 0.15 mM NADPH was added last to start the reaction. The decrease in absorbance due to NADPH oxidation was measured at 340 nm. Oxidation not due to GTR was assessed by excluding GSSG from the assay and corrections were made for this in the calculations. Activity was calculated using the extinction coefficient (6.22 mM cm^{-1}) and expressed on a protein basis.

3.2.2.3 Total glutathione : This assay was modified from Griffith (1980), as described by Hull (1991). A sample of plant material (1 g) was ground with a chilled pestle and mortar in 10 ml of 5% (w/v) aq. 5-sulfosalicylic acid and centrifuged at 1000 g for 10 minutes. A 1 ml aliquot of supernatant was added to 1.5 ml of 0.5 M potassium phosphate (pH 7.5) to neutralise it. The assay is based on an enzyme recycling procedure shown in Figure 3.5. The 1 ml reaction mixture consisted of 0.5 ml potassium phosphate (pH 7.5) containing 5 mM DTPA, 6 mM DTNB (2-nitrobenzoic acid), 2 mM NADPH, 1 unit of GTR and100 µl extract. The rate at which DTNB was oxidised to 2-nitro-5-thiobenzoic acid was followed at 410 nm and the glutathione content was calculated by comparison with a standard curve. The results were expressed on a fresh weight basis.

3.2.3.4 The effect of safeners on GST activity *in vitro*: The protein content in extracts from susceptible and resistant plants was determined and the extracts diluted with assay buffer to give a protein content of 1 g l^{-1}. Stock solutions of benoxacor and fenclorim were made by dissolving in DMSO and adding distilled water to give a final concentration of 1 mM. The final DMSO concentration was ≤ 1% (v/v). A stock solution of 1% (v/v) DMSO was used as a control. The GST assay as performed as before, but 100 µl of assay buffer was replaced with 100 µl of either benoxacor, fenclorim, DMSO or distilled water. The effect of incubating the extract with safener was also investigated. 750 µl of assay buffer, 50 µl of extract and 100 µl of stock solution or distilled water was incubated in a 1.5 ml Eppendorf tube for 30 min at 20 ^{0}C. This was then emptied into a cuvette, the substrates added and GST activity measured as before.

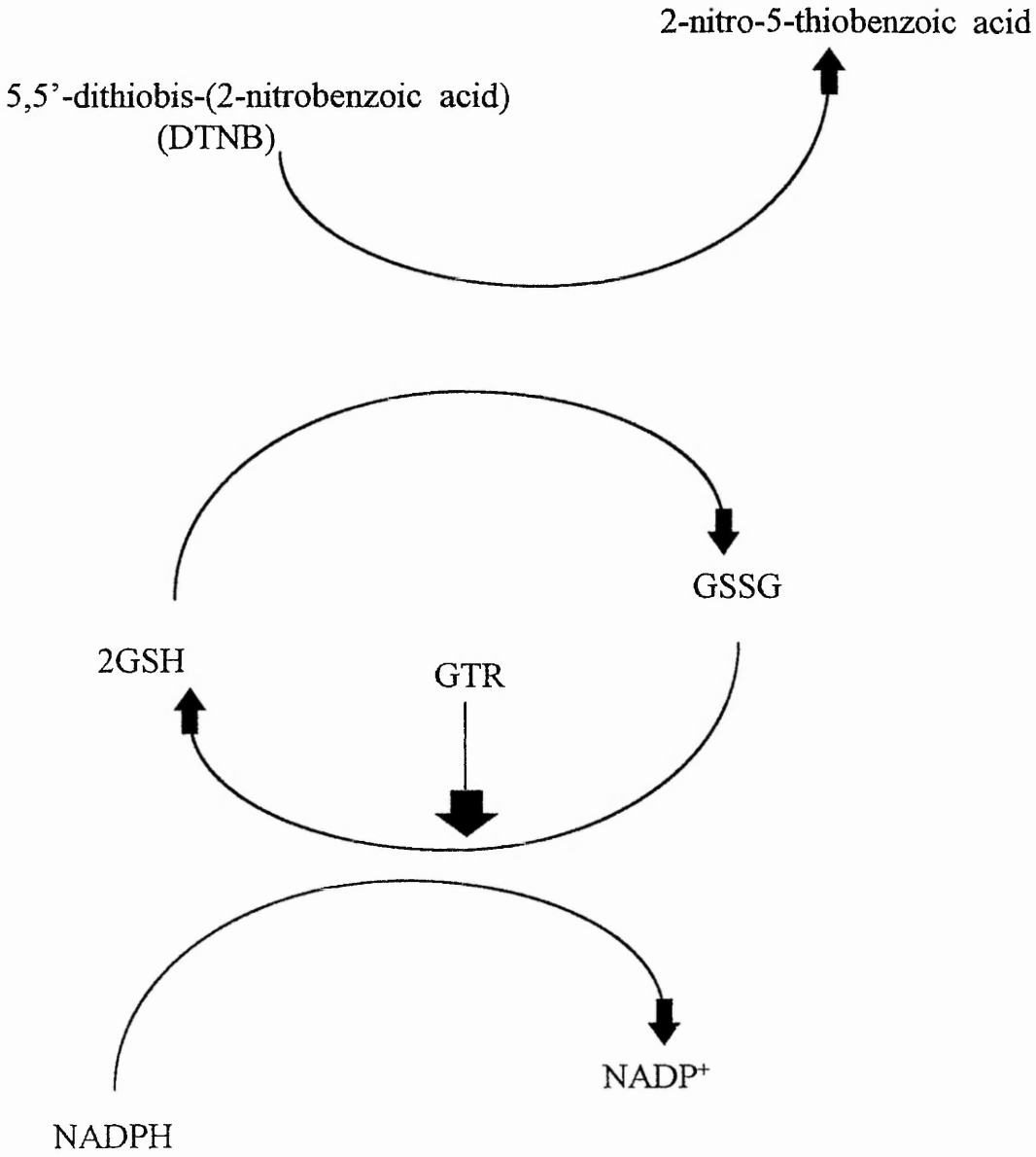


Figure 3.5 Enzymic recycling system used to measure glutathione content. Glutathione is cycled between its reduced form (GSH) and its oxidised form, glutathione disulfide (GSSG).

3.2.3 The exposure of black-grass plants to safeners and synergists using a hydroponic system

3.2.3.1 The hydroponic system: Synergist and safeners were obtained in their technically pure, unformulated state and so could not be applied using the standard spraying method. To deliver small amounts of chemical to the roots, the plants were grown in a hydroponic system. Several experimental designs were investigated to establish the optimum growth of black-grass in hydroponics, including the size and shape of the container, the strength of the nutrient solution, the proportion of time the plants grew in hydroponics compared with compost and the age of the plants when they were treated. This eventually resulted in the protocol below.

The containers used were clear plastic storage boxes (124 x 82 x 22 mm, Fisons Scientific Equipment, Loughborough) with detachable lids in which 14 holes (10 mm diameter) were made to allow 7 susceptible and 7 resistant plants to be grown in each one. This number of plants in each box was chosen because 7 plants were required to give 1 g of tissue which was the amount required for extraction. Each box held 100 ml of nutrient solution and contained a different treatment. The plants were grown as previously described until they had reached the 2 leaf stage when they were transferred to the hydroponic boxes after the roots were carefully washed in water to remove the compost. The plants were supported by a small piece of foam at the base of the stem and by pieces of cotton tied between 2 sticks at either end of each row of plants (Figure 3.6). Each box was filled with half strength Hoaglands solution plus 9.22 μm ferric EDTA (Kemp & Caseley 1987). The nutrient solution was changed every 2-3 days.

Once transferred to the hydroponic system, the plants were grown in growth cabinets (Fytotron, Sanyo, Loughborough) where they received a 14 hour day of 350 μmol photons $m^{-2}s^{-1}$ photosynthetic flux density. Temperature was set at 20 ^{0}C during the day and 12 ^{0}C at night, and humidity was 60% and 40% respectively. However, during the hot summer months of 1995 the cabinets were unable to maintain the temperature which regularly reached 30-35 ^{0}C during the day.

3.2.3.2 Chemical application: The plants were equilibrated in the hydroponic system for 5-7 days before treatment, by which time they had reached the 3 leaf stage. The compounds used were the synergists ABT and tridiphane, and the safeners benoxacor, fenclorim, fenchlorazole-ethyl, flurazole, NA, OTC and oxabetrinil, and their structures were given in Tables 1.4 and 1.5. Since the storage boxes held 100 ml, each unformulated chemical (except naphthalic anhydride, NA) was first dissolved in 0.5-1 ml of DMSO and then made up to 100 ml with nutrient solution, such that the final concentration of each chemical was 100 μM. The final DMSO concentration never exceeded 0.5 % (v/v). Control plants were given nutrient solution containing 0.5 % (v/v) DMSO only. NA and formulated tridiphane were dissolved directly into nutrient solution, again to a final concentration of 100 μM. The nutrient solution in the boxes was replaced by the solutions containing the dissolved safeners and synergists. Plants were harvested 24 hours later, ground in liquid nitrogen and stored at -70 ^{0}C until required.

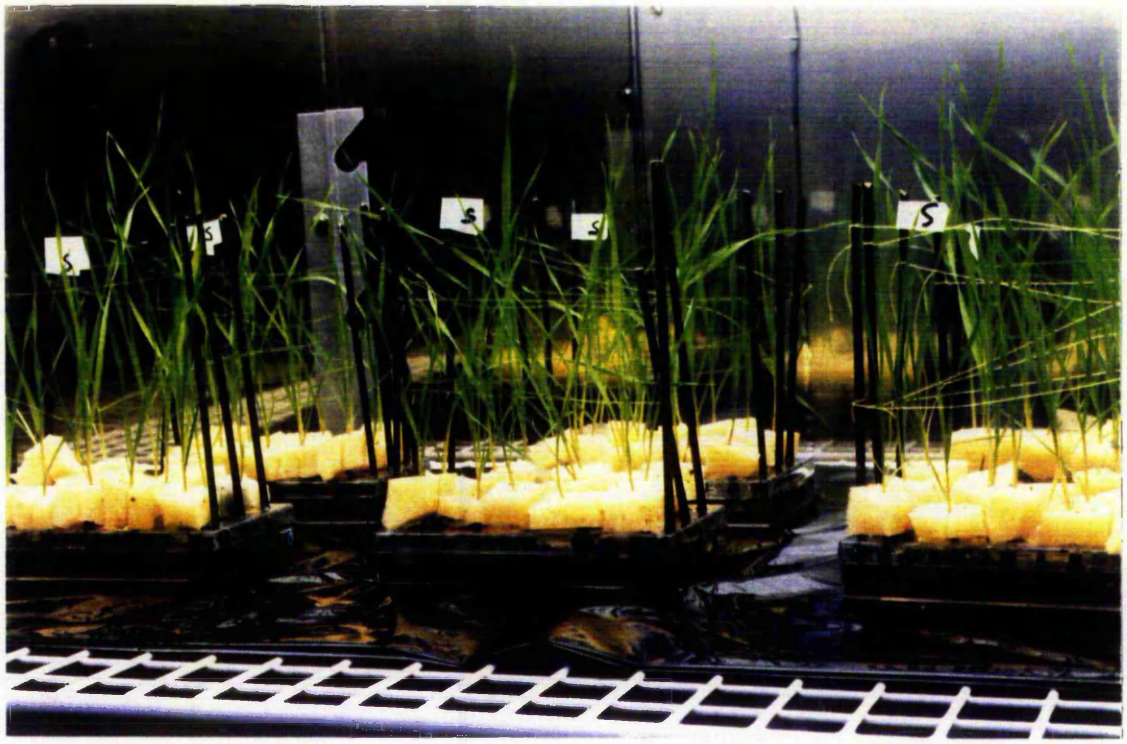


Figure 3.6 Black-grass plants growing in the hydroponic system, shown one third of actual size.

3.2.4 The interaction between chlorotoluron and tridiphane using intact plants

Susceptible and resistant black-grass plants were grown in 7 cm pots as described in Chapter 2. The plants were sprayed at the 2-3 leaf stage with formulated tridiphane at a range of concentrations (0.25 - 1 kg ai ha^{-1}), followed by an application of formulated chlorotoluron (0.88 - 3.5 kg ai ha^{-1}) 24 hours later. Control plants were sprayed with a single herbicide at the same rates. Five pots each containing 6 plants were used for each treatment. Plants were assessed 2 weeks after the first herbicide application by fresh weight. The experimental details of herbicide application were given in Chapter 2 (2.2.2).

3.2.5 The effect of chlorotoluron, safeners and synergists on gaseous exchange

The plants were left in the hydroponic system for 5-7 days until they reached the 3 leaf stage. Photosynthesis was then measured using a portable leaf chamber (PLC, ADC Ltd, Hoddesdon, UK) attached to the LCA4 IRGA. The second leaf on each plant was placed in the PLC and allowed to equilibrate for 3 mins prior to data recording. Leaf area was determined by the length and width of leaf in the chamber. Gaseous exchange from leaves of 3 susceptible and 3 resistant plants from each box was measured and then the nutrient solutions were replaced with ones containing dissolved

formulated chlorotoluron at 0.24 μM - 2.4 μM. Photosynthesis was measured again 4 and 24 hours later to establish which dose gave a response most similar to those obtained when whole plants were sprayed with field rate chlorotoluron.

For subsequent experiments each box was pre-treated with either ABT, tridiphane, benoxacor or fenclorim for 24 hours. Gaseous exchange by 5 susceptible and 5 resistant leaves was measured and then the solutions were replaced by ones containing either 2.4 or 4.8 μM chlorotoluron. Photosynthesis was measured 24 hours later for an indication of chlorotoluron activity.

3.3 Results

3.3.1 Glutathione S-transferase activity in susceptible and resistant black-grass.

The mean *in vitro* activity of glutathione S-transferase (GST) in crude cell-free extracts from susceptible and resistant black-grass plants was determined to be 83 and 154 nmol min^{-1} mg^{-1} protein, respectively (Table 3.3). Thus, the endogenous GST activity in resistant plants was twice that in susceptible plants, when expressed on either a protein or fresh weight basis. Glutathione reductase (GTR) activity was much lower than GST activity in both susceptible and resistant plants, 17.8 and 16.2 nmol min^{-1} mg^{-1} protein, respectively. There was no great difference in glutathione content, although slightly more was measured in resistant plants, 42.6 μg g^{-1} fresh weight compared with 36.29 μg g^{-1} fresh weight. The optimum pH for GST activity in extracts from susceptible and resistant tissue was pH 7.5 for both biotypes and so the same assay buffer could be used in all measurements (Table 3.4). Although changing the pH altered the specific activity, the differential between susceptible and resistant biotypes was approximately the same at each pH.

Enzyme kinetics were determined for GST in cell-free extracts from both biotypes. Firstly the CDNB concentration was maintained at 1 mM and the GSH concentration varied and then 2 mM GSH was used with increasing CDNB concentration. Although activity was optimum with 2 mM CDNB (Figure 3.2) this concentration increased the absorbance readings to over one, and thus the results were

Table 3.3 Glutathione content and the specific activity of GST and GTR on a fresh weight (fwt) and protein basis are compared for extracts from susceptible and resistant black-grass plants. Mean values ± SE, n = 4 experiments.

