Mechanisms of Herbicide Resistance in *Lolium rigidum*

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Frontispiece. A view of a rail siding near the town of Toodjay in Western Australia. The siding was part of the rail system which had been treated for ten years with a combination of the herbicides amitrole and atrazine. Within the treated area and along side the tracks there is a near monoculture of herbicide resistant *L. rigidum*. This observation was the starting point of this investigation into the mechanisms of resistance in this and similar biotypes.

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Abstract

Herbicide resistance has become increasingly frequent in *Lolium rigidum* since it was first detected in 1982. Some biotypes are resistant to the triazine herbicides and two such cases are the subject of this study. The first biotype (WLR2) was exposed to ten years of selection pressure from the herbicides atrazine and amitrole. WLR2 is resistant to atrazine and amitrole but is also cross-resistant to the phenylurea herbicides and metribuzin. The second biotype (VLR69) has a history of selection pressure from diuron, chlorsulfuron, atrazine, paraquat and diclofop. VLR69 is resistant or cross-resistant to nine chemical classes of herbicides including the triazines, phenylureas, sulfonylureas and metribuzin. Both of these resistant biotypes were investigated to determine their mechanism of resistance to the triazines, phenylurea and metribuzin while amitrole resistance in WLR2 and chlorsulfuron resistance in VLR69 were investigated separately.

Resistance to the inhibitors of photosystem II was studied using simazine, chlorotoluron and metribuzin to represent the triazines, phenylureas and triazinones respectively. The two resistant biotypes had a similar level of resistance to each of these herbicides. Resistance was not due to changes at the target site of these herbicides, as oxygen uptake by thylakoids isolated from WLR2, VLR69 and a susceptible biotype was similarly inhibited by each class of herbicide. Uptake and distribution of the herbicides was similar in all three biotypes although there tended to be slightly greater uptake by the susceptible in some cases. The resistant biotypes detoxify the herbicides at a greater rate than the susceptible. This detoxification could be inhibited by the mixed function oxidase inhibitor 1-aminobenzotriazole (ABT) which also intensified the symptoms of each herbicide. When [^{14}C]labelled metribuzin or simazine were taken up continuously via nutrient solution, susceptible plants were found to accumulate more of the herbicides in their leaf tissues than the resistant plants. In the presence of ABT, the resistant plants accumulated similar amounts of herbicide to susceptible plants treated with herbicide alone. The effect of ABT on herbicide metabolism and the ability of ABT to overcome the resistance suggests that

differential metabolism is the basis for resistance to photosystem II inhibitors in these biotypes. Resistance to simazine in WLR2 is inherited as a nuclear trait and is probably conferred by more than one gene.

Amitrole resistance was less clearly defined. WLR2 exhibits ten-fold greater resistance to amitrole than the susceptible VLR1. The degree of resistance is similar both when the herbicide is sprayed on established seedlings and when the seedlings are germinated on agar containing amitrole. The specific target sites of amitrole are not known and, therefore, cannot be studied *in vitro*. Pigments were, however, extracted from seedlings germinated on amitrole and these indicated that amitrole was effecting pigment composition in the resistant plants but that a ten times greater dose was required to generate similar effects to those observed in the susceptible seedlings. Uptake, distribution and metabolism of amitrole was similar in both biotypes independent of the means of dosing. Using the agar germination system it was established that seedlings of both biotypes contained similar amounts of amitrole while showing different symptoms, suggesting either that there was a less sensitive primary target or that the herbicide is sequestered away from the target. Amitrole resistance was inherited as a single semi dominant nuclear gene suggesting the possibility of a single mechanism conferring amitrole resistance in biotype WLR2.

Chlorsulfuron resistance in VLR69 appeared to be conferred by enhanced metabolism in the majority of the population, however, a minority of individuals express a less sensitive ALS. These plants were detected by their resistance to sulfometuron, a sulfonylurea analogue that is less amenable to metabolism in graminaceous species. Thus, two mechanisms of sulfonylurea resistance were detected in the VLR69 population.

It is concluded from these studies that enhanced herbicide metabolism may confer cross-resistance in *L. rigidum*. The biotypes studied also possess multiple-resistance through independent mechanisms. This ability to develop cross and multiple-resistance distinguishes *L. rigidum* as a highly variable and adaptable weed species with the potential to develop intractable herbicide resistance.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference had been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for photocopying and loan.

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Date: 8/3/94

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Introduction

The exposure of weed populations to intense and persistent selection pressure by herbicides can lead to the appearance of herbicide resistant weed biotypes. This phenomenon has been documented in more than 100 weed species world wide. Herbicide resistance has occurred in Australia, especially in the out-crossing grass weed *Lolium rigidum* Gaudin. Resistance has developed most commonly following repeated exposure to the selective graminicide, diclofop methyl, however, some biotypes have displayed resistance to many different herbicides.

Two examples of herbicide resistance in *L. rigidum* are the subject of this thesis. The first biotype (WLR2) was collected from railway lines in Western Australia where high rates of amitrole and atrazine were used for season long control of vegetation. This study shows that biotype WLR2 is resistant to amitrole and many triazine herbicides as well as being cross-resistant to phenylurea herbicides and metribuzin which are chemically distinct to amitrole and triazines.

The second biotype (VLR69) considered in this study was collected from a field used for the production of *Lolium perenne* (perennial ryegrass). This field had a history of exposure to five different chemical classes of herbicides in twenty one consecutive seasons. Biotype VLR69 is resistant or cross-resistant to ten different classes of herbicides.

The primary objective of this investigation was to elucidate the mechanisms of resistance and cross-resistance to the triazines, phenylureas and metribuzin in these biotypes. Knowledge of the mechanisms of resistance provides some insight as to how biotypes of *L. rigidum* develop such broad spectrum herbicide resistance. This in turn provides insight into cross-resistance, the phenomenon in which biotypes become resistant to a herbicide as a consequence of selection pressure by an unrelated herbicide. Cross-resistance is of interest because it indicates that structurally distinct herbicides may be susceptible to the same

mechanisms of resistance.

The herbicides that will be considered in detail in the body of the report are the triazines, substituted ureas, metribuzin, amitrole and sulfonylureas. Both biotypes are resistant to the first three classes of herbicide whereas WLR2 is resistant to amitrole and VLR69 is resistant to sulfonylureas.

The patterns of herbicide resistance in Australia are different to those observed in the Northern Hemisphere, largely due to differences in the agricultural systems and weed flora. Herbicide use in Australian agriculture is largely in extensive cereal production in rotation with grain legume crops and pastures. The rapid development of herbicide resistance in *L. rigidum* followed the introduction of the aryloxyphenoxypropionate herbicides which could be used in all phases of the rotation. The use of this class of herbicides in this manner placed sustained and specific selection pressure on *L. rigidum* biotypes. While the onset of resistance was predictable, what was not predicted was the propensity for this species to become cross-resistant to different classes of herbicides due to mechanisms unrelated to target site changes. This phenomenon elevated herbicide resistance to the status of a major management constraint.

In contrast, the majority of cases of herbicide resistance in the northern hemisphere are resistant by virtue of specific mutations of herbicide target sites that are not often associated with cross-resistance. Examples include chloroplastic triazine resistance, dintroaniline resistant β-tubulin mutants and sulfonylurea resistant ALS mutants. These cases were largely caused by persistent use of triazines in corn, dinitroanilines in cotton and chlorsulfuron in wheat, in most cases at high use rates and without seasonal rotation of crops or herbicides. Most of these herbicides have a long soil-half life, which extends the period over which they exert selection pressure on the weed community, exacerbating the potential for resistance to occur. Such agronomic systems provide strong selection pressure for resistance by specific mechanisms.

Notable exceptions to the site of action mutants include triazine resistant *Abutilon theophrasti* and chlorotoluron resistant *Alopecurus myosuroides*. These biotypes are characterised by a low level of resistance, and in the latter case, by the presence of cross-resistance. Although this form of resistance, and especially broad cross-resistance, is unusual in northern hemisphere systems it is more likely to be the norm for herbicide resistance in *L. rigidum.*

Herbicide resistance and cross-resistance in *L. rigidum* is one of the most rapidly developing forms of pest resistance. The mechanisms by which this occurs are the focus of this report. To introduce the various disciplines of herbicide resistance the report begins with a discussion of the biology and agro ecology of *L. rigidum.* This is followed by an introduction to herbicide resistance with a major emphasis being the biochemistry and physiology of cytochrome $P\text{-}450$ mixed function oxidase enzymes. Chapter two provides a brief background to the biotypes under study including their histories of selection pressure and their resistance spectra. The following five chapters examine in detail the mechanisms of resistance in *L. rigidum* to the triazines, phenylureas, metribuzin amitrole and sulfonylureas in that order. Each chapter commences with a review of the salient features of each herbicide class. The report is concluded with a discussion of a model to explain the cross-resistance phenomena observed in the biotypes under study. The major themes of this report are, therefore, variability in *L. rigidum*, the specificity or otherwise of oxidative enzymes and the role of detoxification in herbicide resistance and cross-resistance.

Chapter One

Lolium, Resistance and Metabolism.

1.1 Lolium rigidum

Introduction

Lolium rigidum Gaudin, known in Australia as annual ryegrass, is both a highly productive pasture plant and a serious weed of crops in southern Australia. Its success as a pasture species and its ability to compete in crops are predictably interrelated (Forcella, 1984) and are the product of its highly adaptable nature. The definitive manifestation of this adaptability has been the rapid development of herbicide resistance in this species which is the subject of this report.

L. rigidum originally came from the northern Mediterranean coast and is well adapted to the similar climatic conditions found in southern Australia. It was introduced to the Wimmera in Victoria before 1887 (Mullet 1919, cited by Heap 1988). From there it spread rapidly via deliberate sowings as a pasture species. It is now found in large areas of South Australia, Victoria, southern New South Wales, and Western Australia.

The importance of *L. rigidum* as a weed of cereal crops has lead to considerable efforts to control it in all phases of farm rotations. The advent of selective herbicides revolutionised the control of *L. rigidum*. However, resistance to these herbicides is now becoming apparent. The biology of *L. rigidum* especially in the context of herbicides and herbicide resistance will be the subject of this section of the review.

Taxonomy

There are 5 members of the genus *Lolium* found in Australia, *L perenne*, *L. multiflorum*, *L. temulentum*, *L. rigidum* and *L. loliaceum*. Each of these species is diploid (2n = 14). These species designations are augmented by interspecific hybrids which form quite readily

between the three out crossing wind pollinated species *L. perenne*, *L. multiflorum* and *L. rigidum* (Kloot, 1983). Hybrids are fertile and are able to "continue crossing and backcrossing to yield almost continual variation in every character" (Kloot, 1983). In the present context this observation can probably be extended to herbicide resistance. Identification of these species is also complicated by morphological differences generated under different environmental conditions. Ecotypic variation has led to the recognition of 11 subspecific taxa in *L. rigidum*, of which the highly productive cultivar, Wimmera, is but one (Kloot, 1983).

The important diagnostic features of *L. rigidum* are as follows: "It is an annual to 120 cm; stems reddish even at maturity; leaf blades to 20 cm x 8 mm, acute, glabrous beneath, glabrous or (occasionally) scabridulous above, generally shiny in appearance; spike 3 - 30 cm; rhachis flexuose, slender to somewhat rigid, to 1.8 mm diam.; spikelets 4-18 x 2-5 mm with 3 -11 fertile and 0 - 2 rudimentary florets; glumes lanceolate or narrowly oblong, about the same length as the spikelet; lemma unawned or occasionally with an awn to 3 mm" (Kloot, 1983).

Control of *L. rigidum* in Crops

Cultural techniques

L. rigidum can be controlled by repeated cultivation especially when an early cultivation has stimulated germination. Conventional cultivation techniques exploit aspects of the seed germination dynamics of *L. rigidum*. Seeds which are buried deeply tend to suffer enforced dormancy, possibly caused by changes in gaseous exchange (Gramshaw and Stern, 1977b) Deep burying of seed, by mouldboard ploughing, usually precludes germination and emergence (Reeves and Smith, 1975) whereas shallow seed burial and soil disturbance tend to stimulate germination (Pearce and Holmes, 1976). Control of *L. rigidum* in crops is essential to competition with the crop in the current season and to minimise seed production to reduce infestations in subsequent seasons (Reeves and Smith, 1975). Stubble burning

also reduces *L. rigidum* seed carry-over from the crop phase. Total fire bans and the need for soil conservation have limited the use of fire as a seed control measure. However, where practical, burning is very effective, especially when the seed remains on the plant or at the soil surface (Pierce and Holmes, 1976). Autumn burning overcomes problems of fire bans but requires sufficient standing dry matter to be effective (Reeves and Smith, 1975). Other techniques used to reduce seed burdens include slashing, hay cutting or grazing. Grazing pastures heavily prior to the cropping phase reduces seed set, but sheep are only able to graze the seeds that remain unshed and therefore accessible to grazing. Seeds are destroyed by ingestion enhancing the efficacy of this method of control (Gramshaw and Stern, 1977a).

Chemical techniques

The realisation that conventional cultivation techniques and stubble burning were causing erosion has reduced the use of these practices. Instead, they have been largely replaced with minimum tillage and the use of herbicides to control weeds. One use of herbicides is spraytopping of pasture. This technique relies on close grazing and then removal of stock in spring to synchronise flower production. Herbicides, either paraquat or glyphosate, are then applied to reduce seedset. This practice is normally carried out in the year prior to cropping.

Chemical control techniques available for use in most crops include the use of non-selective herbicides such as glyphosate, amitrole and paraquat prior to planting or crop emergence. These herbicides will only be effective on that part of the seed bank which has germinated and emerged up until crop planting. *L. rigidum* germinating and emerging with or after the crop can only be controlled by selective herbicides. Trifluralin is used pre-emergence and provides adequate but inconsistent control of *L. rigidum* at 400 g ai /ha. A major problem with trifluralin is that its selectivity mechanism relies on placement of the wheat seed under the herbicide band (depth protection). It also requires incorporation into a fine seed bed to reduce loss by volatilisation. Trifluralin has, therefore, become less commonly used with

the advent of minimum tillage. The trend toward earlier planting and extended crop rotations has increased reliance on in-crop herbicides. Most farmers utilise a selective herbicide at the beginning of the season either pre- or post-emergence. Chlorsulfuron or triasulfuron may be applied early to provide a short preplanting fallow or as a post-emergent treatment on young weeds. Wheat is able to rapidly metabolise chlorsulfuron and triasulfuron but may have limited capacity under stress conditions (Beyer *et al.*, 1988). Diclofop is usually applied as a postemergence treatment at 375 g ha^{-1} and is the preferred herbicide for the control of *L. rigidum* and *A. fatua* in wheat. Alternative herbicides for *L. rigidum* control in cereals include atrazine, pendimethalin, diuron and fenoxaprop (Australian Weed Control Handbook, 1992).

In crops other than cereals there is a greater range of herbicides available for grass control. In legume crops trifluralin and simazine are widely used for broad spectrum weed control and to gain moderate control of *L. rigidum*, however, crop tolerance and efficacy is marginal. Now available are a range of aryloxyphenoxypropionate and cyclohexanedione herbicides including fluazifop, haloxyfop, quizalafop and sethoxydim. Soil applied herbicides that may also be effective include prometryne, cyanazine, pendimethalin and metribuzin.

Competition between *L. rigidum* and wheat

L. rigidum competition with wheat can lead to large yield losses (Reeves, 1976, Pearce and Holmes, 1976). Spatial arrangement of wheat is less important than total wheat density in determining the competitiveness of wheat with *L. rigidum* (Medd, 1985). This may indicate that competition for light is not the critical constraint in competition in some environments.

L. rigidum competes effectively with wheat for nitrogen especially after wheat has reached the four-leaf stage (Forcella, 1984) and may have a greater over all ability to take up nitrogen (Reeves, 1976). Competition for nitrogen is thought to be a major cause of yield loss in wheat, especially under adequate moisture conditions (Reeves, 1976). The observation that

competition between seedlings, when light is less important, can significantly reduce yield supports the importance of nitrogen, especially if soil moisture is not limiting.

L. rigidum competition manifests itself in lower wheat dry matter production and fewer fertile tillers and spikelets. Wheat yield loss is directly proportional to the square root of the *L. rigidum* density (Reeves, 1976) although at high *L. rigidum* densities intraspecific competition tends to reduce the competitive effect (Pearce and Holmes, 1976). This indicates that marginal gains in productivity from herbicide use will not be great unless *L. rigidum* populations are reduced to a low level. Economic imperatives for good weed control, therefore, lead to strong selection pressure for herbicide resistance.

Seed yields by *L. rigidum* (on an area basis) in a wheat crop were found to be unresponsive to total *L. rigidum* density from 33 to 900 plants m^{-2}. Seed yields above 30 000 /m^2 were recorded for all densities in these experiments (Rerkasam *et al.,* 1980 a). This, and the competition data above, reflect the strong plasticity in this species. These data have significant implications for the seed bank dynamics of a cropping system. It is implied that very high levels of weed control would be required to maintain a low level of seed input into the seed bank during the cropping phase. Here again a high selection pressure would be required to reduce input to the seed bank.

These two sets of data provide a basis for assessing the viability of *L. rigidum* control. The objective of such control should be to reduce competition with the crop at the seedling stage and to prevent weed seed production. The plasticity of the species ensures that marginal gain from control measures is only positive for reductions of weed density from high to moderately low levels. Extreme control is impractical in an extensive production system but it is clear that relatively small populations of *L. rigidum* can cause reductions in crop yield and produce large numbers of seed which enter the seed bank.

L. rigidum has a short seed bank life with the majority (ca 80 %) of seed germinating in the

season following seed production. Seed is unlikely to survive longer than three seasons (Monaghan, 1980). Rapid turnover of the seed bank may be one of the mechanisms by which *L. rigidum* populations adapt to changing environments. Similarly, the short seed bank life would enhance the rate at which the frequency of resistant individuals would increase in a population subject to herbicide selection pressure. Thus, the rapid turnover of the seed bank in *L. rigidum* is likely to be one major reason for the rapid onset of herbicide resistance in this species. This effect is further enhanced by the plasticity in seed production which allows a low density of plants to produce a large quantity of seed. Resistant survivors can, therefore, make a large contribution to the seedbank. The only drawback for *L. rigidum* is that it is cross pollinated and low weed densities may reduce pollination efficiency.

Root disease

The realisation that grass components of a pasture phase could be hosts to pathogens of subsequent graminaceous crops has also increased selection pressure on *L. rigidum* out of the cropping phase. *L. rigidum* is thought to be an alternate host to *Rhizoctonia solani* (Roget, 1987). To increase the "disease break" effect of a legume based pasture, selective herbicides such fluazifop have been used to control graminaceous weeds and volunteer cereals known to be disease hosts. Coupled with the use of diclofop in cereal crops, and sethoxydim or fluazifop in grain legume crops, the use of an aryloxyphenoxypropionate or cyclohexanedione in the pasture phase means that different herbicides of the same chemical class with the same mode of action may be used in succession. This has prompted an extension effort to raise farmer awareness of the herbicide classes and the implications for resistance.

L. rigidum as a pasture plant

L. rigidum is easily established and has vigourous autumn and winter growth (Kemp, 1988). For these reasons, *L. rigidum* is one of the most productive pasture grasses. Early yields are enhanced by dense swards but later yields are not responsive to density (Donald,

1951). This is presumably due to more rapid nutrient depletion in dense swards or plastic response in the less dense swards leading to similar total biomass production. *L. rigidum* displays moderate salt tolerance when compared with *Elytrigia pontica* (tall wheat grass, tolerant) increasing the utility of *L. rigidum* in moderately salt effected land (Marcar, 1987).

In some areas a disease called annual ryegrass toxicity (ARGT) occurs. ARGT is caused by infection of *L. rigidum* heads by a nematode, *Anguina funesta* , which forms a gall, which is in turn infected by a bacteria of the genus *Corynebacterium* (McKay *et al.*, 1982). When infected by a particular bacteriophage the *Corynebacterium* produces a toxin in a manner analogous to the production of the diphtheria toxin in humans infected with pathogenic *Corynebacterium diphtheria.* The infection in *L. rigidum* is manifested as a slime on the spikes (in extreme cases) of the infected plant. If sufficient galls are ingested, a grazing animal will die. The disease is traditionally controlled by ensuring sufficient grazing pressure is placed upon the grass stand to reduce the numbers of mature spikes (Pearce and Holmes, 1976). Insufficient stock or the need for stands of dry feed confound this technique. ARGT has caused some graziers to attempt to rid their pastures of *L. rigidum* by using graminicides in both the cropping and pasture cycles. This is at cost of reduced winter feed. Spray topping with glyphosate or paraquat may be employed to reduce seed set and is quite effective in reducing nematode populations (McKay, 1982). As for the cereal disease noted above, ARGT causes selection pressure to be placed on *L. rigidum.*

History of herbicide use on *L. rigidum*

Thc first selective herbicides used to control *L. rigidum* in the crop phase were diallate and triallate, trifluralin, and metoxuron. Diclofop-methyl was introduced in 1977 and provided excellent post-emergence control of *L. rigidum* in wheat. Chlorsulfuron was introduced in 1982 and provides good *L. rigidum* control if used while the seedlings have fewer than three leaves. Fluazifop-butyl was released in 1984 for control of grasses in broadleaf crops. Since 1987 triasulfuron has been registered to control weeds (including *L. rigidum*) in wheat crops. Currently 31 herbicides are registered in Australia for selective and non-

selective control of *L. rigidum* in agricultural, horticultural, and industrial applications (mixed formulations have not been counted). These are amitrole, atrazine, propazine, hexazinione, simazine, metribuzin, cyanazine, prometryne, trifluralin, benfluralin, pendimethalin, bromacil, chlorsulfuron, triasulfuron, diclofop-methyl, fluazifop-butyl, haloxyfop-ethoxyethyl, diuron, ethidimuron, fluometuron, methazole, karbutilate, chloroxuron, metoxuron, 2,2-DPA, oxyfluorfen, propyzamide, glyphosate, paraquat, propachlor, sethoxydim, tralkoxydim, and triallate (Australian Weed Control Handbook 1987).

Herbicide resistance in *L. rigidum*

In 1982 John Heap and Ron Knight reported the occurrence of a biotype of *L. rigidum* (SLR5) resistant to the herbicide diclofop-methyl. Further studies revealed that the biotype was also cross-resistant to chlorsulfuron (Heap and Knight, 1986). In 1986 another biotype (SLR31) was collected from the same farm as SLR5 which was also diclofop resistant (Heap and Knight, 1990). These were the first biotypes in which resistance to diclofop-methyl was accompanied by resistance to the sulfonylurea herbicides. It has since been established that SLR5, and the closely related SLR31, are resistant to members of the aryloxyphenoxypropionate, cyclohexanedione, sulfonylurea, and dinitroaniline herbicide families (Heap and Knight, 1990). The history of herbicide application to these biotypes and their resultant resistance spectra are listed in Table 1.1 and 1.2 along with data for other biotypes mentioned in this study.

The mechanisms of resistance in SLR5 and SLR31 have not yet been fully established. Diclofop uptake is similar and there are only slight differences in the detoxification of diclofop acid (Holtum *et al.*, 1991; Shimabukuro and Hoffer, 1991). A major target of these herbicides is the enzyme acetyl-CoA-carboxylase (ACCase) (Rendina *et al.*, 1988). ACCase extracted from SLR31 is similar to that of susceptible plants in its sensitivity to inhibition by these herbicides *in vitro*. The only major difference between SLR31 and the susceptible biotypes is in the response of membrane potential to diclofop. AOPP and CHD

herbicides act as protonophores, causing depolarisation of plant membranes (Wright and Shimabukuro, 1987; Weber *et al.*, 1988). Many AOPP resistant biotypes of *L. rigidum* are able to recover membrane potential once the herbicide has been removed whereas susceptible plants do not recover membrane potential (Häusler *et al.* 1991; Shimabukuro and Hoffer, 1992). The mechanistic basis of this difference in response is not known but enhanced sequestration has been proposed as a likely mechanism (Häusler *et al.* 1991).

Cross-resistance to chlorsulfuron in SLR31 is not related to differences in the target site of these herbicides, acetolactate synthase (ALS), (Matthews *et al.*, 1990). Instead, the mechanism of cross-resistance appears to be related to a higher rate of chlorsulfuron metabolism (Christopher *et al.*, 1991). It is not known whether this is the only mechanism in operation, however, it was the only difference detected.

Most biotypes collected from Australian agricultural areas have a history of exposure to trifluralin and diclofop (Table 1.1). Resistance to diclofop is the most commonly observed form of resistance and the degree of this resistance is generally correlated with the amount of diclofop applied to the population (Knight, 1990).

The most diclofop resistant biotype is WLR96 which was exposed to ten successive generations of selection pressure by diclofop. WLR96 has a high level of resistance to the aryloxyphenoxypropionates and limited cross-resistance to the cyclohexanediones (Heap and Knight, 1990; Holtum and Powles, 1992). ACCase extracted from WLR96 is less sensitive to inhibition by the AOPPS and CHDS and this is thought to be the major mechanism of resistance (Holtum and Powles, 1992).

A biotype similar to WLR96 is SLR3 (Table 1.1, 1.2). SLR3 was subject to three generations of selection pressure by sethoxydim. It also expresses an ACCase less sensitive to inhibition by both the AOPP and the CHDs (Tardif *et al.*, 1993). SLR3 exhibits much higher resistance to AOPP herbicides than it does to CHDs despite being selected by

Table 1.1. Herbicide histories of *L. rigidum* biotypes discussed in this thesis. Data is recorded up to, and including, the last year of seed harvest (Heap and Knight, 1986; Heap and Knight, 1990; Tardif and Powles, 1992; J. Matthews, J. Christopher and I. Heap pers. com)

Year	Herbicide used[a], kg ha^{-1}									
	Biotypes									
	VLR1	SLR2	SLR3	WLR96	WLR2	NLR12	VLR69	SLR10	SLR31	SLR5
1989	-	-	trf 0.60 sth 0.13 bxl 0.30 mcp 0.38	-	-	-	chl 0.02 atz 2.0	-	-	-
1988	-	-	trf 0.60 sth 0.19 bxl 0.40 mcp 0.40	-	-	-	diu - pqt - dqt -	lin 1.3	pqt - lin - car - sth -	-
1987	-	-	trf 0.60 sth 0.14 bxl 0.40 mcp 0.40	-	atz 2.6 amt 2.6	-	chl 0.02 pqt 0.13 dqt 0.08	trf 0.40 sth 0.09 fzp 0.07 mcp 0.75	met - sth - oxy -	-
1986	-	-	trf 0.60 gly -	dic 0.47	atz 2.6 amt 2.6	dic 0.56	diu 3.0	trf 0.40 fzp 0.11 mcp 0.75	chl 0.03	-
1985	-	-	-	dic 0.56	atz 2.6 amt 2.6	dic 0.56	diu 1.5 chl 0.02 atz 0.9	diu 2.00 pqt 0.38 dqt 0.23	met -	-
1984	-	-	-	dic 0.56	atz 2.6 amt 2.6	dic 0.56	diu 1.0 chl 0.02 atz 0.75	pqt 0.31 dqt 0.18	chl 0.03	-
1983	-	-	-	dic 0.56	atz 2.6 amt 2.6	dic 0.45	diu 2.7 chl 0.02	-	cpm -	-
1982	-	-	-	dic 0.56	atz 2.6 amt 2.6	dic 0.45	diu 2.7 dic 0.30	-	trf - dic -	fzp 0.42
1981	-	-	-	dic 0.56	atz 2.6 amt 2.6	dic 0.45	atz -	-	trf - dic -	-
1980	-	-	-	dic 0.56	atz 2.6 amt 2.6	dic 0.45	atz -	-	trf - dic -	trf -
1979	-	-	-	dic 0.56	atz 2.6 amt 2.6	-	diu 1.4 dic 0.40	-	trf - dic -	-
1978	-	-	-	dic 0.56	atz 2.6 amt 2.6	trf -	diu 1.4	-	trf - lin -	dic 0.75
1977	-	-	-	dic 0.56	-	-	diu 1.4	-	trf -	dic 0.75 trf -
1976	-	-	-	-	-	trf -	diu 1.4	-	trf -	trf -
1975	-	-	-	-	-	trf -	diu 1.4	-	trf -	-
1974	-	-	-	-	-	trf -	diu 1.4	-	trf -	trf -
1973	-	-	-	-	-	-	diu 1.4	-	trf -	trf -
1972	-	-	-	-	-	-	diu 1.4	-	trf -	trf -
1971	-	-	-	-	-	-	diu 1.4	-	trf -	trf -
1970	-	-	-	-	-	-	diu 0.90	-	trf -	trf -
1969	-	-	-	-	-	-	diu 0.90	-	trf -	trf -

a Herbicides are recorded by three letter codes as follows: atz, atrazine; amt, amitrole; bxl, bromoxynil; car, carbetamide; chl chlorsulfuron; cpm, chlorpropham; dic, diclofop-methyl; diu, diuron; diquat; fzp, fluazifop-butyl; gly, glyphosate; lin, linuron; mcp, MCPA; met, metribuzin; oxy, oxyfluorfen; pqt, paraquat; sth, sethoxydim; trf, trifluralin; "-" denotes no application or unknown rate where listed with a herbicide.

Table 1.2. Resistance status of each *L. rigidum* biotype for each chemical class of herbicide. "S" indicates susceptibility to all members of a class tested so far, "R" indicates resistance to at least one member of the chemical class while "CR" denotes cross-resistance and "-" indicates that the combination has not been tested. VLR1 is the biotype used as the reference for designations of resistance or susceptibility. Sources are the same as those cited in Table 1.1.

Biotype	Herbicide groups[a]									
	AOPP	CHD	Sulfonyl.	Imidaz.	Dintro.	Triazines	P. Urea	Metrib	Amitrole	Chloroacet.
VLR1	S	S	S	S	S	S	S	S	S	S
SLR2	S	S	S	S	S	S	S	S	S	S
SLR3	CR	R	S	S	S	S	S	-	-	S
WLR96	R	CR	S	-	-	-	-	-	-	S
WLR2	S	S	S	-	S	R	CR	CR	R	S
NLR12	R	CR	CR	-	R	-		-	-	CR
VLR69	R	CR	R	CR	S	R	R	CR	S	CR
SLR10	R	R	-	-	R	-	S	-	-	CR
SLR31	R	R	R	CR	R	S	S	-	-	CR
SLR5	R	R	R	-	R	-	-	-	-	CR

a Herbicide groups are coded as follows: AOPP, aryloxyphenoxypropionates; CHD, cyclohexanediones; Sulfonyl., sulfuonylureas; Imidaz, imidazolinones; Dinitro., dinitroanilines; P. urea, phenylureas; Metrib., metribuzin; chloroacet, chloroacetamides

sethoxydim (Tardif *et al.*, 1993). This exemplifies what appears to be a general trend amongst AOPP resistant biotypes for there to be much lower resistance to the CHDs, even in cases where the mechanism is not a change in ACCase such as SLR31.

WLR96 and SLR3 are distinct from the other cases of AOPP resistance in *Lolium* by virtue of the mutation of ACCase they possess. These biotypes exhibit a very high level of resistance to AOPPs and lower resistance to the CHDs but do not exhibit broad cross-resistance. It would therefore appear that the mechanism selected was specific to the herbicides applying the selection pressure. Such specific resistance mechanisms render the biotypes amenable to control by alternative herbicides.

Another biotype to develop resistance due to a target site mutation is WLR1. WLR1 was selected with seven consecutive applications of chlorsulfuron and expresses an ALS isozyme less sensitive to inhibition by the sulfonylureas and imidazolinones (Christopher *et al.*, 1992). WLR1 also exhibits moderately enhanced metabolism of chlorsulfuron, although this must be a subsidiary mechanism. The diagnostic feature of all ALS mutants of *L. rigidum* characterised thus far is that they are resistant to the non-selective sulfonylurea analogue sulfometuron. Unlike chlorsulfuron, sulfometuron is thought to be less amenable to metabolism (Sweetser, 1985), hence a target site mutation is the most likely mechansim of resistance to this herbicide. This characteristic has been used to identify ALS mutants from the VLR69 population where they were present at a frequency of 4 % (Chapter seven).

The target site mutants of *L. rigidum* are the product of repetitive and stringent selection, a regime which was, until recently, unusual in Australian agriculture (Table 1.1). The result is a biotype that is not broadly cross-resistant and which may be easily controlled by alternative herbicides. The more insidious form of resistance is that in which biotypes are exposed to a variety of herbicide selection pressures and respond by developing mechanisms with broad effects (Table 1.2). SLR31 and VLR69 appear to be of this type. Such biotypes are able to accumulate resistance to many different herbicides leading to an

intractable resistance problem. This mixture of multiple and cross-resistance is, therefore, of great concern to farmers and advisers.

Metabolism of herbicides by *Lolium* sp.

Most studies of the uptake and translocation of herbicides have been conducted in crop plants or in weeds common in the Northern Hemisphere. Thus, there is relatively little information on the uptake and metabolism of herbicides by *Lolium* sp. *L. perenne* metabolises chlorotoluron by N-demethylation, however this proceeds at a relatively (cf wheat) low rate and produces the semi-phytotoxic mono-de-methylated metabolite (Ryan and Owen, 1982). *L. rigidum* metabolises diuron by N-demethylation resulting in a similar semi-phytotoxic metabolite and the non-phytotoxic di-demethylated metabolite (De Prado *et al.*, 1989). There is some indirect evidence of *L. multiflorum* metabolising EPTC (Barta and Dutka, 1991) but this would not be unusual amongst grass species. Metabolism of diclofop and chlorsulfuron by *L. rigidum* produces the same metabolites as those observed in wheat suggesting that similar detoxification reactions occur in both species (Holtum *et al.*, 1991; Christopher *et al.*, 1991). Resistance to diclofop-methyl in *L. rigidum* biotype SLR31 cannot be fully explained by differential uptake or metabolism (Holtum et al 1991), although a 15% increase in metabolite production was observed in the resistant biotype. In general, *Lolium* sp. have not been used as systems for the study of herbicide metabolism and information on this aspect of their biology is scant. Where they have been studied, the pathways of herbicide metabolism appear similar to those of other graminaceous species.

Genetics

Knowledge of the genetics of herbicide resistance in *L. rigidum* is limited to four separate unpublished studies of which this report is one. *L. rigidum* has a high level of self incompatibility which facilitates crossing but prevents "selfing" for the production of homozygous lines. This complicates genetic studies because parent lines are not necessarily homozygous at all loci. To overcome this problem, parent plants must be carefully selected

and screened for resistance, many different individuals must be crossed and their progeny tested separately.

Initial studies on diclofop resistance indicated that resistance was inherited both maternally and paternally (McCarthy, 1987). In many cases of diclofop resistance there is reason to suspect that resistance is a quantitative trait which develops in response to the directional selection applied in the paddock. A prediction derived from this theory is that the degree of diclofop resistance would be proportional to the total selection pressure applied. In most instances this is the case (Knight, 1990). The mechanism of resistance in these biotypes is unknown, but they are likely to be similar to SLR31.

Studies on the inheritance of resistance by Matthews and Christopher also provides some insight into cross-resistance phenomena. Selecting resistant parents from an already resistant population using diclofop resulted in progeny more resistant to both chlorsulfuron and diclofop. Selecting parents using chlorsulfuron had the same effect, suggesting that the resistance phenomena were genetically linked or conferred by the same genes (Christopher, unpublished data; Matthews, 1991). The same result followed selection of parents from susceptible populations using diclofop (Matthews, 1991). Thus the inheritance of resistance in these biotypes suggests that cross-resistance is due to either same or closely linked mechanisms. These studies further support quantitative inheritance of diclofop and chlorsulfuron resistance in SLR31 (Christopher, unpublished data).

In contrast, resistance to haloxyfop in WLR96 is inherited as a single dominant locus (authors unpublished data, see Appendix 1). A similar observation was made on the inheritance of diclofop resistance in *L. multiflorum* collected from Oregon (Betts *et al.*, 1992). In both cases the evidence of a single major gene conferring resistance correlates with the observation that the major mechanism of resistance is an insensitive ACCase. Amitrole resistance in WLR2 is inherited as a single semi-dominant gene (Chapter six), however, the mechanism encoded is not known.

The genetic basis for resistance, therefore, varies for different biotypes with different mechanisms of resistance. Both the nature of the mechanism of resistance and the genes encoding it may be determined by the selection pressure. It appears that diversity of herbicides used in agriculture may select quantitative traits while repetitive selection pressure with a single herbicide may result in single mechanisms encoded by a single genc.

Conclusions

L. rigidum is the weed species with the greatest range of herbicide resistance found in Australia. The prevalence of *L. rigidum* in cropping areas in Australia, its variable nature, high seed production and short seedbank life, all contribute to the ability of this species to rapidly develop resistance to herbicides. Biotypes have been collected which are resistant to all major selective herbicides used in crop production. Mechanisms of resistance that have been elucidated include changes to the target site of the herbicides and increased detoxification with sequestration suspected in some cases. Some biotypes possess all of these mechanisms and have broad cross-resistance. The ability of *L. rigidum* to develop non-specific mechanisms of resistance is probably the basis of cross-resistance in many cases. It is this propensity for biotypes to develop cross- and multiple-resistance that distinguishes *L. rigidum* from other species with herbicide resistance.

1.2 Herbicide Resistance

Introduction

Weeds are the latest of the major agricultural pest organisms to develop resistance to chemical control agents. For this reason there is much to be learnt from previous studies of other resistant pest species. Foremost of these is the study of insecticide resistance. Insects differ from plants in many obvious ways but there now appear to be similarities in their respective resistance phenomena.

Unlike the diversity of modern herbicides, there are only four major classes of novel synthetic insecticides. These are the organochlorines, organophosphates, carbamates and synthetic pyrethroids (Brattsten *et al.*, 1986). Insects have developed resistance to all of these classes. The mechanisms of resistance employed by insects also fit into five broad categories: enhanced detoxification, amplification of arylester hydrolases, altered target sites (acetylcholine esterase), reduced uptake or activation of the insecticide and behavioural changes to avoid contact with the insecticide (Brown, 1990; Brattsten *et al.*, 1986).

Enhanced detoxification is generally associated with an increase in cytochrome P-450 mixed function oxidase (MFO), glutathione transferase (GST), hydrolase or esterase activity (Brattsten *et al.*, 1986; Lamoureux and Rusness, 1989; Georghiou, 1990; Brown, 1990). In some cases it is associated with the presence of a whole new size class of P-450 protein (Sundseth *et al.*, 1989). Insects are thought to have a constitutively high capacity for oxidation of dietary toxins to which they have been forced to adapt in order to consume plants replete with toxic secondary metabolites (Nebert and Gonzalez, 1987). Thus the introduction of insecticides is thought to only be an extension of the intense evolutionary battle between plants and insects. Biotypes with resistance due to MFOs are generally identified by their susceptibility to the insecticide in combination with an MFO inhibitor such as piperonylbutoxide (PBO) (Brown, 1990). This may be misleading given that oxidative enzymes may also activate some insecticides to their toxic forms and that some oxidative

enzymes may be less sensitive to PBO. MFO expressed in insecticide resistant biotypes appears to have high non-specific affinity for many lipophilic substrates. Consequently, many insects with this mechanism of resistance are resistant to more than one class of compound (Brattsten *et al.*, 1986). This appears analogous to the cross-resistance observed in *L. rigidum.*

Insects may also, but less commonly, become resistant through modifications to isozymes of acetylcholine esterase, the target protein of some insecticide classes. Variation in the nature of this mutation effects the resistance phenotype and there appear to be many different mutations with different resistance spectra (Brattsten *et al.*, 1986).

A salient feature of insecticide resistance is that a biotype may possess both major types of mechanism. Thus an insect biotype may have both a specific target mutation and a broad spectrum detoxification mechanism which in combination confer resistance to many different insecticides. Such biotypes with multiple mechanisms of resistance are termed "multiple-resistant" while "cross-resistant" is reserved for biotypes with a single mechanism that effects more than one class of substrate. The terminology used to describe different forms of herbicide resistance do not make any assumptions about the mechanisms of resistance involved. Rather, the terms used in this report describe similar phenomena but are based on the nature of the selection pressure that lead to the resistance. This is further explained in the section on terminology.

Insecticide resistant biotypes tend to be less ecologically fit than susceptibles immediately following selection. However, biotypes generally regain fitness if maintained in competition with other insects (Mc Kenzie and Game, 1987; Clarke and McKenzie, 1987). Other ecological principles of insecticide resistance differ from the situation for plants. In particular, insects are mobile and many have multiple generations per year. Insects are also most troublesome in situations where the potential for crop rotation is limited such as orchids and intensive horticulture. In these situations the populations may be subject to repeated use

of particular insecticides enhancing selection pressure for resistance (Georghiou, 1990).

Herbicide resistance differs in that plants generally have only one generation per year and are not mobile in the vegetative stage although they may travel in the form of seeds, pollen or as "tumble weeds". Plants also store seed in the soil as a "seed bank" thus creating a reservoir of susceptible (or resistant) individuals during the selection process. While mechanisms of resistance may be similar in principle to those in insects, plants have tended to adapt more often by target site mutation than by increases in herbicide metabolism (Le Baron and Macfarland, 1990).

To understand the general principles in herbicide resistance it is first necessary to define the nomenclature that will be used in this review as it differs from that used in the insect resistance literature noted above. Furthermore, an attempt will be made to describe what a herbicide is, how its properties and uses are determined, and to emphasise that resistance, tolerance, and selectivity are all relative terms.

Definitions and concepts in herbicide resistance

Susceptibility to herbicides means that representative plants of a species are killed by the rate of herbicide application that is deemed useful and efficient in the environment and under the conditions in which the target species grows.

Tolerance to herbicides is defined as the case where a population of a species is not killed by a rate of herbicide that kills another population of the same or of a different species. Tolerant populations have not been selected by the herbicide and, therefore, the tolerance is a normal characteristic of the population. This is a consequence of the "natural variation from species to species, and population to population, in many characteristics including herbicide sensitivity" (Le Baron and Gressel, 1982). It should be noted, that most cases of "tolerance" are really "tolerance to a given rate of application" with crop damage occurring at

higher doses. This term has recently been made redundant in this context by the Weed Science Society of America (WSSA), however, use of this term will be made in this report.

The redundancy of "tolerance" derives from its use by some authors to describe resistance resulting from increases in normal plant defences such as detoxification. These authors reserved "resistance" for the dramatic changes associated with mutated target sites. The term also has its origins in plant pathology in which it describes a plant which is susceptible to infection but which withstands the effect of a pathogen. This is supposedly analogous to a plant being slightly affected but not killed by a herbicide.

Resistance to herbicides is defined as a decrease in response to a normally effective rate of herbicide in a population of a species that has been exposed to selection pressure by that herbicide. Essential elements of this definition are: the exposure to selection and the ability of a proportion of the population to survive and reproduce after being treated with the herbicide. Designation of resistance must be made in reference to a wildtype susceptible population.

Multiple-resistance is the phenomenon where resistance to two or more distinct classes of herbicides is conferred as a consequence of exposure to, and selection by, both herbicides.

Cross-resistance is the case where resistance to a distinct class of herbicides is conferred after selection by a herbicide of an unrelated chemical class.

What makes a chemical a herbicide?

Herbicides are usually synthetic organic molecules which inhibit plant growth, or cause plant death. A compound is not considered a herbicide until it has been demonstrated to kill plants at "realistic" rates of application. Furthermore, a substance can only be considered a herbicide for a particular target species if it controls wildtype populations in the field at rates

which are economically and environmentally desirable. Sodium chloride is quite an effective herbicide, however, it must be used at high rates, it is non-selective and rather too persistent. Thus sodium chloride is neither economic or environmentally sound as a herbicide.

Modern herbicides are usually applied at rates between 5-3000 grams per hectare. The actual rate used is usually the one which will give the most reliable and economic control of the target species under the most commonly encountered circumstances, whilst ensuring crop tolerance (if required). It is these rates upon which terms like resistance and tolerance hinge. After the appropriate rate has been decided the manufacturer and marketer set a price (on a per hectare basis) that will: a) ensure a market share for the product; b) reflect the utility of the product and c) provide the maximum return to research and development costs.

Herbicide Resistance - Population Dynamics and Fitness

The study of herbicide resistance is concerned with weed populations which emerge after herbicide selection. These plants are derived from populations that were susceptible to the herbicide at the rate being applied. These "resistant " weeds are thus, representatives of a genotype that existed in the wildtype population at a frequency that was essentially undetectable in early usage.

The efficient application of a herbicide to a population allows only individuals possessing rare, herbicide resistance genes to survive. These genes may be the result of recent spontaneous mutations or they may be the remnant of adaptation to an environmental stress which either no longer exists or is sporadic in its occurrence. In field crops any individual carrying such resistance genes must not only survive but also compete with the crop at the vegetative stage and produce viable seed. Thus, although emerging populations may be less fit, there is immediate selection pressure for increased fitness. This second phase of evolution is one in which the plant evolves "modifiers" to mitigate the effects of resistance genes (Holt, 1990; McKenzie and Game, 1987). The compensation provided by these

modifiers improves fitness. In the case of triazine resistance where PS II is less efficient, modification of the chlorophyll a/b ratio is observed indicating a change in the proportion of PSI and PS II reaction centres (Vencill *et al.*, 1987; Holt, 1990). The effect of modifiers may be determined by the reproductive time elapsed since the resistance emerged. If the population survives a number of seasons of intense intraspecific competition under herbicide selection one might expect fitness levels to change while the plants remain herbicide resistant. While this has not been documented with plants there are examples in cases of insecticide resistance in which the integration of a resistance trait into a modified background returns fitness to normal levels (McKenzie and Game, 1987).

Fitness of resistant plants is usually measured in comparison with susceptible plants of the same species derived from the same environment. Ideally, isogenic plants that differ only in the gene for resistance should be used to measure the effect of the resistance gene (Holt, 1990; see also Roush and McKenzie (1987) for equivalent observations in insects). The few studies with near isogenic resistant and susceptible plants indicate that there is some cost associated with resistance to triazines (Forcella, 1987). The magnitude of this cost is, however, influenced by the environment (Holt, 1990). Electron transport in chloroplasts with the common triazine resistant mutation is more sensitive to high temperatures than is electron transport in wild type chloroplasts. Conversely, at low temperatures there is little difference between the two (Holt, 1990). This is consistent with many other studies of plant mutants whose mutant phenotype (which may also be less fit) is only apparent in a specific environment.

Differences in fitness manifest themselves in lower dry matter productivity and fertility. This is of concern both for the elimination of resistant weeds from crop land and for the maintenance of productivity in herbicide resistant crops. Theoretically resistant weeds should be removed from the population by competition with fitter susceptibles in the absence of herbicide selection pressure, however, the effect of modifiers noted above will reduce this effect. Similarly the deleterious effect of a resistance gene in a crop plant may be reduced

by breeding and selection after the introduction of the resistance trait.

Herbicide selectivity and crop tolerance

As already noted, species with herbicide tolerance, exist independent of herbicide selection. For example, the tolerant status of wheat to many herbicides is largely the result of the herbicide research and development process. Potential herbicides are tested against a range of crop species to determine their effect. If a crop species is unaffected by the herbicide at rates that control one or more weed species, then the species is deemed tolerant to the herbicide and the herbicide termed selective in that crop. If the herbicide damages all crop species, it is termed non-selective. Until recently major crops (wheat, maize, soybean) were bred for characters other than herbicide tolerance. Thus the tolerance to herbicides displayed in crop species was, and is, determined by herbicide synthesis, and not by selection and breeding. Wheat, therefore, appears to be cross-resistant to a wide range of herbicides. However, this is the serendipitous result of evaluating millions of potential herbicides for selectivity (Gressel, 1990). This serves as an example of how tolerance to a wide range of herbicides can exist in one species without selection by each herbicide. There is no indication of whether tolerance in a crop species to dissimilar herbicides is due to mechanisms in common but this may be the case for some herbicides.

Resistance and Selection Pressure

Herbicides represent a dramatic change in the environment for the weed population, and in most situations, they probably represent the single limiting factor for growth of a plant in that environment. Herbicides, therefore, provide extreme and quite specific selection pressure on the plant populations to which they are applied. Herbicide resistance is, therefore, the change in the balance of the population in favour of resistant individuals.

Target Site Resistance

Resistance to many different classes of herbicides has developed as a consequence of mutations of the genes encoding their protein targets. This mechanism of resistance is often

associated with large decreases in sensitivity to the herbicide both *in vitro* and *in vivo*. Plants with this type of resistance are rarely cross-resistant to other classes of herbicide unless the herbicides have a binding site in common. Where herbicides do have a common target site these mutations provide an opportunity to determine the effect of different amino acid residues on herbicide binding. Such studies have been instructive in the elucidation of the binding sites of the 32 kd protein in PS II (Arntzen *et al.*, 1982). Different target site mutations will be discussed separately and in order of their frequency of occurrence.

Triazine resistance

The intensive use of chloro-s-triazines lead to the development of resistance which was first formally identified in 1970 (Ryan, 1970). Widespread reports of atrazine resistance in maize fields followed in both North America and Europe (Bandeen *et al.*, 1982; LeBaron and McFarland, 1990). Atrazine resistance also arose in other areas where it had been intensively used, such as roadsides and olive groves (Gressel, 1982).

Early studies revealed that there were no differences between resistant and susceptible biotypes in uptake, translocation or metabolism (Radosevich *et al.*, 1977). Examination of isolated thylakoids revealed that atrazine did not bind the target site in the resistant plants (Radosevich *et al.* 1977; 1979). The difference in binding is caused by the substitution of serine for glycine in the target protein (Hirschberg and Mc Intosh, 1983). This difference is the mechanism of resistance in all but a few cases of triazine resistance that have been investigated (Darmency and Gasquez, 1990; Gronwald, 1989). The alteration causes changes to the binding of other inhibitors such as metribuzin, little effect on inhibition by diuron and increased binding of pyridate (Arntzen *et al.*, 1982). The mutation also confers a temperature sensitive fitness difference due to changes in the rate of electron transport through PS II (Stowe and Holt, 1988). Triazine resistance is discussed in more detail in Chapter three.

Sulfonylurea resistance.

The sulfonylurea herbicides such as chlorsulfuron, metsulfuron and sulfometuron inhibit the enzyme acetolactate synthase (ALS, E.C.4.1.3.18), a key enzyme in the synthesis of the branched chain amino acids, valine, leucine and isoleucine (La Rossa and Schloss, 1982; Ray 1984). Initially the herbicide chlorsulfuron was restricted in its patterns of use because of its long half life in alkaline soils (Beyer *et al.*, 1988). More recently, the use of chlorsulfuron in North America has been restricted by the development of resistance in weed species. There has been a rapid increase in the number of cases of sulfonylurea resistance in North America with 143 identified thus far (Thill *et al.*, 1991). Sulfonylurea resistance has been observed in *Kochia scoparia* L., *Lactuca serriola* L., *Salsola iberica, Lolium perenne* L., *Stellaria media* and *L. rigidum* (Mallory-Smith *et al.*, 1990; Christopher *et al.*, 1992; Saari et al, 1989; Saari *et al.*, 1992). In most cases the selective agent was chlorsulfuron used in continuous wheat production. Thus there has been rapid development of sulfonylurea resistance following persistent use of these herbicides.

Where studied the mechanism of resistance observed in the above resistant biotypes is an alteration of the target enzyme, ALS. The predominant mutation found in plants is a substitution for proline at position 194 in the amino acid sequence of the protein. The substituted residues include serine, threonine, histidine, glutamine and alanine (Haughn *et al.*, 1988; Guttieri *et al.*, 1992; Lee *et al.*, 1988). The portion of the protein containing the mutation and presumably the herbicide binding site is highly conserved between species and this may explain the toxicity of sulfonylureas to a wide range of plants and microorganisms. The binding site may also have some regulatory function as indicated by competition between sulfonylurea inhibitors and leucine, a feedback regulator of ALS (Subramanium *et al.*, 1991). Such a regulatory function would explain the conservation of this site. Different mutations of ALS differentially affect the binding of various sulfonylurea herbicides and other ALS inhibitors such as the imidazolinone herbicides (Winder and Spalding, 1988). This capacity of the target enzyme to tolerate a variety of mutations must increase the probability of resistance occurring.

Aryloxyphenoxypropionate resistance

Biotypes of *L. rigidum*, *L. multiflorum*, *Avena sterilis* and *Avena fatua* have all developed resistance to this class of herbicide by changes to the target site, acetyl-CoA carboxylase (ACCase) (Tardif *et al.*, 1993; Gronwald *et al.*, 1989; Holtum and Powles, 1993; J. Holtum personal communication). Little is known about the structure of the enzyme or the nature of the mutation conferring resistance. Resistance by mechanisms other than a change to the ACCase target site has also been observed. In some *L. rigidum* and *A. fatua* biotypes the only difference observed in the resistant biotypes is in the response of the cell membrane to the herbicide (Holtum *et al.*, 1991; Hall *et al.*, 1992; Shimabukuro and Hoffer, 1992; Häusler, 1991). Differential repolarisation is associated with resistance that is not due to changes in ACCase and it may reflect an alternative, as yet unidentified mechanism of resistance associated with the membrane (Wright and Shimabukuro, 1987; Shimabukuro, 1990), however, this is not yet confirmed.

Dinitroaniline resistance

Biotypes of *Eleusine indica*, *Setaria viridis*, *Alopecurus myosuroides Amaranthus palmeri* and *L. rigidum* have developed resistance to the dinitroaniline herbicides (Mudge *et al.*, 1984; Morrison *et al.*, 1989; Moss and Cussans 1991; Gossett and Murdock, 1992; Heap and Knight, 1986). Resistance to trifluralin was first reported by Mudge et al (1984) in *Eleusine indica* after continued application of trifluralin within cotton (*Gossypium hirsutum*) monoculture. Only in this *Eleusine* biotype has the mechanism of resistance been confirmed. The biotype is in the order of 10,000 times more resistant to dinitroanilines than the susceptible. The resistant biotypes express an altered form of β tubulin which is able to polymerise into microtubules in the presence of the herbicide *in vitro* (Vaughn and Vaughn, 1990). The altered β tubulin appears to form a microtubule that is more stable than the wild type form of the polymer and this stability is thought to confer a fitness disadvantage. The resistant biotype is hypersensitive to some anti-microtubule drugs (Vaughn *et al.*, 1987) which provides confirmatory evidence that resistance is related to the site of action.

1.3 Resistance due to Enhanced Detoxification

Detoxification is the major cause of herbicide tolerance in crop species and is also responsible for herbicide resistance in some weed species. Detoxification reactions are of two major types known as phase I and phase II metabolism. Phase I metabolism is generally the initial oxidation of a herbicide molecule. This may detoxify the herbicide or it may activate the molecule to its toxic form (Hutson, 1987). Phase II reactions are usually conjugation reactions in which the molecule is completely detoxified by the attachment of either glutathione or a sugar residue (Shimabukuro, 1985; Jones 1991). Some herbicides such as the triazines may be directly conjugated while others such as chlorsulfuron need to be hydroxylated prior to conjugation (Beyer *et al.*, 1988). These two different types of detoxification reaction will be considered separately.

1.4 Oxidative Enzymes and Herbicide Metabolism

Oxidative enzymes catalyse a range of different reactions including, hydroxylation, sulfoxidation, aliphatic oxidation and dealkylation of herbicides (Jones, 1991). The most commonly studied form of these are the cytochrome P-$_{450}$ mixed function oxidase enzymes. Enzymes in this class are implicated in the mechanisms of resistance to many different insecticides and herbicides. While MFOs from plants and animals function in a similar manner, their major physiological roles are thought to be different.

An important function of animal cytochrome P-$_{450}$ is the oxidation of lipophilic molecules to polar compounds which can then be excreted, a capacity concentrated in the liver of mammals (Nebert and Gonzalez, 1987). The origin of this capacity for oxidative detoxification is suggested to be continual adaptation of animals to consume toxic plant species. As a consequence animals have developed oxidative enzymes with broad specificity for hydrophobic molecules to allow them to metabolise the variety of dietary toxins they ingest. In contrast plant MFO is thought to be adapted for the synthesis of

essential plant metabolites. Consequently, plant MFO systems are considered to have greater substrate specificity but lower concentration in tissue due to their primarily biosynthetic function (Jones, 1991). This theory could be profitably modified to allow for the fact that synthetic oxidative reactions also occur in animal cells while detoxification is largely a hepatic or epithelial function (Jones, 1991; Nebert and Gonzalez, 1987). Plants have no need for an organ equivalent to the liver (as they excrete to the vacuole) and therefore plant MFO generally has a low concentration in tissue and specialised biosynthetic function (as would many non-hepatic animal cells). It would seem that perceptions of MFO are biased by the ease of extraction of these enzymes from the liver or haemolymph of rats and insects respectively.

Function in normal plant metabolism

Cytochrome P-$_{450}$ enzymes are primarily found in the endoplasmic reticulum of the plant cell (Soliday and Kolattukudy, 1978). They participate in the oxidation of cinnamic acid, flavinoids, terpenoids and fatty acids (Hagmann *et al.,* 1983; Soliday and Koluttkudy, 1978; Jones 1991). In particular, cytochrome P-$_{450}$s play a role in the synthesis of cytokinins, gibberellins and abscisic acid (ABA) (Lenton, 1987). These are only a few of the oxidative reactions that could be performed by cytochrome P-$_{450}$ or other oxidative enzymes, however, difficulties associated with extraction and low concentration in tissue hinder demonstration of their involvement in many reactions.

The catalytic cycle of cytochrome P-$_{450}$ is illustrated in Figure 1.1 A. The cycle requires the substrate to bind the enzyme, after which one electron is passed from NADPH via NADPH-cytochrome P-$_{450}$ reductase to the haem centre where it reduces Fe (III) to Fe (II). Molecular oxygen is introduced and substrate-Fe^{3+} and O_2^- are formed. A second electron is donated, possibly by NADH, either via NADPH-cytochrome P-$_{450}$ reductase, NADH cytochrome b_5 or NADH cytochrome c reductase (it is uncertain which), to the haem centre which yields Fe^{3+} and O_2^{2-}. With the introduction of two hydrogen ions the O-O bond is split and the highly reactive oxidant $[FeO]^{3+}$ (complexed with the substrate) is formed along

with H_2O. The substrate is then oxidised and the haem returns to the Fe (III) state (Jones, 1991; Mc Murray and Groves, 1986).

Inhibition of cytochrome P-$_{450}$

Within this reaction sequence there are opportunities for inhibition of the enzyme system. Some inhibitors, including cytochrome c, divert electrons away from the NADPH cytochrome P-$_{450}$ reductase whereas antibodies inhibit its function directly. Inhibition by antibodies emphasises the importance of the reductase in electron transfer (Benveniste *et al.*, 1986). Other inhibitors can be divided into three classes; those which bind reversibly to the protein, those which bind to the haem and those which bind irreversibly to either the protein or the haem. The latter generally requires catalytic activation by the enzyme while the first is exemplified by competition between substrates with differing affinity and reactivity (Ortiz de Montellano and Reich, 1986).

An important inhibitor is carbon monoxide (CO), which inhibits most cytochrome P-$_{450}$ enzymes. This inhibition in combination with reversal by light is a diagnostic characteristic of this class of enzyme *in vitro*. CO competes with oxygen giving rise to an absorbance maximum at $_{450}$ nm on which the name cytochrome P-$_{450}$ is based (Jones, 1991). The binding of substrates to the enzyme leads to maximum at 390 nm and a minimum at 420 nm which is known as a type I difference spectrum. Inhibitors which bind both a lipophilic region of the protein (like a substrate) and which also act as strong ligands for iron cause absorbance characteristics known as type II difference spectra with a peak at 420 nm (Jones, 1991). Compounds containing nitrogen heterocycles (imidazoles and pyrimidines) such as metyrapone are effective in producing this type of inhibition (Ortiz de Montellano and Reich, 1986).

Some reactions have been characterised which appear to be mediated by cytochrome P-$_{450}$ enzymes but which exhibit atypical binding spectra or insensitivity to CO (Soliday and Kolattukudy, 1978). The basis for this deviation from established patterns is not known.

Some hydroxylation reactions, however, may be catalysed by peroxygenase enzymes (Ishimaru and Yamazaki, 1977). Such enzymes incorporate oxygen derived from hydroperoxides and not O_2.

Many inhibitors are oxidised by their target enzyme before reacting with it to cause irreversible inhibition. These inhibitors are of interest because they must bind to the enzyme without initial inhibition and they must be activated by the enzyme before irreversibly binding to it. These constraints suggest that inhibitors of this type may be relatively specific to different cytochrome $P\text{-}450$ enzymes. The reactive intermediate formed by the action of the enzyme may bind either to the protein or to the haem. Binding to the protein is generally less destructive to enzyme activity than is binding to the haem which is essential to catalytic activity. The haem is also nearest to the activated inhibitor and therefore most vulnerable to the reactive intermediate that is generated in this type of inhibition (Ortiz de Montellano and Reich, 1986).

Compounds containing N-N functions such as substituted hydrazines inhibit cytochrome $P\text{-}450$ by covalent binding to the haem. An example of this is inhibition by 1-aminobenzotriazole (ABT). ABT is oxidised to form the highly reactive intermediate, benzyne (Figure 1.1 B, C), which then forms covalent bonds across the haem leading to the loss of iron and inactivation of the enzyme (Figure 1.1 C) (Ortiz de Montellano and Reich, 1986). ABT also inhibits enzymes other than cytochrome $P\text{-}450$ mixed function oxidase such as sulfoxidase (Blee and Durst, 1987), thus inhibition by ABT is not conclusive evidence of cytochrome $P\text{-}450$ involvement in a reaction.

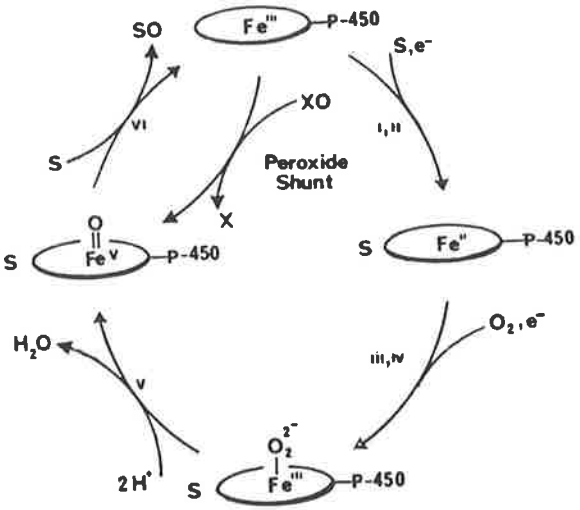
While ABT is not highly toxic to animals or plants, other inhibitors of cytochrome $P\text{-}450$ can produce toxic effects. An example is the azole fungicides like triadimenol whose

Figure 1.1. A. The catalytic cycle of cytochrome P-450 (from McMurray and Groves, 1986).

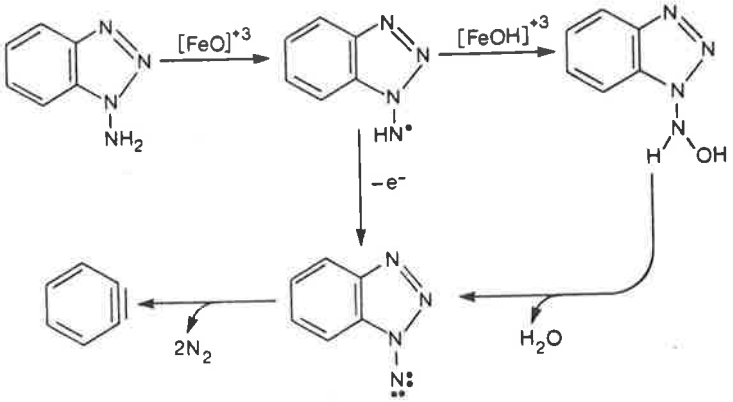
B. The oxidation of ABT to benzyne by cytochrome P-450 enzymes (from Ortiz de Montellano and Reich, 1986).

C. The various means by which the highly reactive benzyne binds to the haem to remove iron (from Ortiz de Montellano and Reich, 1986).

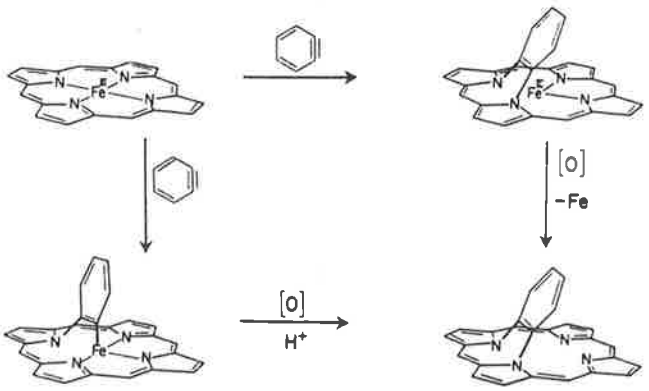
A


SO
Fe'''
P-450
S,e⁻
S
VI
XO
I,II
Peroxide
Shunt
X
O
Fe V
P-450
S
S
Fe''
P-450
H2O
O2,e⁻
III,IV
V
O2 2⁻
2H⁺
S
Fe'''
P-450

B


[FeO]+3
[FeOH]+3
NH2
HN•
H
OH
-e⁻
2N2
H2O
N:

C


Fe
[O]
-Fe
[O]
H+

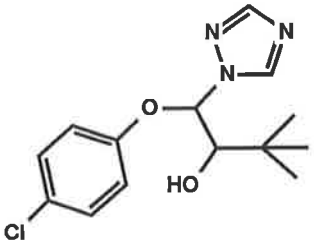
enantiomers may also act as growth regulators in plants (uniconazole is one such commercial compound as are the experimental compounds BAS110 and 111) (Köller, 1987; Lenton 1987). These compounds contain a 1,2,4-triazole ring attached to a hydrophobic backbone (Figure 1.2). This structure is analogous to that of the metyrapone type inhibitors noted above and it is thought that the triazole coordinates to the haem while rest of the molecule binds to the substrate binding site (Lenton 1987). The hydrophobic portions of the molecule mimic those of the substrates they replace, supporting the replacement model (Figure 1.2).

In sensitive fungal species, azole fungicides inhibit demethylation of 2,4-methylene dihydrolanisterol (Figure 1.2) in the synthesis of ergosterol, an essential precursor in cell membrane biosynthesis (Lenton, 1987). The enantiomers of many azoles also retard plant growth by inhibiting ent-kaurene oxidase, an essential enzyme of gibberellin biosynthesis. Some analogues also effect sterol synthesis in some plant systems but this appears to be less important than the effects on gibberellin synthesis (Köller, 1987; Lenton, 1987). The 1,2,4-triazole of these compounds is also present in amitrole which has dramatic affects on plant growth (Carter, 1975). Although amitrole may inhibit mixed function oxidase enzymes in mammalian systems (Koop, 1990) this type of inhibition has not been implicated in its mode of action in plants (see Chapter six for details). It does appear, however, that 1,2,4-triazole containing compounds may have affinity for metalloproteins.

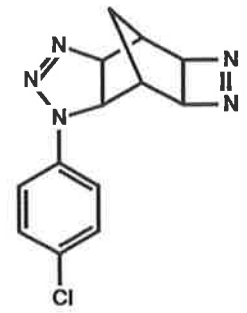
Specificity of inhibition

Many studies of the effect of inhibitors indicate that different enzymes appear to have differential sensitivity to inhibitors. This is taken to reflect the specificity of the enzymes. Some of the inhibitors appear to be very general in their effects (ABT, metyrapone, PBO and tetcyclasis) while others are very specific (triadimenol) (Ortiz de Montellano and Reich, 1986; Lenton 1987). The use of both general and specific inhibitors can, therefore, be used to indirectly discriminate different cytochrome P-$_{450}$ enzymes.

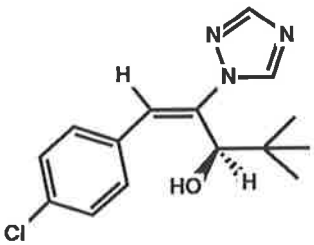
Figure 1.2 The structures of: the cytochrome P-450 substrates, the triazole growth regulators triadimenol (A), tetcyclasis (B) and R/S uniconazole (C and D), in comparison with the endogenous substrates of the enzymes inhibited by the triazoles, ent-kaurene (E) and 2,4-methylene dihydrolanosterol (E), cinnamic acid (F) and lauric acid (G),. Arrows indicate the substituents or positions that are oxidised.



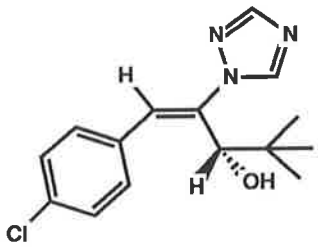
A. Triadimenol



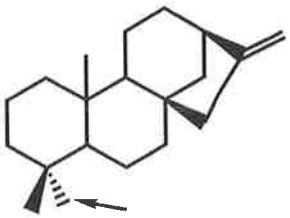
B. Tetcyclasis



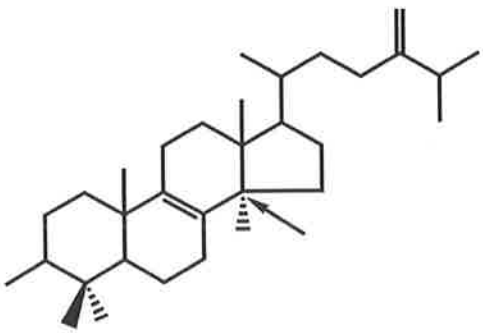
C. Uniconazole (R)



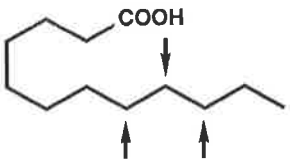
D. Uniconazole (S)



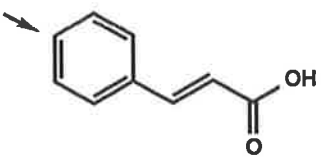
D. ent-kaurene



E. 2,4-methylene dihydrolanosterol



F. Lauric acid



G. Cinnamic acid

Even general inhibitors like ABT do not appear to inhibit all cytochrome P-450 enzymes as indicated by *in vitro* studies of enzyme inhibition. Cinnamate-4-hydroxylase is sensitive to ABT *in vitro* while lauric acid hydroxylase is less affected (Reichhart *et al.,* 1982). Tetcyclasis is a potent inhibitor of diclofop metabolism in microsomes of wheat while ABT is less effective (McFadden *et al.,* 1989). Similarly, tetcyclasis and paclobutrazole inhibit both N-demethylation and ring methyl hydroxylation of chlorotoluron in wheat while ABT is a preferential inhibitor of ring methyl hydroxylation (Canivenc *et al.,* 1989; Cabanne et. al., 1987; Gonneau *et al.,* 1988). Characterisation of a range of different herbicide detoxification reactions *in vitro* with respect to their relative inhibitor sensitivity demonstrates that different reactions have a different spectrum of response to the inhibitors (Moreland and Corbin, 1992).

Differential response to inhibitors following various induction treatments can further distinguish separate enzymes. A combination of induction and inhibition studies provides evidence for three isoforms of the enzyme dealkylating 7-ethoxycoumarin (Werck-Reichhart *et al.,* 1990). The same type of evidence suggests that wheat microsomes contain many different cytochrome P-450 dependent monooxygenases catalysing the oxidation of diclofop, chlorsulfuron, linuron and 2,4-D (Frear *et al.,* 1991). This differentiation on the basis of inhibitor sensitivity provides further evidence for separation of function in this class of enzyme. Thus the potency of an inhibitor varies with different enzymes from different sources.

Substrate competition

An alternative means of determining specificity is competition between substrates. Some substrates with similar structures (chlorsulfuron, triasulfuron) are competitive *in vitro* (Frear *et al.,* 1991). Competition between substrates would indicate that they were detoxified by a common enzyme(s). In the system used by Frear *et al.* (1991) triasulfuron, chlorsulfuron and diclofop hydroxylase activity was induced in wheat seedlings, cv Olaf, by phenobarbital and some degree of competition was observed between all three substrates. In contrast,

phenobarbital induced wheat microsomes prepared by Zimmerlin and Durst (1991) hydroxylate diclofop but show no competition or inhibition with diuron, chlorsulfuron or 2,4-D despite the fact that the herbicides are all potential substrates. These microsomes do not oxidise chlorsulfuron, 2,4-D, bentazon or chlorotoluron indicating that the diclofop system is distinct from those oxidising other herbicides (Zimmerlin and Durst, 1991). Given the high level of induction gained from phenobarbital it is possible that this apparent specificity for diclofop is derived from both the specificity of the reaction and the specificity of induction. It is possible that other treatments may induce isozymes that also react with other herbicides. This may be the case for uninduced microsomes in which chlorsulfuron and diclofop were competitive (McFadden *et al.*, 1989). Alternatively diclofop oxidation may be specific to one enzyme. The general proposition that distinct enzymes oxidise various herbicide substrates in wheat is supported by observations of inhibition, competition and induction responses for other herbicides (Frear *et al.*, 1991). Differences between experimental materials and systems may account for differences in the extent of substrate competition, however, there remains the possibility of overlapping substrate specificity in enzymes degrading herbicides.

Interactions of inhibitors with herbicides

The effect of inhibition of plant cytochrome P-450 enzymes is evident in the synergy of herbicides normally detoxified by these systems. Inclusion of ABT with chlorotoluron increases the toxicity of chlorotoluron to wheat (Gaillardon *et al.*, 1985). Wheat is normally able to rapidly detoxify chlorotoluron, a function which is sensitive to inhibition by ABT, hence the synergistic interaction (Cabanne *et al.*, 1987). A similar interaction between ABT and chlorotoluron may be observed in chlorotoluron resistant *A. myosuroides*, suggesting that metabolism of the herbicide is an important factor in herbicide resistance in that species (Kemp and Caseley, 1987). Tetcyclasis, another cytochrome P-450 inhibitor, can also cause this effect on bentazon metabolism in soybean and maize suspension cultures (Leah *et al.*, 1989, Gronwald and Connelly, 1991). The initial reaction detoxifying bentazon is an oxidation which is sensitive to tetcyclasis *in vitro*. Triazole based molecules, BAS 110 and

BAS 111 also inhibit the metabolism of bentazon and intensify its effect in soybean (Leah *et al.*, 1989).

Induction of cytochrome P-$_{450}$ enzymes

The converse of herbicide synergy is antagonism. There are two forms of herbicide antagonism related to the activity of cytochrome P-$_{450}$ enzymes. One is where a herbicide must be oxidised to an active form, as in EPTC, for which inhibition of oxidation may protect the plant from the active species of the compound (Barta and Dutka, 1991). The other is the effect of a xenobiotic in inducing defences such as detoxification enzymes. This effect is also referred to as "safening". This form of antagonism is best understood for herbicides detoxified by glutathione conjugation but it may also apply to detoxification by oxidation (Sweetser, 1985).

Induction of cytochrome P-$_{450}$ enzymes occurs in response to light, wounding, pathogenesis, xenobiotics (which may also be substrates) and endogenous substrates (Reichhart *et al.* 1986). Wounding induces many enzymes including both cytochrome P-$_{450}$ and NADPH cytochrome P-$_{450}$ reductase (Reichhart *et al.*, 1980). This response is associated with increasing cinnamate-4-hydroxylase and in-chain-lauric acid hydroxylase activity. The induction of the former, but not the latter, can be repressed by the presence of 4-hydroxy cinnamate, suggesting the possibility of feed back regulation of expression by the enzyme product and differential regulation of the two enzyme activities (Salaün *et al.*, 1981). The converse of this is that the absence of the product through inhibition of the enzyme might aid in the induction of the enzyme in some cases.

Initiation of pathogenesis results in a series of inductions of enzymes involved in the biosynthesis of terpenoid and flavinoid phytoalexins. Elicitors extracted from the pathogens may also have the same induction effect (Hagmann *et al.*, 1983). This indicates that the regulation and expression of cytochrome P-$_{450}$ enzymes is specifically controlled.

The effect of xenobiotics is of more interest, given their status as inducers, synergists antagonists and herbicides. Many plant tissues have low extractable cytochrome P-450 activity that is not readily detected by conventional extraction (Salaün *et al.*, 1981). To overcome this it is necessary to incubate plant tissue with an inducer prior to making the extraction. Commonly used inducers include phenobarbital, napthalic anhydride, ethanol, cyometrinil, dichlormid, benoxacor and clofibrate (Reichhart *et al.*, 1986; Saläun *et al.*, 1986). The effect of the inducers depends on many factors including tissue type, plant age, wounding, handling procedures and basal activity. The greatest responses to induction appears to occur in enzymes with very low levels of expression, while enzymes that are expressed constitutively are less responsive to induction. Variation in expression of cytochrome P-450 enzymes with age is also observed in mammals (Nebert and Gonzalez, 1987) and it is to be expected that plants would also vary in their expression of these enzymes with developmental stage. The effect of phenobarbital in inducing diclofop metabolism is influenced by plant age with young seedlings being most responsive (Zimmerlin and Durst, 1991). This is significant given than much of the knowledge of cytochrome P-450 in monocots is derived from young etiolated seedlings. Here again specific procedures and tissue sources may bias perceptions of these enzymes.

1-Napthalic anhydride, a commercial herbicide safener, is a commonly used inducer of these enzymes. Pretreatment of wheat or corn with napthalic anhydride increases their capacity to metabolise sulfonylureas (Sweetser, 1985). Napthalic anhydride also induces the enzymes that oxidise bentazon but not cinnamate-4-hydroxylase in corn, indicating some specificity of induction (Haack and Balke, 1992). Treatment of the plant material with a protein synthesis inhibitor, cycloheximide, prevents the increase in metabolism. This suggests that increased rate of metabolism is derived from the *de novo* synthesis of detoxifying enzymes (Sweetser, 1985; Hendry and Jones, 1984). Treatment with gabaculine, an inhibitor of haem synthesis, also prevents formation of cytochrome P-450 containing enzymes (Werck-Reichhart *et al.*, 1988). The reduction in cytochrome P-450 concentration that occurs following treatment with gabaculine indicates not only that the production of cytochrome P-

450 containing enzymes is inhibited but that they have a short half-life in plants (Werck-Reichhart *et al.*, 1988; Gasser *et al.*, 1982). This enzyme turnover could allow rapid recovery of enzyme activity following treatment with labile inhibitors. It may also allow the system to respond rapidly following induction of specific isozymes.

The various inducers have different effects in different species, while within a species they also vary in their ability to induce specific enzymes. An example of this phenomenon between species is the very different response of laurate hydroxylase to clofibrate in *Vicia sativa* and *Helianthus tuberosus* tubers (Salaün *et al.*, 1986). Within a species, phenobarbital has a greater capacity to induce diclofop hydroxylation and laurate hydroxylase than cinnamate-4-hydroxylase (Zimmerlin and Durst, 1991). These differences in response to inducers again demonstrate that different enzymes are regulated separately and that the nature of enzymes extracted from induced tissue will depend on a range of factors including the inducer.

The mechanisms of induction are not clear. Induction may be a general stress response or it may be more specific. Some compounds that induce cytochrome P-450 also induce glutathione transferase (Lamoureaux and Rusness, 1989), indicating that they are not specific inducers but may be generating a stress response. The mechanism for more specific induction is not known but it may be related to the slight inhibitory activity of some inducers (Canivenc *et al.*, 1989). If an inducer forms a stable complex with a specific cytochrome P-450 enzyme it may cause induction of that enzyme either through a buildup of substrate or a deficiency of product. The converse has been observed in which 4-hydroxy cinnamate repressed cinnamate-4-hydroxylase activity (Salaün *et al.*, 1981). Such a mechanism might apply to inhibitors of cytochrome P-450 that may also act as antagonists. It is possible that inhibition of the enzyme results in induction which in turn confers resistance if the enzyme is normally expressed at a low level.

Substrate induction has been demonstrated in hepatic systems and in plants with endogenous

substrates (Hendry and Jones, 1984), however, the role of this phenomenon in herbicide metabolism is unknown. In mammalian model systems, cytochrome P-450 genes include upstream regulatory sequences that interact both with xenobiotics and with the reaction products of the enzyme they encode (Nebert and Gonzalez, 1987). The latter interaction provides for the negative autoregulation or feedback function referred to earlier using 4-hydroxy cinnamate as an example. The specificity of this function in liver cannot be too great as many (over 300) different substances, such as phenobarbital, induce enzymes catalysing both the oxidation of the inducer and unrelated chemicals (Nebert and Gonzalez, 1987). The best studied gene for a cytochrome P-450 enzyme, that of $P_{1,450}$, exhibits both a negative control site and a receptor (Ah) dependent enhancer (Nebert and Gonzalez, 1987). The receptor is postulated to be soluble in the cytosol where it binds to xenobiotics entering the cell. The receptor xenobiotic complex then moves into the nucleus, binding to the enhancer element and causing the induction of $P_{1,450}$ and the related $P_{3,450}$. This system, therefore, relies both on broad specificity in the receptor and of the enzyme. This model explains the specificity of some induction processes.

Various inducers, such as phenobarbital, lead to increases in transcription of P-450 genes. This is known as transcriptional activation. High cytochrome P-450 activity is associated with high levels of mRNA for the enzyme (Nebert and Gonzalez, 1987) which is required to maintain translation to compensate for the short half life of these enzymes (Gasser *et al.*, 1982). Thus an inducer would be required to maintain transcription in order to retain high enzyme activity.

Specificity of induction has practical applications in the use of safeners formulated with a herbicide. These safeners are applied both to the crop and to the target weeds but their effect is specific to the crop. One such example is the triazole safener, HOE 70542, which is used to safen wheat against fenoxaprop (Bieringer *et al.*, 1989). The structure of this safener is analogous to the triazole fungicides and growth regulators (Figure 1.2) and it is possible that its safening mechanism is related to an inhibitory effect associated with the

triazole structure or the affinity of the triazole-backbone for enzyme like receptors.

Inhibitors as Inducers

The similarity of inhibitors and safeners like HOE 70542 raises the possibility that mechanisms of induction and inhibition may be interrelated. Chlorotoluron metabolism by cell suspension cultures of wheat may be induced by pretreatment with 2,4-D, cyometrinil and dichlormid. Each also acts as a weak inhibitor (or competitor) when applied simultaneously with chlorotoluron (Canivenc *et al.*, 1989) suggesting that pretreatment is required to allow synthesis of new protein. Support for this hypothesis is derived from the observation that napthalic anhydride and dichlormid may also inhibit cytochrome P-450 enzymes, albeit at relatively high concentrations (Barta and Dutka, 1991). Similarly, ABT, an inhibitor, is also able to act as a safener of EPTC, metolachlor and diclofop (Barta and Dutka, 1990, authors unpublished observations). These observations raise the possibility that some safening effects may be due to competition with substrates for specific enzymes leading to overproduction of the enzymes. However, no evidence has been presented for an increase in gross cytochrome P-450 content in response to these chemicals (Barta and Dutka, 1991). This could be due to the induced enzymes being only a small fraction of the total P-450 protein. Such a mechanism of induction would only be efficient for constitutively expressed enzymes because they must be present to interact with the inducer. Constitutively expressed enzymes have only small response to inducers whereas the greatest induction responses occur in more selectively expressed enzymes. Therefore large induction responses, such as safening effects, may be caused by specific mechanisms similar to the Ah system described above. Thus, there are many potential mechanisms of induction from those which act directly on the regulation of expression to interactions with particular detoxifying enzymes leading to their induction.

Herbicide metabolism and crop tolerance

Herbicide metabolism is a very import practical function of cytochrome P-450 enzymes. In many crop species these reactions confer herbicide tolerance to the crop. This capacity is

often lower or nearly absent in susceptible weed species. The mechanistic basis of these differences is not clear. The greater tolerance of the crop plants may be due to either greater copy number (polyploidy) or expression of genes for cytochrome P-450 enzymes, alternatively it may be due to the affinity of a specific isozyme. The differential response to inhibitors observed between species may indicate that variation in enzyme affinity is an important factor. In some cases this tolerance is conferred by a single gene suggesting that it might be a function of a single enzyme or regulatory process. Chlorotoluron resistance in wheat cv Cappelle-Duprez and metribuzin resistance in soybean cv. Tracy M (Hatzios, 1988) are two examples of this.

The capacity for detoxification may apply only to specific analogues of a herbicide as is the case for the wheat-selective sulfonylureas and aryloxyphenoxypropionates. Diclofop and metsulfuron are readily metabolised by wheat while haloxyfop and sulfometuron are not. In contrast, wildtype *L. rigidum* is able to carry out some degree of metabolism of both diclofop and haloxyfop but this is insufficient to confer tolerance. Thus, the selectivity of diclofop in wheat is based on a difference in the rate of metabolism between the crop and the weed rather than the absence of the mechanism in the weed. These subtle differences again point to the specificity of cytochrome P-450 within plant cells.

Purification of cytochrome P-450 dependent enzymes.

The most definitive means of resolving the identity and specificity of particular cytochrome P-450 enzymes is to study them in their pure form. The enzyme normally functions as part of a membrane bound complex with NADPH-cytochrome P-450 reductase in the endoplasmic reticulum. The reductase normally serves a number of P-450 enzymes (Nebert and Gonzalez, 1987). It is the fragility of this complex arrangement which is postulated as the reason for the low recovery of enzyme activity from plant extracts. In order to purify the enzyme, the protein must be released from the membrane with the use of detergents after which it can be subject to conventional chromatographic separation. To regain activity, the protein must be reconstituted with a reductase. While a common procedure with hepatic P-

$_{450}$, reconstitution in an active form has not been frequently reported for plant enzymes (O'Keefe and Leto, 1989). Reconstitution of a known function has been achieved for cinnamate-4-hydroxylase and digitoxin hydrolase (Benveniste *et al.*, 1986; Petersen and Seitz, 1988). Cytochrome P-$_{450}$ from avocado (*Persea americana*) mesocarp has also been purified and the N-terminal portion sequenced, however, the function of this enzyme *in vivo* is not known (O'Keefe and Leto, 1989). Purification and reconstitution leads to large losses of activity, thus a rich source of enzyme such as avocado mesocarp or *Helianthus tuberosus* tubers is an essential starting point. Even if starting activity against an endogenous substrate is high, the affinity of these enzymes for herbicides may be two orders of magnitude lower (Haack and Fuerst, 1992), thus complicating attempts to ascribe herbicide detoxification functions to specific enzymes in their purified form.

An alternative to enzyme purification as a means of determining the number and relationship of different cytochrome P-$_{450}$ isozymes is to exploit the conservation evident within specific regions of genes encoding these enzymes. The haem binding region, in particular, is highly conserved across many different organisms and serves as a useful PCR priming site (R. Hyde, Rothamsted Experiment Station, personal communication). Similarly the leader sequences of some cytochrome P-$_{450}$ isozymes is similar to that of mammals reflecting similar targeting within the cell (O'Keefe and Leto, 1989). Given that in mammals, genes for these enzymes comprise a multi-gene family (Nebert and Gonzalez, 1987), it is expected that the same will be true for plants. Thus a molecular approach might provide a rapid means of estimating the potential diversity of cytochrome P-$_{450}$ protein in plants. However, unless the genes can be cloned and expressed in eurcaryotic systems their function will remain unclear.

Conclusions - oxidative detoxification

The study of cytochrome P-$_{450}$ within plants has been hampered by the low concentration of these enzymes in plant tissue, their lability in extraction and the difficulties associated with interfering pigments. As a consequence much of the knowledge of cytochrome P-$_{450}$ in

plants is derived from studies of tubers and etiolated seedlings, both of which lack pigments. Mechanistically, plant cytochrome P-450 appears to be similar to that of animals, however, specific responses to various chemicals are not always in common. This difference may be due, in part, to the different functions of hepatic and plant cytochrome P-450. There is much evidence to suggest that the cytochrome P-450 enzymes studied in plants have specific functions. The effects of various inhibitors, substrate specificity and response to inducers all differentiate the enzymes. This specificity complicates the advancement of general propositions about these enzymes in plants, especially with regard to means for their inhibition. Variation in specificity also causes crop selectivity for some herbicides because they are not rapidly detoxified by weed enzymes but are readily oxidised by the crop plants. Herbicides are not the normal substrates for these enzymes and their metabolism is not a selective advantage until herbicides are present. Thus, herbicide metabolism in tolerant species is a consequence of the presence of oxidative systems that have evolved with specific biosynthetic and regulatory functions and their activity against herbicides is coincidental. In contrast, plants that have adapted to herbicide selection pressure by enhancing oxidative activity represent the only instances where the enzyme systems have been altered to increase the utilisation of herbicides as substrates.

1.5 Conjugation of Herbicides

Introduction

Conjugation of herbicides with either glycosides or glutathione acts to fully detoxify the herbicide and dramatically increase water solubility in many cases. This may be a direct conjugation as in the detoxification of triazines and chloroacetamides by glutathione-S-transferse or it may be a phase II reaction following from the initial oxidation discussed above (Shimabukuro, 1985). This section will consider the direct reactions in most detail. These are usually the conjugation of the herbicide with glutathione, or homoglutathione in legumes, by the enzyme glutathione S-transferase (GST).