	$\mu g\ g^{-1}$ fwt	nmol min^{-1} mg^{-1} fwt		nmol min^{-1} mg^{-1} protein	
	GSH content	GST	GTR	GST	GTR
Susceptible	36.3 ± 5.3	4.7 ± 0.3	1.0 ± 0.1	82.7 ± 4.0	17.7 ± 1.5
Resistant	42.6 ± 3.9	9.6 ± 1.4	1.1 ± 0.1	154.0 ± 21.0	16.2 ± 1.3
r:s ratio	1.2	2.0	1.1	1.9	0.9

Table 3.4 The effect of assay buffer pH on GST activity (nmol min^{-1} mg^{-1} protein) measured in extracts from susceptible and resistant plants. Mean values ± SE, n = 2 experiments.

	pH 6.0	pH 6.5	pH 7.0	pH 7.5	pH 8.0
Susceptible	14.4 ± 1.3	29.6 ± 1.6	34.4 ± 1.5	44.6 ± 9.3	25.7 ± 16
Resistant	33.7 ± 3.1	50.6 ± 3.1	75.1 ± 3.0	87.9 ± 6.4	65.8 ± 4.2
r:s ratio	2.3	1.7	2.2	2.0	2.5

fwt = fresh weight.

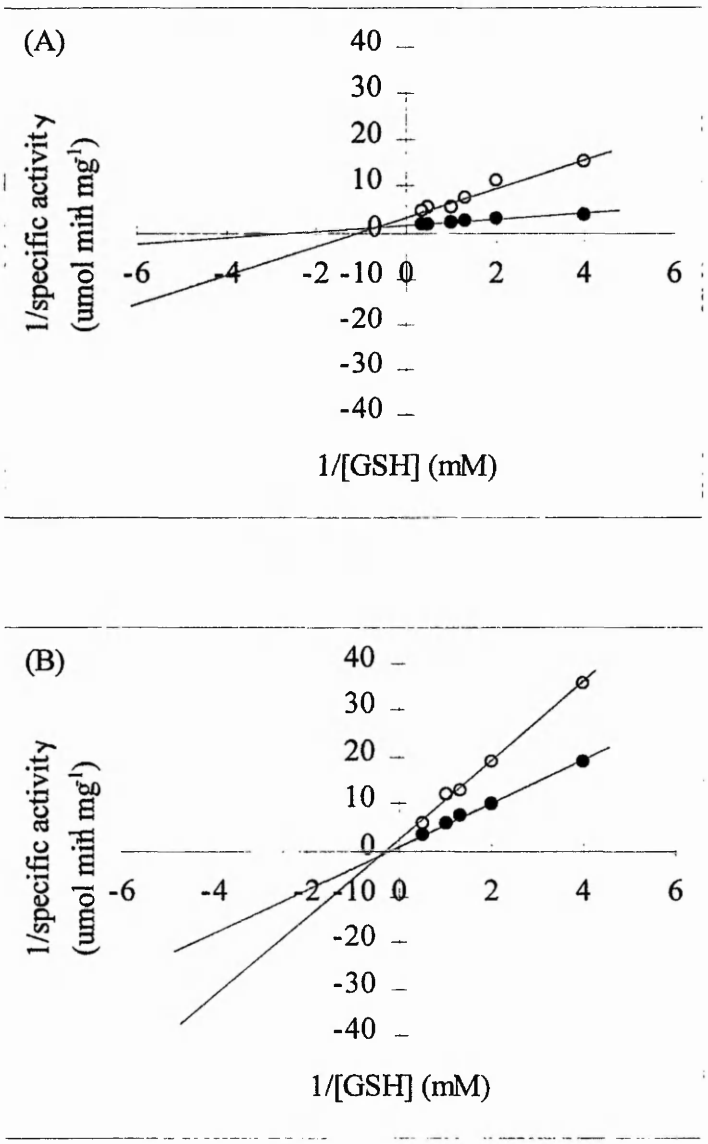


Figure 3.7 Lineweaver-Burke plots for the *in vitro* activity of GST at varying GSH (A) and CDNB (B) concentrations in extracts from susceptible (○) and resistant (●) tissue. Each point is a mean of 2 extracts.

inaccurate. For this reason 1 mM CDNB was used in all experiments. Lineweaver-Burke plots were made to estimate the K_m and V_{max} values for GST from both biotypes as shown in Figure 3.7. The K_m for GSH was calculated to be 0.8 mM for susceptible and 0.3 mM for resistant plants, hence GST in resistant plants has more than twice the affinity for GSH than GST in susceptible plants. The V_{max} values for GST from susceptible and resistant plants were calculated to be 400 and 750 nmol min^{-1} mg^{-1} protein, respectively (Table 3.5). This again gave a 2 fold difference between the biotypes. GST from both biotypes had the same affinity for CDNB (K_m of 3.4 mM), and the V_{max} values were calculated to be 260 and 560 nmol min^{-1} mg^{-1} protein for susceptible and resistant plants respectively. These V_{max} values were lower than the previous ones, which may be because the CDNB concentration was limiting activity. However the r:s ratio for V_{max} was approximately 2 in both sets of experiments. Both biotypes had a low affinity for CDNB which may be a consequence of CDNB not being the natural substrate.

Table 3.5. Enzyme kinetics for GST from susceptible and resistant black-grass.

	Constant [GSH]		Constant [CDNB]	
	K_m CDNB (mM)	V_{max} nmol min^{-1} mg^{-1} protein	K_m GSH (mM)	V_{max} nmol min^{-1} mg^{-1} protein
Susceptible	3.4 mM	260	0.8 mM	400
Resistant	3.4 mM	560	0.3 mM	750

3.3.2 The effect of chlorotoluron on glutathione S-transferase, glutathione reductase and the glutathione pool

Figure 3.8 shows the effect of field-rate chlorotoluron on GST activity over a 24 hour period after treatment. The herbicide did not alter the measurable GST activity in extracts from either susceptible or resistant plants. Glutathione reductase activity and glutathione content were also measured over the same time course (Figure 3.9) and no significant differences were observed in either biotype.

3.3.2.1 The effect of safeners on GST activity *in vitro*: The direct effect of benoxacor and fenclorim on GST activity was assessed by either adding then to the *in vitro* assay immediately before measuring activity, or incubating the cell-free extract with the safener for 30 min before measuring activity. The addition of 100 μM benoxacor or fenclorim to the GST assay had no effect on the *in vitro* activity obtained from extracts of susceptible or resistant plants (Figure 3.10). Pre-incubation with benoxacor and no effect on *in vitro* GST activity and fenclorim caused a slight decrease in activity. However, the pre-incubation period increased activity in all extracts from resistant and susceptible plants by 2 and 6 times, respectively. This indicates that the elevations in GST activity observed are not due to a direct interaction between the safener and endogenous GSTs.

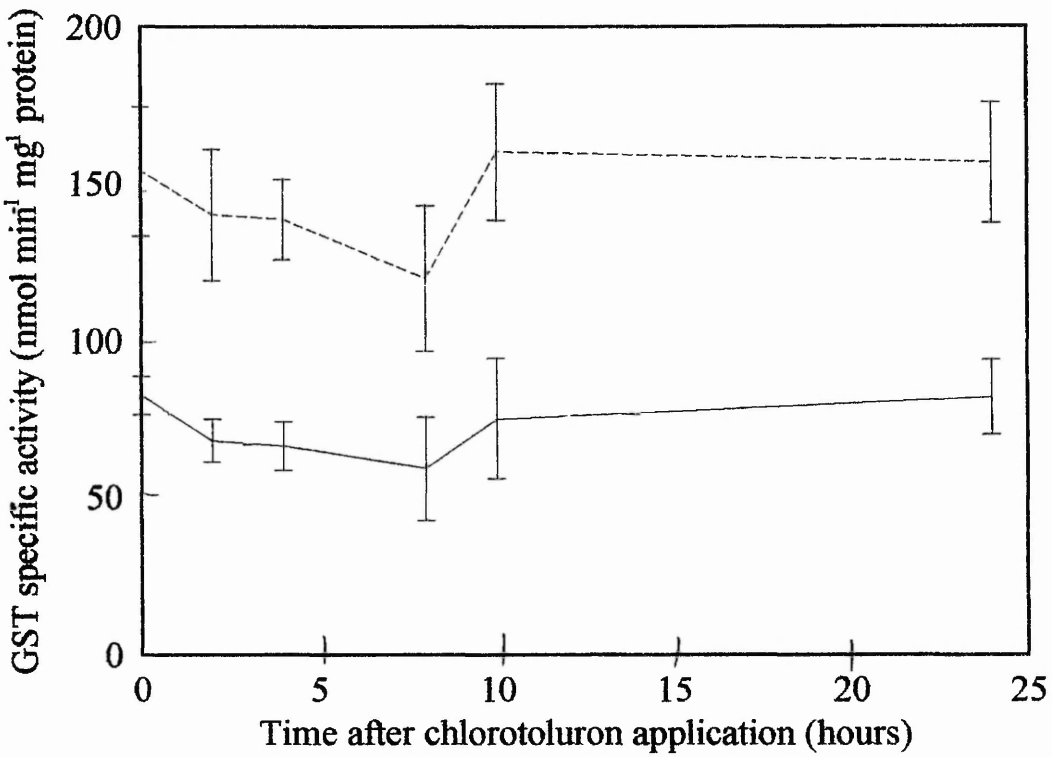


Figure 3.8 GST activity measured in susceptible (—) and resistant (---) black-grass over a 24 hour time period after they were sprayed with field rate chlorotoluron. Values represent means of 4 separate experiments ± SE.

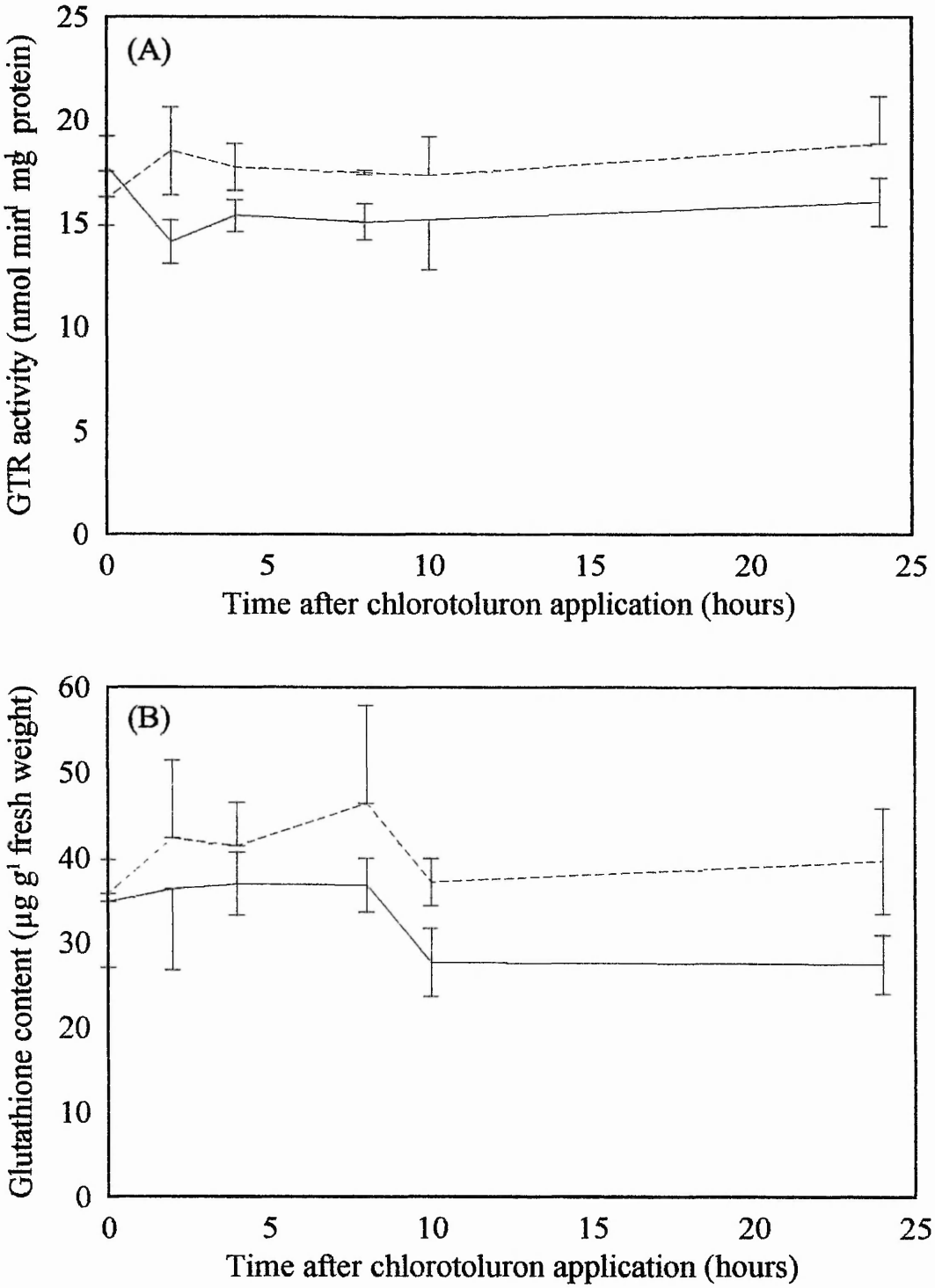


Figure 3.9 GTR activity (A) and glutathione content (B) measured in susceptible (——) and resistant (- - -) black-grass over a 24 hour time period after they were sprayed with field rate chlorotoluron. Values represent means of 4 separate experiments ± SE.

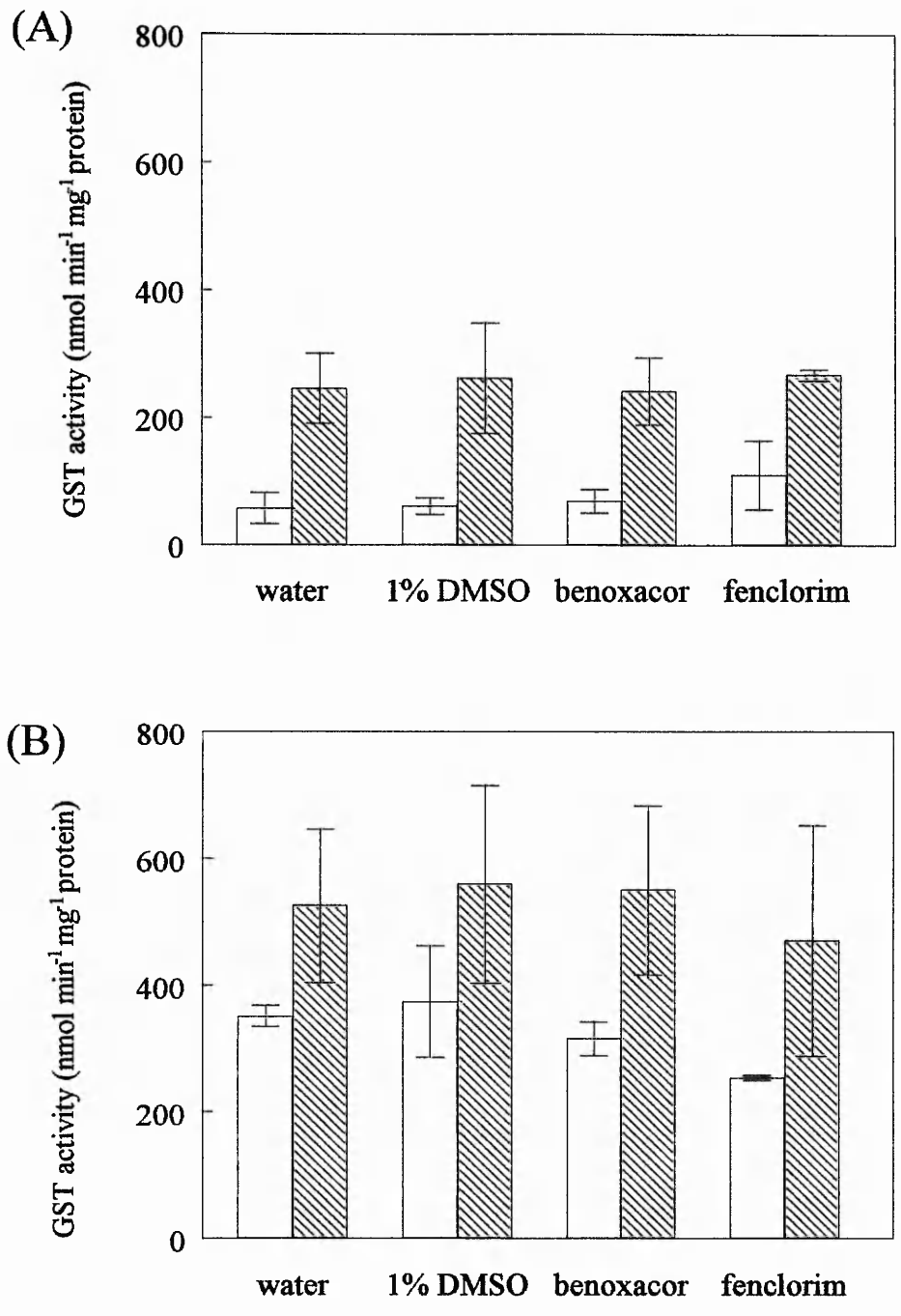


Figure 3.10 The effect of adding benoxacor or fenclorim to the GST assay reaction mixture (A) immediately before measuring activity, and (B) pre-incubating the chemicals with the extract for 30 mins before measuring activity. The results shown are means (± SE) of 2 extracts from either susceptible (▨) or resistant (▭) plants.