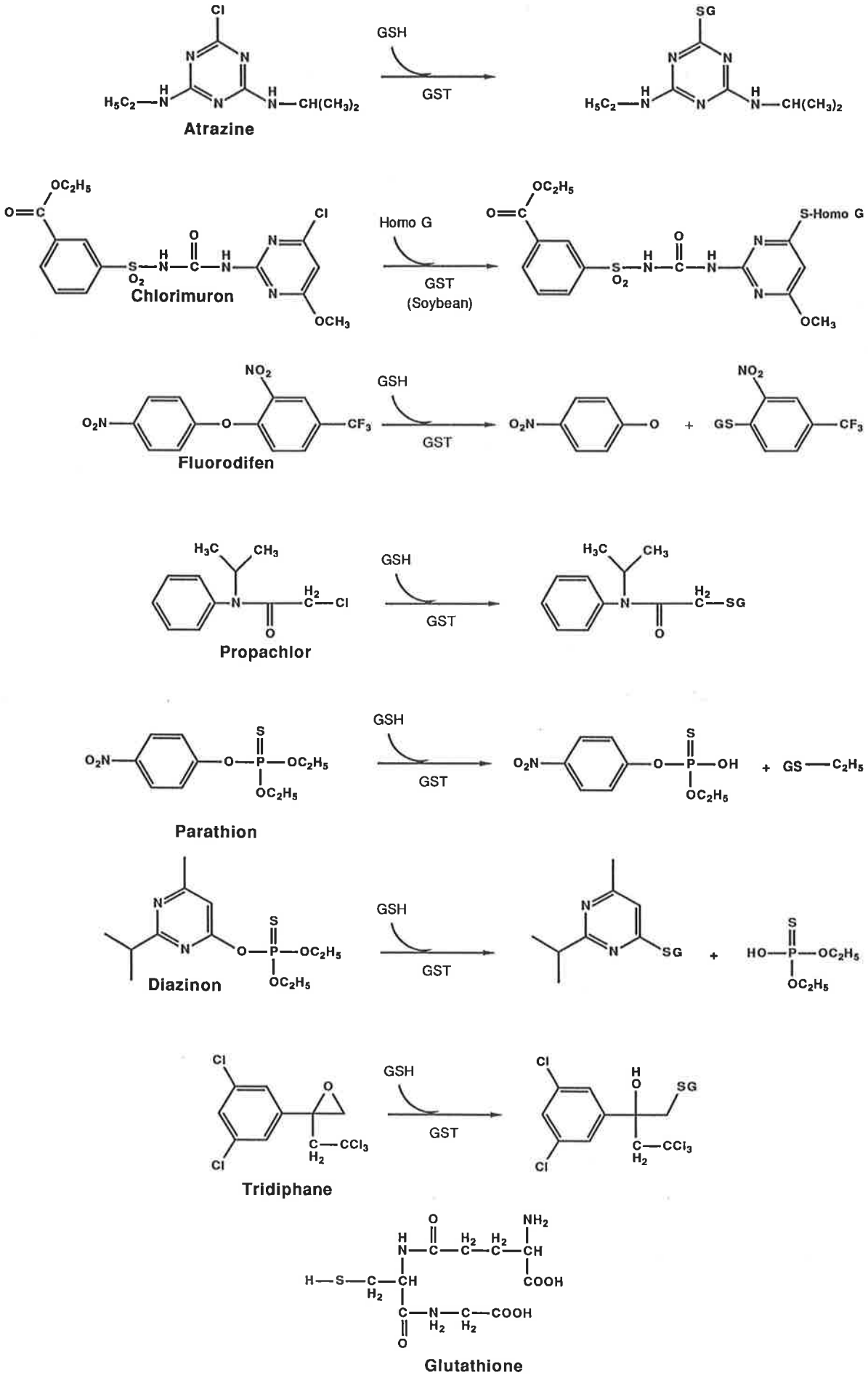
Glutathione-S-transferase

GST is part of a group of enzymes which includes glutathione reductase which are found in many different organisms. Part of their function *in vivo* is thought to be the scavenging of radicals. GST may detoxify xenobiotics in both plants and insects and these enzymes have an important role in herbicide tolerance and resistance in plants (Lamoureux and Rusness, 1989).

In insects glutathione conjugation detoxifies lindane, diazinon and parathion analogues amongst others (Lamoureux and Rusness, 1989). The simplified reactions are illustrated in Figure 1.3. The importance of glutathione conjugation as a direct means of detoxification is unclear in many cases because the compounds are also subject to oxidation prior to further metabolism.

In plants, GST catalyses the direct detoxification of many herbicides. Glutathione directly displaces the chlorine at the 2-position of atrazine forming a water soluble conjugate (Figure 1.3) (Shimabukuro *et al.*, 1973; Shimabukuro, 1985). Similarly, the chlorine group of the chloroacetamides is directly displaced by glutathione (Figure 1.3) (Fuerst, 1987). Chlorimuron selectivity in soybean is conferred by the direct displacement of the pyrimidine

Figure 1.3. The glutathione reactions detoxifying the herbicides chlorimuron, fluorodifen, atrazine, propachlor, tridiphane and the insecticides, parathion and diazinon. The structure of glutathione is shown (bottom). Abbreviations, GSH = glutathione, Homo G = homoglutathione, GST = glutathione transferase. Redrawn from Lamoureaux and Rusness (1989).


Atrazine
GSH
GST
Chlorimuron
Homo G
GST
(Soybean)
S-Homo G
Fluorodifen
GSH
GST
Propachlor
GSH
GST
Parathion
GSH
GST
Diazinon
GSH
GST
Tridiphane
GSH
GST
Glutathione

chlorine by homoglutathione (Figure 1.3) (Brown and Neighbours, 1987). In each case the position of the electron withdrawing chlorine atom makes these herbicides vulnerable to this reaction.

In contrast some herbicides need to be first activated by an oxidative reaction. The activated herbicide is the substrate for glutathione conjugation. An example of this is the metabolism of EPTC to the sulfoxide which is both the inhibitory agent and the substrate for GST (Lamoureux and Rusness, 1989). Metribuzin is also metabolised to the sulfoxide which is then conjugated with homo-glutathione in soybean (Hatzios and Penner, 1988). GST can, therefore, confer tolerance to crop species but this level of tolerance may not always be adequate for crop safety.

Herbicide safeners and glutathione conjugation

To reduce the phytotoxicity of some herbicides in particular crop species they may be applied in combination with another compound which acts to protect the crop plants. This is known as "safening" and the concept has gained commercial application with the combination of EPTC and dichlormid for use in corn (Parker, 1983). Both dichlormid and flurazole are able to increase the rate of glutathione conjugation of the herbicides thus reducing injury (Lay and Casida, 1976; Fuerst and Gronwald, 1986). The means by which they increase GST activity is unclear. There are many processes that might be affected. These include the amount of different isozymes of GST, the amount of glutathione reductase and the level of free glutathione (Lamoureux and Rusness, 1989). The safeners are also substrates for glutathione conjugation (Lamoureux and Rusness, 1989) and they are another example of substrate induced detoxification. The safeners may mimic an elicitor and bind to specific receptors or they may exert a mild form of physiological disturbance which, while not phytotoxic, stimulates the synthesis of the enzyme.

Inhibition of glutathione-S-transferase

In contrast to the effects of the safener, tridiphane acts as synergist of atrazine in *Setaria* sp.

(giant foxtail) and of EPTC and alachlor in *Panicum miliaceum* L. (proso millet) (Ezra *et al.*, 1985). This effect is attributed to the inhibition of GST by tridiphane (Boydston and Slife, 1986). Tridiphane is itself a substrate for GST and the resulting glutathione conjugate of tridiphane is a potent inhibitor of atrazine, CDNB, propachlor and fluorodifen conjugation. Tridiphane does not synergise atrazine in corn but this is due to the high levels of glutathione and GST in corn and the greater ability of corn to metabolise the toxic tridiphane-glutathione conjugate (Lamoureux and Rusness, 1986). GST in *Setaria* is also at least five times more sensitive to inhibition by the tridiphane-glutathione conjugate than GST isolated from corn (Lamoureux and Rusness, 1986). This suggests differences between species in the affinity of GST for different inhibitors.

Specificity of Glutathione-S-transferase

GST activity can be measured in a variety of ways including a colourimetric reaction using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. However, contradictory estimates of GST activity derived from reactions with CDNB and labelled substrates point to there being different GST isozymes with differing affinities for these two types of substrate (Anderson and Gronwald, 1991). There is direct evidence of at least three GST isozymes in corn (Lamoureux *et al.*, 1991). The second isozyme, GST(II), is induced by flurazole and has similar properties to that of the constitutive isozyme (Mozer *et al.*, 1983). The safener, benoxacor, induces several GST isozymes in corn with differing affinity for metolachlor, atrazine and CDNB (Irzyk and Fuerst, 1992). Benoxacor induction is prevented by inhibitors of RNA transcription and translation, suggesting that the effect of the safener is to induce de novo synthesis of GST protein (Miller *et al.*, 1992). The differing response of the isozymes to the inducer and their various substrate specificities support the contention that there are a variety of GST isozymes in plants with various activities against different substrates.

Variation between species is apparent with peas (*Pisum sp.*) expressing GST activity but unable to conjugate atrazine. At least three isozymes of GST are found in peas one of

which detoxifies fluorodifen (Frear and Swanson, 1983). The specificity of this enzyme is such that it will not accept the related diphenylether herbicide acifluorfen as a substrate (Lamoureux and Rusness, 1989).

Enhanced activity of GST isozymes is also the mechanism of atrazine resistance in a biotype of *Abutilon theophrasti* selected with triazine herbicides. This biotype overproduces two GST isozymes with activity against atrazine which are present only at low levels in the susceptible biotype (Anderson and Gronwald, 1991). Enhanced glutathione transferase activity in the resistant plants could not be detected using CDNB as a substrate, however, when elution profiles were assayed with [^{14}C]atrazine, GST peaks with enhanced activity against atrazine became apparent. This reaffirms the importance of the substrate when determining a particular GST activity (Anderson and Gronwald, 1991). Atrazine resistance in this biotype is inherited as a single partially-dominant nuclear gene (Anderson and Gronwald, 1987), yet two gene products appear to be involved in conferring resistance. This could be explained by alterations to the regulation of the genes leading to increased expression (Anderson and Gronwald, 1991). Mutation of a regulatory gene or sequence could affect two nearby genes and segregate as a single locus.

Other evidence for variation in GST within and between species may be found in differential response to inhibitors, inducers and herbicides noted above. This variation within and between species may be explained by GSTs being encoded by a multigene family (Lamoureux *et al.*, 1991). Multiple gene copies allow the evolution of variants while still maintaining viable copies. While differences between isozymes induced by different treatments have been studied in some detail in maize the molecular basis of GST diversity is not well understood. Part of this diversity may be accounted for in the formation of heterodimers of different GST subunits which have different properties to homodimers (Lamoureux and Rusness, 1989). Thus, GST diversity may be generated by post translational processing as well as by different genes

Conclusions - glutathione-S-transferase

GST is found in plants insects and mammals where it detoxifies radicals and xenobiotics either by conjugation with glutathione or by dealkylation and dehalogenation as a consequence of reaction with glutathione. GSTs vary within and between organisms in their capacity to conjugate various substrates. This manifests itself as differential herbicide tolerance in plants between different plant species. GSTs may be induced or inhibited and this has been exploited commercially with safeners and the synergist tridiphane.

While GST directly conjugates chloro-s-triazines, chloroacetamides, chlorimuron, diphenylethers and tridiphane there are other xenobiotics which must be first oxidised prior to conjugation. GST participates in some of these phase II reactions. The significance of the conjugation in detoxifying herbicides varies because some herbicides are fully detoxified by the initial oxidation while others retain toxicity. In many species GST and oxidative systems are important components of the plants defence against xenobiotics with the contribution by each system varying with the herbicide and species.

1.6 Conclusions

L. rigidum has many of the characteristics appropriate to its status as a weed of crops in southern Australia. Amongst these, its rapid seedbank turnover, high fecundity and variability have accelerated its response to herbicide selection pressure. The response to diclofop in particular has been rapid and this may be a function of a high frequency of diclofop resistance genes in some environments (J. Matthews, personal communication). The other notable characteristic of resistance in *L. rigidum* is the prevalence of cross-resistance. This appears to be related to the tendency of this species to adapt with non-specific resistance mechanisms such as differential metabolism (Christopher *et al.*, 1991). This contrasts *L. rigidum* biotypes with the bulk of herbicide resistant biotypes world wide in which adaptation occurs mostly by alteration of the target site (LeBaron, 1991).

The most prevalent forms of herbicide resistance are triazine resistance, sulfonylurea

resistance and most recently, aryloxyphenoxypropionate resistance in both *L. rigidum* and *Avena fatua*. These have largely resulted from the repeated use of the same or closely related herbicides in the same fields. While resistance of this kind has important effects on farm and company revenues it may be controlled relatively easily with alternative measures. The significance of the recent outbreak of sulfonylurea resistance is that it affects the commercial prospects of compounds yet to be released and thus threatens returns to research investment.

There have been a number of instances in which weed biotypes have become resistant through enhanced metabolism. These include chlorotoluron resistant *A. myosuroides*, triazine resistant *A. theophrasti* and *Echinochloa cruss gali*, and chlorsulfuron resistant *L. rigidum*. Differential metabolism generates a low level of resistance and may lead to cross-resistance similar to that found in insects.

Both the oxidative systems and the GST systems of tolerance and resistance by metabolism appear to have characteristics in common. Individual isozymes may have broad specificity but may still catalyse a different complement of reactions to that of another isozyme. Both inhibition and induction of these systems may be achieved to alter plant sensitivity to herbicides. In both cases many inhibitors and inducers are also substrates for the enzymes although they vary markedly in the concentrations and timing of application required to cause specific effects. The basis of induction of either system is not known, however, there are likely to be many different mechanisms of induction given the diversity in other biological systems of this type. However, unlike induction by endogenous substances there are unlikely to be specific receptors that have adapted to interact with the inducing molecule. Inducers may either mimic endogenous compounds when binding to receptors or they may simply stimulate a cascade of non-specific reactions. Low level inhibition of many different isozymes may lead to disruptions of feedback regulation and the induction of many different enzymes thus obviating the need to bind receptors.

Many inhibitors of oxidative enzymes are designed around a terminal nitrogen heterocycle, such as the 1,2,3-triazole of tetcyclasis and ABT or the 1,2,4-triazole of the azole fungicides, growth regulators and amitrole. It appears that a basic heterocycle such as ABT may be a general inhibitor due to reaction largely with the haem centre with little interaction with the substrate specificity determining region of the enzyme. The addition of a hydrophobic backbone appears to increase interaction with the substrate binding site, increasing affinity but also conferring specificity such that stereo isomerism has important effects on inhibitory activity (Lenton, 1987).

GST also demonstrates specificity within and between species. The differential rates of conjugation determine the selectivity of many different herbicides. The differential affinity demonstrated by different response to herbicides detoxified by GST indicates that there is variation in these enzymes which is confirmed by observations of GST activity *in vitro*. The same generalisations could be made for oxidative enzyme systems. This variation may be a foundation upon which weeds become resistant.

Both detoxification systems are encoded by multigene families. These gene families are an elegant mechanism for generating diversity. Both mutation or amplification of the gene or genes for a specific isozyme could lead to changes in herbicide sensitivity. Similarly clusters of genes may show coordinated regulation and changes in regulatory sequences could lead to over expression of many genes. Effects such as this could result in simultaneous increases in metabolism of different herbicide substrates giving rise to cross-resistance.

Chapter Two
Biotype Histories

2.1 Introduction

Two herbicide resistant biotypes of *L. rigidum* (WLR2, and VLR69) are the subjects of this study. They have resistance to the triazines, phenylureas and metribuzin in common but differ in their resistance to other herbicides. Resistance to the inhibitors of photosystem II has been infrequent in *L. rigidum* and these herbicides are likely to gain increased use as alternatives for the control of herbicide resistant biotypes. WLR2 and VLR69 were studied to determine the mechanism of resistance and its implications for the sustained use of these herbicides. There are two objectives of this Chapter. The first is to outline the history of selection pressure that lead to resistance in these biotypes. The second is to define the spectrum of resistance that the biotypes possess.

2.2 Introduction to WLR2

The use of herbicides in non-crop areas places intense selection pressure on the weed populations because high rates of residual herbicides are employed. Residual herbicides are often combined with a herbicide with foliar uptake for control of standing vegetation. A combination of this type is amitrole and atrazine. In Western Australia this combination was used to control weeds on over 5000 km of railroad rights-of-way for 10 years from 1978 to 1988 (Table 1.1) with diminishing control of *L. rigidum* observed during the latter 5 years. At the end of 1987 the decline in control of *L. rigidum* had become evident in many parts of the rail network. Seeds were first harvested in November 1987 from plants growing on a railroad right-of-way near Toodyay in Western Australia. In an attempt to control the burgeoning *L. rigidum* population, diuron (3 kg ai ha^{-1}) was added to the mixture (amitrole 2.56 kg ha^{-1} and atrazine 2.56 kg ha^{-1}) in the 1988 season. No improvement in control of *L. rigidum* was gained from the use of diuron. At the same time experiments under

controlled conditions established that the biotype was cross-resistant to all members of the phenylurea class of herbicides. This was, therefore, a case of herbicide multiple- and cross-resistance. The biotype was eventually designated the code number "WLR2" in the Waite Institute Herbicide Resistant Seed Collection. "W" indicates that the biotype was collected from Western Australia, "LR" stands for *Lolium rigidum* indicating the species of the biotype while the number is an arbitrary identifier.

During 1988 and 1989 WLR2 was subject to dose response experiments to determine its spectrum of cross-resistance. In this section the results of those experiments are discussed.

2.3 Materials and Methods (WLR2)

Seed collection.

Seeds were first harvested in November 1987 from plants growing on a railroad right-of-way near Toodyay in Western Australia. These plants had survived treatment with 2.56 kg ha^{-1} amitrole and 2.56 kg ha^{-1} atrazine (Table 1.1). The susceptible seeds were obtained from a population of *L. rigidum* growing near Bordertown in South Australia, designated VLR1. This biotype has never been exposed to herbicides and is killed by the recommended rates of all herbicides tested against it under normal growing conditions.

Plant growth and herbicide application.

Experiments were conducted over two seasons under the following conditions. For postemergence applications, seeds were pre-germinated on moist filter paper, or 0.8% agar, and transplanted into a soil based on peat and sand. Densities of plants within pots were kept constant within any one experiment. At least 20 plants (usually 63) of each biotype were used for each treatment. Plants were grown outdoors during the normal growing season and post-emergent herbicides were applied at the three to four-leaf stage depending on label recommendation. The herbicides were applied as the formulated product in water with a non-ionic surfactant at 0.2% (v/v). Applications were made with a laboratory

sprayer equipped with two nozzles (moving at 1 m s^{-1}) delivering 113 L ha^{-1}.

For pre-emergent treatments with triazine herbicides, 0.45 g of WLR2 seed or 0.25 g of VLR1 seed was placed on the soil surface in a 0.1 m^2 tray and then covered with 1 cm of lightly compacted soil. The difference in amount of seed was to compensate for differences in germination rates. Herbicides were sprayed onto the soil surface and then leached with the equivalent of 6.5 mm of rainfall applied with a watering can.

Plant mortality and shoot dry weights were determined 30 to 42 d after spraying. Plants were scored as dead if no green tissue was visible. Scoring was delayed until there was minimum ambiguity in determining which plants had survived. Where a calculation of the LD_{50} ratio has been made it is expressed as a single number which is the WLR2 LD_{50} divided by the VLR1 LD_{50}.

2.4 Results and Discussion (WLR2)

Dose response experiments.

Amitrole and atrazine.

Amitrole and atrazine, in combination, were the selective agents applied to WLR2 on the railway rights-of-way. These herbicides interact to produce intense symptoms in the susceptible VLR1 plants whereas the WLR2 plants are up to twelve times less sensitive (Figure 2.1). WLR2 is, therefore, resistant to the combination of the two herbicides, confirming that the failures observed in the field were due to resistance. Further experiments were conducted with the herbicides applied alone.

Amitrole.

Amitrole is one component of the herbicide mixture to which WLR2 was exposed in the field. At the recommended rates of amitrole, 0.75 to 1.5 kg ha^{-1}, less than 10 % mortality

Figure 2.1 The response of the susceptible biotype VLR1 (●) and the resistant biotype WLR2 (O) to amitrole and atrazine applied in combination (1:1 w/w) to plants grown in pots. Each point is the mean of four replicates of 12 plants each and is plotted with the standard error.

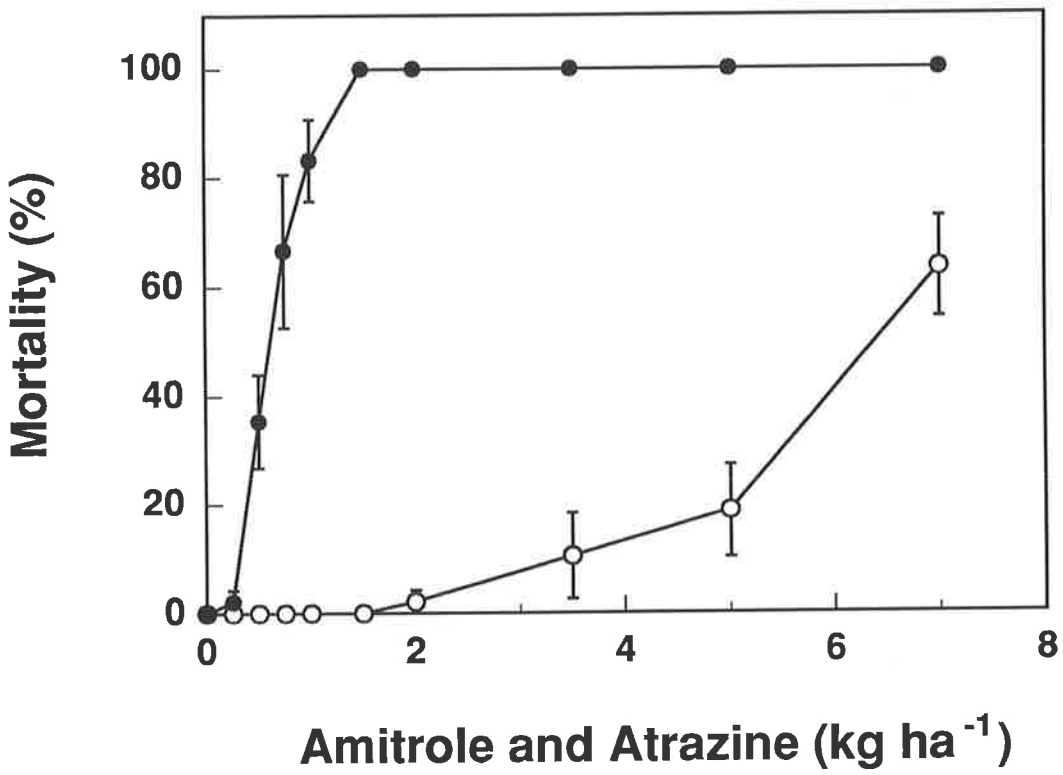

Mortality (%)
100
80
60
40
20
0
0
2
4
6
8
Amitrole and Atrazine (kg ha -1)

Table 2.1. The effect of various herbicides on the WLR2 and VLR1 biotypes as represented by LD_{50}s and LD_{50} ratios for each herbicide based on data of the type shown in Figure 2.1.

Class	Herbicide	LD_{50} (kg ha^{-1})		LD_{50} ratio
		VLR1	WLR2	WLR2/VLR1
Triazines	Simazine	0.30	2.8	9.2
	Atrazine	0.25	0.75	3.0
	Propazine	0.45	1.3	2.9
	Cyanazine	0.15	0.4	2.7
	Prometryn	0.30	1.8	6.0
	Ametryn	1.10	3.4	3.1
Phenylureas	Chlorotoluron	0.25	1.8	7.2
	Isoproturon	<0.50	1.0	>2.0
	Metoxuron	<0.55	1.1	>2.0
	Diuron	0.20	0.4	2.0
	Fluometuron	0.65	1.1	1.7
	Methazole	1.2	2.4	2.0
Others				
	Amitrole	0.5	5.5	11
	Metribuzin	<0.1	0.2	>2.0
	Chlorsulfuron	0.007	0.008	1.1
	Metsulfuron	0.013	0.010	0.8
	Glyphosate	0.70	0.70	1.0
	Oxyfluorfen	0.088	0.097	1.1
	Metolachlor	0.18	0.16	0.9

was achieved in the WLR2 biotype whereas these rates were sufficient to cause high mortality in VLR1 (Figure 6.3). WLR2 was more than ten times less sensitive to amitrole than VLR1 based on a comparison of LD_{50}. Amitrole is, therefore, the herbicide to which WLR2 is most resistant (Table 2.1).

Amitrole inhibits many biological processes and there is little consensus on its primary mode of action in plants. The most obvious symptom of amitrole toxicity is the bleaching of tissue produced after application. This is thought to be due to the inhibition of carotenoid biosynthesis leading to the photobleaching of the leaf tissue after it emerges (Burns *et al.*, 1971). At low doses, the symptoms of amitrole toxicity in *L. rigidum* appear as a band of bleaching perpendicular to the long axis of the leaf blade (Figure 6.6). When amitrole is applied at 0.75 kg ha^{-1}, WLR2 plants display a 1-2 cm band of bleaching on leaves that were emerging at the time of amitrole application, while the majority of VLR1 plants show complete bleaching of all leaves produced after spraying (Figure 6.6). The mechanism of resistance to amitrole will be discussed more fully in Chapter six.

Triazines.

WLR2 was three times less sensitive to atrazine than the susceptible biotype VLR1, based on comparison of LD_{50}s (Table 2.1). This difference in sensitivity was observed whether atrazine was applied pre- or post- emergent (pre-emergent data not shown), suggesting that foliar uptake was not a factor in resistance. The level of resistance is low compared with the resistance to amitrole, given that the two herbicides were applied in equal quantities (by weight) in the field during the selection process. The WLR2 biotype was 9 times less sensitive to simazine (Table 2.1) indicating that the resistance mechanism adapted to atrazine is more effective against simazine. Resistance to the chloro-s-triazines increased in the order (ratio of WLR2 to VLR1 LD_{50}s in parentheses) cyanazine (2.7), propazine (2.9), atrazine (3) and simazine (9.2) (Table 2.1). The mechanism of resistance to simazine is further discussed in Chapter three. WLR2 is also resistant to the thiomethyl-s-triazines (Table 2.1) with resistance to prometryn (6) being greater than that to ametryn (3.1).

Terbutryn was also tested but no mortality was obtained in the WLR2 biotype so no comparison of the degree of sensitivity can be made. Thus the WLR2 biotype exhibited some degree of resistance to all triazines tested.

The low level of resistance relative to other cases of triazine resistance distinguishes WLR2 from most previously reported triazine resistant biotypes. WLR2 is, therefore, unlikely to express an insensitive target site. Given the persistent selection pressure applied in ten consecutive seasons it might seem unusual that a higher level of resistance was not selected. A possible explanation is that the biotype adapted to the combination of herbicides rather than each separately. Thus mechanisms that interacted with herbicides may have been selected. Such an adaptation may be the reason for the greater resistance to the combination of amitrole and atrazine than either alone.

Substituted Ureas.

When it became apparent that a mixture of amitrole and atrazine was not controlling *L. rigidum* on the railway lines, diuron was added in the hope that it would be a cost-effective control for the emerging resistant population. No increase in control was obtained because WLR2 is cross-resistant to the substituted urea herbicides (Table 2.1). Chlorotoluron is the substituted urea herbicide to which the WLR2 biotype is most resistant (Table 2.1, Figure 4.5) and is the subject of studies on the mechanism of resistance in Chapter four. Resistance (in increasing order of resistance) was observed to fluometuron, diuron, methazole, isoproturon, metoxuron and chlorotoluron (Table 2.1) confirming that the WLR2 biotype is resistant to a wide range of substituted urea herbicides. Methazole is classed as an oxadizole herbicide but is thought to exert its phytotoxicity after it is metabolised to a phenylurea analogue (Hutson, 1987). WLR2 was also less sensitive to both linuron and methabenzthiazuron than VLR1 although the LD_{50}s were not reached for either biotype.

Cross-resistance to the substituted ureas was a costly consequence of resistance to atrazine and amitrole because of the loss associated with the attempt to control the population with

diuron. This cross-resistance also forced a change in the control regime to the more costly combination of glyphosate and sulfometuron.

Metribuzin.

While metribuzin binds the same target as the triazines and substituted ureas, it is structurally distinct and has different chemical properties. Metribuzin is thought to have foliar uptake as well as root uptake and is metabolised via a variety of pathways in different species (Hatzios and Penner, 1988). At the recommended rate for metribuzin of 200 - 400 g ha^{-1} the WLR2 biotype survived while VLR1 plants suffered 100 % mortality (Table 2.1, Figure 5.2). WLR2 has never been exposed to selection pressure by metribuzin, thus the reduced sensitivity is an example of cross-resistance.

Other herbicides.

Representatives of other herbicide groups were tested against WLR2 to determine the extent of cross-resistance. There was no resistance to the sulfonylurea herbicides as indicated by LD_{50} ratios for chlorsulfuron and metsulfuron-methyl (Table 2.1). Sulfometuron-methyl at 12.5 g ha^{-1} caused 100% mortality in both biotypes. The recommended rate of sulfometuron in non-crop areas is 250 g ha^{-1}. Complete control was obtained at the recommended rates of the aryloxyphenoxypropionate herbicides fluazifop-butyl and diclofop-methyl, the cyclohexanedione herbicide sethoxydim, and the carbamate herbicide carbetamide. The WLR2 and VLR1 biotypes also appeared equally sensitive to glyphosate and oxyfluorfen (Table 2.1). The current means of control on the railway rights-of-way is a combination of glyphosate and sulfometuron. These results indicate that there is no cross-resistance to these herbicides in the WLR2 population.

Future prospects for control and resistance

If the use of glyphosate and sulfometuron impose a high selection pressure there is a possibility of resistance eventuating, however, resistance to glyphosate does not appear to be

readily selected in the field. Sulfometuron is likely to select for resistance given the apparent frequency of ALS mutations (see Chapter seven) and the strong selection pressure imposed by the residual nature of this herbicide. It would appear unlikely that mutation of the target proteins for both herbicides would occur simultaneously in a single plant, thus ALS mutants present at the time of annual spraying would be killed by glyphosate. Perhaps more likely is the possibility that sulfometuron will move off the sprayed area with rainfall and select for ALS mutants at the margins of the sprayed area. Such mutants may then attempt to recolonise the sprayed area until they have evolved a mechansim of resistance to glyphosate thus accumulating two mechanisms in succession rather than two simultaneously. ALS mutants might also avoid glyphosate through late germination, thus a behavioural mechanism could also lead to an infestation of resistant weeds.

The same model could apply equally to the development of resistance to amitrole and atrazine that has already occurred. Atrazine could also move in runoff leading to mild selection pressure at the margins of the sprayed area. In addition, the sprayed area slopes away from the track surface perhaps further diluting the herbicide dose at the margin. Lower selection pressure which permits some survival may have provided directional selection for the accumulation of quantitative traits conferring atrazine resistance. Amitrole could also have applied reduced selection at the margins because it is water soluble, permitting transport in runoff, whilst also being taken up from soil. As these traits became more effective the biotype would have been able to invade further into the sprayed area where it would have been exposed to greater selection pressure.

The railway rights of way, therefore, constitute a different system from an agricultural field. In a field the distance from the margins to the centre may be very great thus making the effects of the margin less important over the total area of the field. Thus resistance develops from seeds already in the seed bank which are subject to uniform doses. Resistance is observed as emerging from foci which may represent individual mutation events within the field . In contrast, railway rights-of-way are long narrow areas with a succession of

vegetation types from the outer edge into the sprayed area. It is in this situation that the effects of the margin are proposed as being more important for the development of quantitative resistance. Depending on the gradient of herbicide concentration away from the tracks a wide variety of resistant phenotypes could compete in the margin with potential to produce more resistant individuals whose seed might flourish in the treated area. This, however, is only one model that might explain the development of resistance in such a situation. While there is no data from this specific case to indicate which model is more applicable, there are some records of observations made during the onset of resistance. Resistance first appeared as strips along the margins of the tracks. This was initially attributed to the effect of passing trains distributing seeds along the length of the track in the train's slipstream. The disturbance caused by rolling stock may be an important factor in moving seeds into the sprayed area or along the track but the strips of resistance might also be due to the processes of gradual encroachment or succession by the resistant biotype. Succession is a term more commonly associated with the change in species composition at a site over time. In the context of resistance it may be used to describe the gradual invasion of an area by resistant weeds. The greater the level of resistance the greater the extent of the invasion.

2.5 Conclusions (WLR2)

WLR2 has many characteristics which distinguish it from other cases of resistance. It developed resistance to two herbicides which are mildly synergistic and also became cross-resistant to two classes of structurally dissimilar herbicides. WLR2 is therefore both multiple- (amitrole and atrazine) and cross-resistant (phenylureas and metribuzin). The use of mixtures (such as amitrole and atrazine) has been advocated as a means of avoiding resistance (Gressel, 1991) but WLR2 demonstrates that resistance may still develop. To be fair, the selection pressure applied to WLR2 was extreme and would not constitute a rational resistance avoidance strategy. The argument for using mixtures is that the target weed must develop two independent mechanisms to survive the combination of herbicides. In *L. rigidum*, cross-resistance phenomena demonstrate that a resistance response to one herbicide

can protect the plant against a second, thus reducing the utility of some mixtures. If atrazine and diuron were applied as a mixture to a biotype such as WLR2 they would be selecting for the same mechanism and would not provide any advantage over either herbicide alone. Cross- and multiple-resistance mechanisms, therefore, undermine the effect of mixtures in staving off resistance.

Triazine resistance in WLR2 raises other issues associated with resistance because the infested railway lines run through two million hectares of lupin fields where simazine is the principle means of weed control. These circumstances are conducive to the transfer of resistance from a non-crop area to a cropping area. Thus, responsible users of herbicides could have their resistance avoidance strategies undermined by the activities of neighbouring parties. This has always been of concern in the control of noxious weeds and the advent of resistance is only a new aspect of the problem. Fortunately, *L. rigidum* seed and pollen do not appear to migrate over great distances (Heap, 1988) and there is no evidence of resistance escaping from the rights-of-way. Nonetheless, WLR2 illustrates the potential for agricultural practices to be compromised by other herbicide uses.

The essential features of resistance in WLR2 are that it was exposed to ten years selection by amitrole and atrazine which eventually yielded a biotype of *L. rigidum* resistant to those herbicides. The biotype is also cross-resistant to metribuzin and the phenylureas. Cross-resistance necessitated the use of a completely new set of herbicides to control the biotype and illustrates the consequences of generating resistance in *L. rigidum*.

2.6 Introduction to VLR69

While the use of herbicides in non-crop areas places strong selection pressure on weeds, the majority of cases of herbicide resistant *L. rigidum* have occurred in agricultural areas (Powles and Matthews, 1992). The agricultural practices most conducive to the occurrence of herbicide resistance phenomena are those which rely exclusively on herbicides for weed control, require very high levels of weed control and which have a very limited range of useful herbicides (Powles and Howat, 1990). Such a circumstance is the production of certified perennial seed crops. Fields producing seed crops provided the initial cases of herbicide resistance in Australia (Heap and Knight, 1982). Perennial seed crops are not grown as part of short term rotations, may not be cultivated and are of sufficiently high value to permit large amounts of herbicide use. Together these factors act to increase the likelihood of herbicide resistance occurring. The infestation of seed crops with herbicide resistant weeds also has serious implications for the spread of herbicide resistance genes.

In 1989 farmers producing certified perennial ryegrass (*Lolium perenne* L.) seed crops reported intractable infestations of herbicide resistant annual ryegrass. The major means of control in these fields had been the herbicide diuron but a variety of other herbicides had been employed as diuron became less effective. A biotype of *L. rigidum* (VLR69) with extensive herbicide resistance was collected from one such field that had been in seed production for 21 years. In this Chapter the extent and degree of herbicide resistance in this biotype are described

2.7 Materials and Methods (VLR69)

Plant Material.

One susceptible (VLR1) and one resistant biotype were used in this study. The resistant biotype (WIHRSC #s VLR69) was collected from a field used for perennial ryegrass

(*Lolium perenne* L.) seed production near Mansfield, Victoria. The field was sown to perennial ryegrass in 1967 and thereafter subject to annual applications of herbicides for 21 years from 1968 with declining diuron efficacy observed in the latter half of this period. The history of herbicide applications to this field is given in Table 1.1.

Dose Response to Herbicides.

For post-emergent experiments, seeds were germinated on 0.6% (w/v) agar and seedlings transferred to a sterile potting mix based on sand and peat in 18 cm (diameter) pots, 12, 17 or 21 seedlings per pot, with three or four pots per treatment. Plants were grown outdoors during the normal autumn-winter growing season of this species. Herbicides were applied as in section 2.2.

Pre-emergent Experiments.

Seeds (0.15 g) were placed on the soil surface in 18 cm diameter pots and covered with approximately 0.5 cm of soil and lightly watered. For dinitroaniline herbicides the herbicide was then sprayed on to the soil surface and the surface again covered with approximately 1cm of soil which was lightly compacted and watered. For metolachlor and oxyfluorfen the seeds were initially covered with approximately 1cm of soil after which the herbicide was sprayed onto the soil surface and not covered further. Mortality was determined 40-42 days after treatment and expressed as a proportion of the survival in the control pots.

Germination Experiments.

Metolachlor, alachlor, propachlor, trifluralin, pendimethalin and tridiphane were dissolved at varying concentrations in water containing 0.6% (w/v) agar and placed in 9 cm petri dishes (20 ml/dish) to solidify. Seeds (0.13 g) were placed evenly over the agar surface. Dishes were placed in an incubator illuminated with fluorescent lights (photoperiod 12h, light intensity 50 μmol quanta m^{-2} s^{-1}, 20°C light/dark) in a randomised block design with four replicates. Seven days after initiation, germination rate and the proportion in which the first leaf had emerged from the coleoptile were determined.

2.8 Results and Discussion (VLR69)

History and selection pressure.

The selection pressure placed on VLR69 is recorded in Table 1.1. The most frequently used selective agent was the herbicide diuron which was initially very successful in controlling *L. rigidum.* After 11 years of diuron application, greater rates of diuron and alternative herbicides were necessary to control *L. rigidum* in the field. Diclofop was introduced in combination with diuron in 1979 soon after its release in Australia. Diclofop was then removed from the program after 1982. Atrazine was used sporadically from 1980 on five occasions. After initial use of atrazine a higher rate of diuron was employed in 1982 before the introduction of chlorsulfuron in 1983. In 1987 the bypyridilium herbicides paraquat and diquat were introduced. The history reveals a steady increase in the rates of diuron, chlorsulfuron and atrazine being employed. Reduction in control was observed from 1980, after which a succession of different herbicides were introduced. From 1987 harvested seed could not be sold as perennial ryegrass because of high contamination by *L. rigidum* seed.

Herbicides to which there is resistance.

Phenylureas.

Despite the long history of selection pressure by diuron, VLR69 has only three- to four-fold greater tolerance to this herbicide (Table 2.2). Biotype VLR69 does, however, have eight-fold greater tolerance to chlorotoluron, an analogue of diuron (Figure 4.5, Table 2.2). Greater resistance to chlorotoluron than diuron was also observed in WLR2 (Table 2.1). This similarity in level of resistance to both phenylurea analogues provides an early indication that both biotypes may have a similar mechanim of resistance to these herbicides. The mechanism of resistance to the phenylureas in WLR2 and VLR69 is discussed in Chapter four.

Sulfonylureas.

Chlorsulfuron was applied to the field containing VLR69 in six seasons and was the second most commonly used herbicide in the VLR69 treatment history (Table 1.1). The control achieved with chlorsulfuron declined over the last three seasons of use. The resistant biotype VLR69 is more than 20 times less sensitive to chlorsulfuron (Figure 2.2) and triasulfuron (Table 2.2, Chapter seven) than the susceptible biotype VLR1. This is a similar level of resistance to that observed in other biotypes with sulfonylurea resistance such as WLR1 and SLR31 (Christopher *et al.*, 1992; 1991). The biotype also has resistance to the non-selective sulfonylurea herbicide sulfometuron (Table 2.2, Chapter seven). While there is a 7.5 fold shift in the LD_{50} for sulfometuron it remains very low at 7 g ai ha^{-1}. There is, however, a small proportion of the population which survive 30 to 90 g ha^{-1} sulfometuron with only minor symptoms suggesting that more than one mechanism of sulfonylurea resistance may be operating in VLR69. The mechanisms of sulfonylurea resistance that have been identified in VLR69 and are fully discussed in Chapter seven.

Triazines.

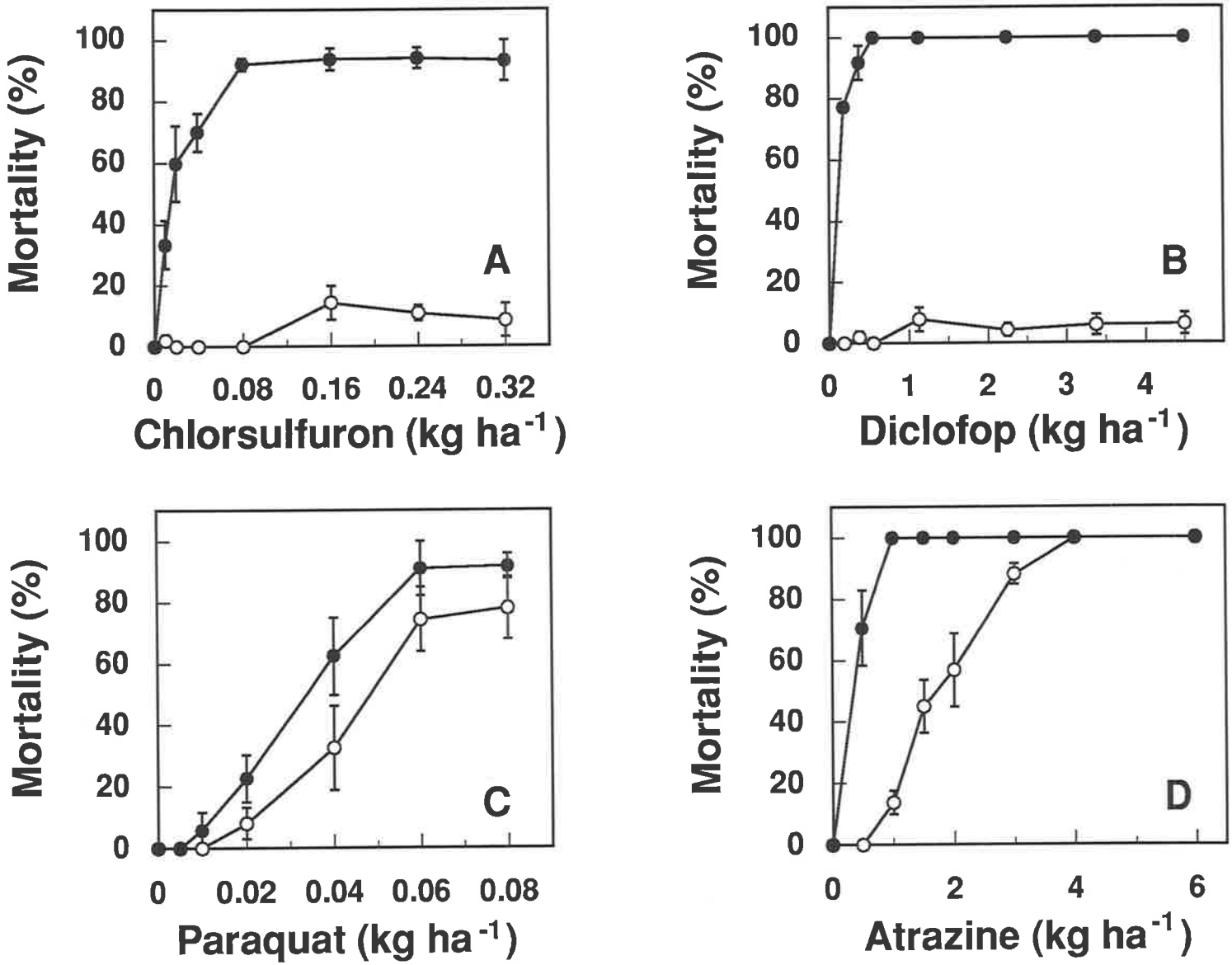
VLR69 exhibits resistance to atrazine (five fold) (Figure 2.2) simazine (six fold) and ametryne (ten fold) (Table 2.2). This is a slightly greater level of resistance to that previously described in biotype WLR2. The biotypes are also similar in that they are both more resistant to simazine than they are to atrazine (Table 2.1, Table 2.2). This similarity suggests that the biotypes may employ a similar mechanism of resistance to the triazines. The mechanism of resistance to the triazines is the subject of the following Chapter

Table 2.2. The LD_{50} values of the resistant biotype VLR69 for various herbicides compared with those of VLR1, the control susceptible.