3.3.3 The exposure of black-grass plants to safeners and synergists using a hydroponic system

Hydroponically grown susceptible and resistant plants were pre-treated with one of 7 safeners and 2 synergists for 24 hours before cell-free extracts were made. The GST activity in these extracts is shown in Figure 3.11 as a percentage of control values. Specific activities from control plants were between 47 and 86 nmol min^{-1} mg^{-1} for susceptible and 157 to 273 nmol min^{-1} mg^{-1} for resistant plants. GST activity was increased in susceptible plants by 150% or more by benoxacor, flurazole, oxabetrinil and fenclorim, whilst GST activity in resistant plants was only increased by this amount by benoxacor. The maximum rates obtained with 100 μM benoxacor were 138 nmol min^{-1} mg^{-1} for susceptible and 323 nmol min^{-1} mg^{-1} for resistant plants. Treatment with 200 μM benoxacor caused a further increase in activity in both biotypes (Figure 3.12), although the 50 μM dose increased activity in susceptible plants. All the safeners which increased GST activity had a relatively greater effect on the susceptible plants. Tridiphane, ABT, fenchlorazole ethyl and OTC had no significant effect on GST specific activity in either biotype.

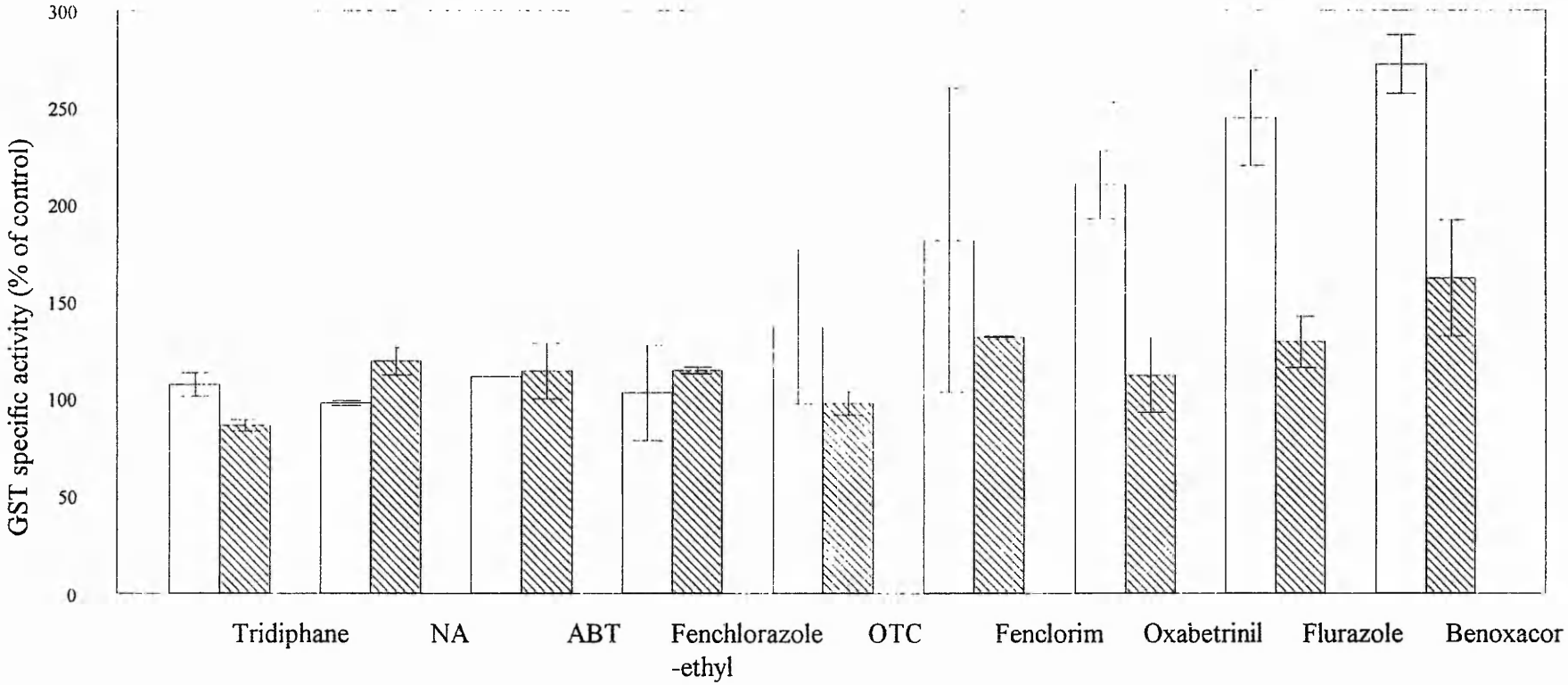


Figure 3.11 The effect of treating susceptible (▭) and resistant (▨) plants with 100µM of a chemical safener for 24 hours before extraction. Values represent means from at least 2 separate experiments, ± SE.

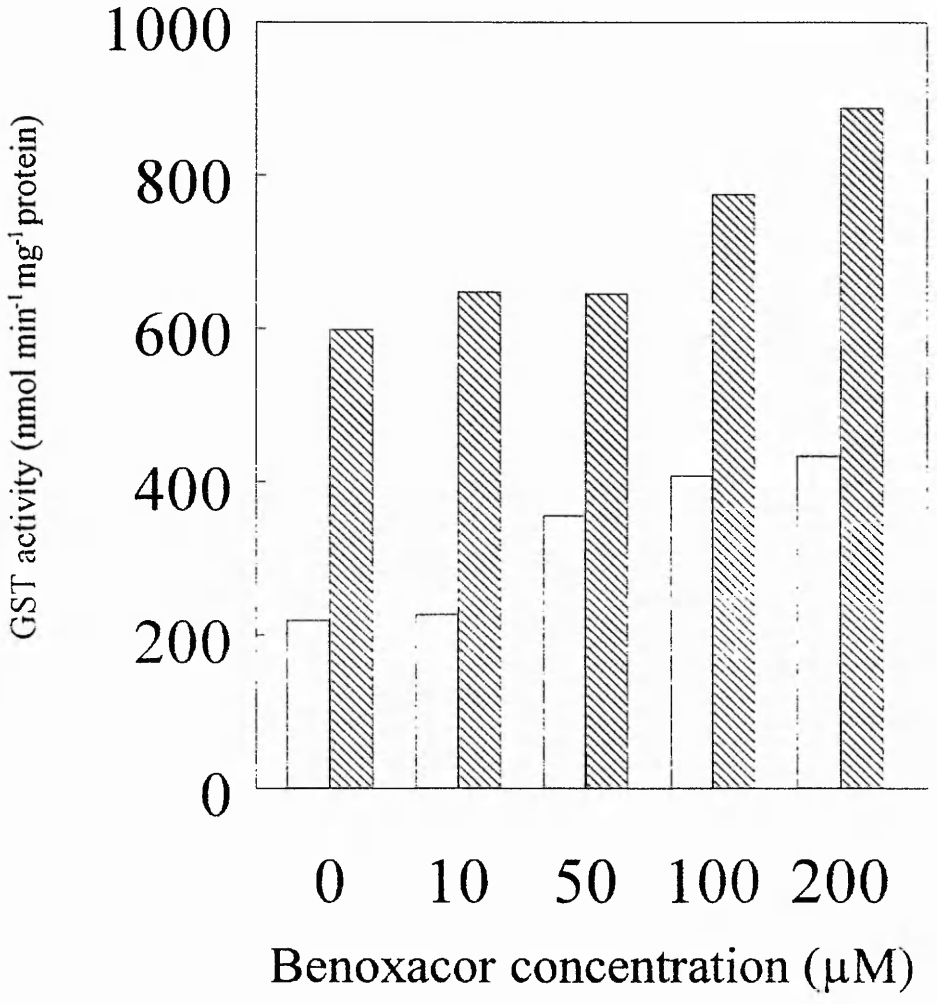


Figure 3.12 The effect of increasing benoxacor concentration on GST activity in susceptible (▭) and resistant (▨) plants. Values presented are from one typical experiment.

3.3.4 The interaction between chlorotoluron and tridiphane using intact plants

The synergists ABT and tridiphane had little effect on GST activity when added to the nutrient solution in the hydroponic system, although tridiphane did appear to depress GST activity in resistant plants by about 15% (Figure 3.10). A possible interaction between tridiphane and chlorotoluron when sprayed on whole plants was also investigated. Two compounds are said to be synergistic if their combined action is greater than the sum of their individual activities. Thus, for each combination of herbicides used, the expected reduction in fresh weight was calculated from the individual results and these were compared with the observed values.

Table 3.6 shows the interaction of 3 rates of tridiphane with field rate chlorotoluron (3.5 kg ai ha^{-1}). Because field rate chlorotoluron alone reduced the dry

weight of susceptible plants by 90% and all the rates of tridiphane used reduced it by over 30%, the predicted activity of the herbicide combinations were all over 100% and so any synergism would not be detected. All 3 combinations reduced growth by about 90% and, given that even dead plants have a certain weight, these results are inconclusive. When the compounds were combined at lower concentrations the predicted reductions in dry weight were obviously lower, as shown in Table 3.7. At two of the rates used (0.88 and 1.75 kg ai ha^{-1} of chlorotoluron each with 0.5 kg ai ha^{-1} tridiphane) the activity was twice that predicted and the activity of the other combination was a third more than expected. This indicated that there was a synergistic interaction between chlorotoluron and tridiphane at the rates used on susceptible plants.

As expected, chlorotoluron at field-rate and below had a minimal effect on resistant plants, but the maximum rate of tridiphane used (1 kg ai ha^{-1}) reduced dry weight by 50%. Most of the herbicide combinations reduced dry weight by amounts similar to those predicted (Table 3.6 and 3.7) with the exception of 3.5 kg ai ha^{-1} chlorotoluron with 1.0 kg ai ha^{-1} tridiphane (the highest amount used) which was 33% less active than predicted.

There are inconsistencies between the two tables of results. For example, 3.5 kg ai ha^{-1} chlorotoluron reduced the growth of susceptible plants by 89.4% in Table 3.6 but only by 51.9% in Table 3.7. Tridiphane is also less active in the second experiment, ie compare Tables 3.7 and 3.6. The plants used in the second experiment were more mature than those in the first, which may explain why they were generally less susceptible to the herbicides, but the results were normalised by their expression as

Table 3.6 The percentage reduction in dry weight caused by field-rate chlorotoluron (ctu) and 3 doses of tridiphane (td) alone and in combination (mean values where n=7-9 plants).

Treatment kg ai ha^{-1}	Susceptible		Resistant	
	Observed	Predicted	Observed	Predicted
3.50 ctu	89.4		8.52	
0.25 td	31.9		7.96	
0.50 td	64.4		42.5	
1.00 td	70.0		50.6	
0.25 td + 3.5 ctu	91.6	121	11.1	16.5
0.50 td + 3.5 ctu	88.2	154	33.2	50.0
1.00 td + 3.5 ctu	90.4	159	65.2	59.1

Plants were sprayed with tridiphane 24 hours before chlorotoluron. Expected values were calculated as the sum of the percentage reduction caused by the 2 chemicals when applied alone.

Table 3.7 The percentage reduction in dry weight caused by field-rate tridiphane (td) and 3 doses of chlorotoluron (ctu) alone and in combination (Mean values where n=7-9).

Treatment kg ai ha^{-1}	Susceptible		Resistant	
	Observed	Predicted	Observed	Predicted
0.50 td	4.6		32.9	
0.88 ctu	25.7		-5.28	
1.75 ctu	32.0		4.88	
3.5 ctu	51.9		-6.12	
0.88 ctu + 0.5 td	59.2	30.3	36.4	27.6
1.75 ctu + 0.5 td	72.3	36.7	39.3	37.7
3.50 ctu + 0.5 td	80.8	56.6	32.8	26.8

Plants were sprayed with tridiphane 24 hours before chlorotoluron. Expected values were calculated as the sum of the percentage reduction caused by the 2 chemicals when applied alone.

percentages of the control plants. Hence, this data suggested that at the rates used, chlorotoluron and tridiphane acted in a synergistic manner on susceptible but not on resistant plants.

3.3.5 The effect of chlorotoluron, safeners and synergists on gaseous exchange.

In Chapter 2 it was demonstrated that when black-grass plants were treated with field-rate chlorotoluron, susceptible plants had ceased to perform photosynthesis within 24 hours after application, whereas photosynthesis by resistant plants was not affected. This characteristic was used to provide a quick method of determining the efficacy of chlorotoluron when applied alone or in combination with safeners and synergists. In contrast to the work in Chapter 2, photosynthesis was determined using a portable leaf chamber enclosing a section of attached leaf (a non-destructive assay) and chlorotoluron was added to the hydroponic solution. To estimate the molar concentration required to produce the same response as aerially applied field-rate chlorotoluron, susceptible and resistant plants were exposed to a range of doses (0, 1.2, 2.4, 4.8, 9.6 μM) in the hydroponic system.

Figure 3.13 indicates the rates of photosynthesis by these plants at 0, 4, and 24 hours after treatment with chlorotoluron. At the lowest concentration used (1.2 μM) photosynthesis by susceptible plants had halved after 24 hours. Chlorotoluron at 2.4

μM had stopped photosynthesis after 24hours and at 4.8 and 9.6 μM it was completely inhibited after 4 hours. Photosynthesis by resistant plants was not affected by 1.2 or 2.4 μM, but 4.8 μM reduced this process by about a third after 4 and 24 hours. At 9.6 μM chlorotoluron photosynthesis was completely inhibited after 4 hours, but had partially recovered after 24 hours. This partial recovery by resistant plants may have resulted from enhanced ability to metabolise chlorotoluron, detoxifying it to the extent that the effective concentration within the plant was reduced. Thus, it was concluded that 2.4 μM approximated to field-rate chlorotoluron because photosynthesis by susceptible plants stopped after 24 hours, but resistant plants were not affected.