Herbicide Class	Herbicide	LD_{50} (kg ha^{-1})		LD_{50} ratio
		VLR69	VLR1	
Phenylureas	Diuron	3.2	1.0	3.2
	Chlorotoluron	4.2	0.5	8.1
Sulfonylureas	Chlorsulfuron	> 0.32	0.016	> 20.0
	Triasulfuron	> 0.48	0.019	> 25.0
	Sulfometuron	0.007	0.0009	7.6
Imidazolinones	Imazaquin	0.60	0.080	7.5
	Imazapyr	0.008	0.003	2.5
Triazines	Atrazine	1.70	0.32	5.2
	Simazine	4.30	0.66	6.4
	Ametryne	3.20	0.32	10.0
Bypyridiliums	Paraquat	0.046	0.034	1.4
Aryloxyphenoxy-propionates	Diclofop	> 4.50	0.122	> 37.0
	Fluazifop	1.80	0.015	> 100.0
	Haloxyfop	0.7	<0.013	> 54.0
Cyclohexanediones	Tralkoxydim	> 0.20	0.021	> 9.5
	Sethoxydim	0.024	0.013	1.8
Triazinones	Metribuzin	1.10	0.12	8.7
Chloroacetamides	Metolachlor	0.29	< 0.18	> 1.7
	Metolachlor*	0.37 µM	0.16 µM	2.3
	Alachlor*	0.65 µM	0.53 µM	1.2
	Propachlor*	1.15 µM	0.45 µM	2.6
Oxiranes	Tridiphane*	0.10 µM	0.17 µM	1.6
Diphenylethers	Oxyfluorfen	0.19	0.23	0.82
Dinitroanilines	Pendimethalin	0.091	0.12	0.76
	Pendimethalin*	0.45 µM	0.045 µM	1.0
	Trifluralin	0.07	0.07	1.0
	Trifluralin*	0.70 µM	1.05 µM	0.67

* Tests were conducted in an agar germination medium, hence LD_{50} values are in µM.

Figure 2.2. The response of the susceptible biotype VLR1 (●) and the resistant biotype VLR69 (O) to chlorsulfuron (A), diclofop (B), paraquat (C), and atrazine (D). Each point is the mean of three pots each containing 17 plants and is plotted with the standard error. These four herbicides were components of the selection pressure imposed on VLR69 in the field.


Mortality (%)
100
80
60
40
20
0
A
0
0.08
0.16
0.24
0.32
Chlorsulfuron (kg ha-1)
Mortality (%)
100
80
60
40
20
0
B
0
1
2
3
4
Diclofop (kg ha-1)
Mortality (%)
100
80
60
40
20
0
C
0
0.02
0.04
0.06
0.08
Paraquat (kg ha-1)
Mortality (%)
100
80
60
40
20
0
D
0
2
4
6
Atrazine (kg ha-1)

Bypyridiliums.

There is a small reduction in sensitivity to paraquat (Figure 2.2, Table 2.2) and this is not likely to be agronomically important. A mixture of the bypyridyl herbicides paraquat and diquat were applied in three consecutive seasons (Table 1.1) after other herbicides became ineffective. This selection pressure may have been responsible for the small difference in sensitivity that is observed, however, differences of this scale may also be ecotypic effects. Paraquat resistance has not been reported in *L. rigidum* but has been observed in 11 other species following more persistent selection pressure (Fuerst and Vaughn, 1990).

Aryloxyphenoxypropionates.

VLR69 is resistant to diclofop (Figure 2.2) and the related herbicides fluazifop and haloxyfop at a very high level (Table 2.2). Diclofop was the least frequently applied herbicide having been used twice in four seasons. The degree of resistance observed was not expected given that there were eight generations between the last application of diclofop and the assessment of the population. The flat dose response observed in VLR69 suggests that the population is uniform in its response to diclofop (Figure 2.2). Uniformity also indicates that resistance was maintained in the population at a high frequency in the absence of selection pressure by diclofop. It would seem unlikely that such uniformity would be generated after only two exposures over four seasons. Either the population has a very narrow genetic base allowing traits to be fixed or diclofop resistance was maintained by the selection pressure provided by other herbicides. The latter suggestion might be plausible if there was a diclofop resistance mechanism in common with another herbicide or if the mechanisms were linked. Previous studies have suggested a cross-resistance link between chlorsulfuron and diclofop (Christopher *et al.* 1991, Matthews personal communication). Such a link may have maintained diclofop resistance in the population given that chlorsulfuron was employed in the years following diclofop use.

Diclofop is a member of the aryloxyphenoxypropionate chemical class. These herbicides inhibit the enzyme acetyl coA carboxylase (Secor *et al.,* 1989) and also perturb proton gradients across membranes (Shimabukuro, 1990; Häusler *et al.,* 1991). The degree of resistance observed in VLR69 is similar to that observed in other biotypes of *L. rigidum* with diclofop resistance such as SLR31 (Heap and Knight, 1990). The mechanism of resistance to diclofop has not been elucidated. VLR69 does exhibit repolarisation of membrane potential after depolarisation by diclofop in a similar manner to SLR31 (Häusler *et al.,* 1991). The rate of this recovery is, however, lower than that of SLR31.

Herbicides to which there is cross-resistance.

Cyclohexanediones.

Although never exposed to cyclohexanediones, VLR69 exhibits cross-resistance to tralkoxydim and a small change in sensitivity to sethoxydim. The pattern of resistance to the cyclohexanediones in VLR69 (resistance to tralkoxydim but only a small change for sethoxydim) is similar to that of other biotypes with resistance to the aryloxy-phenoxypropionates such as WLR96 and SLR31 (Heap and Knight, 1990, Holtum and Powles, 1992; Häusler *et al.,* 1991). The mechanisms of resistance in VLR69 have not been established, however, slow recovery of membrane polarity after depolarisation by sethoxydim has been demonstrated, suggesting that the mechanism of resistance may be similar in part to that in SLR31 (Häusler *et al.,* 1991).

Imidazolinones.

VLR69 is resistant to imazaquin (7.5 fold) and exhibits a small shift in LD_{50} for imazapyr (2.5 fold) (Table 2.1). Both of these herbicides are members of the imidazolinone class which, like the sulfonylureas, are inhibitors of ALS (Shaner *et al.,* 1984). The response to imazapyr is similar to the response to sulfometuron in that there is a small shift in the LD_{50} of the bulk of the population but there is also a small component of the population which

survives and maintains a high dry weight at rates above the LD_{50}. This phenomenon is discussed in Chapter seven. The mechanism of resistance to imazaquin in the majority of VLR69 plants is not known. A previously reported case of sulfonylurea resistance in *L. rigidum*, that of SLR31, also exhibited resistance to the imidazolinones, especially those which are selective in crops such as imazamethabenz or imazaquin (Christopher *et al.*, 1991). Thus it is not unusual to observe cross-resistance to imidazolinone herbicides in *L. rigidum* where the biotype has become resistant to the sulfonylureas.

Metribuzin.

VLR69 exhibits an eight-fold increase in resistance to the triazinone herbicide metribuzin (Table 2.2, Figure 5.2). Metribuzin is an inhibitor of photosystem II as are triazines and phenylureas (Hatzios and Penner, 1988). In VLR69 there is a very similar level of resistance to metribuzin, chlorotoluron, and simazine as is the case for WLR2. It is proposed that resistance to metribuzin may be linked to resistance to the other PS II inhibitors given that there are two very similar instances of cross-resistance. The mechanism of metribuzin resistance in WLR2 and VLR69 is the subject of Chapter five.

Chloroacetamides.

There is a small but perceptible shift in the sensitivity of VLR69 to metolachlor applied either to the soil or to the agar germination medium of the seeds (Table 2.2). This small difference is also consistently observed with the other chloroacetamides alachlor and propachlor (Table 2.2). The agronomic implications of this shift are minor as metolachlor is not used in the area from which VLR69 was collected but this reduced sensitivity is sufficient to undermine the feasibility of using metolachlor as an alternative control. Chloroacetamide cross-resistance appears to be common in herbicide resistant biotypes of *L. rigidum*, especially those with diverse herbicide histories (Table 1.1, 1.2).

Tridiphane.

Tridiphane is a herbicide synergist used with atrazine and not a herbicide used against *L.*

rigidum, however, it is highly phytotoxic to seedlings during germination. VLR69 was consistently found to be less sensitive to the effects of tridiphane in the germination system (Table 2.2). It is suggested that changes in the physiology of a weed that effect its response to a particular herbicide may also alter the its response to other xenobiotics. The observation of decreased sensitivity to tridiphane illustrates that the extent of cross-resistance in these biotypes may be limited only by the capacity to test herbicides or xenobiotics against them. Thus, an awareness of the potential for cross-resistance has increased the probability of its detection in *L. rigidum*.

Herbicides to which there is no resistance.

Dinitroanilines.

There was no evidence of resistance to these herbicides in VLR69 (Table 2.2). VLR69 responded to trifluralin in a similar manner to the susceptible control VLR1. While VLR69 had a slightly lower LD_{50} for pendimethalin in the soil system it had a similar response to VLR1 in the agar system. Trifluralin would not be a suitable alternative control in the perennial crop because of its requirement for incorporation.

Diphenylethers.

The only diphenylether herbicide tested was oxyfluorfen. It was applied as a pre-emergent treatment to soil without incorporation. VLR69 was not resistant to oxyfluorfen (Table 2.2) and this herbicide may have potential as an alternative control. It has been used experimentally in production of perennial ryegrass seed with no end of season yield penalty (Mueller-Warrant, 1990). The lack of a requirement for incorporation is an advantage if it is to be used in a perennial crop.

2.9 Conclusions (VLR69).

Rigid ryegrass biotype VLR69 was placed under selection pressure by herbicides in five chemical classes (Table 1.1). In response, it is less sensitive to ten classes of herbicides

(Table 2.2). The salient features of herbicide resistance in VLR69 are the rapidity with which it became multiple- and cross-resistant and the breadth of its final resistance spectrum.

VLR69 has resistance to the triazines, phenylureas and metribuzin in common with WLR2. They are similar in their degree of resistance to each of these classes and in their resistance to the various analogues within the groups. They are also cross-resistant to metribuzin, further reinforcing the similarity and leading to the inclusion of both biotypes in investigations to determine the mechanisms of resistance to the three classes of PS II inhibitor.

The case of VLR69 also illustrates the potential extent of herbicide resistance in *L. rigidum* biotypes. The wide range of different resistance phenomena in VLR69 is likely to be the product of the diverse selection pressure placed on the biotype and the many different mechanisms of resistance that were selected as a result. While a number of alternative herbicides are listed as remaining effective, it would be difficult to justify the continued use of herbicides alone as a control measure because their effectiveness in the long term would be doubtful. This case study illustrates that the sustained use of a number of herbicides may result in an intractable resistance problem.

2.10 Conclusion

Two biotypes of *L. rigidum*, WLR2 and VLR69 have been exposed to selection pressure by a range of herbicides including the triazines and phenylureas. Although both biotypes have different histories of selection pressure and different spectra of resistance, they have in common cross-resistance to metribuzin and resistance to the triazines and phenylureas. The biotypes were isolated from sites on opposite sides of the Australian continent, yet they exhibit very similar responses to the herbicides in these categories. These biotypes have, therefore, been chosen as the systems for the study of the mechanisms of resistance to the inhibitors of photosystem II in *L. rigidum*.

Chapter Three

Triazine Resistance in *L. rigidum*

3.1 Introduction

History and Applications

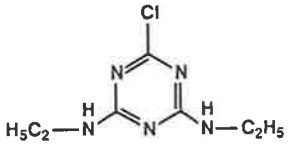
s-Triazine herbicides were first patented in 1954 and their herbicidal properties were widely reported in 1955 (Esser *et al.*, 1975). These investigations were prompted by observations from the dye and pharmaceutical industry that triazine- and urea-based molecules had similar properties. At that time the herbicidal properties of the substituted ureas were being established and it was reasoned that triazines might share this property as well (Ashton and Crafts, 1981). As a result of this program a wide range of herbicidal triazines were developed with both selective and non-selective properties. The structures of the triazine herbicides are given in Figure 3.1, and the numbers following a name indicate where to find that structure in the figure.

Atrazine (2) is the best known of the s-triazines. Atrazine controls a broad spectrum of weed types and has been extensively used in maize production in the Northern Hemisphere. The selective properties of the triazines were manipulated by the substitution of different groups at the 2-position (see later section on chemistry). This affected selectivity by altering both the metabolism of the herbicide in plants and its behaviour in soil. As a result the various triazine analogues found uses as both selective and non-selective herbicides.

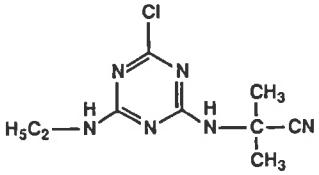
Chemistry

The general structure of a triazine herbicide is given in Figure 3.2A. The s in s-triazine stands for "symmetrical", which describes the distribution of the three nitrogen atoms in the triazine ring. This is in contrast to the as-triazines (asymmetrical) as in metribuzin (Figure 5.1).

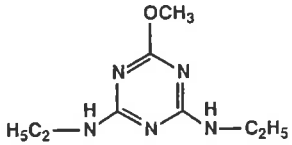
Figure 3.1. The structures of the various triazine herbicides discussed in the text. They are grouped on the basis of the substituent at the 2-position and in order of increasingly complex N-sidechains.



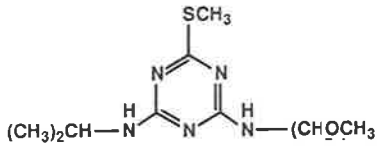
1 Simazine



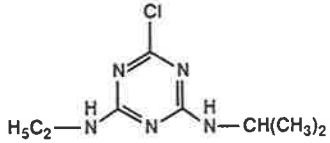
5 Cyanazine



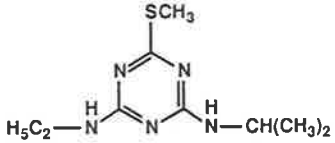
9 Simatone



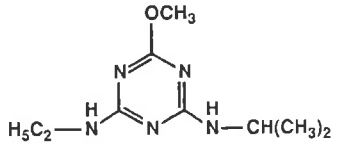
13 Metoprotryne



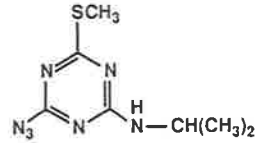
2 Atrazine



6 Ametryne



10 Atratone



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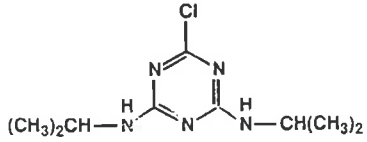
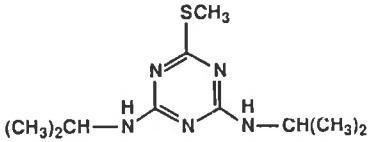
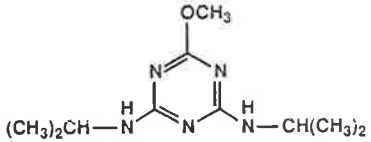


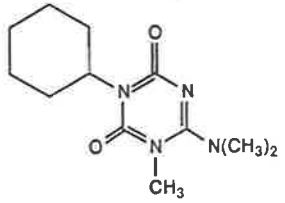
3 Propazine



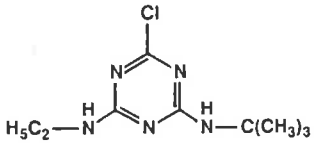
7 Prometryne



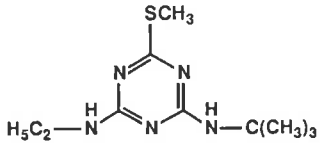
11 Prometone



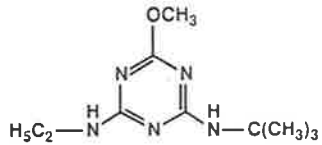
15 Hexazinone



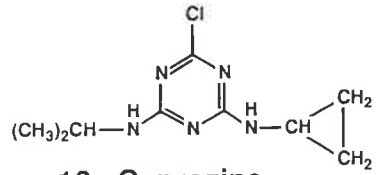
4 Terbuthylazine



8 Terbutryne



12 Terbumetone



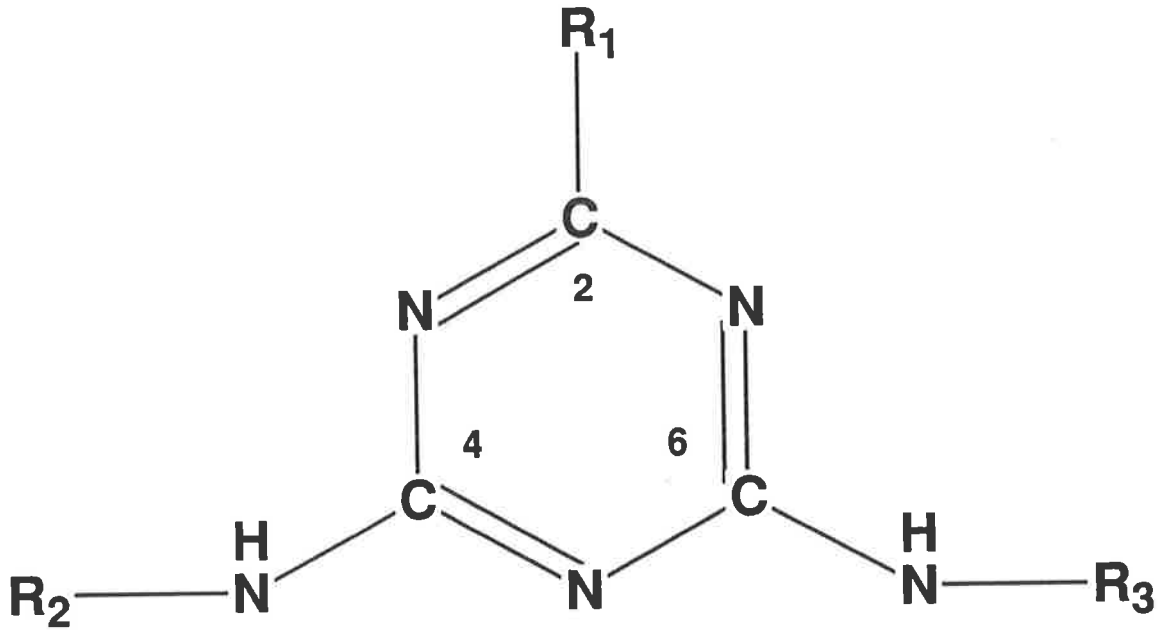
16 Cyprozine

There are three classes of bis-alkylamino s-triazine herbicides denominated by the nature of the substituent at the 2 position. This substituent may be either a chloro, thiomethyl or methoxy group. Since this substituent governs many properties of the molecule the common names of the herbicides are given a suffix to indicate which substituent they contain. Thus -azine (atrazine) indicates a 2-chloro-triazine, -tryne (ametryne (6)) a 2-thiomethyl-triazine, and -tone (atratone (10)) a 2-methoxy-triazine. The prefix of the herbicide common name also indicates its structure. In the previous example each herbicide had a 4-ethylamino group and a 6-isopropylamino group, which was indicated by the similar prefixes. Another example is propazine (3), prometryne (7) and prometone (11); pro- indicating that they have 4,6-bis-isopropylamino groups. An exception to this rule is hexazinone (15) which was developed later and while similar in behaviour to other triazines, differs markedly in structure.

The triazine ring is a very stable structure and is similar in some ways to the benzene ring. The triazine ring is stabilised by delocalisation of its π electrons, however, unlike benzene the triazine ring is heterocyclic and the nitrogen atoms have a greater electro-negativity. As a consequence, charge is unevenly distributed around the ring giving rise to a polar mesomeric form of the ring (Figure 3.2B) (Esser *et al.*, 1975). This renders the ring carbons susceptible to nucleophilic attack, and it is these reactions that are exploited in the synthesis of the triazines. Electron withdrawing substituents like chloro groups tend to exacerbate this property and are readily displaced by other nucleophiles, a property which aids detoxification in plants. Electron donating groups like amino groups render the carbon less susceptible to nucleophilic attack hence the lack of triazine deamination in plants (Esser *et al.*, 1975). The practical implications of this are seen both in the synthesis of triazines and in the treatment of plant extracts in metabolic studies. To avoid displacement of the 2-group, care should be taken to avoid exposing the plant extracts to extreme alkaline or acid conditions. Otherwise a misleading level of hydroxylated herbicide will be observed.

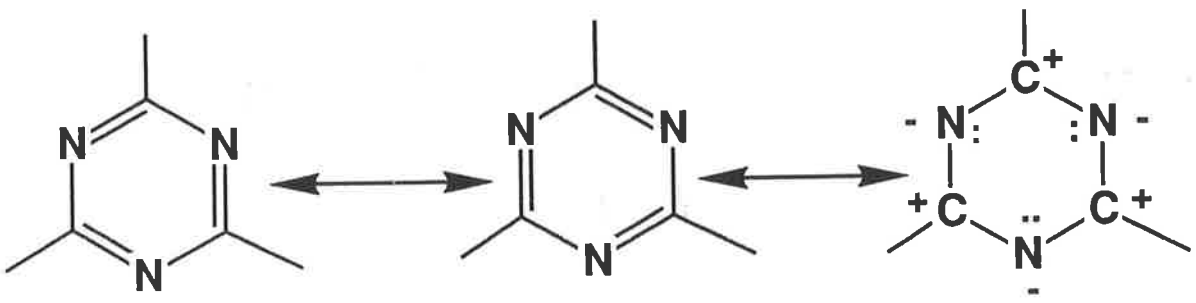
Figure 3.2 A. The general structure of the s-triazine herbicides.

Figure 3.2 B. The resonance structure of the triazine rings



R_1 = Cl, OCH_3, SCH_3

R_2, R_3 = Alkyl groups



The water solubility of the s-triazines is determined by the 2-substituent, with solubility increasing in the order 2-chloro (more electron withdrawing), 2-thiomethyl, 2-methoxy (more electron donating) (Esser *et al.*, 1975). In general, longer alkylamino sidechains lower solubility while asymmetrical alkylamino groups increase solubility. Thus atrazine is more soluble than propazine. The pH of the solution affects solubility. Solubility tends to increase as conditions become more acidic, however, bis-alkylamino-s-triazines have a low solubility in water overall (Esser *et al.*, 1975).

Uptake and Translocation

s-Triazine herbicides are taken up through the roots and foliage, however, the degree of foliar uptake is often dictated by the structure of the molecule (Esser *et al.*, 1975). Ametryne, which has a high water solubility enters foliage readily whereas simazine which has a low water solubility has virtually no foliar uptake (Ashton and Crafts, 1981). Foliar penetration of the triazines is enhanced by the use of non-ionic surfactants. Unlike the triazines, hexazinone has relatively high foliar uptake (Herbicide Handbook 1983).

Root uptake of s-triazines occurs in both resistant and susceptible plants (Esser *et al.*, 1975) and differential root absorption has not been implicated in resistance mechanisms (Cole, 1987). When the plant roots are placed in nutrient solution there is a period of rapid absorption associated with the filling of exchange sites within the roots. Thereafter, uptake and translocation are proportional to transpiration (Ashton and Crafts, 1981). Upon return to nutrient solution roots both release absorbed herbicide into the solution and act as a reservoir of herbicide continuing its translocation to the leaves (Esser *et al.*, 1975).

Uptake from soil may be limited by adsorption of the triazines to soil constituents. The differential adsorption and mobility of the triazines is exploited in the use of herbicide banding as a means of selectivity. The low water-solubility of simazine (1) renders it comparatively immobile in soil. This makes it suitable for surface banding for control of shallow-rooted weeds in deep rooted crops. The more water soluble methoxy triazines are

more mobile and unsuitable for this application (Esser *et al.*, 1975). Instead they are used in non-selective industrial weed control.

Once the triazine has entered the plant, translocation occurs via the apoplast. If plants are removed from the source of the triazine herbicide there is little basipetal translocation of the herbicide to new growth (Esser *et al.*, 1975). Herbicide tends to accumulate in the leaf margins causing rapid chlorosis of the tips and margins. Because translocation of triazines is largely a function of transpiration rates, environmental factors which influence transpiration also affect triazine uptake and translocation (Ashton and Crafts, 1981). These include humidity, temperature, soil moisture and stomatal aperture. Atrazine has been observed to cause stomatal closure in the presence of CO_2 but not in CO_2-free air. This is presumably a consequence of increased CO_2 partial pressure within the leaf following inhibition of photosynthesis (Ashton and Crafts, 1981).

Mode of Action

The triazine herbicides inhibit electron transport in photosystem II. The target protein is the same as that bound by the phenylurea herbicides as indicated by the competitive binding between the two (Tischer and Strotman, 1977). The target protein was identified as the 32 kd protein in PS II using photo-affinity labelling studies with [^{14}C]azidoatrazine (Steinback *et al.*, 1981; Pfister *et al.*, 1981). This site of action was confirmed by the observation that resistance to the triazines in higher plants was caused by mutations of the gene (psb A) encoding this protein (Arntzen *et al.*, 1982). While the immediate effect of the triazines is to inhibit photosynthesis the cause of plant death is related to the effects of light. When atrazine treated plants are grown in the light their chloroplasts are disrupted. This is not observed when they are kept in the dark after treatment (Ashton and Crafts, 1981), suggesting that plant death may be a consequence of light mediated photo oxidation of the chloroplasts. Other sites of action have been proposed for the triazines. However, the resistance caused by mutation of the psb A gene is sufficient to cause complete resistance to high rates of triazine application, indicating that the other sites of action are less important.

Triazines, in common with eleven other classes of photosystem II inhibitors, share a common structural element. This is an alkyl substituted NH group attached to an electron deficient carbon (double bonded to an oxygen or nitrogen) (Hansch, 1969). Ideally, this combination should be attached to a lipophilic moiety and a polar functional group. Triazines fit this description. The structural requirements for the binding of s-triazines to the 32 kd target peptide (D1 protein) are not stringent. The major requirement is the need for one relatively small sidechain (eg. NHEt), with the size of the other sidechain being relatively unimportant (Shimizu *et al.*, 1988). This is thought to be related to the size of the receptors that each side chain enters. The smaller side chain enters a small "pouch" (hence the size limitation), the larger enters an open ended cleft.

The elucidation of the amino acid sequence of triazine resistant D1 protein revealed which residues were important for atrazine binding. Substitution of glycine (GGT) for serine (AGT), at position 264, reduces the binding of atrazine but not diuron. The absence of a hydroxyl group which normally forms hydrogen bonds with atrazine is the apparent reason for atrazine not binding. Substitution of threonine (ACT) for serine confers resistance to both diuron and atrazine due to conformational change in the binding site (Sigematsu *et al.*, 1989).

Arguments that suggest other sites of action of triazines (mostly atrazine) are based on the observations that triazines can stimulate growth at low doses (Ashton and Crafts, 1981). Attempts have been made to link this phenomenon with nitrogen metabolism. Uptake of mineral nutrients can be increased in triazine treated plants when nitrogen is provided as nitrate at low levels with some of these effects possibly linked to enhancement of nitrate reductase activity (Ashton and Crafts, 1981). Herbicidal triazines (at low rates) produce increases in protein and dry matter in both resistant and susceptible crops whereas non-herbicidal triazines do not produce this effect (Esser *et al.*, 1975). This may suggest that, through interactions with photosystem II, the triazines also effect chloroplastic nitrogen

metabolism and that the secondary effects are a consequence of this.

Triazine herbicides may also affect acid phosphatases in corn roots (Scarponi and Perucci, 1986). The effect depends on the structure of the triazine and is influenced largely by the 2-substituent . Both increases and decreases in activity were observed which may suggest that it is a secondary effect.

Metabolism in Plants

The selectivity of triazines is due to the ability of some plant species to metabolise triazines to a greater extent than others. This is particularly true of the chloro-s-triazines. In general, metabolism of the chloro-s-triazines occurs by three mechanisms: non-enzymic hydroxylation at the 2-position, conjugation to glutathione at the 2-position by glutathione transferase (Figure 1.3) and mixed function oxidase dependent dealkylation of the side chains. It is important to note that the first two mechanisms are ineffective in the detoxification of thiomethyl and methoxy s-triazines (Esser *et al.*, 1975). Sulfoxidation of the thiomethyl group detoxifies the thiomethyl-s-triazines in wheat, thus wheat is tolerant of terbutryne (8) and prometryne (7) (Montgomery and Freed, 1964). Hexazinone is detoxified by hydroxylation of its cyclohexyl group in *Solidago fistulosa* (Baron and Monaco, 1986) and also by demethylation in soils (Roy *et al.*, 1989).

Non-Enzymic Hydroxylation at the 2-Position

Early studies on the detoxification of atrazine revealed that cell free extracts from maize could rapidly hydroxylate chloro-s-triazines at the 2-position to form a non-phytotoxic metabolite. The active component was identified as 2,4-dihydroxy-7-methoxy-1,4(2H)-benzoxazin-3(4H)-one (DIMBOA). Rye plants contain a similar compound but without the 7-methoxy group (DIBOA). The presence of high levels of these compounds correlated to resistance in several grass species (Cole, 1987; Hamilton, 1964), but could not account for resistance in sorghum which does not form large quantities of hydroxylated metabolites (Shimabukuro, 1968). When methoxy-s-triazines are exposed to corn extracts they form

hydroxy derivatives at less than 20% of the rate of the chloro-s-triazines, while thiomethyl-s-triazines are not hydroxylated (Montgomery and Freed, 1964). This suggests that only the chloro-s-triazines are readily detoxified by this reaction. The importance of this reaction in maize appears to depend on the mode of uptake of the herbicide. When atrazine is taken up via the roots, hydroxylation occurs, however, conjugation with glutathione is the major reaction in leaves (Esser *et al.*, 1975).

Glutathione conjugation

The most important mechanism of chloro-s-triazine detoxification in maize, sugar cane and sorghum is glutathione conjugation via glutathione transferase (Frear and Swanson, 1970). This detoxification mechanism is less effective for the 2-thiomethyl, 2-methoxy, or 2-hydroxy s-triazines. Mono-dealkylation reduces the affinity of the enzyme for the triazine whereas asymmetrical alkyl substitution increases affinity (Frear and Swanson, 1970). Thus atrazine and cyprozine (16) are more readily detoxified than propazine and simazine. The glutathione conjugate is further metabolised to other amino acid-containing conjugates (Lamoureux and Rusness, 1989). Glutathione conjugation is the major detoxification mechanism in many grasses and the rate of formation of these conjugates correlates well with the degree of tolerance (Weimer *et al.*, 1988).

N-dealkylation

Degradation of s-triazine herbicides is also achieved by cleavage of the N-alkyl groups. This is an oxidative reaction which most plants carry out to some extent (Esser *et al.*, 1975; Shimabukuro, 1967). It is analogous to the N-demethylation in the phenylureas in as much as monodealkylation only partially reduces phytotoxicity. N-dealkylation is generally, a less efficient detoxification pathway and may predominate in plants that are considered only intermediate in their susceptibility to triazines (Esser *et al.*, 1975). The short chain alkyl groups tend to be cleaved preferentially with many species producing a greater quantity of de-ethyl atrazine than de-isopropyl atrazine (Shimabukuro and Swanson, 1969). This is also the case for the metabolism of terbutryne, where the ethyl group is preferentially cleaved

in wheat (Shimabukuro, 1967). N-dealkylation is also an important mechanism conferring selectivity of ametryne in sugar cane (Esser *et al.*, 1975). N-dealkylation does not depend on the nature of the substituent at the 2-position and hydroxylated triazines are still subject to N-dealkylation (Montgomery *et al.*, 1969).

Triazine metabolism - conclusions

The detoxification mechanisms discussed are summarised in Figure 3.3. Removal of the 2-substituent is the most rapid and effective means of detoxifying triazines. This occurs most readily for chloro-substitutions but to a much lesser extent for methoxy and thiomethyl triazines. Plants that are intermediate in their tolerance of triazines tend to rely on N-dealkylating mechanisms. Differences in absorption and translocation appear only to be secondary mechanisms with limited influence on tolerance.

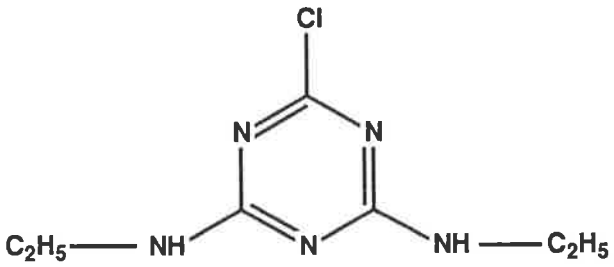
Triazine Resistance.

Resistance to triazine herbicides comprise the majority of reports of herbicide resistance in weeds. The first report was that of Ryan (1970) who described a biotype of *Senecio vulgaris* resistant to simazine. This initial report was followed by many others. Triazine resistance generally developed in areas where there had been repeated use of triazine herbicides. This type of resistance has been intensively studied both in terms of its agro-ecology and the mechanisms which confer resistance.

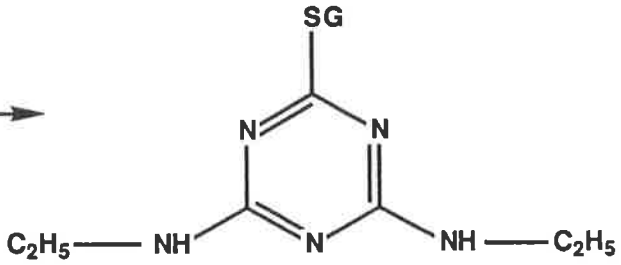
Mechanisms of Triazine Resistance

Triazine resistance in weeds has occurred by two mechanisms. Firstly, and most commonly, by changes in the amino acid sequence of the target site of triazine herbicides, and secondly by enhanced metabolism. The latter mechanism will be discussed in later sections.

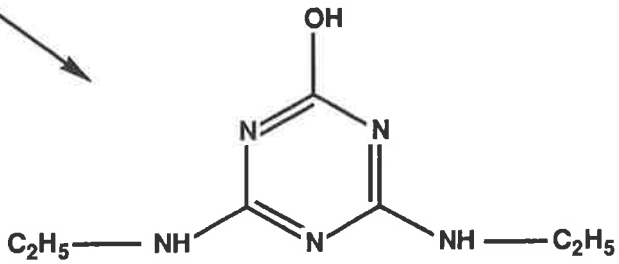
Figure 3.3. The major mechanisms of simazine detoxification in plants (Esser *et al.*, 1975).



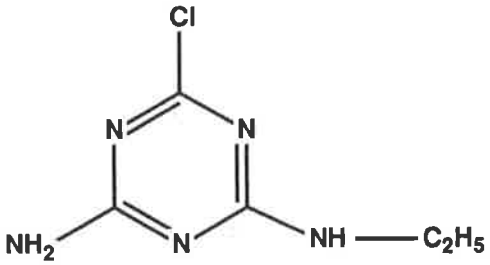
simazine



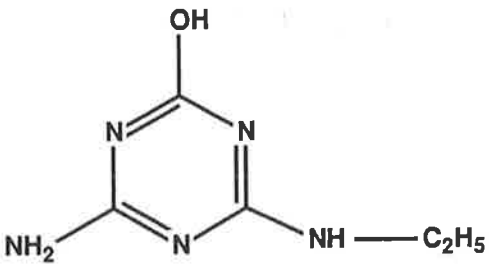
glutathione conjugate



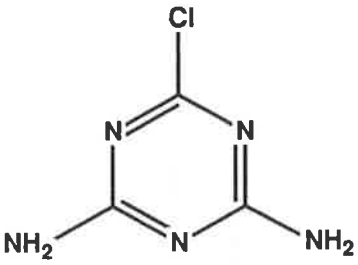
hydroxysimazine



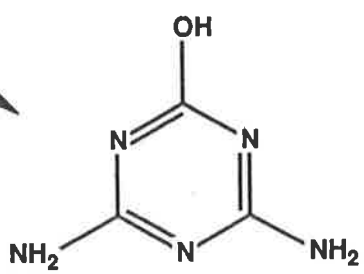
de-ethyl-simazine



de-ethyl-hydroxysimazine



di-de-ethyl-simazine



di-de-ethyl-hydroxysimazine

Initial studies on triazine resistance in biotypes of *Chenopodium album* and *Amaranthus retroflexus* revealed no differences in rates of herbicide uptake and metabolism. However, atrazine inhibited photosynthesis by susceptible chloroplasts but not resistant chloroplasts (Radosevich *et al.*, 1977). It was then established that "chloroplastic" resistance to atrazine in *S. vulgaris* also reduced the binding of other triazine analogues and bromacil but not diuron (Radosevich 1979; Arntzen *et al.*, 1982). This type of resistance is inherited maternally indicating that the resistance is controlled by a cytoplasmic gene (Darmency and Gasquez 1981, Darr et al 1981).

The site of triazine binding was established using photoaffinity labelling with azidoatrazine. UV irradiation of chloroplasts in the presence of azidoatrazine leads to the covalent bonding of azidoatrazine to the target protein (Pfister *et al.*, 1981; Steinback, 1981). By this method it was established that the target was a 32 - 34 kd protein, and that protein derived from resistant plants has less affinity for atrazine. Substitution of serine for glycine at position 264 in the amino acid sequence of this protein causes reduced binding by atrazine (Hirschberg and McIntosh, 1983). This is the only mutation detected in triazine resistant weed species studied thus far (Darmency and Gasquez, 1990). Why only this mutation has been found in higher plants is unknown.

The sequence of the 32 kd protein is highly conserved from algae to higher plants. Changes in the amino acid sequence of 32 kd protein from *Chlamydomonas reinhardtii* show that alterations in the binding of atrazine and diuron depend on the position and the amino acid substituted. Substitution of isoleucine for valine at position 219 reduces diuron binding, tyrosine for phenylalanine at position 255 reduces atrazine binding but increases diuron binding, alanine for serine at position 264 reduces binding of both diuron and atrazine, and glycine for serine at position 264 causes reduced atrazine binding while not affecting diuron binding (Erickson *et al.*, 1985). Similar studies have shown that positions 251 and 275 are important for triazine binding. The importance of position 264 is further reinforced by the observation that substitution of threonine for serine at position 264 reduced binding of both

diuron and atrazine (Sigematsu *et al.*, 1989). All of these positions are thought to be in the membrane spanning portion of the protein (Erickson *et al.*, 1985). Thus triazine resistance and the many variations of triazine and phenylurea resistance can be accounted for by changes in the structure of the target protein.

These studies have also facilitated the modelling of the binding sites of the PS II inhibitors. Arntzen *et al.* (1982) describe a model of herbicide binding which accounts for the competitive binding by the herbicides but also accounts for the specific resistances caused by some mutations. In their model each of the herbicides share a common binding domain but also bind to a unique region of the protein. The common domain is thought to interact with the "essential element" of the PS II inhibitors, namely the -NH-CO- or -NH-CN- moieties (Arntzen *et al.*, 1982, Ducreut and De Prado, 1982, Hansch, 1969). These models have now been improved with the elucidation of the structure of the PS II reaction centre in *Rhodopsuedomonas viridis* (Michel *et al.*, 1986). Triazine herbicides bind the same site as Q_B, hence the reduction in electron transport. The binding of the triazines relies on the presence of appropriate amino acid sidechains for hydrogen bonding to take place between the herbicide and the binding site. In the absence of the sidechain, the triazine binding energy increases and binding is less likely to occur (Oettmeier and Stevens, 1992). Other mutations change the conformation of the site leading to broader effects on the binding of other herbicide classes (Sinning, 1992).

Competitiveness of Triazine Resistant Biotypes

The fitness of triazine resistant biotypes has been extensively studied because of observations, at the chloroplast level, that indicated that photosynthetic electron transport was altered in plants with the psb A mutation (Holt *et al.*, 1983). The change in the Q_B protein is thought to change the redox equilibrium between the reducing and oxidising sides of photosystem II (Ireland *et al.*, 1988; Holt *et al.*, 1983). Studies of whole leaf photosynthesis show that resistant biotypes from a number of species tend to have a 10 - 20 % lower net CO_2 exchange rate (Van Oorschot.and Van Leeuwen; 1984, Ireland *et al.*,

1988; Donnelly and Hume, 1984; Janson *et al.*, 1986; Holt *et al.*, 1981). This reduction in carbon fixation in the resistant biotypes is thought to be largely caused by the changes in the redox equilibrium in PS II (Ireland *et al.*, 1988).

Given that resistant plants have, as a consequence of the altered 32 kd target protein, a reduced capacity for net CO_2 fixation, it is likely that they will also be less competitive than susceptible biotypes in the field. Dry matter production by resistant biotypes is lower than that of susceptible biotypes of *S. vulgaris* and *Amaranthus retroflexus* under both competitive and non-competitive conditions (Conrad and Radosevich, 1979; Holt, 1988). Analysis of F_1 hybrids between susceptible and resistant biotypes of *S. vulgaris* revealed that the hybrids produced greater biomass than the resistant parent line, even when the maternal line of the hybrid was resistant (Stowe and Holt, 1988). This indicates that reduced fitness is not totally mediated by the psb A mutation.

There are two reports of triazine resistance caused by psb A mutation which appear not to be associated with reduced fitness. In *Phalaris paroxida* a resistant biotype has reduced quantum yield but apparently normal growth under non competitive conditions. Growth under competitive conditions was not investigated. This contradiction is possibly explained by the unusual observation of an increase in light saturated electron transport in the resistant biotype (Schonfeld *et al.*, 1987). Alternatively, the system under which growth was measured may not have been limited by photosynthetic activity. A resistant biotype of *C. album* also has reduced quantum yield without reduction in net CO_2 fixation or growth rate (Jansen *et al.*, 1986). The contradiction between these latter results and those from other studies has not been explained although species differences, environmental effects and adaptation may be important. If growth is not being limited by electron transport but by another physiological factor, the effects of the mutation may not be apparent. Lower temperatures, for example may ameliorate the effects of the mutation (Holt, 1990).

Alteration in the Q_B protein also causes greater heat sensitivity in the photosynthetic

apparatus of the resistant plants. Electron transport in resistant chloroplasts is also more sensitive to ferricyanide, high pH, and temperatures between 25 and 35 ° C indicating that electron transport in the resistant chloroplasts was less stable than in the susceptible chloroplasts (Ducruet and Lemoine, 1985). Using nearly isogenic lines of *Solanum nigrum* Ducruet and Ort (1988) further characterised the nature of this heat sensitivity. At high temperatures electron transport in PS II is halted and photosynthesis is inhibited indicating that enhanced heat sensitivity could cause reduced fitness in the resistant biotype. These data explain the observation of declining fitness in these biotypes with increasing temperature in the field.

Changes in Ultrastructure and Lipid Composition

Triazine resistance is also associated with changes in the ultrastructure of chloroplasts and lipid composition of membranes. Chloroplasts from *C. album* and *Brassica campestris* exhibit larger grana stacks, lower chlorophyll a/b ratio and less starch (Vaughn and Duke, 1984, Burke et al 1982; Vencill, 1987). Chloroplasts from susceptible plants could be altered to appear like the resistant chloroplasts by sub-lethal treatments with DCMU or Bentazon, however, this did not confer resistance to atrazine. The rationale for the sub-lethal doses of PS II inhibitors was to test the hypotheses that the ultrastructural changes were brought about by changes in electron transport in PS II. The effect of sub-lethal treatments, therefore, supports the proposition that ultrastructural changes in the resistant plants are secondary effects of the altered electron transport system (Vaughn and Duke, 1984).

Changes in the proportions of chloroplast lipids has been reported in many triazine resistant biotypes. Increases in the proportion of monogalactosyl diglyceride and decreases in the proportion of digalactosyl diglyceride are reported in resistant biotypes of *S. vulgaris*, *C. album*, *A. hybridus*, *A. retroflexus* and *C. canadensis*. (Pillai and St. John, 1981; Polos *et al.*, 1985, Vencill *et al.*, 1987). Burke et al (1982) observed an increase in linolenic acid concentration and lower levels of oleic and linoleic acid in a resistant biotype of *B.*

campestris. Treatment of the algae *Spirodela oligorrhiza* with sub-lethal doses of atrazine brings about a similar change in lipid composition (Mattoo *et al.*, 1984) and ultrastructural changes very similar to those described in *B. campestris* (Vaughn and Duke, 1984). This indicates that changes in the lipid composition and ultrastructure of the chloroplast may be directly related and that these changes occur in response to alterations in electron transport in PS II. Thus it appears that changes in ultrastructure and lipid composition may be secondary effects of reduced electron transport resulting from the PS II mutation.

Triazine resistance by enhanced metabolism

A minority of cases of triazine resistance are due to increased detoxification of the herbicides. The best characterised example is that of *Abutilon theophrasti* which has an enhanced capacity for glutathione conjugation of atrazine (Gronwald *et al.*, 1989). *Echinochloa crussgalli* collected from a site in Europe is also resistant to triazines because of enhanced metabolism (Gressel *et al.*, 1982). Triazine resistant biotypes of *C. album*, *C. strictum* and *Amaranthus powellii* with psbA mutations also have a greater capacity to detoxify atrazine than susceptible biotypes from each species (Khan *et al.*, 1985). The contribution of detoxification to the resistant phenotype is unlikely to be important given the effect of the psbA mutation. It is also possible that the lack of inhibition of photosynthesis allows detoxifying systems to operate more effectively. Jensen *et al.* (1977) reported differential abilities to metabolise atrazine amongst the festucoid, panicoid and eragrostoid grasses. The panicoid grasses were most tolerant and include the Genera *Digitaria*, *Panicum*, and *Setaria*. In most cases tolerance was correlated with the ability to form glutathione conjugates although other triazine metabolites are produced. Thus tolerance and resistance in weeds may be conferred by enhanced metabolism of the triazines.