A crude estimation of ED_{50} values based on photosynthetic rates was 0.8 and 6.5 μM for susceptible and resistant plants, respectively. This gave a Resistance Factor of 8 which was somewhat lower than the 42 calculated in Chapter 2. This reflects the difference in parameters measured as well as differences in growth medium and herbicide application. The susceptible and resistant control plants showed a reduced photosynthetic rate after 4 hours relative to that at 0 and 24 hours. This reduction at 4 hours was also seen in several of the non-lethal treatments. This could have been caused by the natural diurnal rhythm of stomatal opening and closing according to the photoperiod. Times 0 and 24 hours after application were in the middle of the day, when photosynthesis is presumed to be maximal, but 4 hours after application occured late in the day.

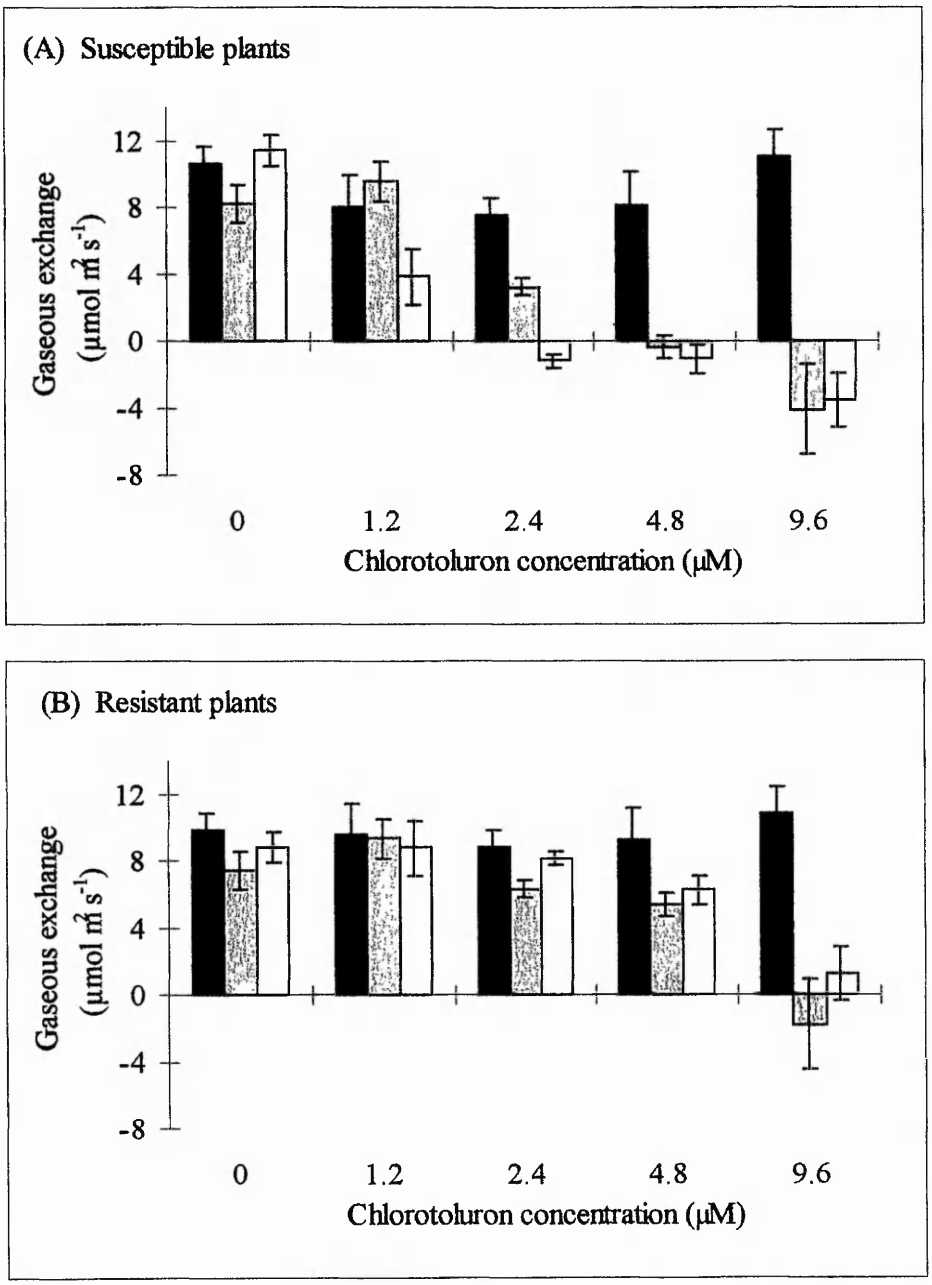


Figure 3.13. The effect of chlorotoluron on photosynthesis by susceptible (A) and resistant (B) black-grass plants when grown in the hydroponic system. Measurements were taken 0 (■), 4 (▩), and 24 (□) hours after treatment using a portable leaf chamber.. Values expressed as means ± SE where n = 3.

It was also necessary to establish how photosynthesis by susceptible and resistant plants was affected by the safeners and synergists under investigation, namely the synergists ABT and tridiphane, and the safeners benoxacor and fenclorim. Incubation with 100 μM of each chemical for 24 hours had no significant effect on photosynthesis by susceptible and resistant plants (Table 3.8). There was an indication that tridiphane and ABT may slightly increase photosynthesis by both biotypes, but the large standard errors and small number of replicates prevent any conclusions being drawn.

Susceptible and resistant plants were pre-treated with each chemical for 24 hours and then supplied with 2.4 μM chlorotoluron for 24 hours. Chlorotoluron alone inhibited photosynthesis by susceptible and resistant plants by 65% and 25% respectively (Figure 3.14), but pre-treatment with benoxacor reduced these values to 47% and 0%. This indicated that there was an interaction between the two reducing chlorotoluron toxicity, which was presumed to be by benoxacor stimulating chlorotoluron metabolism. Fenclorim also reduced chlorotoluron toxicity to both biotypes, but the effect was not as pronounced. Figure 3.15 demonstrates that similar results for benoxacor and fenclorim were obtained when a higher concentration of chlorotoluron was used.

ABT is well known to be synergistic with chlorotoluron in black-grass but did not have any effect in this experiment (Figure 3.14). This was because ABT is only active at high doses and a 100 fold increase in dose would be required to obtain a significant synergistic response (Kemp & Caseley 1987). Tridiphane did not alter the effect of chlorotoluron on susceptible plants, and appeared to slightly decrease its effect

on resistant plants. This may be because the concentrations used were too low or may indicate the tridiphane does not inhibit chlorotoluron metabolism.

Table 3.8 Photosynthesis (μmol CO_2 m^{-2} s^{-1}) by susceptible and resistant plants 24 hours after treatment with benoxacor (n=15), fenclorim (n=10), tridiphane (n=5) and ABT (n=5). Values represent means ± SE (control, n=15).

	Susceptible	Resistant
Control	11.1 ± 0.4	11.9 ± 0.4
Benoxacor	10.3 ± 0.7	11.0 ± 0.5
Fenclorim	11.5 ± 0.6	11.3 ± 0.5
Tridiphane	13.8 ± 1.4	11.6 ± 1.0
ABT	13.8 ± 1.1	13.1 ± 0.7

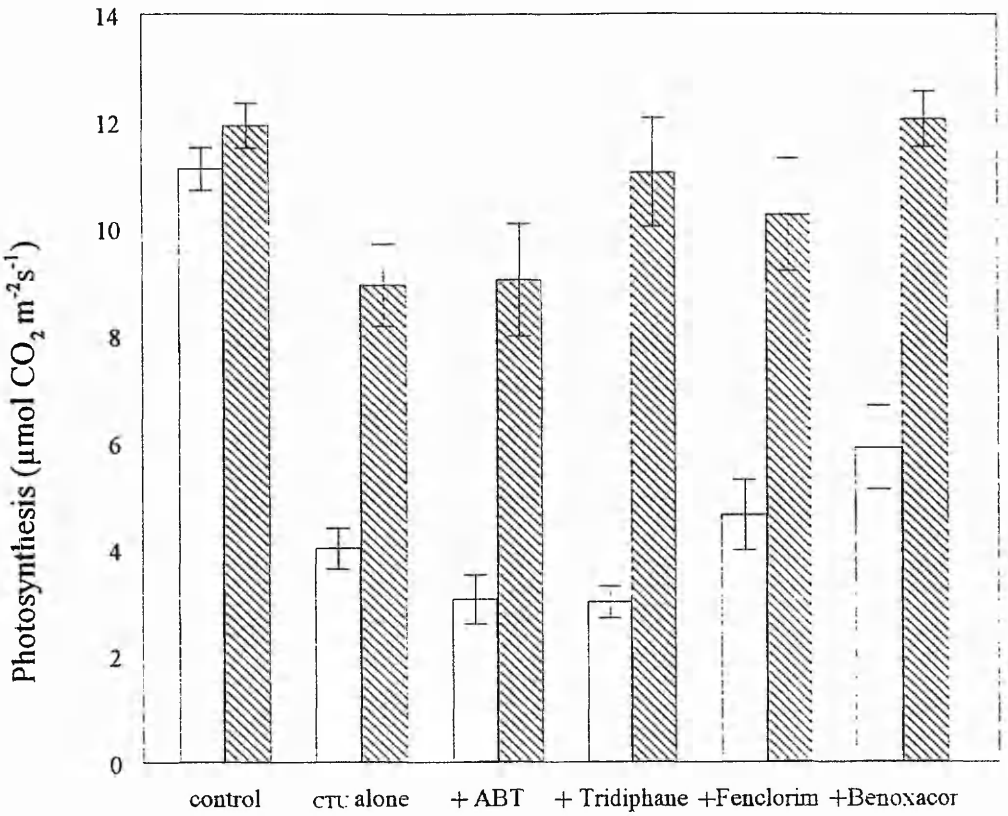


Figure 3.14 The effect of various chemicals on the reduction in photosynthesis caused by chlorotoluron in susceptible (▭) and resistant (▨) black-grass. Plants were pre-incubated with a 100 µM solution of a chemical for 24 hours, then treated with 2.4 µM CTU. The graph shows photosynthetic rates 24 hours after CTU treatment. Values given as means ± SE where n=10 (control, CTU alone and benoxacor) or 5 (ABT tridiphane and fenclorim).

n = number of plants

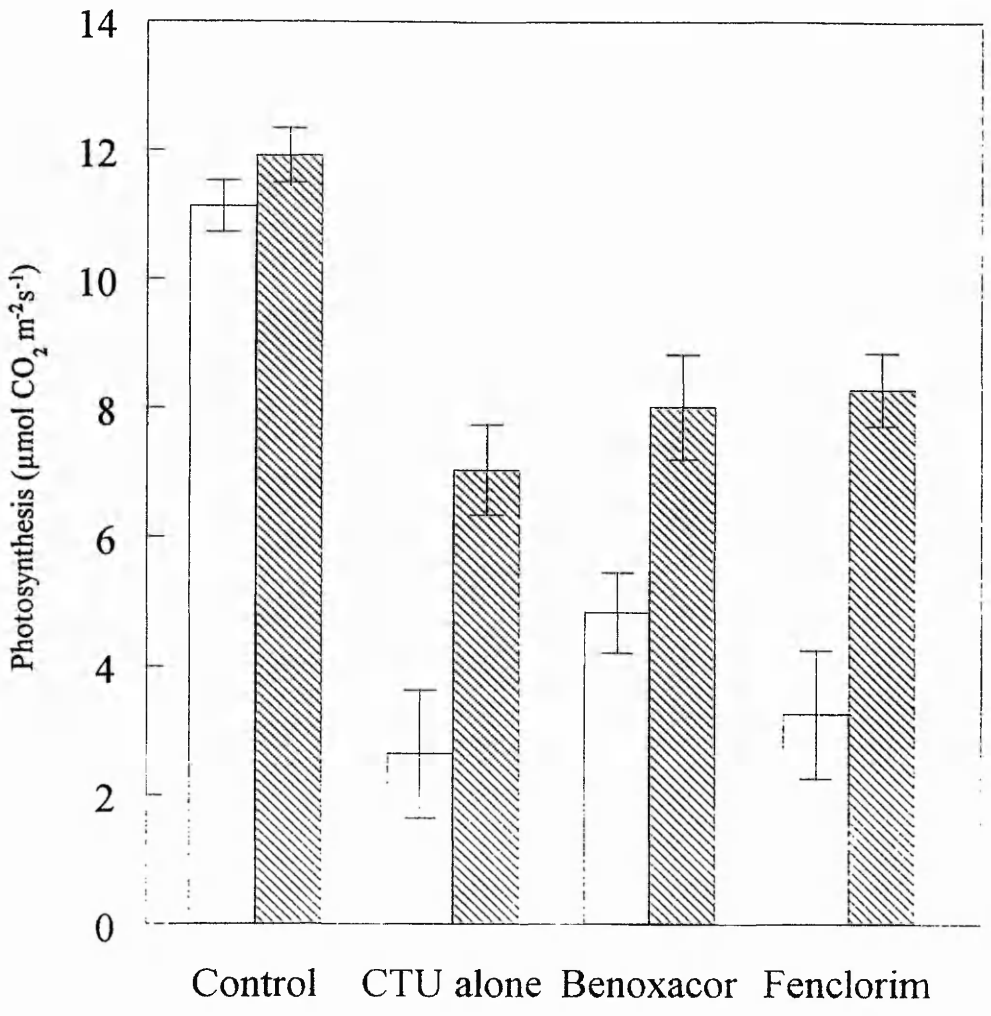


Figure 3.15 The reduction in photosynthesis by susceptible ( ) and resistant ( ) plants when treated with 4.8 µM CTU, with and without pretreatment with 100 µM benoxacor or 100 µM fenclorim. Values represent means ± SE, n = 5.

n = number of plants

3.4 Discussion

The endogenous specific activity of GST from resistant plants was consistently at least twice that of GST from susceptible plants, irrespective of whether activity was expressed on a protein or fresh weight basis. This 2:1 ratio of GST activity was maintained even when the pH was altered and different concentrations of substrates were used, in contrast to Fuerst *et al* (1993) who increased the r:s ratio by elevating the concentration of metolachlor in the assay medium. Variations in GST activity between plant species are known to occur naturally, but very few studies have been carried out to assess differences between biotypes of the same species. GST activity and GSH content in two maize hybrids tolerant and sensitive to acetochlor were found to be higher in the tolerant cultivar, providing an intrinsic protective mechanism (Jablonkai & Hatzios 1991). A similar study of 11 maize cultivars also demonstrated that higher GST activities were characteristic of the metolachlor-tolerant cultivars, although it failed to prove any correlation between glutathione content and herbicide tolerance (Cottingham *et al* 1993). A study of associated weed species correlated GST activity with herbicide injury but again there was no relationship between glutathione availability and herbicide selectivity (Hatton *et al* 1995). This corresponds well to the findings presented here that differences in glutathione content between susceptible and resistant black-grass were not significant and is consistent with the lack of difference in glutathione reductase activity. The values determined for GST specific activity in black-grass were lower than those quoted for maize (Ekler *et al* 1993), but weed species are reported to have GST activities of 3 to 10 fold less than maize. It is also difficult to make direct comparisons

when different procedures and enzyme purities are used. For example, GST activity in sorghum has been recorded as 275 and 830 nmol min^{-1} mg^{-1} protein (Gronwald *et al* 1987, Dean *et al* 1990). The glutathione levels recorded for susceptible and reisistant black-grass, 36 and 42 $\mu g\ g^{-1}$ fresh weight respectively, were similar to reported values of 54 $\mu g\ g^{-1}$ in wheat and 57-110 $\mu g\ g^{-1}$ in maize (Jablonkai & Hatzios 1991).

The difference in specific activity of GST from susceptible and resistant plants can be related to the affinity of the enzyme for glutathione. The K_m for GSH was calculated to be 0.8 mM for susceptible and 0.3 mM for resistant plants, which is similar to the published figure of 0.29 mM for GST IV in maize (Irzyk & Fuerst 1993). This may explain how a higher rate of conjugation could be maintained without a corresponding increase in glutathione content. However, GST in both biotypes had the same affinity for CDNB (K_m 3.37 mM susceptible and 3.36 mM resistant) when GSH concentration was held at 2 mM. The isozymes GST I and II in maize had a K_m of 3.0 and 2.8 mM respectively when CDNB was used as the substrate (Mozer *et al* 1983). This relatively low level of affinity indicated that CBNB is not the natural substrate for GST. It is possible that there are either different endogenous GST isozymes in susceptible and resistant black-grass, or that the isozymes are not expressed in the same concentrations in each biotype, which would provide one explanation for the difference in enzyme kinetics.

Despite the extensive studies of GST and safeners in crops, relatively little is known about the effects of safeners in weeds. The safeners used belonged to diverse chemical groups and so its not surprising that they differed in efficacy. The safeners may be categorised into 2 groups according to their effects on black-grass in the

hydroponic system. Fenclorim, oxabetrinil, flurazole and benoxacor increased the *in vitro* GST activty whereas naphthalic anhydride, fenchlorazole-ethyl and OTC had little effect. The active safeners may be ranked according to their ability to elevate GST activity as benoxacor > flurazole > oxabetrinil > fenclorim. This trend reflects the efficacy of safeners in sorghum which were determined as flurazole > oxabetrinil > naphthalic anhydride (Gronwald *et al* 1987, Dean *et al* 1990). However it must be remembered that these results are based on the amount of GST activity which can be measured *in vitro* using CDNB as the substrate. In maize, safeners often induce the activity of an isoenzyme specific for the conjugation of a particular herbicide substrate. This isozyme would not be detected using this method but any such isozyme induction was always accompanied by an increased ability to conjugate CDNB. Therefore, the increased GST activity may be indicative of a much higher *in vivo* increase. This is also true for the endogenous activity of susceptible and resistant black-grass. Thus, much higher r:s ratios are obtained when a herbicide substrate such as metolachlor is used instead of CDNB (Fuerst *et al* 1993).

The safeners all increased GST activity far more in susceptible than resistant black-grass, although specific activity was always higher in resistant plants. This could indicate that there are more inducible isozymes in susceptible plants which can conjugate CDNB. Alternatively, it may indicate that the activity of endogenous GST (which can use CDNB) is elevated more in susceptible plants, whereas specific isozymes are elevated in resistant plants which cannot be detected. There may be a threshold level beyond which activity cannot be improved but the results do not indicate that, or that it is due to limitations of the assay.

Similar arguments may be put forward to explain why chlorotoluron did not increase GST activity in either biotype of black-grass over the time period used. There are several incidences where herbicides have been shown to induce GST isozymes specific for their own metabolism (Flurry *et al* 1995, Uotila *et al* 1995), which may be due to substrate induction. Metolachlor increased its own conjugation with glutathione *in vivo* but without any increase in GST activity towards CDNB (Dean *et al* 1990), which could indicate that chlorotoluron induced an isozyme which was not active towards CDNB. However, there is currently no proof that chlorotoluron can conjugate with glutathione and thus it may well have no effect on GST enzymes.

It was not surprising that naphthalic anhydride had no effect in this study because it is known to have only a low level of activity in a certain number of species (Gronwald *et al* 1987). Its exact mode of action is not known and there are several reports indicating that it elevates cytochrome P450 activity (Barret & Maxon 1991, Zimmerlin & Durst 1992), although this is not always associated with an increase in herbicide metabolism (McFadden *et al* 1990). Flurazole, oxabetrinil and fenclorim were all active and are known to increase GST activity in maize, sorgum and rice (Mozer *et al* 1983, Dean *et al* 1990, Han & Hatzios 1991). Flurazole also increased glutathione content and has at least two possible modes of action. It can be conjugated with glutathione itself by a GST-mediated reaction, and it was proposed that this conjugate can inhibit the feed-back inhibition of glutathione biosynthesis. The resulting increase in glutathione content would indirectly elevate GST activity (Breaux *et al* 1989). However, flurazole is also known to increase the mRNA coding for the subunits of GST

I in maize (Weigland *et al* 1986). This apparent complexity and lack of basic understanding demonstrates the difficulties encountered when trying to elucidate the interactions of safeners with plant metabolism.

The interaction between chlorotoluron and tridiphane in whole plants was synergistic in susceptible but not in resistant black-grass, although this may have been due to the low rates of chlorotoluron used. The synergism indicated that tridiphane inhibited the enzymes responsible for chlorotoluron metabolism. Tridiphane is a good inhibitor of GST enzymes (Mozer *et al* 1983, Zorner 1985, Lamoureux & Rusness 1986) but has also been shown to inhibit cytochrome P450s (Moreland *et al* 1989). Chlorotoluron is metabolised by cytochrome P450s but there may be some involvement of GST, thus, the results are difficult to interpret. Caseley *et al* (1991b) concluded that the interaction between these herbicides indicated that tridiphane inhibited cytochrome P450s, but this has yet to be proved.

Benoxacor was the most effective safener in black-grass, elevating GST activity by 1.5 and 3 times in resistant and susceptible plants respectively. It was also able to reduce the toxicity of chlorotoluron to both biotypes of black-grass. Benoxacor can protect maize from herbicide injury by elevating GST activity 4 fold, with selective enhancement of the constitutive GST isozymes and differential induction of new isozymes (Dean *et al* 1991, Fuerst *et al* 1993). Again, there is more than one possible explanation for how benoxacor can elevate GST activity. It may be by increasing the biosynthesis of glutathione since heightened APSSTase and ATP-sulfurylase activity has been recorded (Farago & Brunold 1990), but this may be an indirect consequence of

elevated GST activity, since it was not considered to by the main mechanism by other workers (Cottingham & Hatzios 1991). Benoxacor was also demonstrated to induce the *de novo* synthesis of GST mRNA transcripts (Miller *et al* 1994). This elevation of GST activity was correlated with a reduction in chlorotoluron injury to black-grass, but does not prove that this is due to the conjugation of chlorotoluron with glutathione. Indeed it is possible that benoxacor is also elevating the action of cytochrome P450s, since it was shown to double the P450 content of maize and increase primisulfuron metabolism (Fonne-Pfister *et al* 1990). Thus, the interaction between chlorotoluron and benoxacor may depend on one or other or both of the two main groups of herbicide detoxifying enzymes.

3.5 Conclusion

The endogenous GST activity in resistant black-grass is twice that in susceptible plants when measured *in vitro* although glutathione concentrations are the same. The GST activity can be further elevated in both biotypes by pre-treatment with certain safeners. The increase in GST activity due to pre-treatment with benoxacor can be correlated with a reduction in chlorotoluron injury to the plants. However, this relationship cannot be proved since the GST assay only measures the activity of isozymes which can utilise CDNB and the exact mode of action of benoxacor is not known.

Chapter Four

General Discussion

4.1 Introduction

The results from this study of herbicide resistant black-grass are presented in Chapters 2 and 3. The Peldon population had a resistance factor of 42 and resistance was not associated with a reduction in growth or photosynthesis. The resistant black-grass plants had 2-3 times more endogenous GST activity than the susceptible population and the activity could be modulated by various safeners and synergists. As with all scientific investigations, these results were gained under specific experimental conditions and using certain pieces of equipment. It is conceivable that different experimental procedures or even alternative equipment could produce results of a different nature. Therefore, it is important to consider the appropriateness of the techniques used. The conclusions put forward here do not exist in isolation from other studies of herbicide resistance and metabolism, and need to be considered from other points of view. It is only then that a hypothesis can be put forward to identify the possible roles of elevated GST activity in black-grass. Finally, the results gained so far point to an incomplete understanding and so there is much scope for further work in this important area of weed science.

4.2 A critique of the techniques and experimental procedures used

4.2.1 The use of whole plants

The work in this thesis is dependant on the use of whole plants, either in the measurement of total growth or photosynthesis, or to provide plant material for assays

of metabolism. The advantages of whole plants over isolated plant organs, cell cultures or isolated organelles such as chloroplasts, is that any model will most closely resemble the real field situation. The amount of herbicide reaching the target site is governed by a complex interaction of the physiology of the plants, physical properties of the chemicals and the soil and the influence of the environment, as considered in Chapter 2. The advantages of having an overall picture resulting from all plant processes must be balanced against the fact that the contribution of each individual process cannot be quantified. For example, the sensitivity of the target sites to chlorotoluron cannot be accurately compared as the amount reaching there is unknown and may not be the same in both biotypes. However, if chlorotoluron never reaches the target site in resistant whole plants, then the sensitivity of the target site is irrelevant. Many other systems have been used to study the action of photosynthesis inhibiting herbicides such as measuring electron transport in isolated chloroplasts (Devine *et al* 1993), chlorophyll fluorescence (Habash *et al* 1985), analysis of chlorophylls and carotenoids by HPLC (Young *et al* 1989) and photofluorography (Yanase & Andoh 1992). A recent review of the techniques used to determine herbicide resistance concluded that glasshouse pot grown plants still provided the most appropriate test, although chlorophyll fluorescence of intact leaves was much quicker and the ED_{50} values related well to those from whole plant fresh weight (Moss 1995).

There is a trend towards using routinely maintained cell cultures for providing quick results in many areas of herbicide research. The herbicide can easily enter the cell to interact with target sites and metabolic enzymes, but there is no differentiation or often chlorophyll, and metabolism may not always be the same as in whole plants. The

usefulness of this approach depends on the type of results desired and the resources available. Experiments with cell cultures save time and space, and are much less labour- intensive than growing pots of whole plants. The dose response curves from whole plants and isolated cells are of a similar shape and the potency rankings of herbicides are usually the same, but cells are usually more sensitive than would be expected for plants (Olofsdotter *et al* 1994). It has been noted that low or sub-lethal herbicide doses often stimulate cell growth which makes the results more difficult to interpret. The differences between resistant and susceptible biotypes can be less marked in cell culture. For instance, a 180 fold resistance to chlorsulfuron was observed using whole plants compared with a 30 fold difference in cell cultures (Swisher & Weimmer 1986). There can also be problems with chemical shifts as compounds are more likely to be hydrolysed in an aqueous than in a solid growth medium.

There have been many studies of herbicide metabolism in achlorophyllous cell cultures which depend on complex carbohydrates as a carbon source in the medium. The composition of the nutrient media will affect the speed of cell growth and their ability to metabolise herbicides. The metabolism of chlorotoluron by cotton and maize was shown to occur at different rates according to the media composition, but the species specific metabolites produced were the same as in plants (Cole & Owen 1987a). The age of the cell culture can have a more significant effect, for example, when cultures of *Lactuca sativa* were over five years old they lost the ability to perform ring-methyl hydroxylations (Cole & Owen 1988). Cells also show no differentiation and some herbicide resistance mechanisms partly depend on differentiated structures. The metabolism of 3, 4-dinitroaniline was directed towards different end products in leaves

and isolated cells because the whole plants made use of the extensive cell walls for the deposition of residues. The cells where unable to compartmentalise the metabolites and so formed soluble end products instead (Gareis *et al* 1992).

Whole plants were used to provide the plant material for the GST and glutathione assays in this study for much the same reasons. A maize cell line was generated from plants known to actively conjugate atrazine to glutathione but the cell cultures lost their activity towards atrazine during primary dedifferentiation (Edwards & Owen 1988). Other differences in herbicide conjugation pointed to the presence of different GST isozymes in plants and cell cultures (Edwards & Owen 1986). Thus, there needs to be further characterisation of the GST isozymes and their activities in cell cultures before they can be used to provide information about whole plants. In conclusion, whole plants provide the best system in which to study growth, photosynthesis and metabolism.

4.2.2 The measurements of photosynthesis

The gas exchange system used was an "open system" which was chosen for its advantages over the closed and semi-closed systems. In the closed system, air is continually recycled and the carbon dioxide concentration steadily increases with time. It is simple to set up and requires no measurement of the flow rate of air, which can be a large source of error if not monitored accurately. The main disadvantage is that photosynthesis does not reach a steady state, which creates two sources of error. Photosynthesis only increases in a linear fashion with carbon dioxide concentrations in the range of 100-200 $\mu l\ l^{-1}$, and it is very difficult to define the exact concentration at

which measurements were taken. Stomata open and close according to carbon dioxide concentrations, but they respond very slowly and there can be a delay of up to 60 min in response depending on the prevailing environmental conditions. Thus, this method can only be used when the changes in carbon dioxide concentration are very small (Sestak *et al* 1971). With all experimental designs air leaks are a major source of error, but in the closed system this error is compounded with each circulation of air and huge errors can result. In this study measurement were taken over a relatively long period of time and the treatment of some plants resulted in large changes in carbon dioxide concentration which meant that this method was unsuitable. The modern IRGAs, such as the LCA4 (ADC), are able to accurately monitor the flow rate so it is no longer an advantage not to require flow rate measurements.

The open system allows continuous readings of small fluctuations of carbon dioxide with reasonable accuracy and there is no recirculation of the air stream. It also has the advantage that simultaneous measurements of several plants can be made and transpiration and stomatal conductance can also be monitored. In some systems water vapour is removed to prevent condensation in the chambers, but this was not considered necessary in this case because no condensation was observed in the chambers used. The open system is most accurate when a differential of 20 ppm of carbon dioxide between the reference air and air from the plant chamber is obtained, and the flow rate can be adjusted for the speed of photosynthesis (Sestak *et al* 1971). This differential was difficult to obtain due to the simultaneous measurements of plants conducting photosynthesis at different rates. The differential was normally between 10 and 20 ppm, but to obtain a higher differential would have necessitated using a lower flow rate and

this may have increased boundary layer resistance. Another major advantage of the open system is that if the air is pushed, not sucked through the system, then a positive pressure will build up in the chamber and any leaks will be outwards, thus air-tight seals are not so important. This is a particularly good way to compensate for the almost inevitable leak around the stem or petiole where the plant or leaf enters the chamber. It also ensures that the air pressure in the chambers is close to atmospheric pressure. Unfortunately, the system used in this study involved a gas handling device with its own internal and external pumps, and to push air through the system would not have been compatible with them. The air was sucked through the system by these pumps and the internal pumps in the IRGA. However, tests showed that, on the whole, the system was free of leaks and a good seal was obtained around the plant stem.

Having considered the system as a whole, the other major consideration is the type of plant chamber. The main problem with any gaseous exchange chamber is temperature control. The ideal material would be one which transmits all light, absorbs no water or carbon dioxide and can provide good heat exchange. The whole plant chambers designed for these experiments were constructed out of Perspex which uniformly transmits 92% of light waves in the 400-800 μm range (photosynthetically active radiation), has a high capacity to adsorb water and low thermal conductivity (Hall *et al* 1993). Glass is equally good at transmitting light, absorbs less carbon dioxide and provides good heat exchange, but it is not as easy to manipulate. The main source of heat is the overhead sodium lamp and some large scale systems have been built whereby temperature was regulated with tanks of circulating water under the lamps (Louwerse &

Oorschot 1969). This was not practical in our laboratory and there are risks associated with leaving experiments with running water unattended.