3.2 Triazine Resistance in *L. rigidum*.

It is apparent from Chapter two that *L. rigidum* biotypes WLR2 and VLR69 exhibit resistance to the triazine herbicides. In both cases they were subject to selection pressure by atrazine but are resistant to all triazine analogues tested against them. They are resistant to both chloro-s-triazines and thiomethyl-s-triazines suggesting that mechanisms based on removal of the 2-substituent are unlikely to cause resistance. Both biotypes are also more resistant to simazine than atrazine with the level of triazine resistance in both biotypes being less than ten times that of a susceptible.

On the basis of dose response to various herbicides described in Chapter two, these biotypes appeared to be distinct from most other reports of triazine resistance. The objective of the work reported in this Chapter was to elucidate the mechanism of triazine resistance in *L. rigidum* biotypes WLR2 and VLR69.

3.3 Materials and Methods

Dose response to simazine

Plants were maintained and treated with herbicide according to the method in Chapter two.

Isolation of thylakoids and measurement of oxygen uptake.

Plants were grown in a growth room with a 14 h photoperiod (15°C) at 230 μmol m^{-2} s^{-1} PAR (400-700 nm) and a 10 h dark period at 11°C. Fully expanded leaves (5 to 7 g) were extracted twice for 5 s in 40 mL of extraction buffer (400 mM sorbitol, 5 mM $MgCl_2$, 200 mM NaCl and 50 mM Tricine, pH 7.5) in an Omni-mixer (Sorvall Instruments). The homogenate was filtered through two layers of Miracloth and centrifuged for 5 min at 1500 g. The pellet was washed in 30 mL of resuspension buffer (extraction buffer without sorbitol) and centrifuged as above. The pellet was resuspended in 1 to 1.5 mL of resuspension buffer. The chlorophyll content of the thylakoid suspension was determined

according to Arnon (1949). Extractions were conducted at 4°C.

Oxygen uptake with methyl viologen as an electron acceptor was measured at 25°C with a Clarke-type oxygen electrode. The reaction mixture consisted of 3.9 mL of assay buffer (50 mM Tricine, 50 mM KCl and 5 mM $MgCl_2$, pH 7.6), 20 µM methyl viologen, 5 mM NH_4Cl, 50 µL of thylakoid suspension (final chlorophyll concentration was 5 µg mL^{-1}) and 10 µL of technical grade simazine in ethanol or an ethanol control. The chamber was illuminated at a photon flux density of 2000 µmol m^{-2} s^{-1} by a slide projector passing light through a 15-cm-diameter spherical water filter .

Effect of 1-aminobenzotriazole (ABT) on growth

Seeds were germinated on 0.6% (w/v) agar. After 7 days seedlings were transferred to polystyrene foam trays and grown hydroponically in Hoagland's nutrient solution (KH_2PO_4 0.5 mM, K_2SO_4 0.4 mM, $MgSO_4$ 1 mM, $Ca(NO_3)_2$ 1.67 mM, KNO_3 1.67 mM, EDTA Na_2 64 µM, $FeSO_4$ 72 µM, $CaSO_4$ 800 µM, Na_2MoO_4 0.25 µM, $CuSO_4$ 0.16 µM, $ZnSO_4$ 0.38 µM, $MnCl_2$ 4.6 µM, H_3BO_3 23 µM) which was changed every ten days. The trays were maintained in a growth room with a 16 h photoperiod (20°C) at 230 µmol m^{-2} s^{-1} PAR (400-700 nm) and an 8 h dark period at 17°C. After 21 d, seedlings were transferred to 75 mL polycarbonate vials, 7 seedlings per vial. The vials were wrapped in aluminium foil and maintained in a growth room (as above but with light intensity 320 µmol m^{-2} s^{-1}). Nutrient solution (as above) was added daily. Transpiration rate was determined gravimetrically. Vials with extremely high or low rates of water loss were discarded after plants had been given 48 h to acclimate to the vials. Treatments applied to the plants were as follows: simazine (3 µM), ABT (70 µM) or ABT (70 µM) in combination with simazine (3 µM). The control and all treatments contained 0.1 % (v/v) ethanol. Treatments were applied in triplicate and the vials were randomly distributed within the experimental area. Plants were maintained in these solutions for 7 d after which the roots were washed twice with deionised water, returned to nutrient solution for a further 7 d before harvest and fresh and dry weight measurements.

12 hour dosing experiments

Plants were placed in vials as above. After 48 h, nutrient solution containing 22 μM [^{14}C]simazine (specific activity 32.6 MBq mmol^{-1}, uniformly ring labelled) was added to drained vials. Plant roots were maintained in the [^{14}C]simazine solution for 4, 8 or 12 h during the light period in the growth room (when present the final concentration of ABT was 2 mM). Vials were then drained and roots were washed 4 times with 10 mL aliquots of deionised water after which nutrient solution was added to the vials. Plants were harvested up to 50 h after the initiation of the treatment period. Roots were washed and separated from the shoots. Fresh weight of both shoots and roots was recorded and tissue wrapped in aluminium foil, frozen in liquid nitrogen and stored at -20°C.

Simazine uptake

Hydroponically grown plants prepared as described above, were exposed to 15 mL of 22 μM [^{14}C]simazine (37 MBq mmol^{-1}) for twelve hours. Six replicates of each biotype were used. Tissue was harvested and stored as above.

Extended simazine uptake experiments

Plants were placed in vials as above. The roots were immersed in nutrient solution containing 3 μM [^{14}C]simazine (spec. act. 94 MBq mmol^{-1}) at the commencement of the light period of day 1 of the experiment. Harvests were made 12, 24, 48, 72, 96, 120, 168, 216 and 264 hours after initiation of the dosing period. Nutrient solution containing [^{14}C]simazine was added daily to replace evapotranspiration losses. The 12, 24, 48, 96, 168, 216 and 264 h treatments were also conducted in the presence of ABT (70 μM). After 168 h, roots of remaining plants were washed and immersed in nutrient solution for the remainder of the experiment. Plants were harvested as above.

Extraction, separation and quantification of metabolites

Frozen plant material was ground in methanol:water (4:1 v/v) with a mortar and pestle, then

centrifuged at 14500 g for 30 min at 2°C. The pellet was washed 4 times with methanol:water (4:1 v/v). Recovery of radioactivity in the pooled supernatants was 90 to 95 %. Extracts (4 mL) were concentrated to dryness *in-vacuo* then resuspended in 1 mL acetonitrile:water (1:19 v/v) prior to chromatography.

Metabolites were separated and quantified by reverse phase HPLC on a Brownlee Labs ODS-5A, 250 x 4.6 mm column. The mobile phase was acetonitrile, water and a constant 0.1 % (v/v) acetic acid. Metabolites were eluted with a 5 % to 99 % acetonitrile gradient over 28 minutes (0 min 5%, 3 min 5%, 10 min 20 %, 26 min 70 %, 28 min 99 %) at a column temperature of 39°C and flow rate of 1 mL min^{-1}. Simazine metabolites were detected using an on-line HPLC radioactivity monitor (Berthold LB504) with a solid, cerium-activated glass scintillant cell (Berthold, G650U4). Metabolites were quantified as a proportion of the total radioactivity injected onto the column.

Identification of metabolites

Metabolites were tentatively identified by co-chromatography with standards in both methanol and acetonitrile gradients with two different gradients for each solvent system using the column described above. Metabolites are described according to the method of Esser *et al.* (1975) in which the substituents of the metabolite retain the number given to ligands of the parent herbicide. Hence 2-chloro-4,6-ethylamino-s-triazine (simazine I) becomes 2-chloro-4-amino-6-ethylamino-s-triazine (de-ethylsimazine II) by the removal of one ethyl sidechain. This, in turn, becomes 2-chloro-4,6-amino-s-triazine (di-de-ethylsimazine III) by cleavage of the other ethyl sidechain. For simplicity the names in parentheses are used in the text. Standards of de-ethylsimazine (code number: G - 28'279) and di-de-ethylsimazine (G - 28'273) were supplied by Ciba Geigy Basel, Switzerland.

Inheritance of simazine resistance

Resistant WLR2 plants were selected as parents for use in crossing experiments by exposing them to a selection pressure of 2 kg ha^{-1} amitrole and 2 kg ha^{-1} atrazine. Three surviving

WLR2 plants were selected from a sample of 20 individuals and hybridised with susceptible plants. *L. rigidum* is a self-incompatible wind-pollinated plant (Kloot, 1983). Hybridisation was achieved by placing a pot containing a resistant WLR2 plant and a susceptible VLR1 plant within a sleeve of clear polythene prior to flowering (single plants isolated in this system set zero or negligible amounts of seed). The sleeve was 1.2 m high and open at both ends to reduce humidity. Seeds from crosses were collected separately from each female parent. Plants derived from F_1 seed from each female parent were screened for response to simazine at the rates of 1 and 2 kg ha^{-1} and compared with both the resistant WLR2 and susceptible VLR1 populations. Screening was performed according to the section "dose response to herbicides". Two crosses provided sufficient seed for assessment.

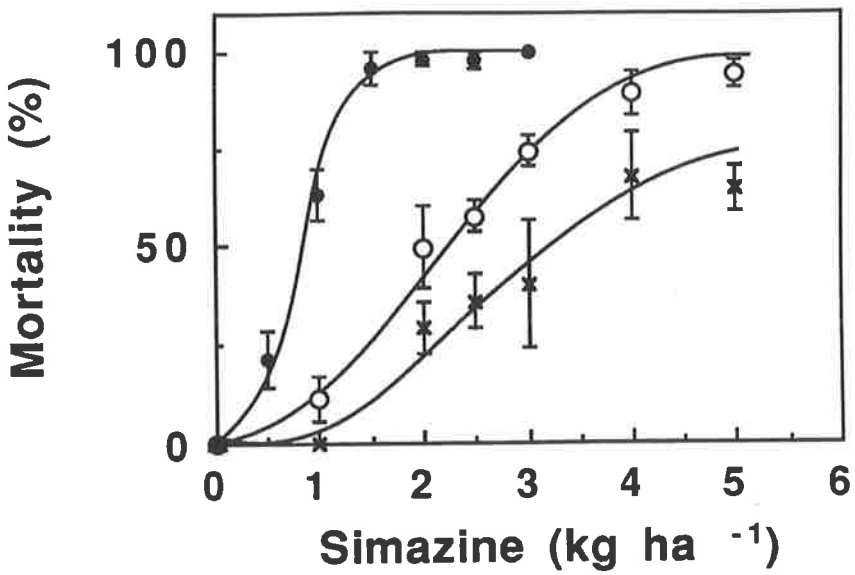
3.3 Results and Discussion

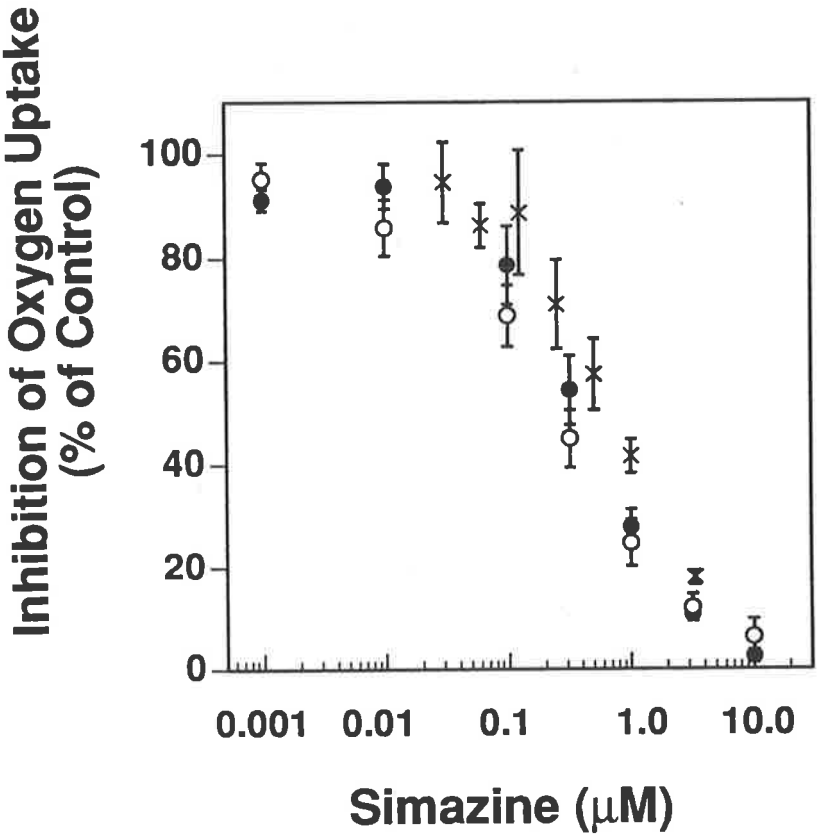
Dose response to herbicides

WLR2 and VLR69 are 2.5 and 3.5 times, respectively, more tolerant to simazine than is VLR1 based on a comparison of LD_{50} values. The experiment was conducted with all three biotypes simultaneously during the winter growing season (Figure 3.4). The degree of resistance increases with increasing temperature as both WLR2 and VLR69 have six to eight fold greater LD_{50} values than VLR1 when experiments are performed during autumn or spring (Table 2.1, 2.2). However, the result reported in Figure 3.3 permits a direct comparison of the relative susceptibility of the biotypes because the biotypes were treated simultaneously whereas other experiments were conducted at slightly different times. In most experiments VLR69 was more resistant to simazine than WLR2. This is also observed in the response of these biotypes to chlorotoluron and metribuzin (Chapters four and five). Any conclusions on the mechanism of resistance to these herbicides in VLR69 and WLR2 should, therefore, explain the relative difference between the biotypes in response to the herbicide at the whole plant level.

Figure 3.4. The response of the susceptible biotype VLR1(●) and the resistant biotypes WLR2 (○) and VLR69 (✕) to increasing doses of simazine sprayed onto 3-leaf plants growing in pots. Each point is the mean of mortality determined in three individual pots each containing 12 plants and is plotted with standard error.

Figure 3.5. The effect of simazine on oxygen uptake by thylakoids isolated from the susceptible biotype VLR1 (●) and the resistant biotypes WLR2 (○), and VLR69 (✕). Oxygen uptake was the parameter measured because paraquat was used as a terminal electron acceptor. Rates are expressed as a percent of control which was at least 230 μmol O_2 mg chl^{-1} h^{-1} with similar rates for each biotype. Each point is the mean of four replicates and is plotted with standard error.


Mortality (%)
100
50
0
0
1
2
3
4
5
6
Simazine (kg ha -1)


Inhibition of Oxygen Uptake
(% of Control)
100
80
60
40
20
0
0.001
0.01
0.1
1.0
10.0
Simazine (μM)

The dose response to simazine for both biotypes adopts a shallow increase with increasing dose suggesting that there is large variation for simazine resistance within the resistant populations. This may reflect quantitative inheritance of simazine resistance or it may indicate that the populations are not yet homozygous for the resistance genes. Determination of the mechanisms of resistance must, therefore, allow for the variation in the population.

Thylakoid studies

Oxygen uptake by thylakoids isolated from WLR2, VLR69 and VLR1 leaves exhibits similar sensitivity to simazine (Figure 3.5). This indicates that simazine resistance in the resistant biotypes is not due to insensitivity of the D1 protein of PS II. This observation immediately contrasts biotypes WLR2 and VLR69 with the majority of previously reported cases of triazine resistance in which the mechanism of resistance was an alteration of the target protein.

Triazine resistant biotypes with a psbA mutation characteristically exhibit a very high level of resistance in the order of 100-fold. At rates of application in the order of 10 kg ha^{-1} psbA mutants show no symptoms of triazine toxicity (Clay, 1987). In contrast, biotypes WLR2 and VLR69, exhibit symptoms such as chlorosis and desiccation of leaf tips and margins at rates in the order of 3 to 4 kg ha^{-1}. These observations of whole plants in which resistant plants display symptoms at moderate doses indicate that simazine is inhibiting PS II *in-vivo* to some extent. This is consistent with the sensitivity of the thylakoids to inhibition. These results, therefore, indicate that the mechanism of resistance in these biotypes of *L. rigidum* is not related to the target site of the triazines.

Simazine uptake

Simazine uptake over a 12 h period for the susceptible VLR1 and resistant WLR2 and VLR69 biotypes was 14.3, 12.2 and 12.4 nmol (g shoot FW)$^{-1}$ respectively (Table 3.1). VLR1 plants had significantly greater uptake ($p < 0.05$) than the resistant biotypes in which

the simazine content of the shoot tissue was approximately 85% of that found in VLR1. This difference is relatively small and is unlikely be a major component of the resistance mechanism. Small differences in uptake are also observed during extended dosing experiments, however, both types of experiments indicate that there is no major barrier to simazine transport to the shoots.

During the dosing period of the pulse chase experiments the roots of all biotypes contained high levels of radioactivity. After the plants were returned to nutrient solution, radioactivity levels in the roots of all biotypes declined to a value of approximately 2 to 5 % of the radioactivity in the whole plant. Some radioactivity was found in the nutrient solution while the majority was found to have been translocated to the shoots. This pattern was the same in all biotypes and is consistent with the observation that simazine is readily translocated in the xylem with some binding to absorption sites in the roots (Esser et al, 1975). Thus there was no evidence of greater accumulation of radioactivity in the roots of resistant biotypes, eliminating another potential mechanism of resistance.

Simazine metabolism

In short dosing period experiments the resistant biotypes WLR2 and VLR69 metabolised simazine more rapidly than the susceptible biotype VLR1 (Figure 3.6). The difference in the rate of metabolism during the dosing period was approximately two-fold. There were also large differences in the final amounts of simazine in the plants. WLR2 and VLR69 plants reduced the amount of the herbicide to approximately 4 to 6 % of the total

Table 1. The root and shoot fresh weights and the amount of simazine taken up by plants after 12 h exposure to nutrient solution containing 22 μM simazine. Values are the means of 6 replicates and are recorded with the standard error. Data is representative of four different uptake and metabolism experiments.

Biotype			Simazine		Radioactivity	
	Shoots	Roots	Shoots	Roots	Shoots	% as Simazine
	(g F.W.)		(nmol g F.W. $^{-1}$)		(% of Total)	
VLR1	3.97 a ± 0.23	2.33 a ± 0.16	14.3 a ± 0.59	4.8 a ± 0.22	84 a ± 0.9	51 a ± 1.0
WLR2	3.43 b ± 0.14	2.25 a ± 0.14	12.2 b ± 0.58	4.9 a ± 0.32	79 a ± 1.6	34 b ± 1.0
VLR69	3.50 b ± 0.15	2.06 a ± 0.15	12.4 b ± 0.45	5.1 a ± 0.45	81 a ± 1.2	30 b ± 2.3

*Figures followed by a different letter are significantly different at the 0.95 level of significance according to a t-test.

radioactivity taken up (Figure 3.6) whereas VLR1 consistently had 40 to 50 % of the radioactivity remaining as simazine at the end of the experimental period (Figure 3.6).

There are a number of constraints on the design of this experiment. Simazine is relatively insoluble in water, thus limiting the maximum concentration that can be supplied in nutrient solution to 22 μM. The low solubility limit necessitates the use of longer periods of exposure to gain physiologically relevant doses of herbicide. On the basis of experiments in which inhibition of photosynthesis by atrazine was monitored using fluorescence, it was decided that 12 h exposure to 22 μM simazine would be sufficient to cause inhibition of photosynthesis in all biotypes. This is approximately equivalent to the 3 h of 120 μM chlorotoluron used in similar experiments described in Chapter four. Triazines are taken up largely in the transpiration stream during light periods. Dosing with simazine took place at the commencement of the photoperiod to ensure that plants were exposed to a full 12 h of uptake. Because uptake is dependent on transpiration it was necessary to combine a number of plants into one vial to gain enough leaf area to generate appreciable transpiration during the dosing period. Combining individual plants within a treatment provides some inherent averaging of the variation in the population with respect to the varying degree of simazine resistance noted in the dose response experiments.

After exposure to ^{14}C simazine, plants were harvested, extracted in methanol:water (4:1 v/v) and the extract injected onto HPLC. The retention times of simazine and its metabolites following separation by HPLC were similar for extracts from VLR1, VLR69 (Figure 3.7) and WLR2 (chromatogram not shown). Under the chromatographic conditions used, simazine, de-ethyl simazine and di-de-ethyl simazine have retention times of 23 min 24 s, 16 min 14 s and 6 min 55 s respectively. Di-de-ethylsimazine is relatively polar and is not well resolved by this chromatographic system.

Figure 3.6. A: Metabolism of simazine by the susceptible biotype VLR1 (●) and the resistant biotype WLR2 (O) expressed as the proportion of the extracted radioactivity remaining as simazine over time. Time 0 is the start of a 12 h pulse of 22 μM [^{14}C]simazine (indicated by a dark bar on the abscissa) after which the plants received no further input of simazine. Data is from one of three experiments and is representative of these.

B: Metabolism of simazine by the resistant biotype VLR69 (✕) and the susceptible biotype VLR1 (●) under conditions similar to those of 3.6A. Data is from one of two experiments and is representative of these.

The recovery of radioactivity in all simazine metabolism experiments was at least 85% for both resistant and susceptible biotypes. There was no difference in recovery between biotypes. Recovery did, however, decline slightly as the period of metabolism increased. This could have been due to the formation of insoluble conjugates or to loss as CO_2.

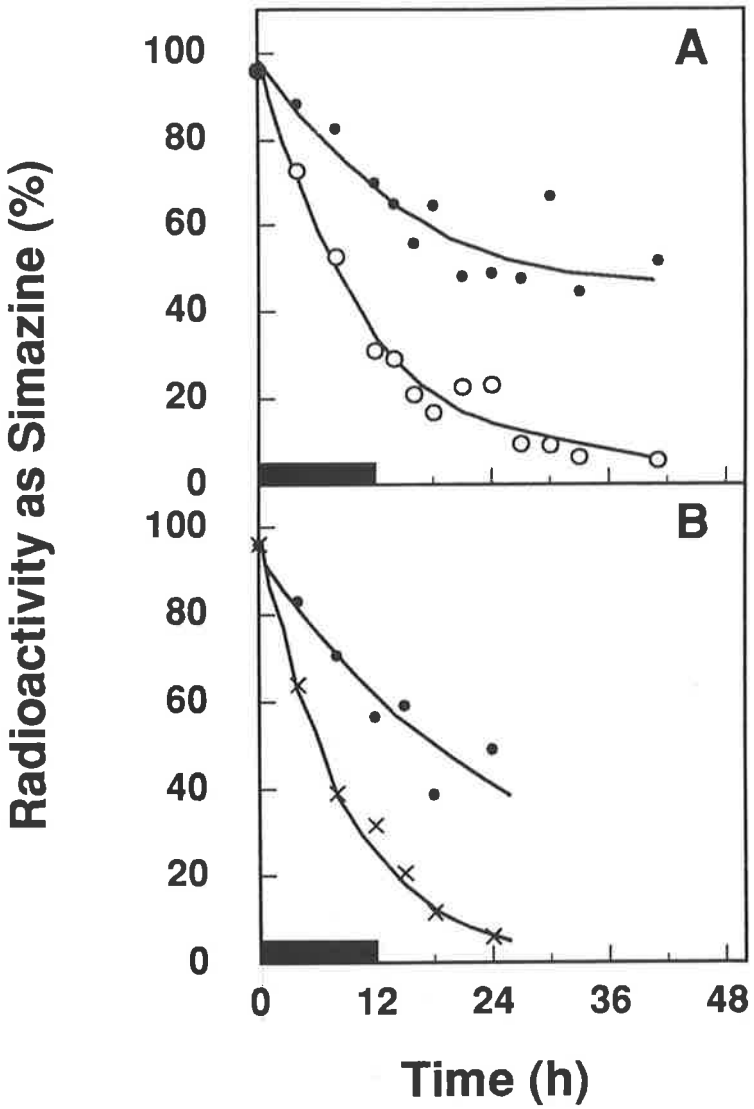

Radioactivity as Simazine (%)
100
80
60
40
20
0
A
B
0
12
24
36
48
Time (h)

Figure 3.7. HPLC radioactivity chromatograms for the resistant VLR69 (A) and susceptible VLR1 (B) biotypes 16 h after the start of a 12 h treatment with [^{14}C]simazine. These can be compared with a UV (254 nm) trace (C) of a mixture of simazine (I), de-ethylsimazine (II) and di-de-ethylsimazine (III) chromatographed using the same gradient and column. Units of radioactivity are counts per second (CPS).

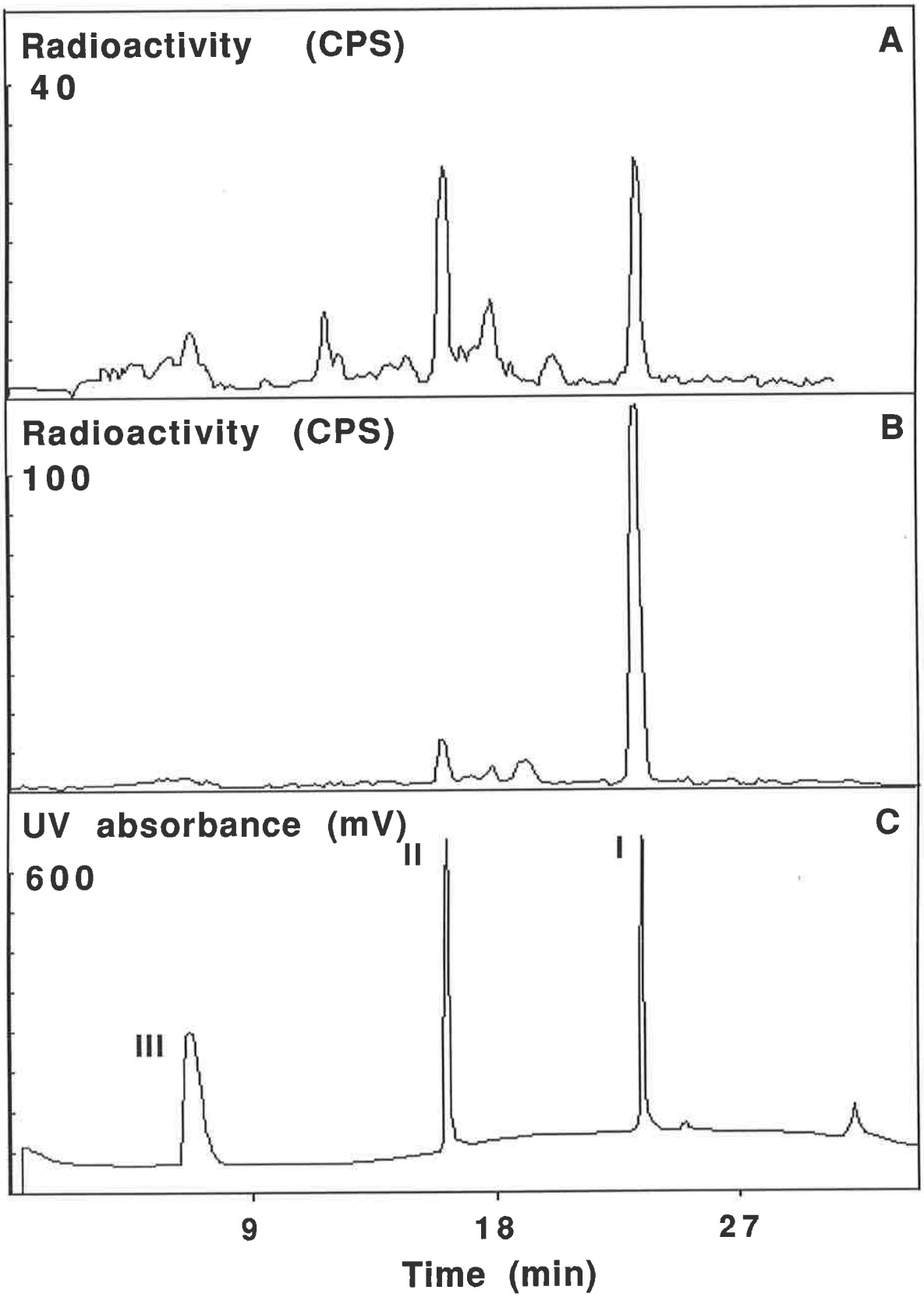
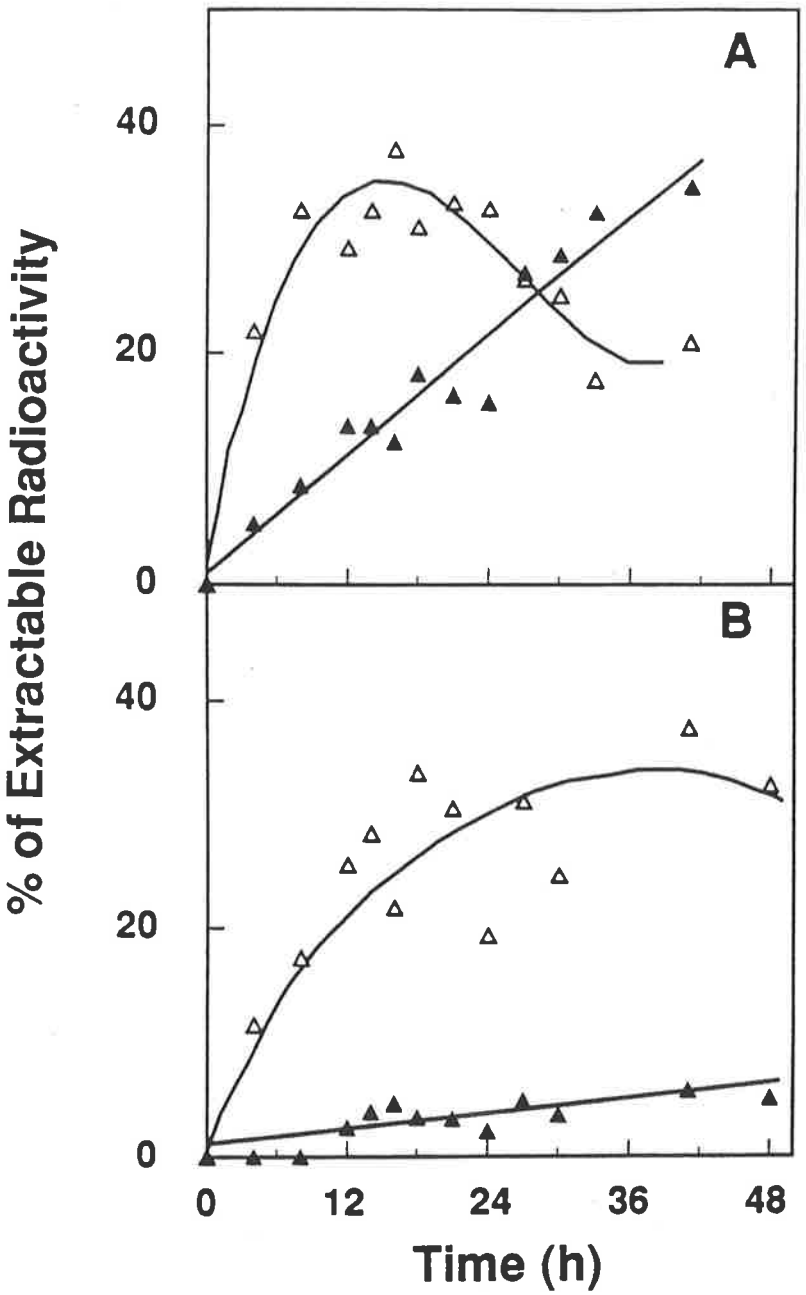

Radioactivity (CPS)
40
A
Radioactivity (CPS)
100
B
UV absorbance (mV)
600
C
II
I
III
9
18
27
Time (min)

Figure 3.8. The proportion of the radioactivity recovered from WLR2 (A) and VLR1 (B) plants found as de-ethyl simazine (Δ) and di-de-ethylsimazine (▲) for the experiment recorded in Figure 3.6A. Data is from one of three experiments and is representative of these.


A
B
40
20
0
40
20
0
0
12
24
36
48
Time (h)
% of Extractable Radioactivity

De-ethylsimazine is the first metabolite observed during the time course with other metabolites such as di-de-ethylsimazine being produced subsequently (Figure 3.7 and Figure 3.8). The time course of changes in the relative proportions of each metabolite is consistent with the interpretation that the primary metabolite is the de-ethyl derivative with subsequent removal of the second ethyl residue (Figure 3.8). Simazine is a symmetric molecule, therefore, cleavage of either side chain yields the same product, the single major intermediate, de-ethylsimazine (Figure 3.7 and 3.3). This pathway (Figure 3.3) is not exclusive and represents only one major pathway of degradation. Other reactions are possible as indicated by the presence of other minor metabolites.

Resistant biotypes differ from the susceptible VLR1 only in the rate of production of these metabolites (Figure 3.6, Figure 3.8). The chromatograms illustrated (Figure 3.7) show the extent of metabolism 16 h after the start of the simazine treatment by which time some metabolites are not yet detectable in the susceptible VLR1 extract whereas most metabolites can be detected in the resistant extract. The chromatogram of a VLR69 extract indicates a predominance of the de-ethyl metabolite with the polar metabolites yet to become a major component of the metabolite pool. The proportion of radioactivity found in each metabolite varies throughout the experiment (Figure 3.8). The amount of the de-ethyl metabolite increases initially and then decreases as more polar metabolites such as di-de-ethylsimazine are formed. The de-ethyl and di-de-ethyl metabolites constitute the majority of the radioactivity found as metabolites and were the only metabolites to be tentatively identified (Figure 3.7).

The observation that dealkylation is the major pathway of simazine metabolism in WLR2 and VLR69 may explain their greater resistance to simazine compared with atrazine. Simazine has two ethyl sidechains, whereas atrazine has one ethyl side chain and one isopropyl side chain. In some species the ethyl side chain of atrazine is preferentially oxidised while the isopropyl side chain is relatively more stable. De-ethylatrazine retains some toxicity (Shimabukuro, 1967), thus the stability of the isopropyl side chain would lead to a build up

of semi-phytotoxic metabolites. Simazine, having two ethyl sidechains, may be more rapidly dealkylated because the second ethyl sidechain is more labile than the isopropyl sidechain of atrazine. This may partly explain why simazine exerts moderate selectivity in legume crops which detoxify triazines by N-dealkylation. (The other important mechanism of selectivity is placement of simazine above the crop root zone). Similarly, biotypes WLR2 and VLR69 appear to employ dealkylation as their primary detoxification reaction and are more resistant to simazine than atrazine. Thus, the proposed mechanism of resistance may explain the relative resistance to the two triazine analogues.

Metabolism of simazine under prolonged exposure.

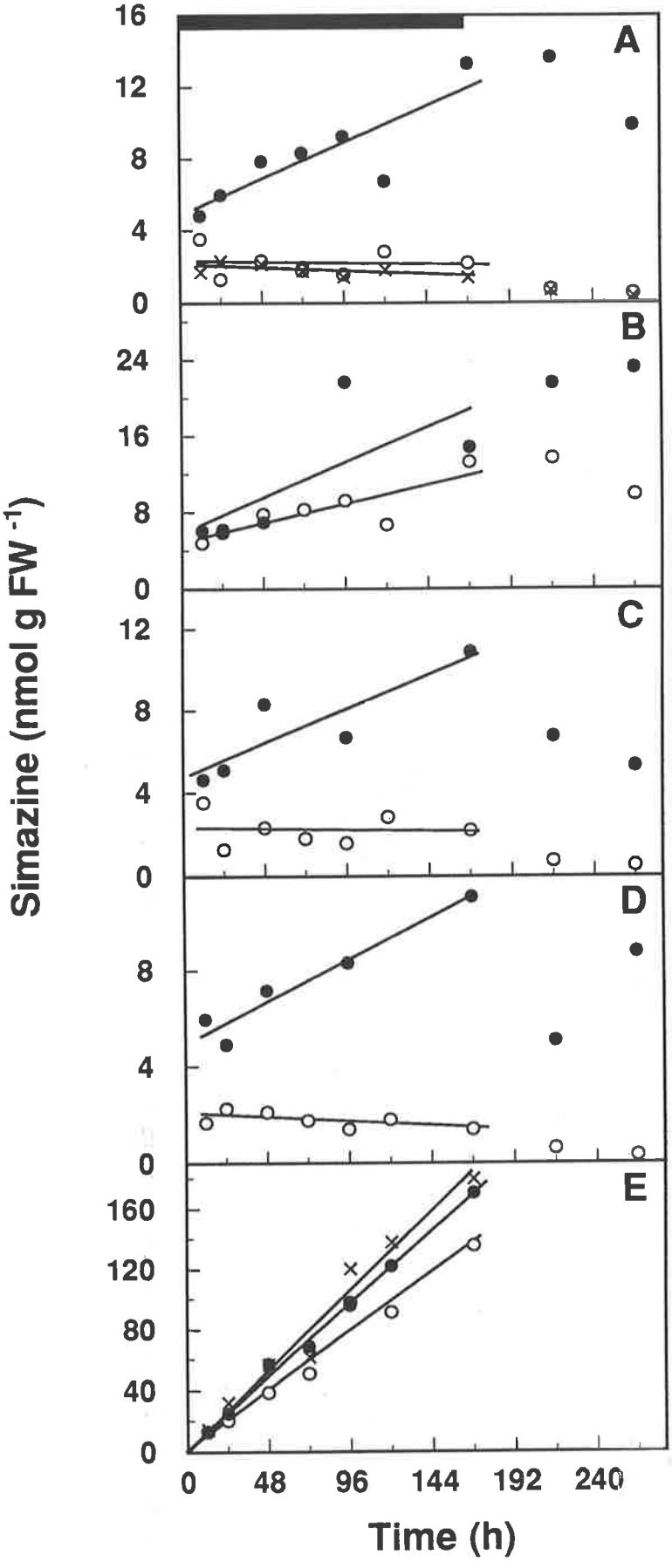
Plants in the field may be exposed to simazine for long periods because simazine can have a relatively long soil half-life (Esser *et al.*, 1975). Simazine is applied to the soil surface and binds within the upper soil layers. Simazine is taken up slowly via the roots of seedlings that are establishing in these upper soil layers. This process is enhanced by rainfall soon after application. Simazine metabolism was, therefore, studied using a prolonged dose to determine whether the different rates of metabolism observed in the short dose studies (Figure 3.3) were sufficient to explain resistance under conditions analogous to those which cause mortality in the field.

While [^{14}C]simazine was present in the nutrient solution there was a linear increase in the amount of radioactivity in the shoots of all biotypes after an initial period of high uptake in the first 12 h (Figure 3.9E). The amount of radioactivity remaining as simazine in the susceptible VLR1 leaf tissue increased linearly with time while the amount of simazine found in the resistant WLR2 and VLR69 leaf tissue remained low (Figure 3.9A). After simazine was removed from the nutrient solution the amount of simazine in the shoot tissue declined, albeit with higher amounts of simazine remaining in the VLR1 tissue at the end of the experimental period. Uptake of simazine under these conditions was linear with time with slightly lower uptake by WLR2 (Figure 3.9E). Therefore, the dramatically reduced

Figure 3.9. A: The amount of simazine in the shoot tissue of VLR1 (●), VLR69 (✕) and WLR2 (○) plants during 168 h of exposure to 3 μM [^{14}C]simazine followed by 96 h of recovery. Lines are fitted by regression. The dark bar above Figure 3.9A indicates the period of dosing after which there is no further input of simazine. Data is from one of three experiments and is representative of these.

B, C and D: The accumulation of simazine in the tissues of the susceptible VLR1 (B), and the resistant biotypes WLR2 (C) and VLR69 (D) in the presence (●) and absence (○) of ABT. Data is from one of three experiments and is representative of these.

E: The cumulative uptake of simazine during 168 h exposure to 3 μM [^{14}C]simazine based on total extractable radioactivity for VLR1 (●), WLR2 (○) and VLR69 (✕). Data is from one of three experiments and is representative of these


A
B
C
D
E
16
12
8
4
0
24
16
8
0
12
8
4
0
8
4
0
160
120
80
40
0
0
48
96
144
192
240
Time (h)
Simazine (nmol g FW -1)

levels of simazine in the WLR2 and VLR69 shoot tissue must be due to a greater rate of metabolism of the herbicide in the resistant biotypes.

Experiments of this type are intended to more closely mimic conditions in the field than pulse chase experiments. The results demonstrate that when the plant roots are immersed in a solution containing a low concentration of simazine the resistant plants have a greater ability to metabolise simazine, preventing its accumulation in the leaves. Where the rate of metabolism is equivalent to, or exceeds the rate of uptake of simazine, the resistant plants are able to maintain a low tissue concentration of simazine. Accumulation of simazine in the susceptible tissues can be explained by the low rate of metabolism in this biotype. The difference in rate of metabolism leads to a divergence between the biotypes in simazine tissue concentration which presumably determines plant survival. Should a higher herbicide concentration be used, it is expected that the rate of uptake in resistant plants would exceed their rate of metabolism eventually resulting in plant mortality. The critical factor for mortality appears to be the period of time that the plant is exposed to tissue concentrations of the herbicide sufficient to cause photo-oxidation. From these results it is apparent that resistant plants would take longer to reach such a concentration and reduce it sooner, once the herbicide was removed from the medium.

The Effect of the Cytochrome P-$_{450}$ Inhibitor, ABT.

1-Aminobenzotriazole (ABT) is an inhibitor of some cytochrome P-$_{450}$ monooxygenase enzymes that are implicated in the detoxification of herbicides (Ortiz de Montellano and Reich, 1986). The mechanism by which this occurs is outlined in Chapter one. Inhibition of metabolism by ABT would indicate that oxidative enzymes were involved in detoxification.

ABT (70 μM) dissolved in the nutrient solution did not significantly affect the growth of any biotype (Table 3.2). This lack of toxicity of ABT for *L. rigidum* is consistent with other reports in which *L. multiflorum* and wheat were grown in the presence of ABT at

Table 3.2. The effect of ABT (70 μM) on shoot dry weight of VLR1, WLR2 and VLR69 when applied alone or in combination with simazine (3 μM) for 7 d followed by a 7 d recovery period in the absence of simazine or ABT. Values are the mean of 3 vials each containing 7 plants and data is from one of two experiments.

	Shoot Dry Weight (g, mean ± se)			
Biotype	Control	70 μM ABT	3 μM Simazine	3 μM Simazine + 70 μM ABT
VLR1 *	1.755 a ± 0.096	2.041 a ± 0.012	0.282 b ± 0.025	0.237 b ±0.015
WLR2 *	1.959 a ± 0.199	1.751 a ± 0.108	0.755 b ± 0.065	0.416 c ± 0.036
VLR69 *	2.048 a ± 0.166	1.761 a ± 0.093	0.595 b ± 0.045	0.348 c ± 0.052

*Means in the same row followed by a different letter are significantly different at the 0.95 level of significance according to a t-test.

similar concentrations without effect (Barta and Dutka, 1991; Cabanne *et al.*, 1985). ABT in combination with 3 μM simazine for 7 d caused a significant reduction in the dry weight of resistant plants compared with simazine alone (Table 3.2, Figure 3.11). Resistant plants treated with both ABT and simazine responded similarly to the susceptible VLR1 plants treated with simazine alone (Table 3.2). The effect of ABT in combination with simazine on VLR1 dry weight was not significant as simazine severely reduced growth of the susceptible biotype when applied without ABT.

ABT added to the dosing solution during pulse-chase experiments reduced the rate of metabolism of simazine in all biotypes (Figure 3.11). ABT was only supplied for the period of the herbicide pulse so the persistent inhibition of detoxification after the end of the pulse suggests that residual amounts of ABT were sufficient to maintain the inhibition of detoxifying enzymes. The effect was less pronounced in VLR1 plants (Figure 3.10), presumably because these plants already have a low rate of simazine metabolism.

ABT also inhibited simazine metabolism in the prolonged dosing experiments (Figure 3.9B,C,D). These experiments were conducted in similar manner to those designed to investigate the effect of ABT and simazine on growth. In each case plants were subject to seven days of continual dosing with simazine. The similarity in the dosing regime and the conditions of the experiment permit the comparison of results obtained from both systems. Resistant plants treated with both simazine and ABT accumulated simazine in their leaf tissue to levels similar to those of susceptible VLR1 plants treated with simazine alone. Thus, in terms of metabolism, ABT is able to convert the resistant phenotype to a susceptible phenotype. Similarly, in terms of dry weight reduction, ABT also converts the resistant phenotype to that of a susceptible. This suggests that the effect of ABT in reducing the dry weight of resistant plants is related to the inhibition of simazine detoxification and the consequent accumulation of the herbicide in leaf tissue. Thus both types of experiment indicate that differential metabolism is a major difference between the biotypes.