The chambers used enclosed the whole plant but most measurements of gaseous exchange are made using leaf chambers. The leaf chambers used by Van Oorschot and Van Leeuwen (1984, 1992) had the advantage that the leaf was flat and perpendicular to the light source, providing optimum conditions for maximum and constant photosynthesis. Photosynthetic rates gained in such chambers are typically higher than those from whole plant chambers. However, plants growing in the field rarely receive such perfect interception, and grasses grow with their leaves at several orientations to the sun. The rates obtained in leaf chambers also depend on which leaf is used. Photosynthetic rate varies according to the degree of leaf expansion, and tends to increase along with chlorophyll content as the leaf matures (Ludlow 1991). Thus, whole plant chambers give a good indication of the overall photosynthetic rate relevant to plants in the field. Although a relatively high light intensity was used the majority of leaves would not have received the full benefit of this. The only unnatural condition was the high temperature which equated to a hot summers day, not to autumn when most seeds germinate. The photosynthetic rate measured in any chamber may be reduced below those in free air by the act of passing a stream of air over the leaf. There is evidence that this can cause the stomata to close, especially if the air is dried, although there is no absolute proof of this (Idso 1992). Small fans are often placed inside the chambers to reduce boundary layer resistance and prevent the build up of humidity. However, in this system there were no difference in the values recorded when chambers with and without fans were compared.

Once the number of chambers and their design was decided, the whole set up such as equipment used and the total length of tubing had to be considered. An artificial air source with known carbon dioxide concentration was considered, but the air drawn from outside the building proved to be of a reasonably stable composition which did not warrant the use of expensive gas cylinders. The gas handling system was set up to switch between chambers every 5 mins with reading taken from each chamber 2.5 to 3.5 minutes after each switch. Therefore, each chamber was monitored for 5 mins out of every 30mins. Calculations based on the flow rate and the length and diameter of the tubing indicated that a maximum of 90 s was required before the air in the IRGA came from the new chamber, which provided an ample margin of error. Throughout the system an attempt was made to keep the total length of tubing to a minimum because some carbon dioxide will be adsorbed onto the surface of the tube.

In the past there was a far greater potential for errors in calculations, especially when the measurement of water vapour had to be corrected for manually. The LCA4 and other modern IRGAs make automatic corrections for water vapour, temperature and air pressure which removes this source of error. The transpiration rates were based on an estimated leaf temperature as only the temperature of the air in the chamber was measured. This was because it was impossible to fix a leaf thermistor on to a leaf without damaging it in the whole plant chamber. Winner *et al* (1989) compared two commercially available gas exchange systems and reported that they gave similar values, although careful interpretation of the results was required. The differences

between plant material, the environment, the operators and how the calculations were made were of much greater importance.

However photosynthesis is measured it has to be related to an appropriate plant characteristic. Leaf surface area is used most frequently but dry weight, fresh weight, number of cells, chlorophyll concentration or protein content can all be used (Sestak *et al* 1971). Clearly, the most useful information will be based on a plant characteristic which limits the process, and this is why the area of one side of the leaf is used as standard. If a flat leaf is equally irradiated from the top and the bottom then photosynthesis would be based on the area of both sides. The problem comes with whole plant chambers where the amount of leaf surface irradiated is unknown and this can be another large source of error in the calculation of photosynthetic rate. Dry weight could be used but this often has little relationship to the radiation absorbed or carbon dioxide uptake. In this study it was assumed that half of the total leaf surface area was irradiated, ie the total area of one side of each leaf was used. Since black-grass leaves are generally errect this may have been an over estimation resulting in the calculation of photosynthetic rates lower than the actual values.

It must be noted that what is actually measured is net or apparent photosynthesis. Some workers calculate gross photosynthesis by adding the respiration rate (measured in the dark) to the net photosynthesis value. This ignores the process of photorespiration which occurs in the light at various rates according to the flux density and plant species, thus gross photosynthesis cannot easily be calculated. The problem with interpreting or predicting any photosynthesis data is that the rates are

simultaneously influenced by many internal and external factors (extensively reviewed in Sestak *et al* 1971). Some of the rate limiting factors are under the genetic control of the plant such as stomatal resistance, photorespiration, the amount and distribution of chlorophylls and associated pigments and the leaf structure. The environmental factors such as the quantity, quality, direction and duration of light, the carbon dioxide supply, air temperature and the availability of water are all significant. The natural diurnal cycle, developmental stage of the plant and leaf maturity must also be considered. In this study, only two biotypes of the same species were compared and it is reasonable to assume that the variables under genetic control would be similar. The environmental conditions were controlled and measurements from both biotypes were made simultaneously which will also have reduced this source of error. However, it is perhaps not surprising that there was a lot of variation in photosynthetic rate between the plants measured.

Whole plant chambers were used to give a good overall indication of photosynthesis by black-grass plants in the field. The disadvantages were that it took a long time to enclose the plants securely in the chambers and that a maximum of six plants could be monitored. When photosynthesis was used to indicate the interaction of chlorotoluron with other compounds, a portable leaf chamber was much more suitable. It provided a repeatable and non-destructive means of measuring a much larger number of plants.

4.2.3 The hydroponic system

Two methods of herbicide application were used, overhead spraying and hydroponics. As was noted previously, the plants were more sensitive to chlorotoluron when applied to the hydroponic system, where there is no soil for chlorotoluron to bind and become unavailable to the plant. The hydroponic units allowed a more uniform distribution of the herbicide and other chemicals at the root zone, which resulted in more uniformity between the units and experiments. The disadvantage is that root growth and herbicide availability may be very different in the field, especially when uptake over time is involved (Devine *et al* 1993). In hydroponics the entire root is exposed to the chemical, whereas only a small proportion absorbs the chemical when plants are grown in the soil. The water status of the plants and the growth medium will also be different.

Hydroponics has the advantage of being able to control the duration of exposure to the compound. The safeners could be added to the nutrient solution for a precise period after which they were removed and chlorotoluron was added, which prevented any interaction between the chemicals outside of the plant. Safeners are usually applied as a dry seed dressing as some are relatively insoluble in water. Some of the nutrient solutions appeared cloudy after the dissolved safener was applied, and this insolubility could have reduced the effectiveness of certain safeners. If the original photosynthesis experiments had used hydroponics the recovery of photosynthesis after the herbicide was removed could have been measured (Van Oorschot & Van Leeuven 1979, 1992). One of the main criticisms of the whole plant spray was that it did not allow for photosynthesis to be measured before herbicide application, which was possible with

hydroponics. The physical problems of allowing the herbicide to dry on the leaves (about 30 min) before moving and the time taken to place 6 plants in their chambers without damage or creating air leaks, meant that the first reading was taken 1-2 hours after application.

4.2.4 The limitations of the GST assay

GST activity was measured *in vitro* using a crude protein extract to which CDNB and glutathione were added. The addition of glutathione was necessary because it was removed along with other low molecular weight thiols by the desalting columns. The first problem with this assay is that CDNB is an artificial substrate and GST activity towards different substrates can vary enormously. The activities realised using CDNB are much higher than when "natural" substrates such as the herbicides metolachlor are used (Gronwald *et al* 1987). In a most extreme case, the rate of atrazine-glutathione conjugation was 3000 times slower than that of CDNB-glutathione (Lamoureuex & Rusness 1986). Although using CDNB gives greater rates of activity which can facilitate the detection of GST, it may be reducing the measurable difference between the two biotypes. For instance, if a specific GST isozyme for metolachlor metabolism is induced in resistant plants, then a much greater difference between GST activity in susceptible and resistant tissues would be detected with metolachlor than with CDNB. It can also fail to give a true picture of safener activity. Safened plants showed a much greater increase in activity with metolachlor and acetochlor than CDNB (Ekler & Stephenson 1991, Jablonkai & Hatzios 1991, Fuerst *et al* 1993). This is expected since safeners induce specific GST isozymes for these herbicides in maize and

sorghum. The constitutive isozymes have activity towards CDNB, but the induced isozymes are specific to certain herbicides and may have little or no affinity for CDNB (Dean *et al* 1990, Cole 1994). Increases in activity due to the induction of specific isozymes may be undetected, but all detailed isozyme studies indicate that safeners also increase endogenous GST activity. Thus, CDNB can only be used for comparative purposes and as an indication, not for exact measurements, of activity.

An obvious disadvantage of the GST assay used is that it only measures the *in vitro* activity which may not correlate to the ability of the plant to form glutathione conjugates *in vivo*. Quantifying the herbicide-glutathione conjugates in the plant would determine the specific activity towards that substrate. Several studies have found a good correlation between *in vivo* and *in vitro* measurements of conjugation (O' Connel *et al* 1988), however in some studies conjugation has been found to be higher *in vitro* than *in vivo* (Hatton *et al* 1995). The activity was determined using crude extracts which may contain molecules which inhibit enzyme activity and may also include proteases which would reduce the total amount of enzyme. Only soluble GST was measured although the existence of microsomal GST has been demonstrated (Diesperger & Sandermann 1979). This method could not be used to identify different isozymes nor to quantify the amounts they were present in. The use of CDNB most likely under estimated the true values of GST activity, especially when safeners were applied, and the difference between susceptible and resistant biotypes is expected to be greater than the values generated suggested.

GST activity was expressed on a fresh weight or protein basis. Although fresh weight is easier to measure, it is more accurate to correlate GST activity to the protein content of the extract. The Bradford (1976) protein assay was used because it is very reproducible and results can be achieved very quickly. The standard Lowry (1951) procedure is more time consuming and there is a greater risk of inaccuracies due to the dye binding to non-protein substances. It is also much more sensitive than the Lowry method yielding a greater change in absorbance per microgram of protein. The only disadvantage is that it stains the cuvettes blue making it difficult to reuse them for other assays (Bradford 1976).

It must be noted that the glutathione assay used provided an estimate of total glutathione content, but initial studies demonstrated this to consist of at least 90% reduced glutathione. The assay may possibly detect other low molecular weight thiols, but it is standard procedure to assume that they contribute a negligible amount to the total thiol content compared with glutathione (Griffith 1980). The effect of safeners and synergists on glutathione content could not be measured as the GST and glutathione assay require a different extraction procedure. The physical constraints of space in the growth cabinets and time prevent the growth of the extra plants required to conduct the glutathione determination.

The possible disadvantage of using whole plants to provide plant material was that growth conditions were not constant, resulting in variations in growth and enzyme activity. Rates of GST activity could only be compared on the basis of percentage of control values in many cases. Plants grown in the hotter weather typically received

temperatures 10 ^{0}C higher than normal which doubled the GST activity in extracts from susceptible and resistant plants. It has been reported that GST activity and metolachlor conjugation in maize also increased with elevated temperature (Kunkel *et al* (1991, Viger *et al* 1991).

Other considerations of the assay include using only aerial tissue and the timings of treatments. No attempt was made to assess GST activity in the roots because it would be impossible to adequately remove the compost of pot grown plants and also because it was not possible to grow the amount of plants required to provide enough root tissue for the assay. However, it has been noted that in maize, GST activity in roots is higher than in the shoots (Jablonkai & Hatzios 1991) and that the ratio of isozymes may also be different in roots and shoots (Holt *et al* 1995). GST activity was always measured 24 hours after chlorotoluron treatment because the photosynthesis data indicated that any resistance mechanism would be fully operational by this time. The pre-incubation with safeners also lasted 24 hours because it was reported that maximum GST activity occurred after 24 hours of treatment with oxabetrinil (Dean *et al* 1990) and benoxacor (Miller *et al* 1994). However, it is possible that in some cases GST activity would have been optimal after a greater period of time.

4.3 The genetics and evolution of herbicide resistance

It was 40 years ago that Harper (1956) observed the problem of resistant organisms being encountered in the fields of medicine, bacteriology and entomology and raised the question of how long it would be before weeds would become resistant to

herbicides. He concluded that action was necessary to prevent the evolution of resistance and proposed methods such as multiple treatments of herbicides with different modes of action may prove important in delaying its occurrence. However, the 1950s and 1960s were a boom time for herbicide development and little was done to address the potential threat to future weed control. Resistance did not quickly follow the introduction of herbicides, as in the case of antibiotic resistance, and 20 years later there were only a few resistant biotypes, and these only occurred in areas of persistent usage of high doses of herbicides (Gressel & Segel 1978). Some of the reasons put forward for this were that the selection pressure of most herbicides was too low, that resistant biotypes had a reduced fitness, and that there existed a large seed reservoir of susceptible weed seeds in the soil. This complacency, however, was seriously misplaced as the enormity of the current resistant weed situation proves. The questions which have to be asked are whether we are now able to make reliable predictions about the future development of resistance and are we in a position to prevent it from happening?

There are now major investigations into most of the resistance situations which have occurred, and these are usually followed by management programmes to prevent the spread of resistance. However, resistance is now more common and there is a need for a more active approach to prevent resistance to new herbicides, and this necessitates that the information required to predict resistance is gathered and preventative measures are practised from the start of use (Shaner 1995). Two processes are required for the evolution of resistance to occur; heritable variation for the trait and natural selection (Maxwell & Mortimer 1994). The rate of evolution will depend on the mode of

inheritance and the intensity of selection pressure. The selection pressure can be determined with relative ease as this is determined by the efficacy of the herbicide, its frequency of use and persistence. It is the mode of inheritance which poses problems to any model attempting to predict the evolution of resistance.

An ideal model to predict the evolution of resistance would be based on the initial frequency of resistance, the mode of inheritance, gene flow between individuals and populations, the breeding system and the relative fitness of resistant and susceptible biotypes (Shaner 1995). The problem with creating such a model for a new herbicide is that many of these factors are unknown. The initial frequency of the gene(s) controlling resistance in the population is rarely known because weed populations are only studied after resistance has been reported and the initial frequency of that mutation cannot be established. The mode of inheritance can only be determined if the possible resistance mechanisms can be accurately predicted and their genetic control known. This can be difficult if more than one resistance trait to a single herbicide is possible, as in the case of resistant *Lolium rigidum*. The relative fitness of susceptible and resistant biotypes can be difficult to establish and is partly dependant on the environment and the vigour of the crop it is competing with. Very few studies follow through the whole life cycle from seed germination to the germination of the next generation. Fitness is also a dynamic condition under the influence of natural selection itself (Maxwell & Mortimer 1994). It must be noted that all models assume that herbicides are selecting for existing resistance genes which naturally occur spontaneously at low rates in a random fashion throughout the weed population. Since the initial frequency of such genes is not known the presumption that herbicides themselves do not cause mutations cannot be confirmed

or denied. If herbicides were demonstrated to cause mutations this would alter any predictive model. Any model of the evolution of resistance would only provide a prediction for one population of weeds, and as most herbicides are active against a range of species this represents a mammoth data collection task just to predict the evolution of resistance to one herbicide.

Similar problems are encountered when considering which methods are the most suitable to prevent the evolution of resistance, as this is dependant on accurately predicting whether the resistance mechanism will be due to a single site mutation or involve multigenic inheritance. If the rate and frequency of herbicide application is reduced it may delay the onset of a resistance mechanism controlled by a single gene, usually a mutation at the target site, but this regime will increase the likelihood of polygenic resistance evolving, which often results in enhanced metabolism (Gressel 1995). Thus, there is no point in altering the herbicide regime until the resistance mechanism is known or at least predicted with a good degree of certainty. Information about this can sometimes be obtained from knowledge about crop selectivity or from creating resistant tissue cultures in the laboratory, however, great care must be taken not to make too many generalisations. For example, atrazine selectivity is due to its enhanced metabolism in maize and sorghum, but most resistant weeds have a target site mutation.