Figure 3.10. The effect of ABT on the metabolism of [^{14}C]simazine under pulse-chase conditions for VLR1 (A), WLR2 (B) and VLR69 (C). Simazine, in the presence (●) and absence (O) of ABT, was applied to the roots of plants for 12 h (indicated by the solid bar on the abscissa) after which there was no further input of either material. Data is from one of two experiments and is representative of these. Experimental conditions were similar to those of experiments reported in Figure 3.6

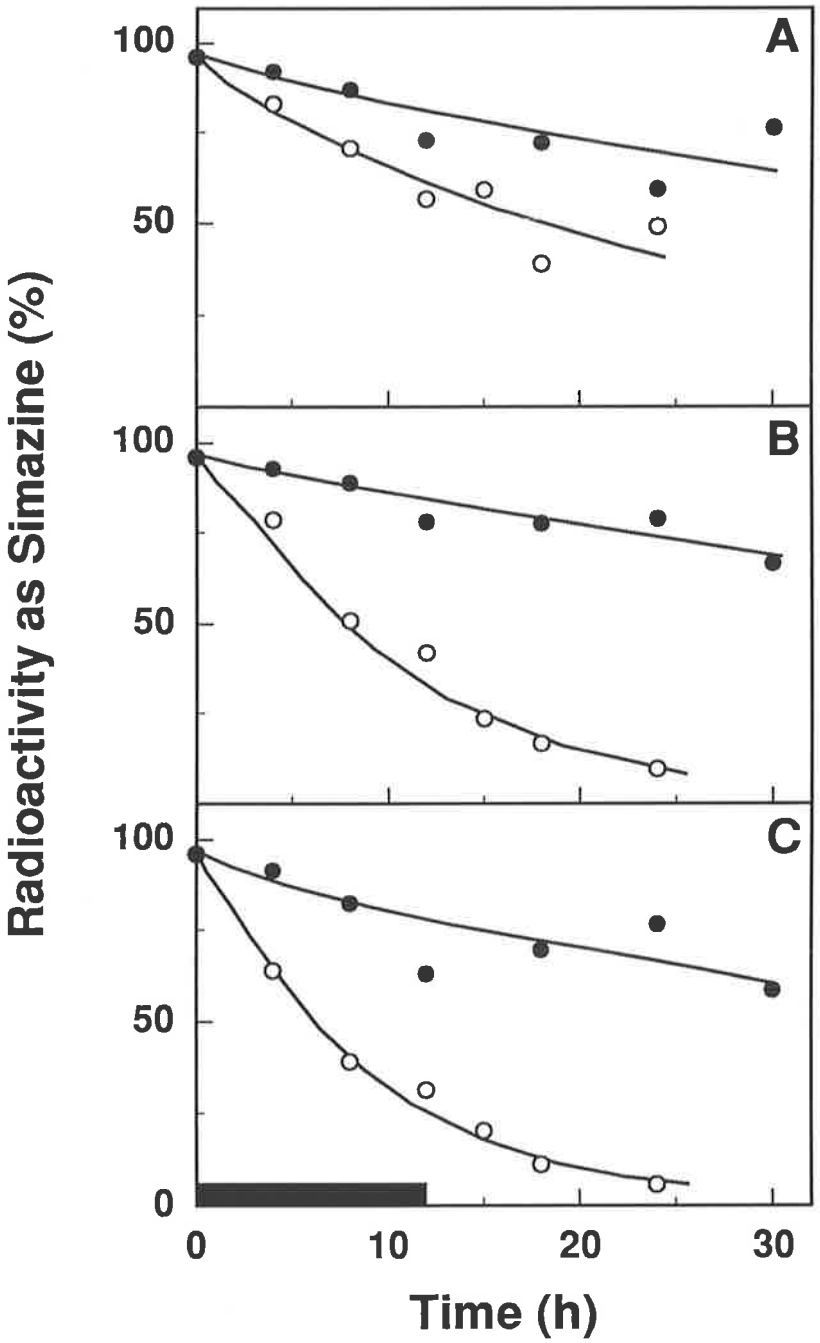
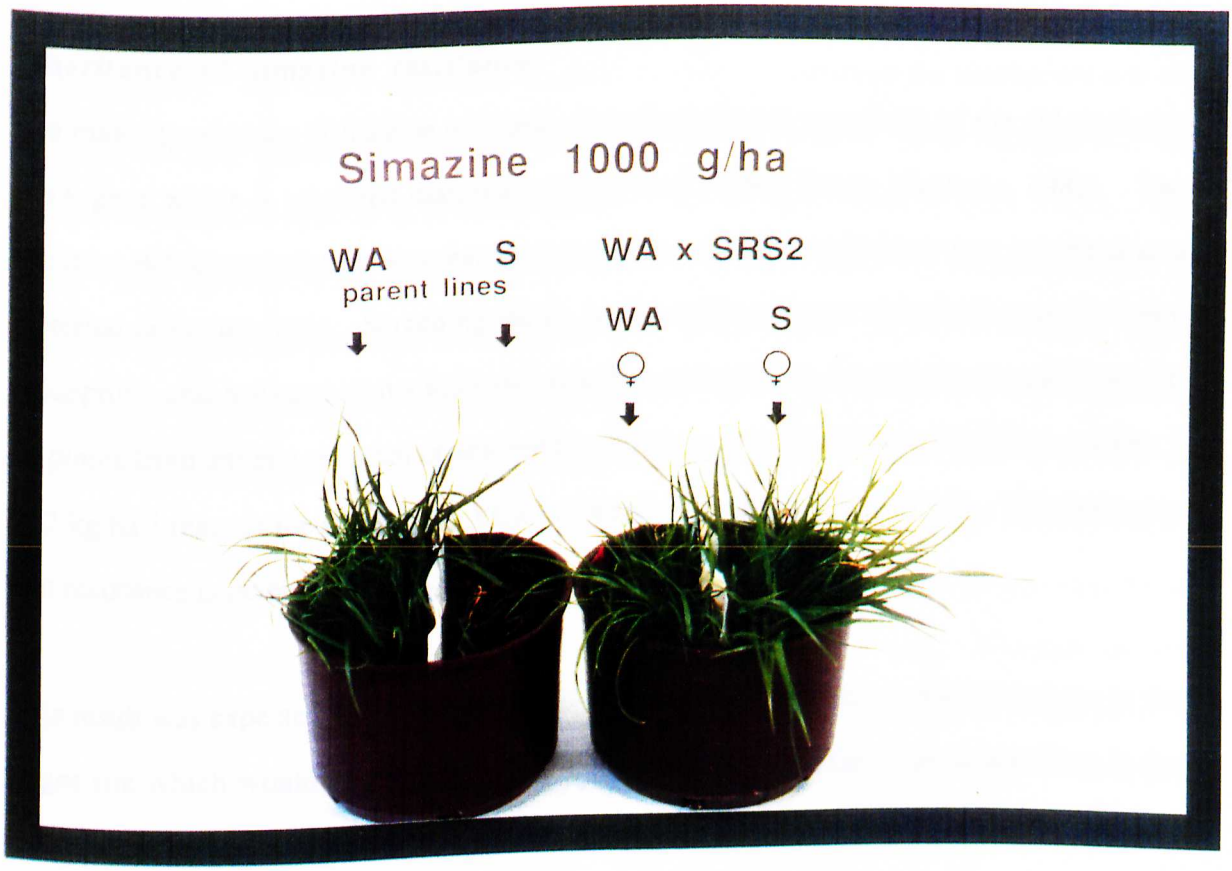

Radioactivity as Simazine (%)
100
50
A
100
50
B
100
50
0
C
0
10
20
30
Time (h)

Figure 3.11. The effects of simazine (second from left) and ABT (right) alone and in combination (third from left) on growth of VLR69 compared with the untreated control (left). "B1" was the designation for VLR69 at the time that the photograph was taken.

Figure 3.12. Response of reciprocal F_1 hybrids (right) to simazine applied at 1 kg ha^{-1} compared with the response of the parent lines (left), WLR2 and VLR1. Hybrids with either a paternal or maternal resistant parent exhibit less mortality following simazine treatment than the susceptible parent generation


B1
Control
B1
3 µM
Simazine
B1
70 µM ABT
+
3 µM
Simazine
B1
70 µM ABT


Simazine 1000 g/ha
WA
S
parent lines
WA x SRS2
WA
S
♀
♀

Although ABT inhibited the formation of all metabolites there is insufficient data to suggest whether all enzymes in this pathway are sensitive to ABT or if only the enzymes performing the initial reactions are sensitive and all metabolites are derived from the initial reactions. It has been proposed that the same enzymes could perform both the first and second de-ethylation thus, inhibition of these enzymes could prevent both major steps of the detoxification (Esser *et al.*, 1975). The inhibition of simazine de-ethylation *in-vivo* by ABT suggests that oxidative enzymes are responsible for this de-ethylation. The effect of ABT is not limited to the enhancement of simazine toxicity in *L. rigidum*. Under similar conditions ABT is also able to intensify the effect of chlorotoluron and metribuzin in VLR69 and WLR2 (Chapters four and five). ABT also inhibits the metabolism of chlorotoluron in wheat leading to reductions in dry weight when given in combination with the herbicide (Cabanne *et al.*, 1987). Similarly ABT increases the toxicity of chlorotoluron in phenylurea resistant biotypes of *A.myosuroides*. These biotypes are resistant due to an enhanced capacity to N-dealkylate chlorotoluron (Kemp *et al.*, 1990).

Inheritance of simazine resistance

The majority of cases of triazine resistance have been due to mutations of the chloroplastic psbA gene which is inherited maternally (Darr *et al.*, 1981; Souza Machado, 1982). The resistant WLR2 population was tested to determine whether resistance was inherited as a maternal or nuclear trait. Screening the F_1 seed resulting from a reciprocal cross between susceptible and resistant plants indicates whether resistance is inherited maternally or not. F_1 plants from either a resistant or susceptible female parent exhibit less mortality at either 1 or 2 kg ha^{-1} than do the susceptible VLR1 controls (Table 3.3, Figure 3.12). This indicates that resistance is inherited as a nuclear trait.

This result was expected given that there was no evidence of resistance due to changes in the target site which would be inherited via the cytoplasm. If differential metabolism is the basis of resistance, it is likely be a nuclear encoded trait. Furthermore, if resistance was polygenic or quantitative the segregation patterns in the F_2 would be expected to be complex.

Screening of the F_2 and backcross progeny with 1.0 and 2.0 kg ha^{-1} simazine resulted in very high mortality. Mortality in parent lines and F_1 plants present for comparison was also higher than expected. This was attributed to the cold conditions that ensued following spraying. Previous dose response experiments had demonstrated that the degree of resistance is reduced in experiments conducted at lower temperatures. The higher than expected mortality at the time of F_2 screening (and limited seed stocks) prevents any conclusions on the inheritance of triazine resistance in WLR2 being made. Observation of the F_2 and backcross plants suggested that response to simazine was variable with the majority of plants less resistant than the parent generation. This may suggest polygenic inheritance.

3.4 Conclusions

Both resistant biotypes, WLR2 and VLR69, exhibit resistance to simazine (Figure 3.4). This is not due to changes in the sensitivity of the target site, the D1 protein, in photosystem II (Figure 3.5). The minor difference in uptake of simazine between the susceptible and the resistant biotypes appears insufficient to confer resistance (Table 1). Both resistant biotypes have an enhanced capacity to detoxify simazine via N-dealkylation (Figure 3.6, 3.7, and 3.8). This detoxification is inhibited by ABT (Figure 3.9 and 3.10) which also acts to enhance the effect of simazine at the whole plant level (Figure 3.12 and Table 2). The observation that the degree of resistance may be reduced by the inclusion of an inhibitor of herbicide metabolism suggests that enhanced metabolism is the basis of the resistance mechanism. The products of this enhanced metabolism are N-dealkyled derivatives, a pathway of catabolism which also predominates in legume species such as peas and weed species such as *Senecio vulgaris* (Esser *et al.*, 1975; Gressel *et al.*, 1983). Observations on the mode of inheritance of resistance (Table 3) indicate that resistance is inherited as a nuclear gene or genes thus differentiating *L. rigidum* from most other triazine resistant species in which resistance is inherited maternally (Souza Machado, 1982).

Table 3. The response of the F_1 progeny of reciprocal crosses between the resistant WLR2 biotype and the susceptible VLR1 biotype when screened with simazine. These are compared with the response of the parent biotypes. Data is from one of two years of testing.

Parents (cross#)*		Simazine rate			
		1 kg ha^{-1}		2 kg ha^{-1}	
male	female	% survival	n	% survival	n
WLR2 (1)	VLR1 (1)	100	16	61	18
VLR1 (1)	WLR2 (1)	100	18	66	18
WLR2 (2)	VLR1 (2)	100	52	100	17
VLR1 (2)	WLR2 (2)	100	17	100	15
Parent lines					
WLR2		100	17	100	17
VLR1		33	18	0	18

*Numbers in parentheses indicate the identity of the individual parent.

While these studies have used simazine as a model system there remains the question of whether the observations made with simazine extend to the other triazine analogues to which there is resistance. Most triazine herbicides including atrazine, which was a selective agent in both cases, can be detoxified by cleavage of the alkyl sidechains (Esser *et al.*, 1975). Given that the triazine analogues to which there is resistance have similar properties of uptake, translocation and metabolism it is likely that conclusions based on simazine will extend to other members of the s-triazine class.

These observations of the mechanism of resistance to simazine are very similar to those made on the mechanism of resistance to the other PS II inhibitors, chlorotoluron and metribuzin, in these biotypes (Chapters four and five). In all cases there is a greater rate of metabolism which is sensitive to inhibition by ABT. The degree of resistance to the herbicides is similar as is the difference in the rate of herbicide metabolism (Chapter four). In each case uptake of the herbicide does not appear to be a major factor in the resistance mechanism. This similarity in results for each herbicide class supports the conclusion that enhanced metabolism is the major mechanism of resistance to these PS II inhibitors.

In contrast to the resistant *L. rigidum* biotypes studied here, the majority of previously reported cases of triazine resistance have an alteration of the PS II target site, a maternally inherited trait that confers a high level of resistance (Bettini et al, 1987, Darmency and Gasquez, 1990). Some biotypes with the altered PS II also display minor increases in the rate of triazine metabolism (Khan et al, 1985). Cases of triazine resistance involving increased detoxification alone have been less common. An example is the recently characterised biotype of *A. theophrasti* that has a ten-fold increase in atrazine tolerance (Gronwald et al, 1989). PS II in the resistant *A. theophrasti* remains sensitive and the trait is encoded by a single nuclear gene. The mechanism in this case is enhanced detoxification by conjugation with glutathione due to overexpression of two glutathione-S-transferase enzymes (Anderson and Gronwald, 1991). This triazine resistant *A. theophrasti* is the

nearest precedent to the type of resistance found in the *L. rigidum* biotypes described in this study.

In conclusion, the studies of triazine resistant *L. rigidum* biotypes reported here show that there is no change in PS II, however, there is an increase in the rate of metabolism via N-dealkylation. Thus, the triazine resistant biotypes of *L. rigidum* appear to be distinct from previously reported cases of triazine resistance by virtue of the difference in the mechanism resistance. The major mechanism of resistance to triazine herbicides in resistant *L. rigidum* biotypes WLR2 and VLR69 is an increased capacity to metabolise these herbicides.

Chapter Four

Substituted Urea Resistance in *L. rigidum*

4.1 Introduction

History and Applications

Substituted ureas are used as fungicides, herbicides and pharmaceuticals. This diversity of applications is, in part, due to the amenability of urea to substitution which provides extensive potential for novel syntheses. Approximately 25 substituted ureas (or phenylureas) are now in use as herbicides although this does not include sulfonylureas. The phenylureas are also notable because they have facilitated studies on the biophysics of photosynthesis. Diuron (3)[1], or DCMU, is a potent inhibitor of electron transport in photosystem II. This effect has ensured that the mode of action of diuron has been widely studied and is now well understood. The first patent for a herbicidal phenylurea, monuron (2), was filed by Du Pont in 1952. This was followed by fenuron (1), neburon (10), and diuron (3) (Geissbuhler et al 1975). Various substituted ureas are now used extensively in cereals in the Northern Hemisphere, as are methabenzthiazuron (14) and metoxuron (5) in Australia.

Chemistry

In this Chapter, the term phenylurea will be used for those compounds conforming to the generalised structure in Figure 4.1 (the numbers following the herbicide name are a guide to identifying the structure in Figure 4.2). The term substituted urea includes phenylureas (Figure 4.1) as well as compounds such as isouron (17) and methabenzthiazuron (14) which are not strictly phenylureas because they lack a phenyl ring. In general the phenylureas have low solubility in water and are not volatile.

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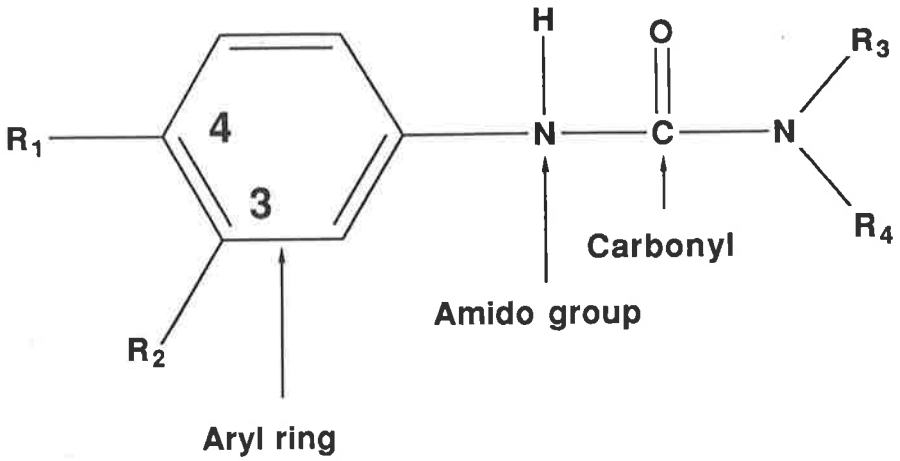
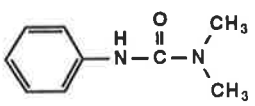
[1]Numbers in parentheses following each herbicide name indicate where to find the structure of that herbicide in Figure 4.2.

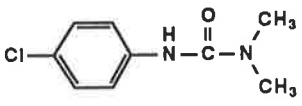
Figure 4.1. The general structure of phenylurea herbicides. R1 is often a chloro or alkyl group while R2 is commonly unsubstituted or a chloro group. R3 and R4 are most commonly methyl groups although they may be substituted with longer chain alkyl groups.

Figure 4.2. The chemical structures of commonly used substituted urea herbicides (continued on following page).

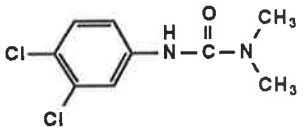




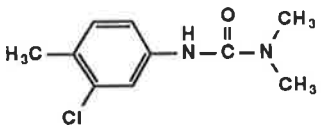
1. Fenuron



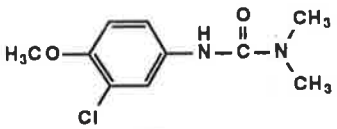
2. Monuron



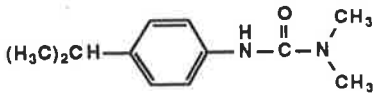
3. Diuron



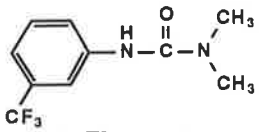
4. Chlorotoluron



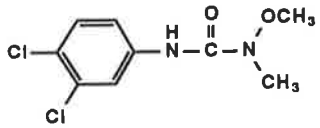
5. Metoxuron



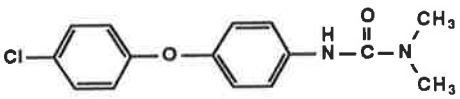
6. Isoproturon



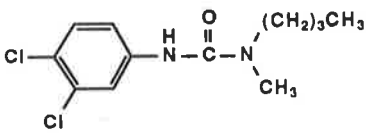
7. Fluometuron



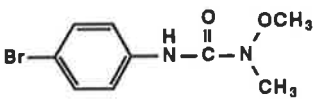
8. Linuron



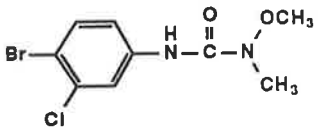
9. Chloroxuron



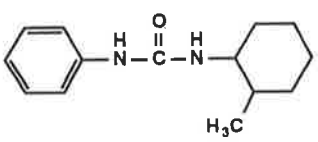
10. Neburon



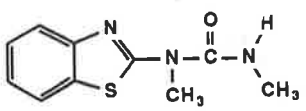
11. Metobromuron



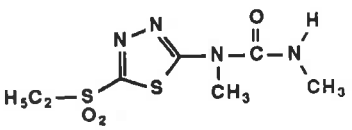
12. Chlorbromuron



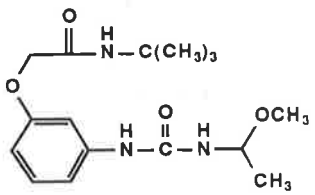
13. Siduron



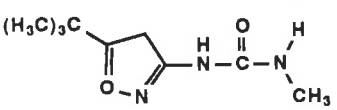
14. Methabenzthiazuron



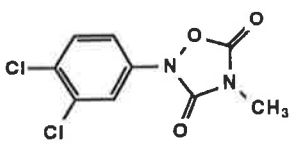
15. Ethidimuron



16. Karbutilate



17. Isouron



18. Methazole

Interaction with The Environment

The behaviour of the substituted ureas in the environment is determined by their substituents. Adsorption to soil particles determines the availability of the herbicide to plants and microorganisms and thus affects the half-life in soil. Halogenation of the aryl ring increases adsorption. The absence of aryl substituents, as in fenuron, reduces adsorption. As a consequence diuron has a longer soil half-life than does fenuron under the same conditions, while monuron is intermediate. Alkoxy substituted ureas like linuron (8) are more strongly bound. Bromine substituents tend to increase adsorption as in chlorbromuron (12) which is more strongly adsorbed than linuron (Geissbuhler *et al.*, 1975).

Substituted ureas are degraded in soil by microorganisms. Some organisms may utilise diuron as a carbon source. Chemical breakdown in the soil is considered to be a less important process than microbial degradation due to the relative stability of substituted ureas in sterilised soil (Geissbuhler *et al.*, 1975). Photochemical degradation is a significant cause of non-biological degradation of substituted ureas (Tanaka and Wien, 1979). Methazole (18) also photodecomposes in sunlight to both phytotoxic and non-phytotoxic products (Ivie *et al.*, 1973).

Uptake and Translocation

Substituted ureas are predominantly taken up passively via roots (Ashton and Crafts, 1981). They are translocated in the apoplast exclusively. As a consequence herbicide transport to leaves is transpiration dependent. The amount of foliar uptake differs between individual herbicides but is not considered to be an important uptake mechanism. The extent of foliar uptake also varies in different species with greater fluometuron (7) absorption by cucumber (susceptible) leaves than cotton (tolerant) leaves (Ashton and Crafts, 1981).

Limited uptake and translocation is considered to be one of the mechanisms by which plants tolerate various phenylurea herbicides. This has been demonstrated in a number of plant

systems. Greater chlorotoluron (4) uptake by *A. fatua* (wild oats) compared with wheat may contribute to the selectivity of chlorotoluron in wheat (Ryan and Owen, 1982). Fluometuron (7) accumulates in the roots and stems of *Citrus aurantium* (sour orange) with relatively little active herbicide reaching the leaves (Goren, 1969). Conversely, fluometuron is well translocated in *Vicia faba* (french bean) relative to chloroxuron (9) and metobromuron (11) (Geissbuhler *et al.*, 1975). Linuron is poorly translocated in carrot yet the near analogue, diuron, is well translocated. The selectivity of linuron was achieved by the substitution of an N-methoxy group for one N-methyl group of diuron. Selectivity obtained with minor substitutions reflects a significant aspect of substituted urea development, namely the compromise between photosynthetic inhibitory power and desirable physico-chemical properties which allow selectivity, or to reduce environmental persistence. Thus uptake and translocation do have some influence on plant response to these herbicides and this can be manipulated by alteration of the basic herbicidal structure.

Mode of Action

The mode of action of the substituted ureas is principally the inhibition of electron transport from photosystem II to photosystem I. The herbicides bind to the 32 kd protein in Photosystem II which is also known as the D1 protein. By binding to the protein the herbicide interferes with the binding of plastoquinone and, therefore, electron transport. Treating thylakoid membranes with trypsin removes part of this protein complex and thus the binding site of diuron (and other substituted ureas, triazines, and uracils) confirming that the target site is a membrane bound protein (Tischer and Strotman, 1977; Böger and Kunert., 1979).

The actual cause of death of the plant is now thought to be photooxidation as a consequence of the block in electron transport system (Ridley, 1977; Barry *et al.*, 1990). Chloroplasts incubated in the presence of diuron and light display loss of β-carotene, chlorophyll a, xanthophylls and chlorophyll b in that order. The importance of light in mediating the toxicity of PS II inhibitors is also demonstrated by the symptoms shown in light and dark

grown plants. Chlorosis and death ensue only in the presence of light. Further evidence for this proposition is the observation that both *Euglena* and leaves of higher plants treated with diuron produce ethane, a product of lipid oxidation (Pallett and Dodge, 1979; Elstner and Pils, 1979). An endogenous supply of electron acceptors can reduce this effect as does the absence of O_2. The effect of light in causing disruption of the photosynthetic apparatus explains the relatively rapid death of treated plants and their chlorotic desiccated appearance.

Extensive studies with isolated chloroplasts and thylakoids have resulted in an understanding of how different substituents affect the inhibitory performance of the herbicide. Dimethyl ureas are the most phytotoxic as are those with aryl substitutions at the 3,4 positions. Halogen substituents on the aryl ring cause the greatest phytotoxicity (Moreland, 1969). Phenyl substituents increase toxicity by increasing the capacity for hydrogen bonding by the carbonyl group (Ducruet *et al.*, 1992). The most inhibitory combination is therefore 3-(3,4-dihalogenphenyl)-1,1-dimethylurea (diuron). Departures from this general formula are less inhibitory but they may be more suitable to some applications through differences in soil mobility, adsorption, degradation, translocation and metabolism. It has been deduced that the amido hydrogen (between the phenyl and carbonyl groups, see Figure 4.1.) must be free to interact with the target site. Hydrogen bonds are also thought to form between the carbonyl oxygen and the target site (Ducreut *et al.*, 1992). Ortho substituents interfere with the amido hydrogen and result in non-phytotoxic compounds (Geissbuhler *et al.*, 1975). Examples of this are the ortho-hydroxy metabolites which are non-phytotoxic.

Substituted ureas inhibit other biological processes, including root growth, and urease activity (Geissbuhler *et al.*, 1975). However, when considering the mode of action of a herbicide the most important target is the one which is affected rapidly at the lowest toxic dose, and for the substituted ureas this is the 32 kd protein in photosystem II. An exception to this is siduron (13) which inhibits root growth of germinating seedlings as well as binding photosystem II (Herbicide Handbook, 1983). The best test for the importance of subsidiary target sites would be to engineer resistance to a herbicide with a single point mutation of the

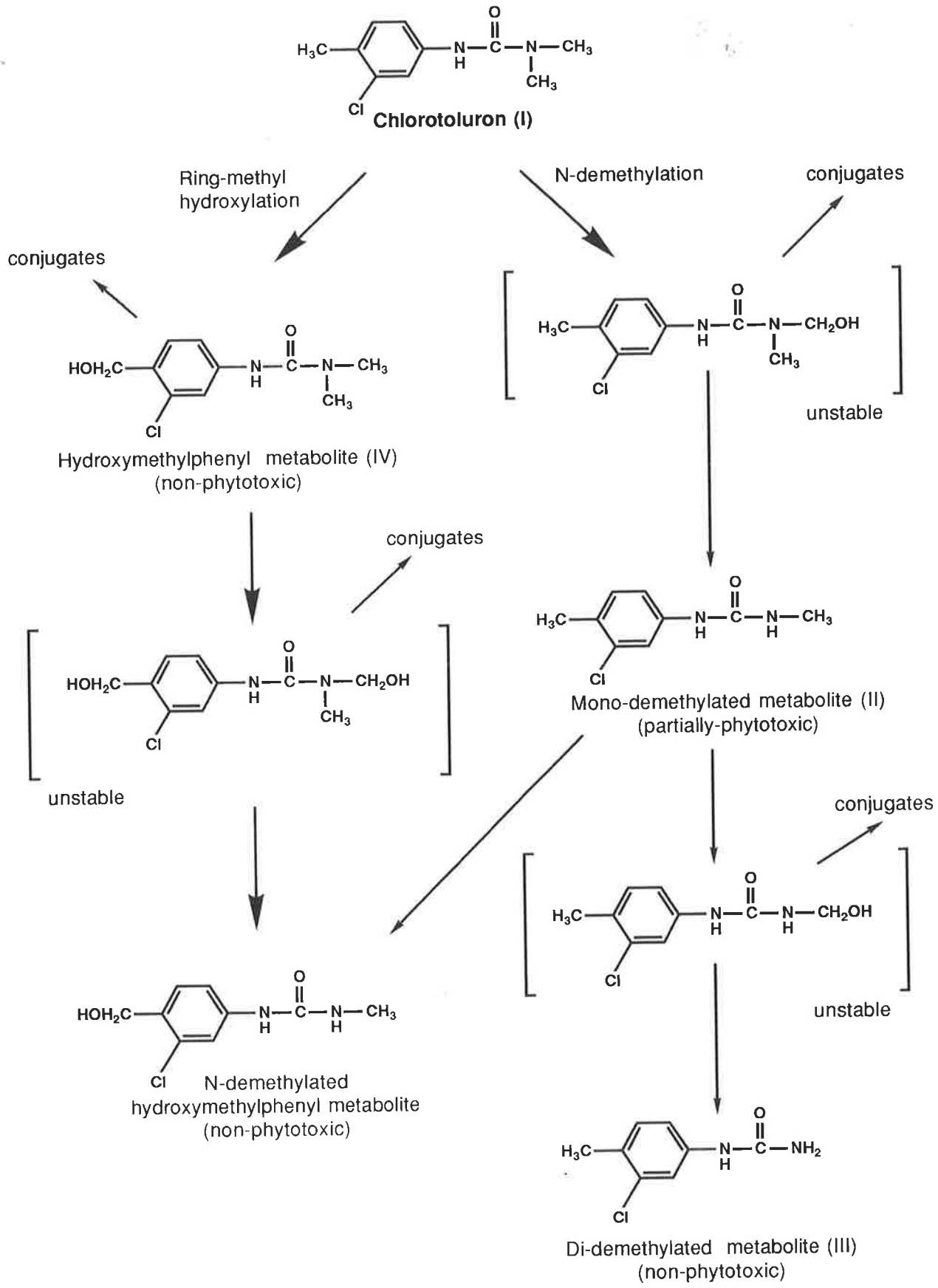
gene encoding the target protein. If subsidiary targets are of consequence, a single mutation will not provide a large margin of protection. If, however, a mutation in a single gene protects against a dose two orders of magnitude higher than previously tolerated it would appear likely that subsidiary mechanisms are less important in causing herbicide toxicity.

Metabolism by Plants

Plants detoxify phenylurea herbicides with oxidative reactions at a number of different sites on the molecule. In general the substituted ureas undergo two reactions. The first is dealkylation of the terminal nitrogen via a hydroxylated intermediate, the second is the hydroxylation of the aryl ring (Frear and Swanson, 1974) or the non-halogen aryl substituents (Gonneau *et al.*, 1988). Dehalogenation of the aryl rings of phenylureas has not been observed in biological systems (Geissbuhler *et al.*, 1975). Alkyl (chlorotoluron (4)) and alkoxy (isoproturon (6), metoxuron (5)) ring substituents are more readily oxidised and this contributes to their selectivity as herbicides. Hydroxylation tends to occur at the ortho position of the aryl ring in the halogenated phenylureas (Geissbuhler *et al.*, 1975). A demethylated metabolite may still undergo ring hydroxylation and vice versa. These results are expressed in the proposed metabolic pathways for chlorotoluron shown in Figure 4.3. All of these sites on the molecule are susceptible to oxidative reactions and similar metabolites are produced in animal systems (Suzuki and Casida, 1981).

The metabolism of the substituted ureas can play a significant role in their selectivity. Chlorotoluron selectively controls *A. fatua* (wild oats), *A. myosuroides* (blackgrass) and *L. perenne* (perennial ryegrass) in wheat crops, because of the greater capacity of wheat to detoxify the herbicide, although *A. fatua* also has greater herbicide uptake (Ryan and Owen, 1982). Differential response to diuron in cotton (tolerant) and soybean (susceptible) is also due to differences in metabolism. Diuron metabolism by cotton appears to take place partly in roots such that less diuron and more di-demethylated metabolite reaches the leaves (Smith and Sheets, 1967). Monuron metabolism in *Phaseolus vulgaris* and *Zea mays* differs in

Figure 4.3. The metabolism of chlorotoluron in wheat (redrawn from Gonneau *et al.*, 1988).

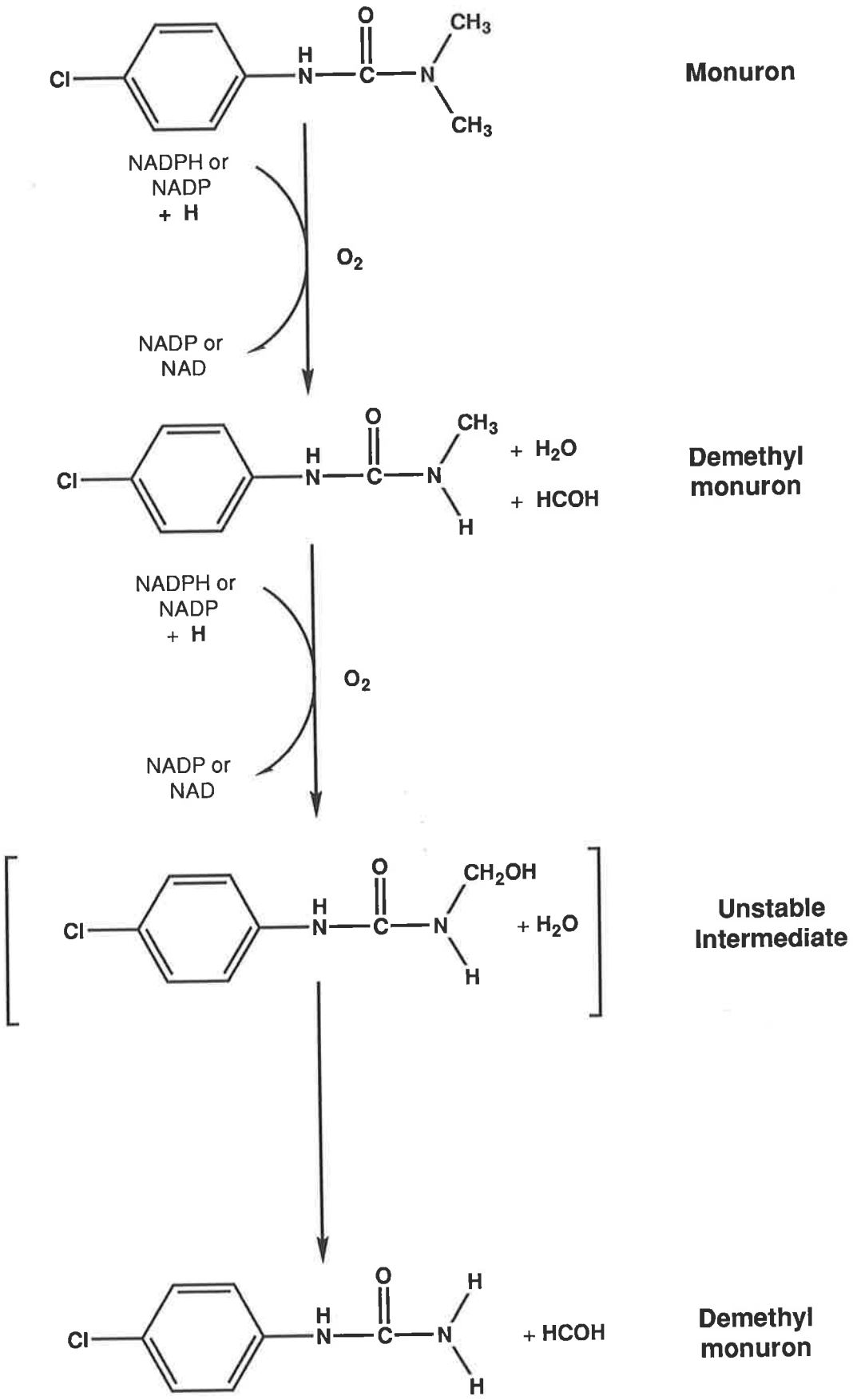

Chlorotoluron (I)
Ring-methyl hydroxylation
N-demethylation
conjugates
conjugates
unstable
Hydroxymethylphenyl metabolite (IV)
(non-phytotoxic)
conjugates
unstable
Mono-demethylated metabolite (II)
(partially-phytotoxic)
conjugates
unstable
N-demethylated hydroxymethylphenyl metabolite
(non-phytotoxic)
Di-demethylated metabolite (III)
(non-phytotoxic)

that the former accumulates aryl-hydroxy-monuron in small quantities, while the latter carries out sequential N-demethylation producing more non-phytotoxic metabolites (Shaw and Fang, 1973). Thus both crops and susceptible weed species produce metabolites but at differing rates and with different metabolites predominating (Ryan and Owen, 1982; Ishizuka *et al.*, 1985; Gonneau *et al.*, 1988).

The best understood mechanism of substituted urea metabolism is N-demethylation. This is an oxidative reaction catalysed by mixed function oxidase enzymes. N-demethylation of monuron was the first oxidative reaction in plants to be characterised *in vitro*. Microsomes prepared from cotton cells required both molecular oxygen and NADPH (or NADH) as cofactors for monuron N-demethylation activity which suggested that the reaction was mediated by a monooxygenase (Figure 4.4) (Frear *et al.*, 1969). The metabolism of chlorotoluron by wheat is also mixed function oxidase dependent. In wheat the dominant metabolite of chlorotoluron is the non-phytotoxic aryl methoxy metabolite (Cabanne *et al.*, 1987). N-demethylation plays a lesser part in wheat but is more significant in some weed species (Cabanne *et al.*, 1987; Gonneau *et al.*, 1988). The production of the aryl-methoxy metabolite in wheat is inhibited by aminobenzotriazole (ABT) while N-demethylation is less sensitive (Cabanne *et al.*, 1987). ABT treatment intensifies the effect of chlorotoluron on wheat confirming the importance of metabolism in the tolerance of wheat to this herbicide (Gaillardon *et al.*, 1985; Cabanne *et al.*, 1985).

The lower sensitivity of chlorotoluron N-demethylation to ABT has prompted investigations into the nature of the enzyme performing the reaction. In suspension cultured cells, N-demethylation becomes a more important means of chlorotoluron detoxification. Microsomes prepared from these cell cultures sustain both ring-methyl hydroxylation and the first N-demethylation in the presence of NADPH and O_2. Both systems are similarly induced by cyometrinil, dichlormid and 2,4-D, and inhibited by tetcyclasis and paclobutrazole, both inhibitors of cytochrome $P\text{-}450$ enzymes (Canivenc *et al.*, 1989). The

Figure 4.4. The mechanism of N-demethylation of Monuron in cotton microsomes. The first unstable intermediate is not drawn for clarity (redrawn from Shimabukuro, 1985).


Monuron
NADPH or
NADP
+ H
O2
NADP or
NAD
+ H2O
+ HCOH
Demethyl
monuron
NADPH or
NADP
+ H
O2
NADP or
NAD
Unstable
Intermediate
+ H2O
+ HCOH
Demethyl
monuron

reactions differ, however, in their response to ABT and carbon monoxide (CO) *in vitro* (Canivenc *et al.*, 1989; Mougin *et al.*, 1990). Inhibition by CO with reversal by light is a defining characteristic of most cytochrome P-450 enzymes. While aryl methyl-hydroxylation conforms to this pattern, N-demethylation does not (Mougin *et al.*, 1990). N-demethylation is also active in the presence of cumene hydroperoxide which suggests that a peroxygenase might sustain this reaction in addition to other enzyme types (Mougin *et al.*, 1990). There are precedents for both of these anomalies in mixed function oxidase extracts; however, these other examples were not conclusively identified as cytochrome P-450 dependent enzymes (Soliday and Kolattukudy, 1978; Young and Beevers, 1976; Ishimaru and Yamazaki, 1977). On balance, N-demethylation in wheat is considered likely to be cytochrome P-450 dependent process given that there are some precedents for such enzymes being less sensitive to ABT or CO, however, the enzyme mediating the reaction is obviously markedly different from that oxidising the ring methyl group (Mougin *et al.*, 1990).

Isoproturon, which differs from chlorotoluron in the presence of an isopropyl group in place of the ring-methyl, and is also detoxified by both N-demethylation and aryl hydroxylation in wheat. This metabolism is sensitive to inhibition by ABT which also intensifies the symptoms of isoproturon in wheat (Gaillardon *et al.*, 1985; Cabanne *et al.*, 1987). Isoproturon metabolism is, therefore, similar in many ways to that of chlorotoluron in wheat.

N-dealkylation appears to be a less efficient means of detoxifying substituted ureas given that tolerant crop species such as wheat preferentially oxidise ring alkyl groups while susceptible weeds species often employ N-dealkylation. The monodealkylated metabolites retain 50 - 70 % of the phytotoxicity of the parent herbicide (Geissbuhler *et al.*, 1975). All other metabolites are essentially non-phytotoxic. In general, it appears that plants more readily perform the first dealkylation than the second, resulting in larger pools of the partially detoxified metabolite (Geissbuhler *et al.*, 1975). Thus plants for which N-demethylation is a major reaction must carry out a second reaction to detoxify the molecule. Reliance on this

mechanism would, therefore, appear to be inefficient. The demethylated metabolites may be further degraded to the parent aniline, however, this is not a major product in corn (Onley *et al.*, 1968).

Hydroxylation of the N-methyl groups by oxidative enzymes can yield the hydroxymethyl intermediate which may be conjugated with glucose. This intermediate is very unstable and conjugation must occur rapidly, otherwise it decomposes to the demethylated metabolite and formaldehyde (Shimabukuro, 1985). Conjugation is thought to be catalysed by glucosyl transferases (Geissbuhler *et al.*, 1975). The conjugates are more polar than the parent herbicide but can be broken by acid hydrolysis or treatment with β-glucuronidase. Endogenous hydrolases may also cause cleavage of the conjugates releasing the semi-phytotoxic demethylated metabolite. Aryl hydroxylations are also subject to glycosylation but only a single conjugation at either end of the herbicide molecule can be made (Geissbuhler *et al.*, 1975). A large proportion of the chlorotoluron metabolites extracted from wheat or *A. myosuroides* is in the form of conjugates. Many of these are formed after oxidation of the ring-methyl group and this particular conjugate comprises 50 % of the wheat conjugate pool (Cabanne *et al.*, 1987). Conjugation is therefore, not a primary mechanism of detoxification of phenylureas but it may influence the toxicity of the semi-phytotoxic mono-demethyl metabolites.

The similarity in the metabolism of different phenylurea analogues means that different herbicides are metabolised to the same semi-phytotoxic metabolite, for example: methazole, diuron, and linuron (and probably neburon) are metabolised to DCPMU (di-chlorophenyl mono-methyl urea) after the first dealkylation or demethoxylation in the case of linuron (Suzuki and Casida, 1981; Hutson, 1987). Given that many different phenylureas have N-methyl groups in common, detoxification at this site on the molecule could protect a plant from a variety of phenylurea analogues. It appears, however, that the affinity of the detoxifying enzymes is influenced by the other substituents leading to varying tolerance to each of the analogues (Geissbuhler *et al.*, 1975).

Resistance to the Phenylurea Herbicides

Resistance to the phenylureas has not occurred as often as resistance to the triazine herbicides. While triazine resistant psbA mutant are generally not cross-resistant to phenylureas, alterations of PS II conferring triazine resistance may have minor effects on resistance to phenylureas. There are some cases where modest resistance to diuron or other phenylureas has been observed as a consequence of triazine selection (Polos *et al.*, 1985). Many studies have examined the inhibition of electron transport by phenylurea herbicides in triazine resistant chloroplasts, however, few report dose response at the whole plant level (Arntzen *et al.*, 1982; Oettmeier *et al.*, 1982). Inhibition of electron transport by diuron is generally unchanged or slightly decreased, however, Oettmeier *et al.* (1982) found that the effects of different phenylurea analogues varied widely in chloroplasts isolated from triazine resistant *Amaranthus retroflexus*. Linuron was 3100 times less inhibitory, diuron was 4 times less inhibitory whereas fluometuron was four times more inhibitory to the resistant chloroplasts. These data demonstrate that generalisations based on diuron may not adequately describe changes in sensitivity to phenylureas following alterations of the target site. Thus multiple-resistance to chlorbromuron and metobromuron in a psbA mutant of *Conyza canadensis* could be due to alterations of PS II (Polos *et al.*, 1985; Polos *et al.*, 1987). It is also possible though, that mechanisms other than target site effects may contribute to phenylurea resistance in such cases. These secondary mechanisms may go undetected due to the extreme effects of the target site mutation, however, their importance in conferring cross-resistance may justify more careful examination of resistant biotypes.