Resistance management programmes often advise the use of herbicide rotations. If resistance is due to enhanced metabolism then the herbicides used in the rotation must be detoxified by a different route. However, this may not work for outcrossing species

such *Lolium rigidum* which can exchange genetic information between populations (Holtum & Powles 1991). If resistance is inherited by a dominant nuclear gene, then the weeds around the field also need to be controlled to avoid the spread of resistance, whereas if resistance is controlled by a recessive trait then leaving strips of susceptible weeds in the field may prevent the selection of resistance in outcrossing species (Maxwell *et al* 1990). Therefore, because of the difficulties in predicting resistance mechanisms, and the need to use to preventative measures specific to that mechanism, it would appear that we still have a long way to go before a new herbicide can be launched alongside the correct information to delay the onset of resistance.

Most of the known resistances are due to a single dominant or semi-dominant nuclear gene controlling altered target site, with a few exceptions such as *Setaria viridis* resistance to dinitroanalines, which is controlled by a recessive nuclear gene, *Eleusine indica* resistance to dinitroanalines, which is multigenic and triazine resistance in many species is determined by a dominant chloroplast gene (Shaner 1995).

4.3.1 The evolution of resistance in black-grass

Chlorotoluron resistance (or tolerance) in wheat is indisputably controlled by a single major gene located on chromosome 6B (Snape & Parker 1988). However, a comparison of two wheat cultivars revealed small but significant differences in response to chlorotoluron which were attributed to the presence of minor "modifier" genes (Sixto *et al* 1995). The majority of wild wheat (*Triticum dioccoides*) populations tested also showed significant variations in their response to chlorotoluron, both between and

within populations (Snape & Parker 1988). The heritability value calculated indicated that wild populations had the ability to rapidly evolve resistance under the selection pressure of chlorotoluron. The fact that herbicide resistant crops cannot be successfully bred in this manner is attributed to the fact that modern crop cultivars have insufficient natural variation to provide new resistant germplasm (Darmency 1994).

Resistant black-grass populations have a variety of cross resistance patterns, and three phenotypes with high, intermediate and no resistance have been categorised according to their ability to metabolise chlorotoluron. The segregation ratio of progeny from crosses between susceptible and resistant plants was approximately 4:11:1 (resistant:intermediate:susceptible). This agrees with the theory that there are two nuclear genes with additive actions, although this remains to be confirmed (Chauvel & Gasquez 1991, Darmency 1994). Errors in the methodology due to the subjective nature of phenotypic determination of the progeny, the rates of herbicide used and the environmental factors can result in false conclusions. The selection pressure of herbicides for the trait of resistance often results in a reduced genetic variability within the population. For example, all the *Chenopodium album* L. plants studied with a mutation in the psbA gene had the same genetic back-ground, indicating that only certain genotypes in the population were capable of evolving resistance (Darmency 1994). A study of several susceptible and resistant black-grass populations revealed that there was no evidence of differences between resistant and susceptible populations in any genetic parameter, and this is considered unique among resistant weed populations after exposure to a selection pressure (Chauvel & Gasquez 1994). Black-grass was also shown to have a high level of genetic polymorphism, which together with the ability of

a wide range of genotypes to evolve resistance, may account for the extensive cross resistance observed, and the different range of cross resistances seen in different resistant populations.

An obvious question to consider is why did chlorotoluron resistance first evolve on Peldon Hall Farm and could this have been predicted? This was the first site to be examined in detail and resistance probably occurred concurrently on other farms with similar soils and cropping practices. Nevertheless, this was the first site to report black-grass resistance and several of the factors which favour resistance came together at this site. The geographical location of the farm was in an area where black-grass was historically a major weed, strongly associated with the heavy clay soil. Black-grass persisted at low levels despite annual herbicide use and rotations including a break grass ley once every three years (Wilson & Brain 1991). A large contributing factor was probably the soil type. Clay has more structured aggregates than sandy or loamy soil, with wide channels between the aggregates. It is able to hold more water, but a significant amount moves in the wider channels between aggregates, so the herbicide often remains bound in fine pores within the aggregates (Caseley & Walker 1990). This will result in a reduced herbicide availability, so although field-rate chlorotoluron was applied, the effective dose was a lot lower. Chlorotoluron resistance is due to more than one gene, and low dose rates favour the evolution of multigenic resistance, so this may well have been a contributing factor. There is also increasing evidence that when reduced rates of herbicide are applied resistance tends to creep up with each selection cycle, especially when multigenic inheritance of enhanced metabolism is involved (Gressel 1995). This process is speeded up when selection pressure is increased, and in

the 4 years previous to resistance the fields as Peldon were sprayed twice a year instead of once (Moss & Cussans 1985). Resistance probably evolved the quickest on this farm because it was one of the first to employ cultural measures which favour black-grass and resistance. Spring sown crops are not profitable on this soil so autumn crops which favour black-grass have always been grown (Henly 1991). Minimum cultivation was introduced in 1972 at a time when the majority of farmers were still ploughing (Elliot *et al* 1979). It should not have been a surprise that resistance developed because the outcrossing nature and genotypic variation of black-grass coupled with the cultural conditions warranted it. However, to have predicted the nature of the resistance would have been much harder. If it had been predicted to be controlled by a single gene like the crop then the wrong management programme may well have been employed.

The second area to consider is does the putative genetic control have any implications for the resistance mechanism and cross and multiple resistance? Crop species tolerate chlorotoluron by elevated metabolism via the cytochrome P450 dependant monooxygenases. Since this mechanism is controlled by one gene in crops (Snape & Parker 1988), it is reasonable to presume that only one gene would be necessary to provide this mechanism in black-grass. Resistance in black-grass is controlled by two genes (Chauvel 1991, Darmency 1994) leaving room for speculation over what the other gene controls. It may be that both are necessary for elevated cytochrome P450 content in black-grass, or that in the light of the results presented in this study, one gene controls the elevation of GST activity. The possible interaction of the P450 and GST enzymes is not known but could provide an explanation for the extensive cross resistance in black-grass.

4.3.2 Recommendations for black-grass control

The UK Weed Resistance Action Group has published guidelines on how to manage resistance, emphasising cultural control measures and a rotation of herbicides based on different modes of action (Moss and Clarke 1994). In fields where resistance has been established it is recommended that trifluralin and/or triallate is used in combination with ploughing, crop rotation, spring sowing or delayed autumn drilling and mechanical weeding where possible. The nature of black-grass resistance to the AOPP and CHD herbicides is uncertain but their use is discouraged because there is increasing evidence that the development of resistance to these herbicides is far more rapid than that to the majority of herbicides (Chauvel *et al* 1992). However, although resistance to fenoxaprop-ethyl can develop rapidly (Clarke & Moss 1994) it does not follow that this will be true for all AOPP herbicides. A recent proposal for controlling black-grass is based on clodinafop-propargyl with trifluralin, rotated with triallate in areas where resistance has been established (Mills & Ryan 1995). There is no evidence of resistance to clodinafop-propargyl after 5 years of use on the same field site.

It was noted that a herbicide rotation based on different modes of action was of little use because resistance is determined by the ability of black-grass to metabolise the herbicide, which is dependant on the molecular structure and not the mode of action (Moss and Clarke 1994). For example, Peldon black-grass is resistant to pendimethalin but not trifluralin, despite both being dinitoaniline herbicides (Kemp *et al* 1990). Pendimethalin has ring-methyl substituents which can be hydroxylated by the cytochrome P450s in the same manner as chlorotoluron, and its this metabolism

pathway which results in resistance. Trifluralin lacks available ring-methyl substituents for catalysis, and thus cannot be detoxified in this way, resulting in toxicity to chlorotoluron-resistant black-grass (James *et al* 1995).

At Peldon Hall farm ploughing has been recommended, but a consistent seed bed is crucial for winter wheat, and the spring-sown break crop suggested is not profitable on the clay soil. It is still more profitable to minimum cultivate and to invest more in herbicides than to plough (Henly 1991). The black-grass control regime now includes delayed sowing so that glyphosate can be applied before sowing, followed by applications of triallate, and then a tank mix of isoproturon and trifluralin a few days later. In some years this may be supplemented by a tank mix of isoproturon and cyanazine later in the year (Henly 1991).

A new approach under investigation will reduce the amount of herbicide used because only areas with a high weed density are sprayed. Black-grass infestations are not uniform but restricted to patches, probably because the majority of seeds are shed close to the parent before harvest. These patches were stable over a 10 year period, even when there is crop rotation with a break year of grass (Wilson & Brain 1991). Early field trials relying on the farmer locating the areas to spray and feeding the information electronically into an experimental patch sprayer are encouraging (Miller *et al* 1995). The effect of patch spraying on the population dynamics of weeds and whether this could favour some type of resistance evolving, is not known.

The prognosis for the future control of black-grass if these measures are taken needs to be considered. Susceptible and resistant black-grass biotypes are equally fit, so if no more chlorotoluron is sprayed the numbers of resistant plants will not be reduced by competition between biotypes. However, it is an outcrossing species so in time the resistance will become diluted, although the "patchiness" of black-grass will make this a slow process. The problem with cross resistance is that the use of other herbicides, even with different modes of action may still exert a selection pressure for chlorotoluron resistant biotypes. No resistance to glyphosate has yet evolved in the field, and the rotation of herbicides with different modes of action should delay resistance. However, if the resistant black-grass biotypes have elevated cytochrome P450 enzymes and elevated GST activity, then all herbicides detoxified by either pathway should not be included in the rotation. There is now a need to be predicting resistance to triallate, trifluralin and the other herbicides now used to control black-grass, as any measure can only delay the evolution of resistance, not provide a permanent solution.

4.4 Putative role for elevated GST activity in black-grass

This study has clearly demonstrated that chlorotoluron resistant black-grass from Peldon has elevated endogenous GST activity, but the significance of this finding remains to be established. GST may have roles in the formation of chlorotoluron-glutathione conjugates, that GST can provide a protective mechanism against free-radical damage, or that GST provides a means by which xenobiotic-glutathione conjugates can be sequestrated into the vacuole. The possible interactions between GST

with other detoxifying enzymes must also be considered, especially in relation to the extensive cross resistance observed in resistant black-grass. Such possibilities are considered in the following discussion.

The GST enzymes could mediate the direct conjugation of chlorotoluron with glutathione by substitution of the aryl chlorine substituent in a manner analogous to the formation of atrazine-glutathione conjugates in maize and sorghum (Cobb 1992). Atrazine has some structural similarites with chlorotoluron and shares the same mode of action (Devine *et al* 1993). Alternatively, chlorotoluron could be hydroxylated or demethylated by the cytochrome P450 monooxygenases and the metabolites produced conjugated with glutathione, as the metabolites may be more reactive with glutathione (ie any more electrophilic) than the parent molecule (Edwards 1995). A further possibility is that both cytochrome P450 and GST are both detoxifying chlorotoluron in parallel, resulting in the production of glucoside and glutathione conjugates. This theory was also put forward to explain the degradation of fenoxaprop-ethyl in wheat and barley (Tal *et al* 1993). The existence of chlorotoluron-glutathione conjugates has not been demonstrated, but interestingly, a large proportion of the radiolabelled chlorotoluron fed to black-grass, 26% in susceptible and 56% in resistant (Peldon), was found to remain in an unidentified conjugate that was resistant to β-glucosidase (Hyde *et al* 1996). This was assumed to consist of oxidised demethylated and ring methyl-hydroxylated metabolites, but could hypothetically include glutathione conjugates, especially in resistant black-grass where this fraction accounted for over half the radioactivity recovered.

GST activity was originally considered only with respect to secondary metabolism, but recent studies have demonstated it to have a much wider role in plants. After plants have detoxified a xenobiotic by rendering it inactive by conjugation to saccharides or glutathione, the majority of these conjugates are sequestered into the vacuole, and GST may have a role in black-grass at this third stage. An investigation into the fate of metolachlor-glutathione conjugates in barley suggested that they were sequestered into the vacuole by a specific ATPase which shows a great similarity to the GS-conjugate pump in the membranes of mammalian liver (Martinoia 1993). The mammalian and plant glutathione S-conjugate ATPase also transports oxidised glutathione into the vacuole with competition between the substates for the target site (Tommasini *et al* 1993). This transport process has been characterised in a range of species to require MgATP and is not dependant on the trans-tonoplast H+ gradient for energy (Li *et al* 1995). Sequestration should be more carefully considered as part of the overall detoxification mechanism which can be modulated by herbicide safeners. Cloquintocet-mexyl doubled vacuolar transport of glutathione conjugates into isolated barley vacuoles with a suggestion that this was due do a higher expression of the transporter (Gaillard *et al* 1994).

A putative role of GST in black-grass is to catalyse the formation of chlorotoluron-GS conjugates which could then be sequestered into the vacuole via this specific ATPase. However, if some or all of the chlorotoluron is metabolised by the cytochrome P450s then the resultant conjugate would be a β-glucoside. There are specific glusoside ATPases which appear to transport other biological glucose conjugates aswell as those of xenobiotics, and this transport pathway can also be

enhanced by cloquintocet-mexyl (Gaillard *et al* 1994). If GST and cytochrome P450 metabolism was concurrent then simultaneous sequestration could also occur which would double the speed of metabolism in resistant plants. However, there is another possibilty which would be analogous to final enzymatic steps in anthocyanin biosynthesis which involves cytochrome P450 monooxygenases, glucosyl transferases and GST, the 3 enzymes involved in xenobiotic detoxification (Marrs *et al* 1995). In this hypothetical pathway, chlorotoluron would be metabolised by the P450s, conjugated with glucose and the glusoside then conjugated with glutathione (like cyanidin-3-glusoside) which provides the transfer intermediate recognised by the glutathione-ATP pump for transport into the vacuole.

Although the role of GST activity in herbicide detoxification is indisputable, these enzymes also form part of the plants natural defences against environmental stresses such as temperature extremes (Esterbauer & Grill 1978), drought (Burke *et al* 1985), ozone pollution (Price *et al* 1990), heavy metal toxicity (Grill *et al* 1989), pathogen attack (Dron *et al* 1988) and viral infection (Gullner *et al* 1995). For example, a GST isozyme was identified in wheat which was quite distinct from the herbicide detoxifying isozymes (Mauch & Dudler 1993). It had a low endogenous expression but was specifically induced by pathogen attack or elevated glutathione levels. Of particular relevance to the stress caused by chlorotoluron is that GSTs have been shown to have glutathione peroxidase activity (Prohaska 1980) in which glutathione is conjugated to hydroperoxide to form an intermediate (sulfenic acid) which then reacts non-enzymatically with glutathione to produce GSSG. This reaction is thought to be important in protecting plants from ozone injury. The toxicity of ozone is largely due to

its oxidising potential and the production of free radicals (Price *et al* 1990). The elevated GST activity resulting from ozone damage may protect the plants from toxic substances such as the hydroxyalkenals which are produced by oxidative stress or they may be limiting lipid peroxidation by removal of acyl peroxides (Price *et al* 1990). Chlorotoluron inhibits electron transfer at PS II which produces free radicals and results in lipid peroxidation, thus high levels of GST in black-grass could reduce the toxic effect of chlorotoluron and provide a basis of increased tolerance. Elevated GST activity and other free radical scavenging enzymes also appear to contribute to resistance to acifluorofen (Gullner *et al* 1991).