The only previously reported case of phenylurea resistance caused by selection pressure by those herbicides is that of *A. myosuroides* (blackgrass) collected at various sites in England and Germany (Moss and Cussans, 1991). One particular biotype collected from a farm near Peldon in Essex exhibits the highest degree of resistance to chlorotoluron amongst the various *A. myosuroides* isolates. The history of herbicide use leading to resistance in this case was dominated by chlorotoluron with 6 other herbicides being used to some extent.

The mechanism of chlorotoluron resistance in the Peldon biotype is enhanced detoxification (Kemp *et al.*, 1990). The degree of resistance can be influenced by treatment with cytochrome $P\text{-}_{450}$ inhibitors including ABT and triadimefon supporting the proposition that metabolism may be involved in the resistance mechanism (Kemp *et al.*, 1990; Kemp and Casely, 1987). Both of these inhibitors intensify the effect of chlorotoluron in the resistant *A. myosuroides*. These *A. myosuroides* biotypes, especially the Peldon biotype are also moderately resistant to a range of other herbicides including simazine, diclofop, pendimethalin and chlorsulfuron (Moss and Cussans, 1991). In this respect there appears to be some similarity between the Peldon biotype and *L. rigidum* biotype VLR69. Both have a long history of exposure to many different herbicides and seem to have accumulated resistance to each of them.

Conclusion

Ureas have been made selective by trading off herbicidal activity *in vitro* for more readily metabolised substituents. The selectivity of the ureas is also based on soil placement, adsorption, and translocation. Tolerance to the phenylureas in crop species such as wheat is largely due to rapid detoxification, although in some species reduced uptake and translocation may confer moderate tolerance. Enhanced metabolism has been proposed as the cause of resistance in some weed species that have been subject to selection pressure by phenylurea herbicides. In others, modest cross-resistance may be derived from triazine resistant mutations of PS II. Given the range of factors which affect phenylurea toxicity in plants, any studies of resistance to the substituted ureas should follow all of these lines of inquiry.

4.2 Substituted Urea Resistance in *L. rigidum*

As reported in Chapter one, two biotypes of *L. rigidum*, VLR69 and WLR2, have developed resistance to the substituted urea herbicides. The biotypes are similar in their level of resistance to diuron and chlorotoluron with the level of chlorotoluron resistance being approximately twice that to diuron (Chapter 2). The biotypes differ, however, in the history of selection pressure that lead to their resistance. Both were subject to selection pressure by atrazine but WLR2 was never selected with substituted ureas making it cross-resistant to these herbicides. In contrast, VLR69 was selected for 17 seasons with diuron. Despite this disparity in selection pressure both biotypes appear to have similar resistant phenotypes. The objective of this study was to determine the mechanisms of resistance and cross-resistance to these herbicides and whether these are similar in the two biotypes.

4.3 Materials and Methods

The plant material, dose response to herbicides, thylakoid extraction and measurement of electron transport are reported in Chapter three.

Chlorotoluron uptake and metabolism

Plants used in chlorotoluron uptake and metabolism studies were grown under the same conditions as those described for studies of simazine metabolism (Chapter 3).

Nutrient solution containing 120 μM [^{14}C]chlorotoluron (specific activity 20.2 MBq mmol^{-1}, uniformly ring labelled, or in experiments with ABT, 23.7 and 13.0 MBq mmol^{-1}) was added to drained vials. Plant roots were maintained in the [^{14}C]chlorotoluron solution for 3 h during the light period (when present, the final concentration of ABT was 6 mM (Table 1) or 0.25 mM (Figure 8)). Vials were then drained and roots were washed four times with 10 mL aliquots of deionised water after which nutrient solution was added to the vials. Plants were harvested at regular intervals up to 51 h after the initiation of the

treatment period. Roots were washed and separated from the shoots. Fresh weight of both shoots and roots was recorded and tissue wrapped in aluminium foil, frozen in liquid nitrogen and stored at -20°C.

Extraction, separation, and quantification of metabolites

Frozen plant material was ground and extracted as described for simazine (Chapter 3). Metabolites were separated and quantified by reverse phase HPLC using the same protocol as that used for simazine separation. Chlorotoluron metabolites were detected using an on-line HPLC radioactivity monitor (Berthold LB504) with a solid, cerium-activated glass scintillant cell (Berthold, G650U4). Metabolites were quantified as a proportion of the total radioactivity injected onto the column and tentatively identified by co-chromatography with standards in both methanol and acetonitrile gradients with two different gradients for each solvent system using the column described above.

Effect of chlorotoluron on CO_2 exchange

Plants were grown hydroponically as described above for chlorotoluron metabolism studies. Forty days after germination, six of the youngest fully-expanded leaves from two plants of each biotype were placed within a 1 L chamber of a LI-COR L6200 portable photosynthesis meter. The same area of leaf was used for each measurement. The roots of the plants were immersed in 120 μM commercially formulated chlorotoluron in nutrient solution for a 3 h treatment period. Following treatment, roots were washed with four changes of deionised water. Treated plants were then placed in trays in which the roots were immersed in 6 L of nutrient solution. Untreated plants were kept in separate trays. Measurements were made every 30 minutes during the treatment period, and thereafter three times a day for the following 5 d. Ambient CO_2 concentration was 410 ± 18 ppm (sd) and light intensity was 200 μmol m^{-2} s^{-1}. The operators breath was vented to the exterior of the growth room to reduce CO_2 accumulation.

Effect of ABT on response to chlorotoluron in nutrient solution

Plants were placed in vials as above and treated with: chlorotoluron (4 μM), ABT (70 μM) or ABT (70 μM) in combination with chlorotoluron (4 μM). All treatments and the control contained 0.1 % (v/v) ethanol. A similar experiment was performed with 3 μM chlorotoluron instead of 4 μM providing the result illustrated in Figure 4.11. Plants were maintained in these solutions for 7 d after which the roots were washed twice with deionised water, returned to nutrient solution for a further 7 d before harvest and measurements of fresh and dry weight. Treatments were replicated three times and the vials were randomly distributed within the experimental area.

4.4 Results

Dose Response to Herbicides

Both resistant biotypes exhibit between a six- and eight-fold higher LD_{50} than the susceptible VLR1 for the phenylurea herbicide chlorotoluron (Figure 4.5, Figure 4.11, colour plate). VLR69 was marginally more resistant than WLR2 in all experiments. Resistance to diuron is also observed but at a lower level for both WLR2 (2-5 fold) and VLR69 (3-6 fold) (Table 1.1, 1.2). Temperature was an important variable with the level of resistance for winter experiments being lower for all herbicides than the level of resistance determined in spring or autumn. When screened against six phenylurea analogues, WLR2 was most resistant to chlorotoluron (Chapter 2), hence the use of chlorotoluron as the model system for this study. The major difference between chlorotoluron and diuron is that chlorotoluron has a methyl group instead of a chlorine at the 4 position of the phenyl ring (Geissbuhler *et al.*, 1975). This methyl substituent renders the molecule more susceptible to oxidative metabolism and thus confers selectivity in some crops (Ryan and Owen, 1982).

Effect on PS II Target Site

Electron transport in thylakoids isolated from leaves of all three biotypes was equally sensitive to inhibition by diuron (Figure 4.6A). Thylakoids from WLR2 and VLR1 also

Figure 4.5. The response of the susceptible biotype VLR1(●) and the resistant biotypes WLR2 (O) and VLR69 (✕) to increasing doses of chlorotoluron sprayed onto 3-leaf plants growing in pots. Each point is the mean of three pots each containing 12 plants and is plotted with standard error.

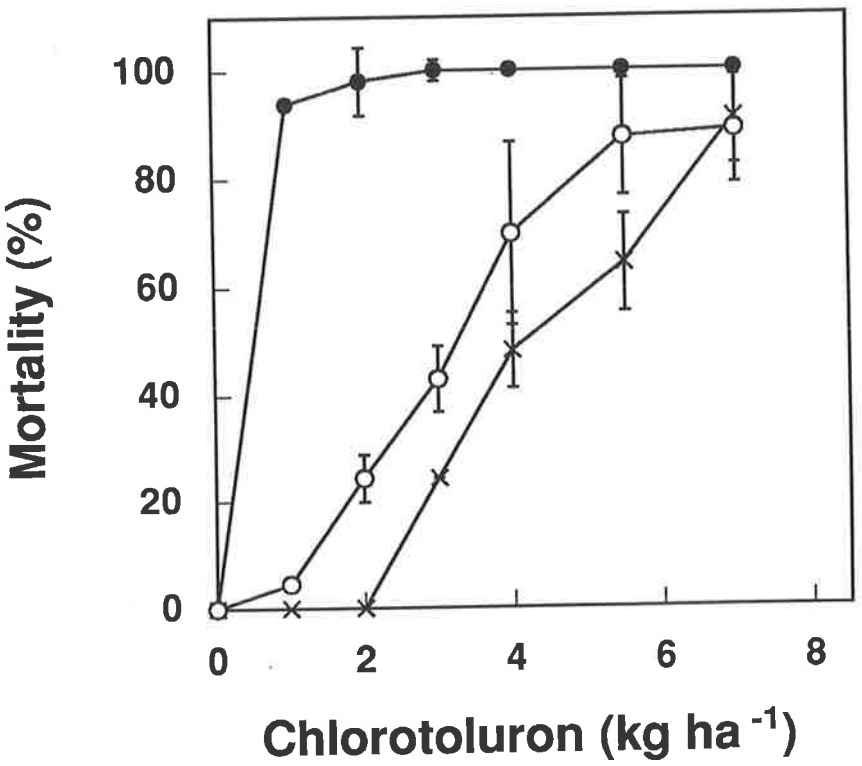

Mortality (%)
100
80
60
40
20
0
0
2
4
6
8
Chlorotoluron (kg ha -1)

Figure 4.6. The effect of diuron (A) and chlorotoluron (B) on oxygen evolution by thylakoids isolated from VLR1 (●), WLR2 (O) and VLR69 (✕) biotypes. Rates are expressed as a percent of control which was at least 230 μmol O_2 mg chl^{-1} h^{-1} with similar rates for each biotype. Each point is the mean of four (A) or six (B) replicates and is plotted with standard error.

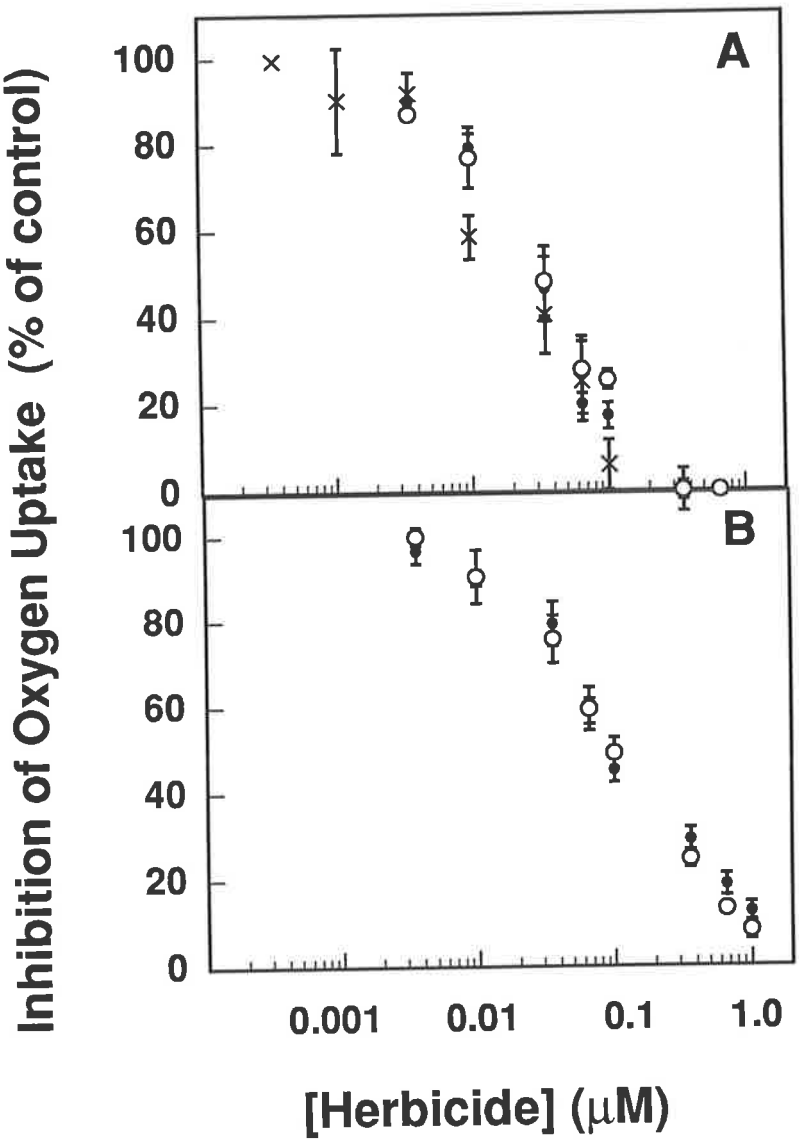

Inhibition of Oxygen Uptake (% of control)
100
80
60
40
20
0
A
B
0.001
0.01
0.1
1.0
[Herbicide] (μM)

exhibit similar sensitivity to chlorotoluron (Figure 4.6B). This indicates that there is no difference between the susceptible and resistant biotypes in the sensitivity of the target site to inhibition by chlorotoluron or diuron.

This result was not unexpected in WLR2 given that the selection pressure had been applied by atrazine without causing any change in the sensitivity of PS II to triazines (Chapter 3). It would seem very unlikely for cross-resistance to the phenylureas in WLR2 to be due to a mechanism not active against the selecting agent. For VLR69 it is possible that 17 generations of selection may have lead to the emergence of a target site mutant, however, the low level of resistance to the substituted ureas made this seem unlikely. The low rates of diuron used in the field may have imposed selection pressure that was not sufficiently extreme to select for a target site mutation. Thus for both biotypes the low level of resistance suggested that the mechanism would not be related to a change in sensitivity of PS II. These data give no indication of whether there are any differences in the transport of the herbicide into the chloroplast but they do indicate that the target site itself is sensitive to the herbicides.

Chlorotoluron Uptake and Transport

Chlorotoluron uptake and transport from nutrient solution by hydroponically grown plants was similar for all biotypes (Table 4.1). This indicates that there is no difference in the amount of herbicide translocated into the shoot tissue of susceptible and resistant plants, however, these data give no indication of the cellular or sub-cellular distribution of chlorotoluron. If there were differences in absorption and translocation of chlorotoluron in the shoots contributing to resistance one would expect significantly lower amounts of radioactivity to be present in the leaves of resistant plants. Since this is not the case, it appears that there is equal uptake of chlorotoluron into the shoot tissue of both biotypes.

Uptake of chlorotoluron may also be observed indirectly in the kinetics of reduction of photosynthetic activity of plants treated with chlorotoluron. A 3 h pulse of 120 μM

Table 4.1. The amount of chlorotoluron taken up from nutrient solution containing 120 µM [^{14}C]chlorotoluron (23.7 MBq mmol^{-1}) after 3 h exposure. Values are the means of 5 treatments recorded with the standard error and are representative of two experiments of this type.

Biotype	Plant Fresh Weight *				Chlorotoluron taken up * (pmol mg FW^{-1})		% in Shoots *	
	Shoots (g)		Roots (g)					
	- ABT	+ ABT	- ABT	+ ABT	- ABT	+ ABT	- ABT	+ ABT
VLR1	5.93 a ± 0.46	5.51 a ± 0.21	1.77 a ± 0.17	1.66 a ± 0.14	25.4 a ± 1.4	25.0 a ± 1.0	92.3 a ± 1.5	95.6 a ± 1.0
WLR2	6.13 a ± 0.39	6.11 a ± 0.15	1.7 a ± 0.04	2.00 a ± 0.31	27.3 a ± 1.4	21.6 a ± 1.9	95.2 a ± 1.3	93.5 a ± 2.3
VLR69	5.17 a ± 0.17	4.98 b ± 0.15	1.50 b ± 0.08	1.46 a ± 0.06	24.5 a ± 4.8	23.4 a ± 2.7	93.9 a ± 0.8	95.6 a ± 0.7

*means in each column followed by a different letter are significantly different at the 0.95 level of significance according to a t-test.

chlorotoluron applied to the roots of hydroponically grown VLR1 and WLR2 plants caused a rapid reduction in photosynthetic carbon fixation such that there was zero net fixation of CO_2 within 2h (VLR1) or 3 h (WLR2) of the treatment commencing (Figure 4.7). These data support the observations of inhibition of electron transport *in vitro* (Figure 4.6) in that photosynthesis is also inhibited *in vivo*. The slightly slower reduction in photosynthetic activity in the WLR2 plants during the uptake phase may be explained by their greater rate of metabolism of chlorotoluron outlined below.

Chlorotoluron Detoxification

Chlorotoluron is metabolised by the resistant biotypes WLR2 and VLR69 more rapidly than in the susceptible VLR1 (Figure 4.8). The conditions under which chlorotoluron metabolism was studied were similar to those used in the study of CO_2 exchange and can be compared with data in Figure 4.7 which show that photosynthetic activity was reduced to zero after a 3 h exposure to 120 μM chlorotoluron. The dose of chlorotoluron used in this system was sufficient, therefore, to inhibit photosynthesis in both biotypes. Within 40 h of chlorotoluron removal, WLR2 plants had recovered net fixation and by 110 h were photosynthesising at rates similar to the controls (Figure 4.7). In contrast, the susceptible VLR1 plants required over 80 h to recover net fixation. From this comparison it can be seen that 75% of the herbicide had been detoxified by WLR2 in the time required to resume net CO_2 fixation. In VLR1 plants the rate of metabolism had become very low after 40 h yet some recovery was evident by 80 h. This gradual recovery may be due both to metabolism and translocation of chlorotoluron to the tips and margins of the leaf and away from the area of the leaf being assayed for photosynthetic activity. For the WLR2 plants, however, the recovery of photosynthesis directly corresponded to the reduction in the amount of herbicide in the shoot tissue.

Figure 4.7. A. CO_2 exchange (at a PFD of 200 μmol m^{-2} s^{-1}) in response to a 3 h pulse of 120 μM chlorotoluron initiated at 0 h for VLR1 (●) and WLR2 (O). Control rates of CO_2 fixation were between 5 (VLR1) and 6 (WLR2) μmol CO_2 m^{-2} s^{-1}. Each point is the mean of two replicates. B. Metabolism of chlorotoluron by the susceptible VLR1 (●) and resistant WLR2 (O) biotypes expressed as the proportion of the extracted radioactivity remaining as chlorotoluron. These data are reproduced from Figure 4.8A to facilitate comparisons between the two types of experiment.

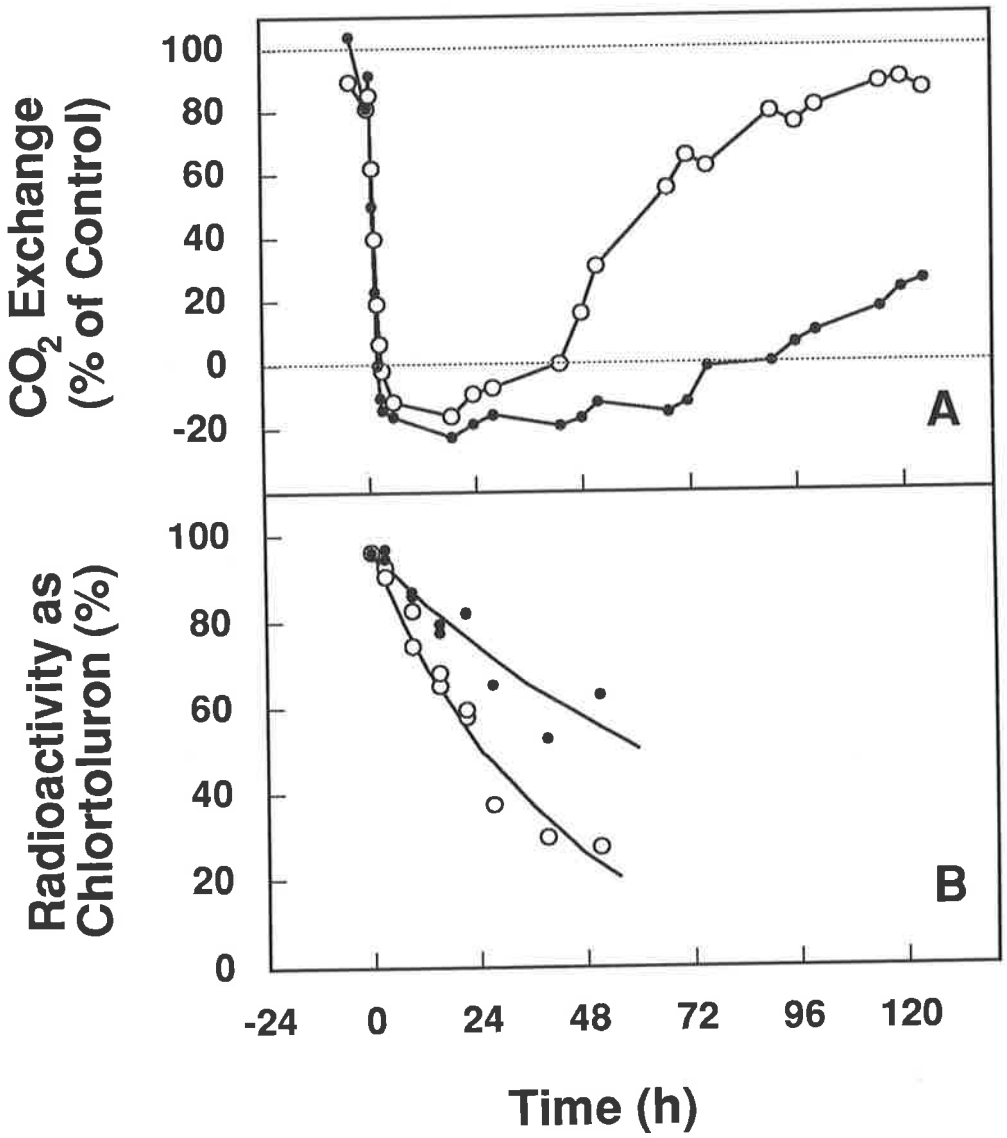
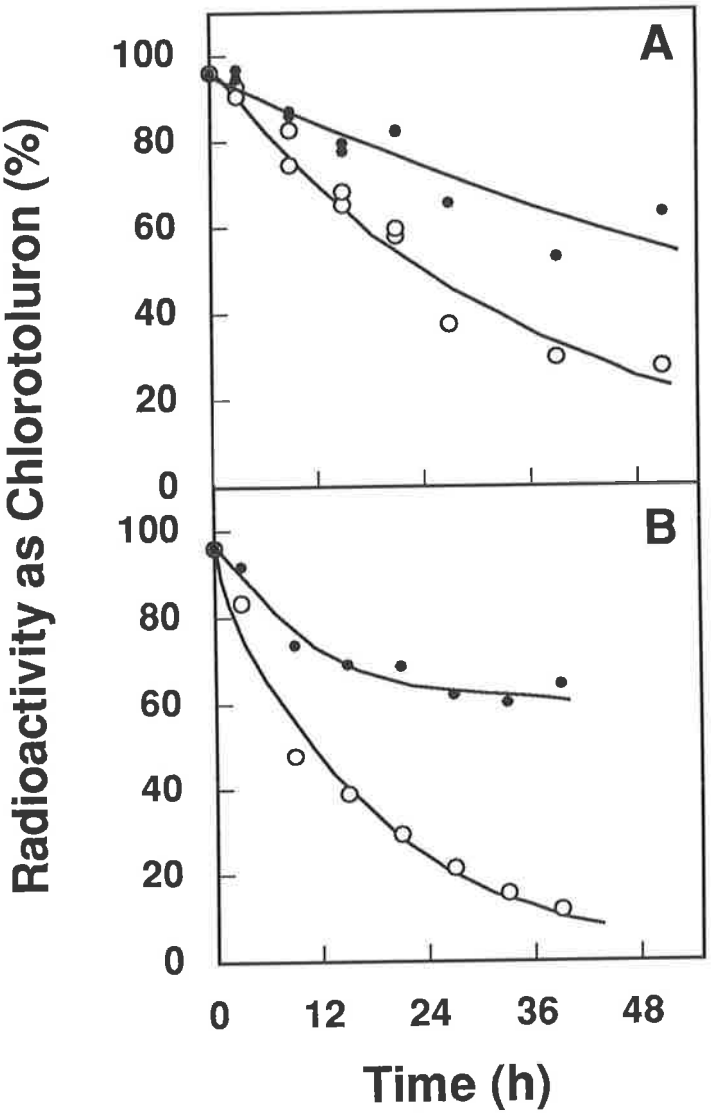

CO_2 Exchange (% of Control)
100
80
60
40
20
0
-20
A
Radioactivity as Chlortoluron (%)
100
80
60
40
20
0
B
-24
0
24
48
72
96
120
Time (h)

Figure 4.8. A. Metabolism of chlorotoluron by the susceptible VLR1 (●) and resistant WLR2 (O) biotypes expressed as the proportion of the extracted radioactivity remaining as chlorotoluron. Treatment conditions were similar to those in Figure 4.7 except for the addition of [^{14}C]chlorotoluron (20.2 MBq mmol^{-1}) to the pulse solution. B. Metabolism of [^{14}C]chlorotoluron (7.8 MBq mmol^{-1}) by VLR1 (●) and VLR69 (O) biotypes under conditions similar to those of Figure 4.7. Data are from one of three experiments and are representative of these.

The recovery of radioactivity in all chlorotoluron metabolism experiments was at least 85% for both resistant and susceptible biotypes. There was no difference in recovery between biotypes. Recovery did, however, decline slightly (90 to 85%) as the period of metabolism increased. This could have been due to the formation of insoluble conjugates or to loss as CO_2.


A
B
100
80
60
40
20
0
0
12
24
36
48
Radioactivity as Chlorotoluron (%)
Time (h)

The difference in the rate of metabolism between the susceptible and resistant biotypes was approximately two-fold. VLR69 appeared to have a slightly greater capacity to detoxify chlorotoluron than did WLR2 (Figure 4.8) which might explain its greater resistance to the substituted ureas (Figure 4.5). The same conclusions were made with respect to simazine resistance where VLR69 has both a greater rate of simazine detoxification and level of simazine resistance compared with WLR2. This further supports the suggestion that the level of resistance is related to the rate of metabolism of the herbicide.

The biotypes did not differ in the variety of metabolites produced given the similarity of HPLC chromatograms of chlorotoluron and metabolites extracted from each biotype (Figure 4.9). VLR1 did, however, form these metabolites more slowly. Metabolites were only tentatively identified by co-chromatography with standards (chemical structures in Figure 4.3). On this basis the first metabolite produced was the N-demethylated metabolite (II). After 3 to 6 h this metabolite was the largest component of the metabolite pool (Figure 4.10). Subsequently the unidentified metabolite (V) is observed. After 6 to 9 h the unknown (V) becomes the major component of the metabolite pool (Figure 4.10). The hydroxymethylphenyl metabolite (IV) and di-de-methylated metabolite (III) were never major constituents of the extracted radioactivity. By 24 h the most polar metabolites were formed (Figure 4.10). When metabolite fractions were reintroduced to the cut shoots of *L. rigidum* plants the unknown (V) was not further metabolised. This suggests that it is an end product of metabolism.

The model for the metabolism of chlorotoluron in *L. rigidum* is that N-demethylation occurs first and the product of this reaction is rapidly converted to the unknown (V) possibly via an intermediate (rt 18.40 min, Figure 4.9). The second reaction does not appear to be another demethylation as there is no evidence of the di-demethyl metabolite increasing as a proportion of the radioactivity. Similarly there is no evidence of ring-methyl hydroxylation being an important initial reaction. There is, however, the possibility that the ring methyl group may be hydroxylated after the first N-demethylation. This would yield a non-

Figure 4.9. HPLC radioactivity chromatograms for VLR1 (A) and WLR2 (B) 24 h after the start of a 3 h treatment with [^{14}C]chlorotoluron. These can be compared with a UV (254 nm) trace (C) of a mixture of chlorotoluron (I), the mono-demethylated metabolite (II), the di-demethylated metabolite (III) and the hydroxymethylphenyl metabolite (IV) chromatographed using the same gradient and column.

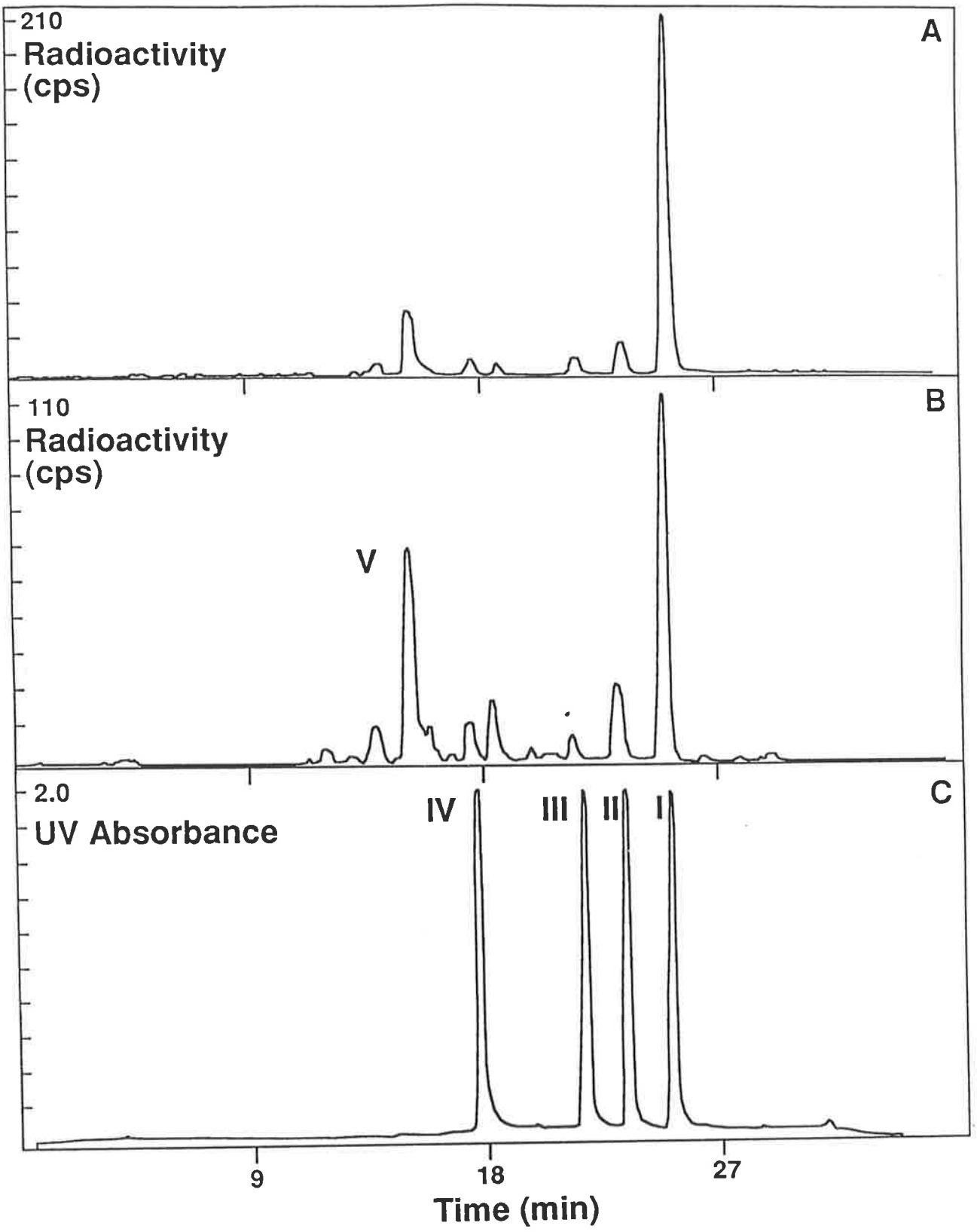
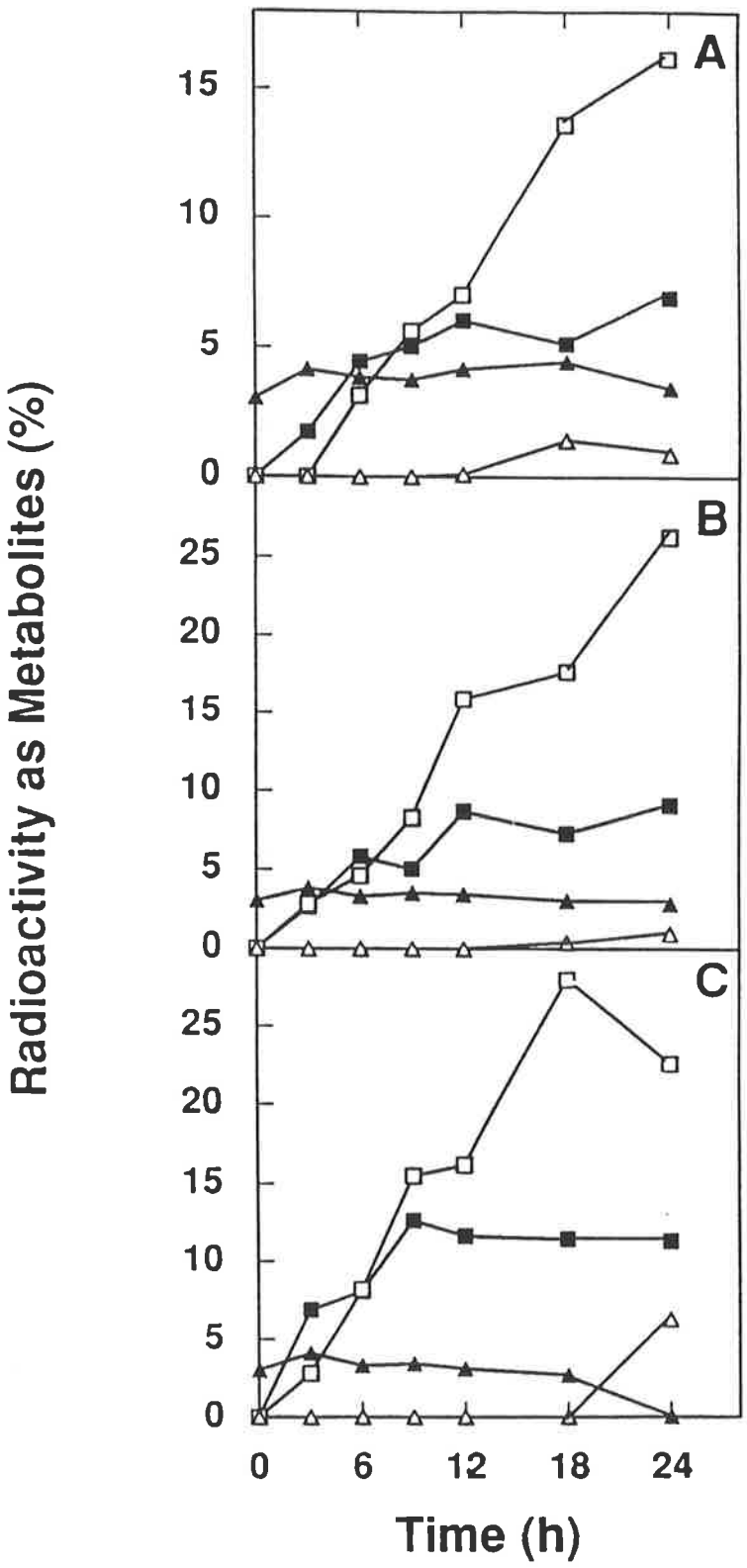

210
Radioactivity
(cps)
A
110
Radioactivity
(cps)
B
V
2.0
UV Absorbance
IV
III
II
I
C
9
18
27
Time (min)

Figure 4.10. Major metabolites of chlorotoluron extracted from the susceptible biotype VLR1 (A) and the resistant biotypes WLR2 (B) and VLR69 (C) as a proportion of total extractable radioactivity after a 3 h dose of [^{14}C]chlorotoluron (23.7 MBq mmol^{-1}). Mono-demethylated metabolite (II) (■), unknown metabolite (V) (□), di-demethylated metabolite (III) (▲) and polar metabolites (Δ). Data is representative of results obtained in other chlorotoluron metabolism studies.


A
B
C
15
10
5
0
25
20
15
10
5
0
25
20
15
10
5
0
0
6
12
18
24
Time (h)
Radioactivity as Metabolites (%)

phytotoxic metabolite from the semi-phytotoxic N-demethylated metabolite (Figure 4.3). An alternative but less likely explanation is that enzymes catalysing other reactions are induced following chlorotoluron treatment leading to a lag in the production of metabolite V. The pathway of chlorotoluron metabolism in *L. rigidum* is not clear but N-demethylation appears to be the initial reaction.

The effect of ABT on chlorotoluron symptoms

ABT is an inhibitor of some monooxygenase enzymes that are implicated in the detoxification of herbicides (Cabanne *et al.,* 1987; Ortiz de Montellano and Reich, 1986). The mechanism of this inhibition requires the monooxygenase to catalyse the oxidation of ABT to benzyne which then covalently binds to the prosthetic haem of the enzyme, rendering it inactive (Ortiz de Montellano and Reich, 1986). Although ABT is thought of as an inhibitor of cytochrome $P\text{-}450$ dependent enzymes it is not entirely specific to this class, thus its effect provides only an indication of the nature of the enzymes detoxifying the herbicide (Blee *et al.,* 1987).

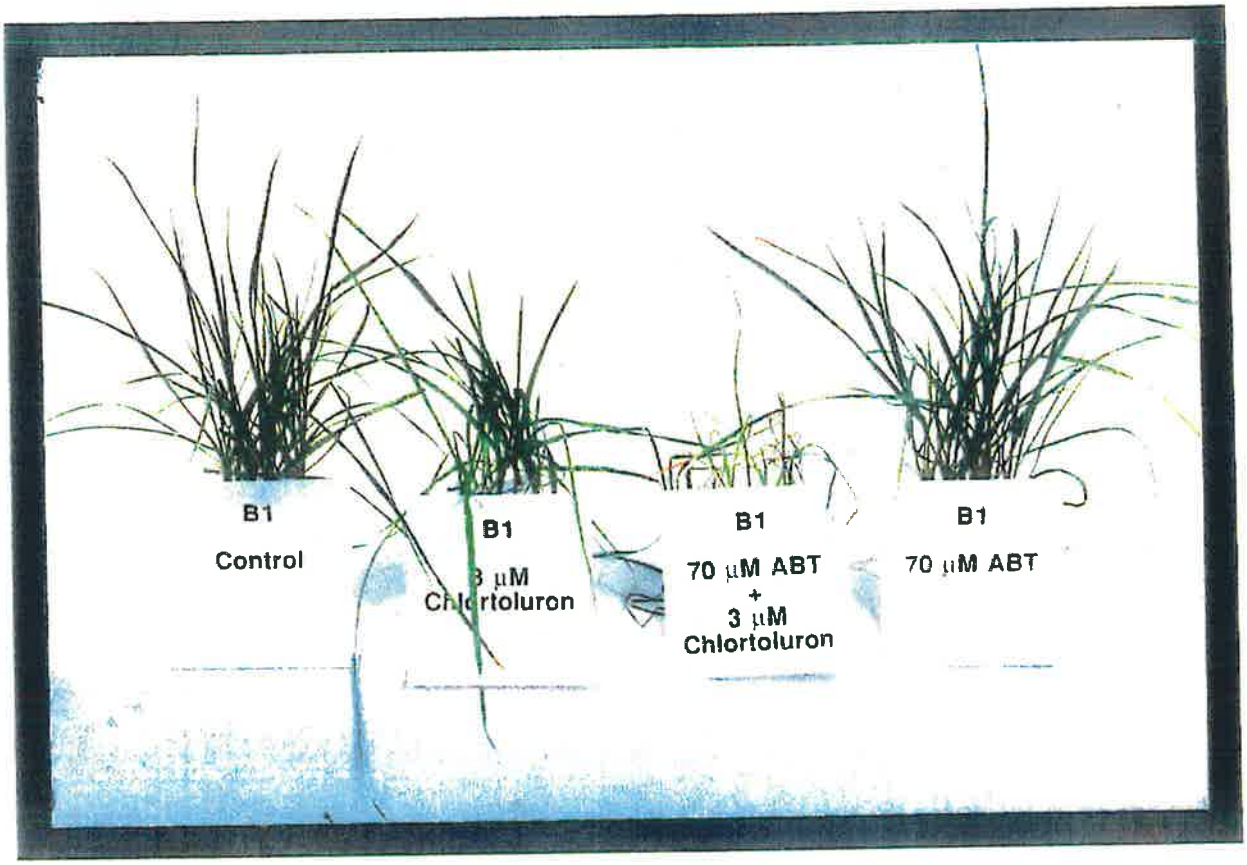
To determine the capacity of ABT to synergise chlorotoluron, resistant and susceptible biotypes were grown in the presence of herbicide ± ABT. ABT (70 μM) did not significantly reduce growth of any biotype (Table II, Figure 4.12). In combination with chlorotoluron, however, ABT caused significant reductions in fresh weight of resistant plants compared with chlorotoluron alone. WLR2 and VLR69 plants treated with both ABT and chlorotoluron had only 15 % of the fresh weight of those plants treated with chlorotoluron (Table II, Figure 4.12). The difference was less marked for the susceptible plants because they exhibited extensive symptoms of chlorotoluron toxicity without the addition of ABT. The inclusion of ABT into the treatment solution did not affect uptake or distribution of the herbicide into the shoots (Table 1).

The concentration of ABT used in this experiment was the same as that used by others to synergise herbicides in graminaceous species (Barta and Dutka, 1989; Cabanne *et al.,* 1987;

Figure 4.11. The response of WLR2 (foreground) and VLR1 (background) to chlorotoluron applied to pot grown seedlings.

Figure 4.12. The effect of ABT (70 μM) alone and in combination with chlorotoluron (3μM) on growth of VLR69 ("B1" was the original designation of this biotype). The experiment was similar to that reported in Table 2.


CHLORTOLURON
CHLORTOLURON
CONTROL
SUSCEPTIBLE
CHLORTOLURON
0.5 kg a.i. ha
SUSCEPTIBLE
CHLORTOLURON
1.0 kg a.i. ha
SUSCEPTIBLE
CHLORTOLURON
CONTROL
RESISTANT
CHLORTOLURON
0.5 kg a.i. ha
RESISTANT
CHLORTOLURON
1.0 kg a.i. ha
RESISTANT


B1
Control
B1
3 µM
Chlortoluron
B1
70 µM ABT
+
3 µM
Chlortoluron
B1
70 µM ABT

Kemp, 1987). A range of ABT doses were applied to the *L. rigidum* biotypes in preliminary experiments and 70 μM was below the limit of toxicity for *L. rigidum*. In experiments with simazine, metribuzin and chlorotoluron, ABT alone did not cause a significant reduction in plant weight. Thus any reduction in weight of the herbicide plus ABT treatment compared with the herbicide alone is likely to be due to the effects of ABT on herbicide metabolism. This is supported by the observation that plants treated with the combination of herbicides exhibited symptoms associated with chlorotoluron toxicity and not those of ABT (Figure 4.12).

A similar type of experiment was performed with piperonyl butoxide (PBO) and chlorotoluron applied to pot grown plants. In combination with chlorotoluron, PBO reduced survival and dry weight of resistant plants compared with chlorotoluron alone (Table 4.3). The symptoms of the seedlings treated with both PBO and chlorotoluron were similar to that associated with a high rate of the herbicide, suggesting that inhibition of chlorotoluron detoxification had occurred. The synergy observed with both ABT and PBO provides evidence that differential metabolism is the major difference between the susceptible and resistant *L. rigidum* biotypes.

PBO is an inhibitor of many mixed function oxidase enzymes in insects and mammals (Brown, 1990; Ortiz de Montellano and Reich, 1986). PBO is not a potent inhibitor of many plant cytochrome $P\text{-}_{450}$ enzymes (Benveniste, 1978), however, both PBO and ABT synergise the activity of chlorotoluron and isoproturon in wheat cv. Clement whereas metyrapone and 2,4-dichlorophenoxypropyne do not (Gaillardon *et al.*, 1985). The observation of interactions between PBO and chlorotoluron in Clement provides a precedent for the effect of PBO in combination with chlorotoluron on a graminaceous species. It is likely that in both Clement and *L. rigidum* the effect of PBO is, at least in part, related to the inhibition of chlorotoluron detoxification although this has not been demonstrated directly (Gaillardon *et al.*, 1985).

Table 4.2. The effect of ABT (70 μM) on shoot fresh weight of VLR1, WLR2 and VLR69 when applied alone or in combination with chlorotoluron (4 μM) for 7 d followed by a 7 d recovery period in the absence of chlorotoluron or ABT. Data is from one of two experiments and is representative of these (see also Figure 4.12).