The metabolism of chlorotoluron by cytochrome P450s as the basis of resistance is not disputed, but there are clear indications that GSTs could work alongside them and other detoxifying enzymes. The extent to which these enzymes interact will be dependent on their sub-cellular location and tissue specificity. The cytochrome P450s are membrane bound and various studies have placed them on the endoplasmic reticulum, in small vesicles, on the plasma membrane and in the mitochondria and glyoxysomes (Donaldson & Luster 1991). The most convincing location to date is the rough endoplasmic reticulum which was demonstrated to support cinnamate 4-hydroxylase and demethylase activities (Young & Beevers 1976). Each study was conducted using a different species, and so the location may be species specific, or depend on the cell type or plant organ. Cytochrome P450s catalyse a wide range of detoxification and biosynthetic reactions and isozymes with different functions may be located on different membranes within the cell.

In contrast to the P450s, GST isozymes are mostly soluble although microsomal GSTs have been identified in legumes with activity towards cinnamic acid (Diesperger & Sandermann 1979, Edwards & Dixon 1991). GST is very hydrophobic and this association with the microsomal fraction may be an artefact due to isolation proceedures, especially as microsomes from maize and *Setaria faberi* do not have GST activity (Edwards 1995). The soluble GST isozymes may be located in the cytosol or in the chloroplast stroma. It has been calculated that the glutathione concentration in the plant cell is approximately 20 μM in the vacuole, 60 μM in the cytoplasm and 2000 μM in the chloroplast (Rennenberg 1982), thus, the activity of GSTs in the chloroplast would not be limited by the availability of glutathione. The chloroplast would be the ideal location for GST isozymes with hydroperoxidase activity because chlorotoluron causes the production of free radicals by inhibiting electron transport across the the thylakoid membrane. As with the cytochrome P450s, different isozymes may be located in different parts of the cell, and the location may be dependent on their specific activity.

A schematic diagram of the metabolism of chlorotoluron within a cell in a resistant black-grass plant, and the hypothetical roles of GST, is shown in Figure 4.1. This is a very general scheme since the amount of enzyme, proportions of isoenzymes and their sub-cellular location may be dependant on the cell/tissue type, age of plant and the presence/abscence of xenobiotics. For example, the activity of maize GST I is higher in the roots than the shoots (Dixon *et al* 1995), and GST II in maize is only present when induced by safeners (Mozer *et al* 1983). Microsomal fractions from tulip bulbs, maize endosperm and pea seedlings contained different amounts of cytochrome

A plant cell.

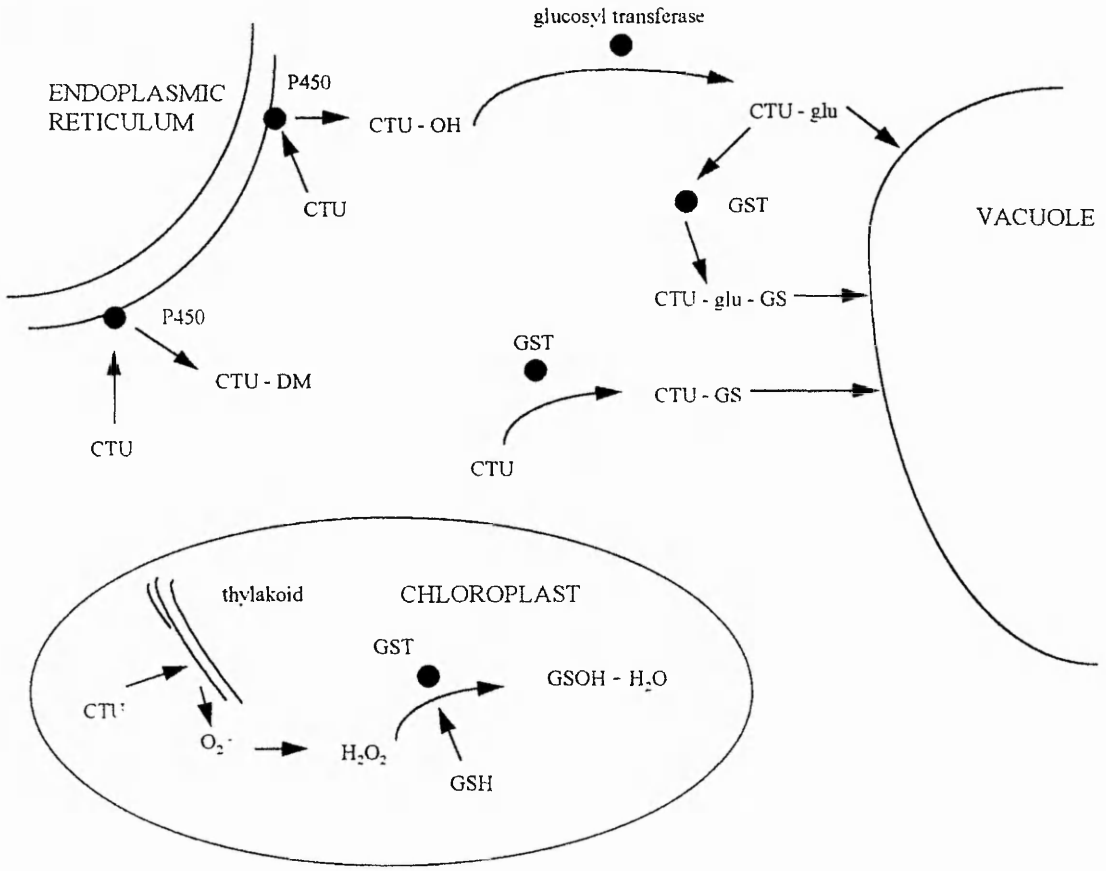


Figure 4.1 The hypothetical roles of GST in chlorotoluron metabolism in black-grass

CTU	chlorotoluron
CTU-DM	demethylated chlorotoluron
CTU-OH	ring-methyl hydroxylated chlorotoluron
CTU - glu	CTU glucoside conjugate
CTU - GS	CTU-glutathione conjugate

P450, and were not all able to support the same reactions (Marabini *et al* 1994). Thus, the relative importance and concentrations of the P450s and GSTs within the cell may vary according to the location of the cell within the plant.

Cross resistance in black-grass is well documented, but the emphasis has always been on resistance to herbicides from different chemical classes with different modes of action (Moss & Cussans 1991). Since target site resistance is not involved this is hardly surprising, but no rigorous efforts have been made to understand the mechanisms behind cross resistance. It has often been stated that cross resistance is due to the ability of the plants to detoxify these herbicides (Kemp & Caseley 1987, Kemp *et al* 1990, Moss & Cussans 1991), but what proof do we have that the elevation of one particular enzyme will enhance the metabolism of all the herbicides black-grass is cross resistant to?

Black-grass was first reported to be resistant to chlorotoluron, which is initially metabolised by ring-methyl hydroxylations and N-demethylations dependant on cytochrome P450 monooxygenases (Gonneau *et al* 1987). Resistant plants metabolise chlorotoluron twice as fast as susceptible plants, produce a higher proportion of ring-methyl hydroxylated metabolites, and have elevated levels of cytochrome P450 (Caseley *et al* 1990). Of the 20 herbicides which Peldon black-grass is resistant to, only chlorotoluron and diclofop-methyl have been demonstrated *in vitro* to be metabolised by cytochrome P450 (McFadden *et al* 1989, Fonne-Pfister & Kreuz 1990, Mougin *et al* 1990, Zimmerlin & Durst 1990), although there is evidence of their involvement in the metabolism of several others (Werck-Reichhart 1995). The validity of the theory of cross-resistance depends on the substrate specificity of the cytochrome P450 isozymes

in black-grass. On the one hand it has been demonstrated that diclofop, chlorsulfuron, linuron and chlorotoluron are catalysed by distinct cytochrome P450s in wheat (Frear *et al* 1991, Mougin *et al* 1991), whilst on the other hand there is evidence that a single, broad spectrum P450 enzyme could metabolise bentazon, imidazolinones, sulfonylureas and chlorotoluron (Barrett *et al* 1995). The biosynthetic P450 isozyme responsible for lauric acid hydroxylase also metabolises diclofop (Zimmerlin *et al* 1990), indicating that herbicides may just fortuitously bind with one of the several P450s involved in the biosynthesis of natural plant products (Werck-Reichhart 1995).

The cytochrome P450s in black-grass have not been characterised, but there is clear evidence from other species that the ring-methyl hydroxylase and N-demethylase reactions in chlorotoluron metabolism are mediated by 2 distinct P450 isozymes (Mougin *et al* 1990). Both these reactions are elevated in resistant black-grass (Caseley *et al* 1990). Thus, it can be predicted that this weed would be resistant to any herbicide which was metabolised by either of these reactions. Dicofop is metablised by aryl-hydroxylation, however, which suggests the invlolvement of at least one more cytochrome P450. The population of chlorotoluron resistant black-grass in Tiptree is not resistant to diclofop-methyl or pendimethalin, and this more specific resistance means that it cannot be assumed that a single detoxification mechanism occurs in all resistant populations (Moss 1990). Finally, some resistant black-grass biotypes are resistant to fenoxaprop-ethyl (Moss & Clarke 1994) which is metabolised at least in part by glutathione conjugation (Tal *et al* 1993). Either cross resistance is due to the elevation of one general cytochrome P450 which metabolises all herbicides, or there is a common mechanism for elevating several specific P450s. If distinct P450s under

separate regulation are involved, then there is a case for this to be re-defined as multiple resistance.

The possible roles of GST in cross and multiple resistance are the same as those for chlorotoluron resistance, forming herbicide-glutathione conjugates (especially with fenoxaprop), protection from oxidative stress or aiding sequestration of conjugates into the vacuole. Again, this will depend on the role and the specificity of the GST isozymes. The evolution of multiple mechanisms is easy to understand in an out-crossing species, where 2 individuals each surviving with a single mechanism cross breed to produce some offspring with both mechanisms and increased survival. However as many enzymes and isozymes are involved, this enhanced ability to catalyse many of the reactions involved in herbicide metabolism means that resistant black-grass has the potential to be resistant to new herbicides which are developed in the future. Thus, one way forward to combat resistant biotypes would be to develop a herbicide which is metabolised by an unusual pathway. If elevated GST activity protects black-grass from oxidative stress, this would provide a mechanism for cross resistance to all herbicides which cause the generation of free radicals, either as a primary or secondary effect, regardless of their metaboic pathway.

Finally, as it has already been alluded to, it is possible that all detoxification enzymes, cytochrome P450s, GSTs, glucosyl transferases and specific ATP vacuolar pumps may all work together and be elevated or stimulated by a common switch. The activity of both GST and cytochrome P450 can be induced by stress (Jones 1991, Edwards 1995) and they have a common biosynthetic role in the production of

anthocyanins (Marrs *et al* 1995). Observations such benoxacor inducing both P450 and GST activity (Fonne-Pfister & Kreuz 1990, Dean *et al* 1991) and that inhibitors of P450s can also promote their activity (Devine *et al* 1993), give credence to a hypothetical scheme of interrelations between safeners, synergists, GSTs and P450s (Fedtke & Trebst 1987). It proposed that the primary action of safeners and synergists was to inhibit monooxygenase enzyme activity which in turn stimulated P450 and GST enzyme induction. The mechanism of induction for both groups of enzymes appears to be by enhanced gene expression (Werk-Reichhart 1995). The elevated endogenous levels of P450 and GST in black-grass could be due to an innate over expression of these genes, possibly mediated by a single cell messenger along with other detoxification enzymes. However, if the role of elevated endogenous GST is to protect black-grass from oxidative stress then there is evidence to suggest that it would be under a distinctly different regulation mechanism, possibly triggered by an increase in glutathione concentration (Mauch & Dudler 1993). Hence, the role of elevated endogenous GST activity has to be established before its induction, regulation and interaction with other detoxification enzymes can be known.

4.5 Future Work

It is a high experimental priority to establish the existance or otherwise of the chlorotoluron-glutathione conjugate. This could firstly be investigated *in vitro* using a cell free extract produced by a method similar to that described in Chapter 3. The extract would be incubated with chlorotoluron for 60 mins after which the reaction

would be terminated by adding HCl. The reaction mixture would then be centrifuged and the supernatant analysed using an HPLC column and monitoring the eluent for UV absorbance at 264 nm, as described by Hatton *et al* (1995). A reference standard for the chlorotoluron-glutathione conjugate could be made by incubating a chlorotoluron solution with glutathione at 30 ^{0}C for 24 hours, and analysing the products by TLC (Hatton *et al* 1995).

The natural progression would be to then establish whether susceptible and/or resistant black-grass plants could produce chlorotoluron-glutathione conjugates *in vivo*. This would necesitate obtaining radiolabelled chlorotoluron and feeding it to plants growing in a hydroponic system for 24-48 hours as described previously. The aerial portions of the plants would then be harvested, homogenised in methanol and filtered. The methanol extract would then be applied to a TLC plate and co-chromatographed with a reference chlorotoluron-glutathione conjugate (Edwards & Owen 1986). If the chlorotoluron-glutathione conjugate cannot be synthesised using this method then TLC should still give some indications of the production of such a product. The demethylated and ring-methyl hydroxylated metabolites would clearly resolve whereas the glutathione conjugate would remain on or near the origin (Hatton *et al* 1995). Chlorotoluron could be eliminated by co-chromatography and the residue tested for sensitivity to cleavage by β-glucosidase. If it proved resistant to degradation by β-glucosidase, it could theoretically be analysed further using methods which can detect the prescence of amino acids or sulphur residues. It is possible that the TLC plate could be scraped, the un-identified conjugate solubilised and then assayed according to Griffith (1980) which identifies glutathione and low molecular weight thiols.

A logical extentsion of the above studies would be to see if GST from resistant black-grass can conjugate fenoxaprop, as this may be the reason why Peldon black-grass is cross-resistant to fenoxaprop-ethyl. It would aslo be interesting to investigate the metabolism of other herbicides such as the chloroacetanilides and atrazine by black-grass GST, and the effect of safeners on this metabolism, since GST in maize is active towards these herbicides (Chapter 1). The major disadvantage with the *in vitro* assay is that it can only measure total GST activity. The GST isozymes in black-grass need to be isolated by anion exchange chromatography which would separate them on the basis of different isoelectric points (Edwards 1995). Once the isozymes were identified it would be possible identify them as endogenous and/or inducible, and to investigte their spectrum of activity. From this their role(s) in herbicide metabolism and resistance could be deduced. This would run concurrent with raising antibodies to the GST isozymes so that sub-cellular immunolocalisation could be performed to identify their precise location.

Our understanding of the sequestration of xenobiotics into the vacuole is limited at present, not least because of the technical difficulties in isolating vacuoles. Methods such as those used by Hull *et al* (1995) could be employed to isolate the tonoplast to investigate the presence and activity of GST specific ATPase pumps. The interactions between cytochrome P450s. GSTs and sequestration ATPases, and the possibility of a common cell signal controlling them also represents a large area of future work.

If the presence of elevated levels of P450s and GSTs could be rapidly dectectd, this could form the basis a diagnostic test for resistance in the field. Currently, farmers suspecting they have resistant black-grass on their farm, have to send seed away for testing, and they often wait for 5 months before receiving the result (Henly 1990). An immunological test would only require plant tissue and the results woud be known within days, allowing the farmer to adjust his weed management programme accordingly. It would also require less man power and resources, although it could be expensive to develop. Some difficulties with this can be envisaged, as the antibodies raised may only recognise on isozyme, requiring the production of several isozymes. The enzyme concentration required to give resistance would have to be determined, and this may not be an absolute characteristic and vary between resistant populations.

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