	Shoot Fresh Weight (g, mean ± se)			
Biotype	Control	70 μM ABT	4 μM Chlorotoluron	70 μM ABT + 4 μM Chlorotoluron
VLR1*	10.52 a ± 0.29	9.75 a ± 0.89	2.06 c ± 0.53	0.95 c ±0.41
WLR2*	9.31 a ± 0.64	9.90 a ± 0.36	5.79 b ± 0.58	0.89 d ± 0.23
VLR69*	9.83 a ± 0.37	8.86 a ± 0.74	7.34 b ± 0.29	1.10 d ± 0.33

*Means in each row followed by a different letter are significantly different at the 0.95 level of significance according to a t-test.

Table 4.3. The effect of PBO 4.2 kg ha^{-1} on shoot dry weight of VLR1, WLR2 and VLR69 when applied alone or in combination with chlorotoluron 0.5 kg ha^{-1}. Data is from one of two experiments and is representative of these.

	Dry Weight per pot (g, mean ± se)			
Biotype	Control	PBO 4.2 kg ha^{-1}	Chlorotoluron 0.5 kg ha^{-1}	PBO 4.2 kg ha^{-1} + Chlorotoluron 0.5 kg ha^{-1}
VLR1*	3.35 a ± 0.40	2.71 a ± 0.11	0.47 c ± 0.26	0.09 c ±0.08
WLR2*	2.59 a ± 0.31	2.75 a ± 0.20	1.24 b ± 0.50	0.27 d ± 0.16
VLR69*	2.64 a ± 0.35	2.73 a ± 0.62	2.25 b ± 0.85	0.90 d ± 0.07

*Means in each row followed by a different letter are significantly different at the 0.95 level of significance according to a t-test.

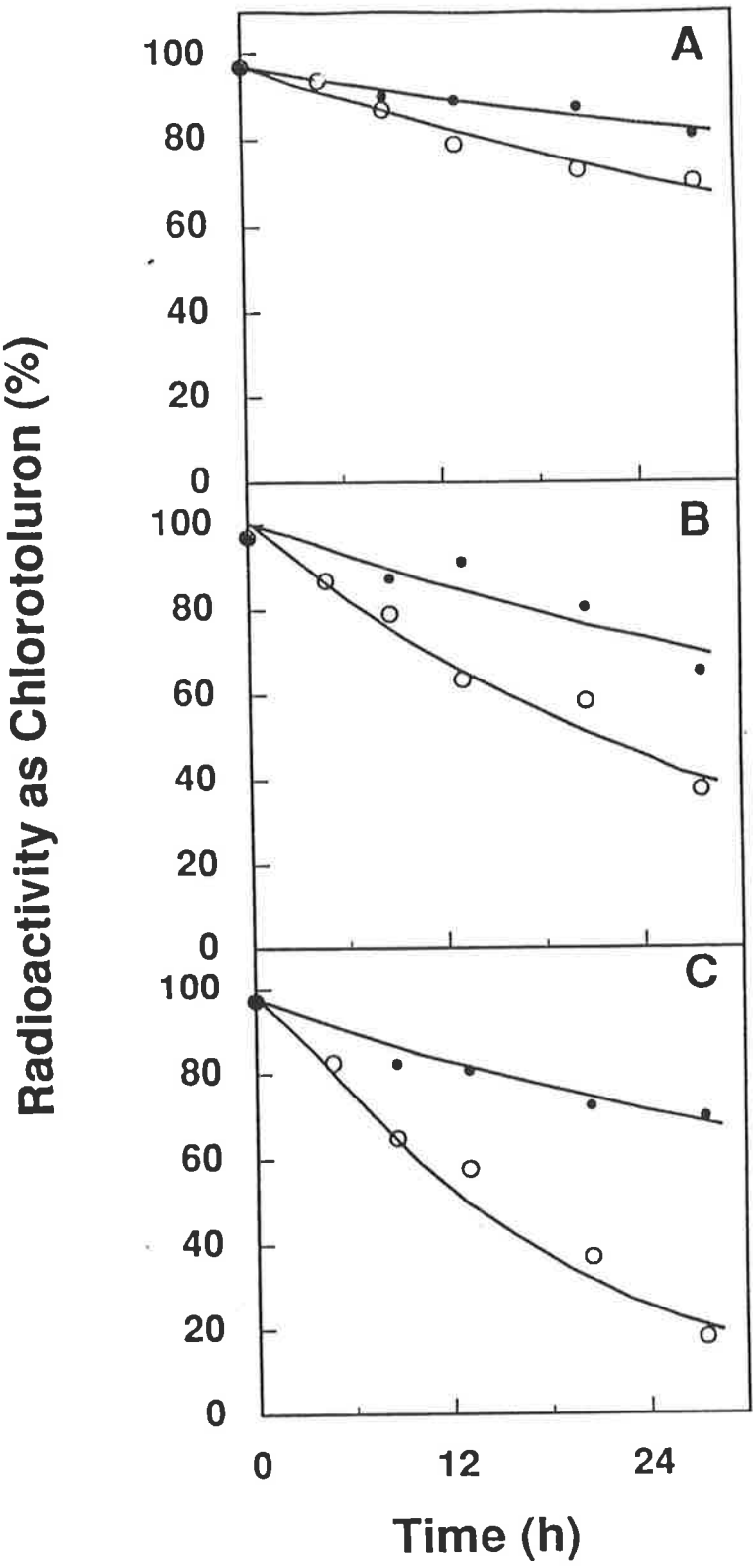
Effect of ABT on chlorotoluron metabolism

ABT inhibited the metabolism of chlorotoluron in *L. rigidum* (Figure 4.13). The effect of ABT was most apparent in the resistant biotypes and least apparent in VLR1 due to the already low rate of metabolism in the susceptible biotype. ABT inhibited the production of all metabolites with no metabolite being produced at the same rate as the controls (no ABT). Either all enzymes metabolising chlorotoluron are sensitive to ABT or the initial reactions are ABT sensitive and all metabolites are derived from the initial reactions. The initial reaction is thought to be N-demethylation. Given that this type of reaction is often oxidative (Frear *et al.*, 1969), inhibition is not surprising. The relative insensitivity of N-demethylation to ABT in wheat may not extend to *L. rigidum*. Alternatively, the relatively high dose of 250 μM ABT for 3 h may have saturated the detoxifying enzymes. Irrespective of the mode of inhibition, chlorotoluron metabolism appears sensitive to ABT.

The inhibition of chlorotoluron metabolism by ABT explains the effect of ABT on shoot fresh weight, when applied with the herbicide (Table 4.2). This supports the proposition that differential metabolism of the herbicide is a major difference between the biotypes. The same type of evidence was used to conclude that a high rate of detoxification was the major cause of chlorotoluron and isoproturon tolerance in wheat cv. Clement (Cabanne *et al.*, 1987).

The effect of ABT in Clement is largely to inhibit the oxidation of the ring-methyl groups of chlorotoluron and isoproturon. N-demethylation of chlorotoluron is relatively less affected although the second demethylation of isoproturon is inhibited (Cabanne *et al.*, 1987; Cabanne *et al.*, 1985; Gonneau *et al.*, 1988). These data raise the question of whether the first and second demethylations may be performed by the same enzyme. In susceptible *A. myosuroides* and *A. fatua* and *L. rigidum* very little didemethylated chlorotoluron is detected (Ryan and Owen, 1982) (Figure 4.10). This may be due to the monodemethylated metabolite being sequestered, further reaction via another mechanism or the inability of the oxidative enzymes in these species to use the metabolite as a substrate. The mono-

Figure 4.13. The effect of ABT on the metabolism of [^{14}C]chlorotoluron (13.0 MBq mmol^{-1}) under pulse-chase conditions for VLR1 (A), WLR2 (B) and VLR69 (C). Chlorotoluron in the presence (●) and absence (O) of ABT was applied to the roots of plants for 3 h after which there was no further input of either material. Exponential curves are fitted to the data. Data is one of two experiments and is representative of these.


A
B
C
100
80
60
40
20
0
0
12
24
Time (h)
Radioactivity as Chlorotoluron (%)

demethylated metabolite retains partial toxicity so its presence may cause some toxic effect on the plant. It is for this reason that species employing this mechanism of metabolism require a second detoxifying reaction to fully detoxify the herbicide. The reactions that follow N-demethylation in these species are not yet clear.

4.5 Discussion and Conclusions

The resistant *L. rigidum* biotypes, WLR2 and VLR69 were approximately 6 to 8 times less sensitive to chlorotoluron, than a susceptible biotype, VLR1, when the herbicide is applied to plants grown in soil (Figure 4.5). Resistance is also expressed when plants are treated with chlorotoluron dissolved in nutrient solution as indicated by the recovery of CO_2 fixation (Figure 4.7) and the differential symptoms of after 7 d exposure to 4 μM chlorotoluron (Table 2).

Resistance is not due to differential sensitivity at the target site (Figure 4.6), or to differences in uptake of chlorotoluron from nutrient solution (Table 1). Resistant plants do, however, exhibit greater metabolism of the herbicide, having at least twice the initial rate of metabolism of VLR1 plants (Figure 4.8). Under similar conditions WLR2 plants were able to reduce the initial dose of chlorotoluron by 75% in the time it took to recover net fixation of CO_2 (Figure 4.7 and Figure 4.8). The slower metabolism of the susceptible VLR1 plants provides some explanation for their greater time required for recovery of CO_2 fixation. Slower recovery in photosynthetic activity has also been correlated with slower metabolism of chlorotoluron in *A. fatua* (Ryan and Owen, 1982). VLR69 exhibits both a greater degree of resistance and a faster rate of chlorotoluron metabolism than WLR2, further strengthening the link between the rate of detoxification and the resistant phenotype.

ABT, an inhibitor of cytochrome $P\text{-}_{450}$ monooxygenase enzymes, inhibited metabolism of chlorotoluron (Figure 4.13). Both ABT and PBO intensified the effects of the herbicide *in vivo* (Table 4.2, Table 4.3, Figure 4.12) in all biotypes. This shows that with chlorotoluron detoxification inhibited, the resistant plants respond similarly to susceptible

plants treated with chlorotoluron alone. Therefore, the greater rate of metabolism in resistant plants is the major difference between the biotypes.

N-demethylation is hypothesised to be the primary detoxification reaction in all three biotypes. This would explain the broad resistance to many phenylurea analogues which have N-alkyl groups in common while varying in their phenyl substituents (Geissbuhler *et al.*, 1975). The 3,4-chloro groups of diuron, for example, are not easily oxidised and species such as *L. rigidum* preferentially oxidise the N-methyl groups over other possible reactions (De Prado *et al.*, 1990). This is in contrast to chlorotoluron where the ring-methyl group can be readily oxidised by species such as wheat (Cabanne *et al.*, 1987; Geissbuhler *et al.*, 1975; Ryan and Owen, 1982). Like *L. rigidum*, substituted urea resistant *A. myosuroides* biotype "Peldon" is resistant to many urea analogues which is partly attributed to the greater ability to perform N-dealkylation (Kemp *et al.*, 1990). Thus the initial reactions detoxifying chlorotoluron in *L. rigidum* and the broad spectrum of resistance to substituted ureas suggest that enhanced N-demethylation is a mechanism of resistance in WLR2 and VLR69.

The observations made on the mechanism of chlorotoluron resistance are very similar to those made on the mechanism of simazine resistance in these biotypes. For both herbicides the biotypes have a similar level of resistance and a similar increase in rate of detoxification of the herbicide. Detoxification of both chlorotoluron and simazine is inhibited by ABT. In both cases the initial detoxification reaction is an N-dealkylation which is an oxidative reaction. Together these observations suggest that the reactions could be catalysed by the same enzymes.

Such a model would explain three phenomena. The first is the cross-resistance to substituted ureas in WLR2. If triazines and substituted ureas are detoxified by the same enzymes then cross-resistance would simply be a consequence of the elevation of activity against the triazines. The second phenomenon is the relatively rapid acquisition of atrazine

resistance in VLR69. While WLR2 took ten consecutive years of treatment to adapt to atrazine, VLR69 required only five seasons of selection over nine years. If the same enzymes can dealkylate both classes of herbicide it would appear that the selection pressure imposed by diuron may have increased the frequency of atrazine resistance genes in the VLR69 population prior to the use of atrazine. The last phenomenon of interest is the similarity in the response of both biotypes to both classes of herbicide. If they have similar mechanisms it would seem likely that they would have similar responses to the various analogues of each class of inhibitor.

The similarity of the mechanisms in the two resistant biotypes may have stemmed from another cause. It is possible that the use of atrazine may have screened the already slightly resistant VLR69 population for only those mechanisms common to diuron (initial selector) and atrazine (subsequent selector). Thus the common element of atrazine selection may have ensured that only a limited set of mechanisms were maitained in the population. Alternatively, the application of diuron may have selected only the N-dealkylation mechanism from the outset of herbicide use.

VLR69 is good example of a multiple resistant biotype. It was subject to a succession of different selection pressures eventually becoming resistant to all herbicides used to control it. The model proposed to explain the rapid onset of multiple-resistance is as follows. The initial selection pressure selects for one or more mechanisms of resistance that may be specific or general. The use of another selector will result in good control if it has no mechanisms of resistance in common with the previous herbicide. If, however, the new selector is affected to some extent by one of the previously selected mechanisms, the population will use this slightly enhanced resistance to respond more quickly to the selection pressure. As the population responds to selection pressure, genes for resistance to both types of herbicide will become fixed within the population due to its small genetic base. Thus any subsequent selection from that population will contain those genes for resistance. The rate of response to the new selector will depend on the extent of the cross-resistance

selected by the previous herbicide. If no cross-resistance is selected then the population should respond like a wildtype population. If extreme cross-resistance is selected the new herbicide will fail.

WLR2 and VLR69 developed under very different selection pressure at sites over 2000 km apart. WLR2 was never exposed to selection pressure by phenylurea herbicides yet it has the same N-dealkylation detoxification mechanism as VLR69 which was exposed to 17 seasons of selection pressure by diuron and 5 season of selection by atrazine. The similarity of the mechanism in WLR2 and VLR69 can be partly explained by the limited range of reactions that may be performed on phenylureas such as chlorotoluron and may be due merely to a generalised increase in oxidative capacity. There is, however, the possibility that the selection pressure provided by atrazine and diuron may select for the same enzyme or group of enzymes as both biotypes also detoxify simazine, a triazine herbicide, more rapidly by N-dealkylation. It is possible that the basis of cross-resistance in WLR2 is that triazine and phenylurea herbicides may be common substrates for one or more oxidative enzymes in *L. rigidum* and that these enzymes have increased activity in the resistant biotype.

Chapter Five

Metribuzin Cross-Resistance in *L. rigidum*

5.1 Introduction

Metribuzin was initially developed by Bayer in the early 1960s and is used for the control of annual grass and broadleaf weeds in a variety of crops including peas, beans, barley, tomatoes and potatoes (Aust Weed Control Handbook). Metribuzin is a member of the triazinone class of herbicides so named for the ketone at the 5-position of the heterocycle. The two other triazinone analogues, metamitron and isomethiozin, have not been widely used and will not be discussed in this review. Metribuzin is also known as an as-triazine or asymmetrical triazine due to the uneven distribution of nitrogen atoms in its heterocycle. Metribuzin has many properties in common with the triazine herbicides including mode of action and uptake. However, it is more potent than triazines, requiring lower application rates in the field for effective weed control.

Metribuzin has not been used extensively in Australia due to both high cost and marginal crop tolerance. It has also not been implicated as a major selective agent in any cases of herbicide resistance in *L. rigidum* and there have been few cases of cross-resistance to metribuzin. Metribuzin is, therefore, a potentially useful herbicide for the control of *L. rigidum* resistant to more commonly used herbicides.

Mode of action

The primary mode of action of metribuzin is to inhibit photosystem II by binding to the 32 kD D1 protein (Hatzios and Penner, 1988). This is the same protein that is bound by both the triazine and phenylurea herbicides which are competitive with metribuzin for inhibition of this site (Buman *et al.*, 1992). While all three classes are competitive, they bind the protein at slightly different domains (Arntzen *et al.*, 1982). This is made apparent by the observation that mutations of the D1 protein leading to triazine resistance usually confer

lower levels of resistance to metribuzin (Arntzen *et al.*, 1982). Chloroplasts from triazine resistant biotypes of *Amaranthus* sp., *Chenopodium album*, *Brassica campestris* and *Ambrosia artimesifolia* are respectively 290, 33, 250 and 21 times less sensitive to inhibition by metribuzin than those from susceptible biotypes. Chloroplasts from the same biotypes vary from being 500 to 1000 times less sensitive to triazines. The variation in degree of resistance between the biotypes suggests that the resistant biotypes may have had other biochemical differences beside that of the mutated D_1 protein (Arntzen *et al.*, 1982). The use of intact chloroplasts, for example, may have exacerbated this effect by introducing herbicide movement through the chloroplast membrane as a potential variable. In contrast to the four examples noted above, is a biotype of *Amaranthus retroflexus* in which chloroplast electron transport is 250 times less sensitive to atrazine but 1500 and 40 times less sensitive to metribuzin and metamitron respectively (Oettmeier *et al.*, 1982). These data indicate that there is some variation in the effects of triazine resistant-target site mutations on the binding of metribuzin, however, in the absence of molecular characterisation of the psb A gene in these biotypes it is not clear whether these result from the same or different mutations. Given that all of the triazine resistant mutations identified thus far in plants have involved a serine for glycine mutation at position 264 it appears likely that alternative explanations will be necessary to explain the variation in PS II sensitivity in triazine resistant biotypes (Darmency and Gasquez, 1990).

Mutations of the D_1 protein with a high level of resistance to metribuzin have been identified in *Chlamydomonas reinhardtii* (251, Ala for Val, 1000 x) and *Anacystis nidulans* (264 Ser for Ala, 5000 x) (Mazur and Falco, 1989). In both cases these mutations have relatively little effect on inhibition by atrazine or diuron. Thus, the different effects of mutations of the D1 protein on metribuzin sensitivity suggest that metribuzin binds both a unique domain in the protein and a domain in common with the triazines and ureas (Arntzen *et al.*, 1982)

Metribuzin Metabolism

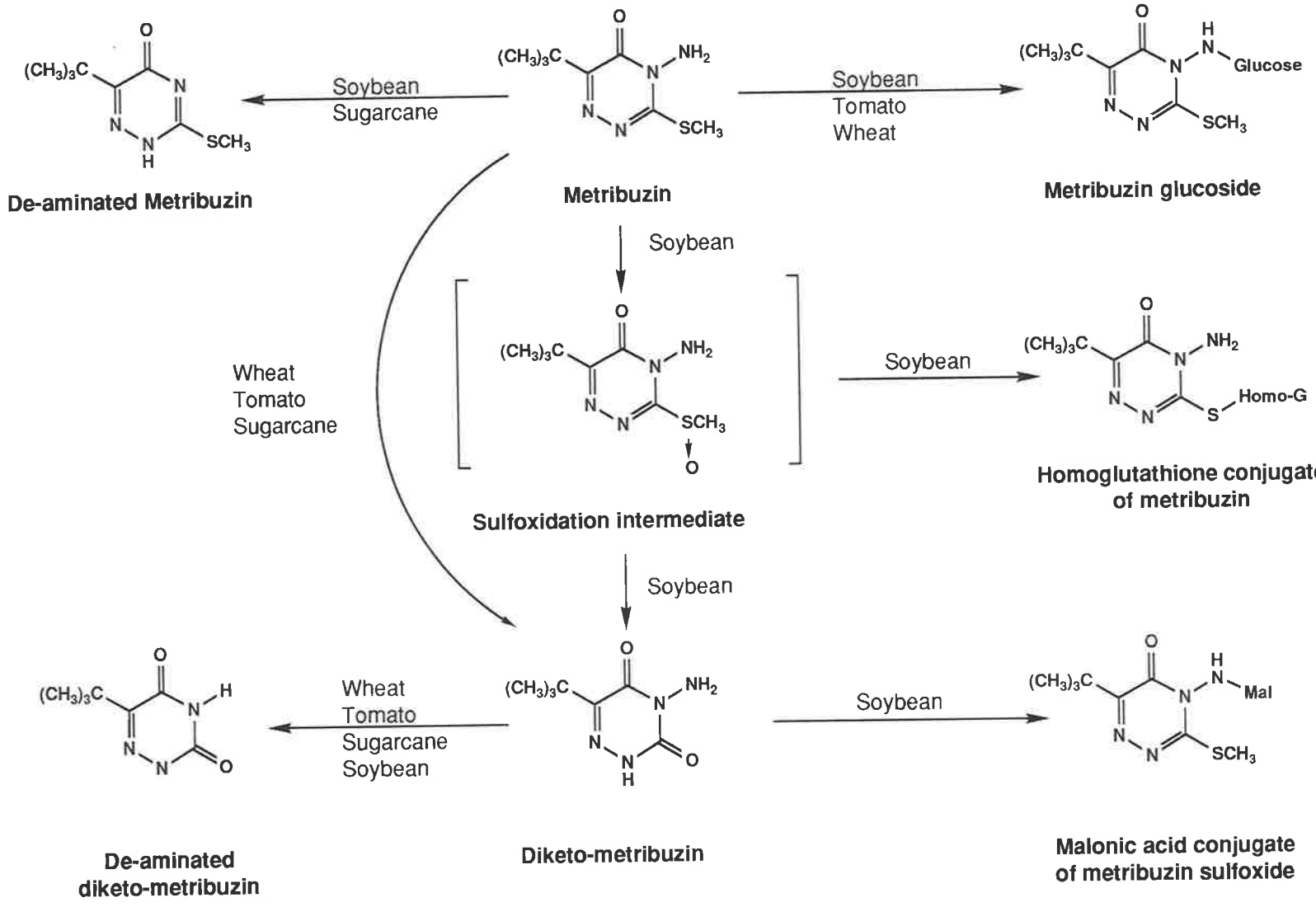
A major restriction on the use of metribuzin is variation in crop tolerance to this herbicide.

Cultivars of the same species may differ in their ability to tolerate metribuzin, which in turn, complicates the use of the product. In many cases these differences are based on differential metabolism of metribuzin. Metribuzin metabolism by crop plants has been studied in *Solanum* sp., barley, wheat, sugarcane and *lycopersicon* sp. The major metabolic pathways of metribuzin metabolism are illustrated in Figure 5.1.

The primary detoxification reactions include deamination, sulfoxidation and glucose conjugation. Deamination occurs both photochemically and biologically. *In vitro* this reaction is mediated by mixed function oxidase enzymes extracted from mammalian tissue but this has not been satisfactorily demonstrated for plants for which there is strong evidence for the participation of peroxysomal enzyme systems (Hatzios and Penner, 1988). The product of sulfoxidation is often referred to as diketo-metribuzin and its production is also thought to be mediated by mixed function oxidase enzymes (Bleeke and Casida, 1984). Metribuzin may undergo both primary reactions to yield deaminated-diketo-metribuzin in wheat, barley, soybean and sugarcane (Hatzios and Penner, 1988). These are followed by secondary reactions such as conjugation to glycosides. The conjugated residue varies between species. In tomato cells, glucose is directly conjugated to metribuzin via an N-glucosyltransferase (Davis *et al.*, 1991). Differential tolerance to metribuzin between some tomato cultivars is due to a difference in the ability to perform this conjugation. In soybean a major detoxification reaction is sulfoxidation followed by glutathione conjugation resulting in a non-toxic metabolite (Frear *et al.*, 1985). There is, however, little consensus on the primary mechanism of metribuzin detoxification in soybean with different cultivars appearing to have different primary metabolites (Hatzios and Penner, 1988).

Metribuzin tolerance also varies between cultivars of barley. Barley cultivar Steptoe is metribuzin resistant while cultivar Morex is susceptible. While there is some difference in the foliar absorption of metribuzin by these cultivars (Gawronski *et al.*, 1986a) the major difference between the two is their ability to detoxify metribuzin (Gawronski *et al.*, 1987). Differences in metribuzin sensitivity between soybean (*Glycine max*) cultivars is also due to

Figure 5.1. The various pathways of metribuzin metabolism in different plant species (redrawn from Hatzios and Penner, 1988).


Soybean
Sugarcane
Soybean
Tomato
Wheat
Glucose
De-aminated Metribuzin
Metribuzin
Metribuzin glucoside
Soybean
Wheat
Tomato
Sugarcane
Soybean
Homo-G
Sulfoxidation intermediate
Homoglutathione conjugate
of metribuzin
Soybean
Wheat
Tomato
Sugarcane
Soybean
Mal
De-aminated
diketo-metribuzin
Diketo-metribuzin
Malonic acid conjugate
of metribuzin sulfoxide

differential detoxification. In soybean cv Tracy M this detoxification activity is controlled by a single recessive gene which confers a ten-fold increase in resistance over susceptible background (Hartwig *et al.*, 1980). It is evident that many different pathways of metabolism have been identified in soybean (Figure 5.1) but the relative importance of these appears to vary between reports. This might be due to differences between cultivars or it may be determined by the *in vivo* metribuzin concentration during an experiment (Hatzios and Penner, 1988). Irrespective to their importance it is apparent that metribuzin is susceptible to many different metabolic reactions in this species.

Metribuzin resistance in soybean may be reduced by the application of organophosphorous insecticides (Waldrop and Banks, 1983). These appear to prevent the metabolism and increase the uptake of metribuzin (Waldrop and Banks, 1983). Tridiphane, an inhibitor of glutathione-S-transferase also inhibits metribuzin metabolism in soybean, presumably due to the reliance on glutathione conjugation in this species although tridiphane may have other effects (Gaul *et al.*, 1985). Metribuzin metabolism may also be prevented indirectly by the synergist picolinic acid t-butyl amide. This compound increases the toxicity of metribuzin in *Ipomoea hederacea* L. Jacq. by preventing the deamination of metribuzin *in vivo* (Klamroth *et al.*, 1989). Inhibition of deamination also affects the level of conjugates detected suggesting that conjugation is a secondary step following deamination in this species. Metribuzin is also metabolised by other weed species including *Bromus tectorum* L., however, the rate of metabolism is still lower than that of wheat. Differential metabolism of metribuzin is the major mode of selectivity of metribuzin when controlling *B. tectorum* in wheat (Devlin *et al.*, 1987a).

Thus differential metabolism accounts for many of the differences in sensitivity observed between plant species and varieties. The mechanisms of metribuzin metabolism vary between species and occasionally within cultivars. These have not been fully characterised in all cases but they include oxidative and conjugation reactions. Differential metabolism is, therefore, a major mechanism of crop tolerance and metribuzin selectivity.

Metribuzin uptake and translocation

Metribuzin is taken up both via the foliage and the soil. The amount of foliar absorbtion varies between species and may be affected by environmental conditions (Hatzios and Penner, 1988). The metribuzin susceptible cultivar of barley, Morex, absorbs more metribuzin through foliage than the resistant cultivar, Steptoe, however, uptake via the roots is similar for the two cultivars (Gawronski *et al.*, 1986). Similarly, *B. tectorum* (downy brome) absorbs more metribuzin than winter wheat (Devlin *et al.*, 1987b). In both of these cases of differential tolerance, differences in uptake were combined with enhanced metabolism of the herbicide. Metribuzin taken up through the foliage is not translocated basipetally suggesting that metribuzin entering newly emerging leaves must be taken up from the roots. In this respect it is similar to the triazines and phenylureas.

Metribuzin uptake from the soil varies with soil conditions. It is weakly adsorbed by clay and organic matter (Harper, 1988) but this depends on soil pH. Under high soil pH, crop injury may occur due to leaching of metribuzin into the root zone with concomitant loss of metribuzin from the shallower surface soils leading to poor weed control. Under conditions of low pH, sorption to clay is higher and again poor weed control will result (Hatzios and Penner, 1988). These data demonstrate that soil placement and soil type will contribute to the selectivity of metribuzin.

5.2 Metribuzin Cross-Resistance in *L. rigidum*

As noted in the introductory Chapters there are two biotypes of *L. rigidum* (WLR2 and VLR69) that are cross-resistant to metribuzin. These biotypes have no history of metribuzin application but do have in common a history of exposure to atrazine and resistance to the phenylurea herbicides. Cross-resistance to metribuzin is unusual and its occurrence in these two biotypes suggests the possibility of a similar resistance mechanism affecting all three classes of PS II inhibitor. The objective of this study was, therefore, to establish the mechanism of cross-resistance to metribuzin in *L. rigidum* and compare it with the

mechanisms of resistance to the other classes of inhibitor.

5.3 Materials and Methods

Dose response to metribuzin

Plants were maintained and herbicides applied under the same conditions as reported in Chapter two. Mortality was determined 24 days after treatment. Plants were scored as dead if they had no green tissue remaining.

Thylakoid extraction and measurement of electron transport

Thylakoids were prepared and O_2 evolution measured as described in Chapter three with metribuzin substituted for simazine.

Metribuzin metabolism and uptake

Plants used in metribuzin metabolism studies were grown under the same conditions as those described for studies of simazine and chlorotoluron metabolism in these biotypes. Seedlings were transferred to vials as previously described. In most cases, experiments with metribuzin were performed at the same time as experiments with simazine or chlorotoluron.

Nutrient solution containing 50 μM [^{14}C]metribuzin (specific activity 48 or 25 MBq mmol^{-1}) was added to drained vials. Plant roots were maintained in the [^{14}C]metribuzin solution for 4 h during the light period in the growth room (when present, the final concentration of ABT in the dosing solution was 320 μM). Vials were then drained and roots were washed 4 times with 10 mL aliquots of deionised water after which nutrient solution was added to the vials. Plants were harvested up to 36 h after the initiation of the treatment period. Roots were washed and separated from the shoots. Fresh weight of both shoots and roots was recorded and tissue wrapped in aluminium foil, frozen in liquid nitrogen and stored at -20°C. Metribuzin uptake was calculated from the total extractable radioactivity of plants used in

metabolism studies. Recovery of radioactivity was the same for all biotypes.

Extraction, separation, and quantification of metabolites

After application of metribuzin plants were harvested at regular intervals and extracted using methanol:water (4:1 v/v) as previously described.

Metabolites were separated and quantified by reverse phase HPLC. HPLC was performed on a Brownlee Labs ODS-5A, 250 x 4.6 mm column. The mobile phase was acetonitrile, water and a constant 0.1 % (v/v) acetic acid. Metabolites were eluted with a 5 % to 95 % acetonitrile gradient over 24 minutes at a column temperature of 39°C and flow rate of 1 mL min^{-1}. A different separation protocol with a gradient from 5 to 100 % acetonitrile in 20 minutes provided the chromatogram in Figure 5.9E. Metribuzin metabolites were detected using an on-line HPLC radioactivity monitor (Berthold LB504) with a solid, cerium-activated glass scintillant cell (Berthold, G650U4). Metabolites were quantified as a proportion of the total radioactivity injected onto the column.

Effect of ABT in combination with metribuzin in nutrient solution

Plants were grown hydroponically and placed in vials as above. Treatments applied to the plants were as follows: metribuzin (0.8, 1.6 and 3.2 μM), ABT (70 μM) and ABT (70 μM) in combination with metribuzin at various concentrations. The control and all treatments contained 0.1 % (v/v) ethanol. Treatments were replicated three times and the vials were randomly distributed within the experimental area. Plants were maintained in these solutions for 7 d after which the roots were washed twice with deionised water, returned to nutrient solution for a further 7 d before harvest and fresh and dry weight measurements.

Extended metribuzin uptake experiments

Hydroponically grown plants were placed in vials as above. Their roots were immersed in

nutrient solution containing 0.4 μM [^{14}C]metribuzin (spec. act. 0.46 MBq mmol^{-1}) at the commencement of the light period of day 1 of the experiment. Harvests were made 24, 48, 72, 96, 120, 144, 168, 192 and 216 hours after initiation of the dosing period. Nutrient solution containing [^{14}C]metribuzin was added daily to replace evapotranspiration losses. Treatments were replicated with the addition of ABT (70 μM).

5.4 Results and Discussion

Dose response to herbicides

The resistant biotypes WLR2 and VLR69 are approximately 8 to 9 times more resistant to metribuzin than is the susceptible, VLR1, based on a comparison of estimated LD_{50} values (Figure 5.2, Figure 5.3). The degree of resistance to metribuzin is similar to the degree of resistance to simazine and chlorotoluron in these biotypes when tested under similar circumstances. VLR69 tends to be slightly more resistant than WLR2 which is also consistent with results with the other herbicides. The rates of metribuzin required to reach the LD_{50} are, however, 5 to 10 times lower than rates of chlorotoluron or simazine required for the same effect. This may be partly explained by the greater affinity of the target site for metribuzin compared with triazines, slower metabolism and more efficient uptake due to foliar absorbtion (Hatzios and Penner, 1988).

The absence of prior selection by metribuzin makes the resistance of these biotypes to metribuzin an instance of cross-resistance. Small changes in sensitivity to metribuzin have been reported in other *L. rigidum* biotypes but they were less than two-fold (Heap, 1988). The occurrence of cross-resistance is of interest because metribuzin has been mooted as an alternative herbicide to control resistant populations of *L. rigidum*.

Figure 5.2. The response of the susceptible biotype VLR1(●) and the resistant biotypes WLR2 (O) and VLR69 (✕) to metribuzin applied to 3-leaf plants growing in pots. Each point is the mean of mortality determined in three individual pots each containing 12 plants and is plotted with standard error.

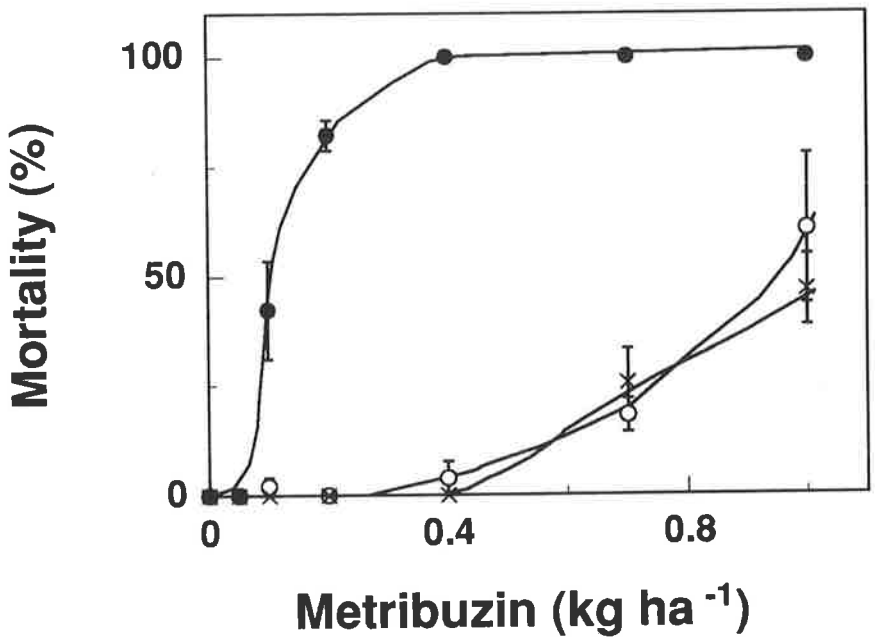

100
50
0
Mortality (%)
0
0.4
0.8
Metribuzin (kg ha -1)

Figure 5.3. The effect of 200 and 400 g ha^{-1} metribuzin on growth of WLR2 (front row) and a susceptible biotype (back row) compared with unsprayed controls 14 days after spraying

Figure 5.4. The effect of ABT in combination with metribuzin compared with metribuzin alone on growth of WLR2 in nutrient solution. (See Figure 3.1 for the untreated and ABT alone controls use in this experiment)


CONTROL
METRIBZIN
200 g a.i. /ha
METRIBZIN
400 g a.i. /ha
CONTROL
RESISTANT
METRIBUZIN
200 g a.i. /ha
RESISTANT
METRIBUZIN
400 g a.i. /ha
RESISTANT

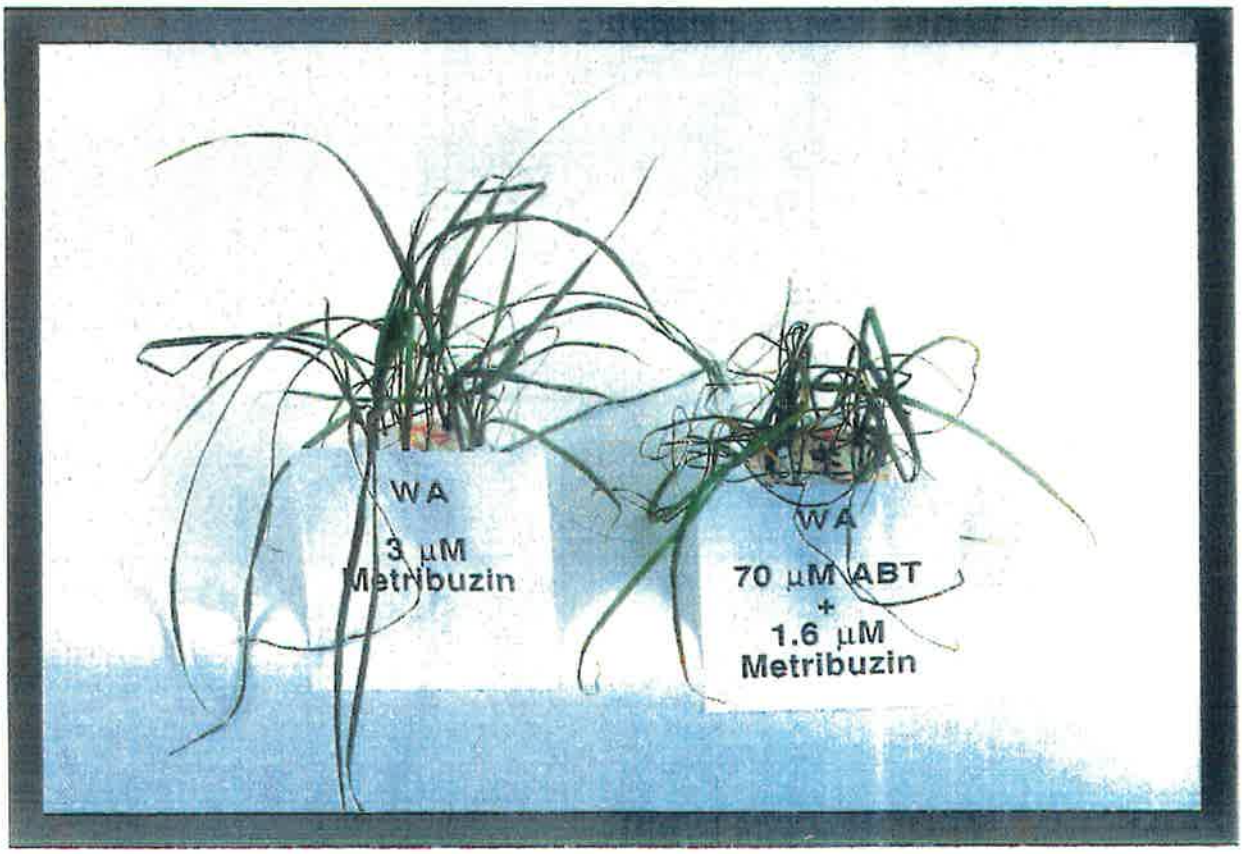
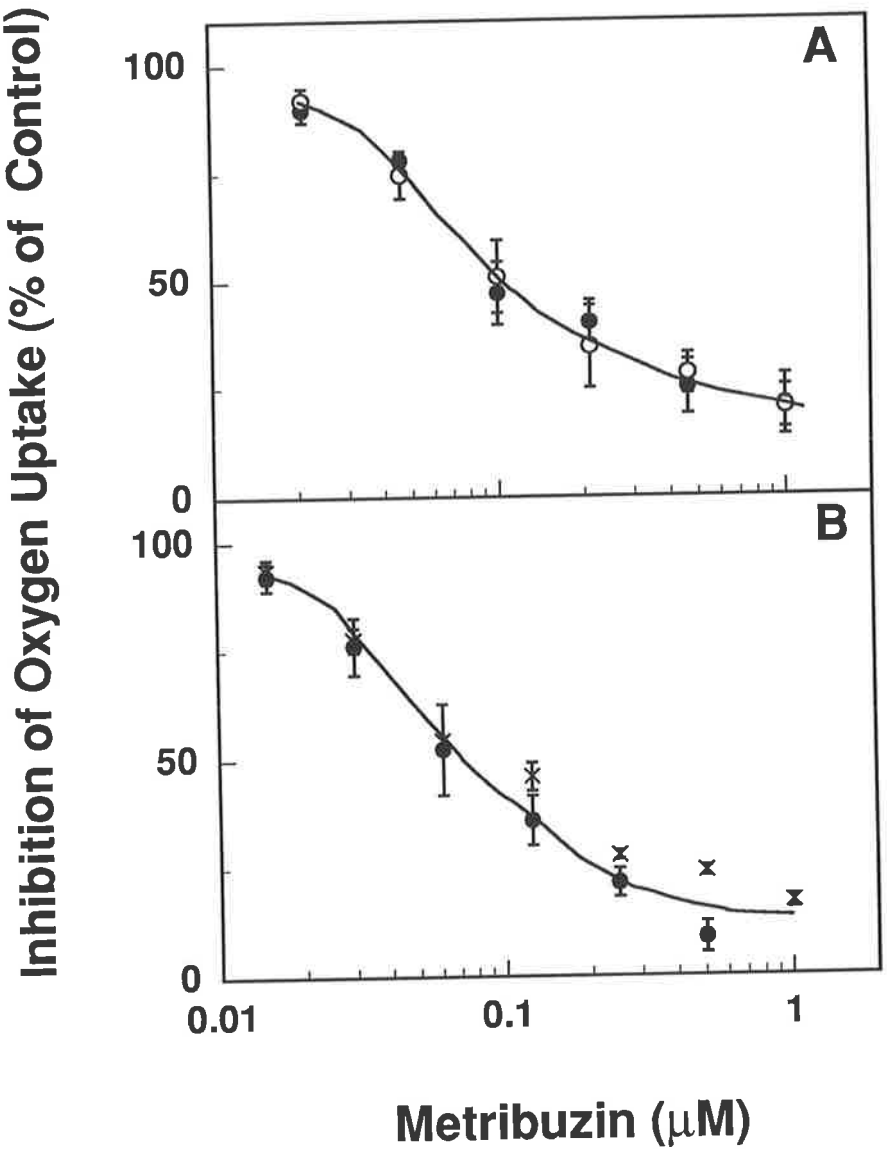

WA
3 µM
Metribuzin
WA
70 µM ABT
+
1.6 µM
Metribuzin

Figure 5.5. The effect of metribuzin on oxygen evolution by thylakoids isolated from: A, the susceptible biotype VLR1 (●) and the resistant biotype WLR2 (O), and B, the susceptible biotype VLR1 (●) and the resistant biotype VLR69 (✕). Rates are expressed as a percent of control which was at least 230 μmol O_2 mg chl^{-1} h^{-1} with similar rates for each biotype. Each point is the mean of four replicates and is plotted with standard error.


Inhibition of Oxygen Uptake (% of Control)
100
50
0
A
100
50
0
B
0.01
0.1
1
Metribuzin (μM)

Target site inhibition

Inhibition of oxygen uptake by metribuzin *in vitro* was similar for thylakoids isolated from WLR2, VLR69 and VLR1 leaves (Figure 5.5). Metribuzin resistance in WLR2 and VLR69 is, therefore, not due to a change in the characteristics of the D1 protein of PS II. This is a similar result to that obtained for the herbicides atrazine, simazine, diuron and chlorotoluron (Chapters three and four; Burnet *et al.*, 1991). These data indicate that resistance is not directly related to the site of action in PS II. The mechanism of metribuzin resistance must, therefore, either prevent the herbicide reaching the target site or reduce the concentration of metribuzin at the target more rapidly than in susceptible plants.

Metribuzin uptake

Metribuzin uptake from nutrient solution by intact seedlings over a 4 h period for the susceptible VLR1 and resistant WLR2 and VLR69 biotypes was 1.67, 1.50 and 1.69 nmol (g shoot FW)$^{-1}$ respectively (Table 5.1). VLR1 plants had similar or slightly higher uptake of metribuzin in all experiments (see also Figure 5.9E). The proportion of the total absorbed radioactivity found in the shoots was similar for all biotypes (Table 5.1).

Differential uptake of metribuzin through foliage is observed in some crop species and may be a factor in crop tolerance of the herbicide (Gawronski *et al.*, 1986). Foliar uptake was not tested in *L. rigidum* because the biotypes express resistance when treated via nutrient solution. Preliminary experiments indicated that the metribuzin LD_{50} for WLR2 was at least 2 fold greater than that of VLR1 in nutrient solution. The similar uptake from nutrient solution by all biotypes indicates that resistance is due to mechanisms acting after metribuzin has entered the leaves and not to barriers to uptake and translocation

The effect of ABT in combination with metribuzin on growth

An inhibitor of oxidative enzymes, 1-aminobenzotriazole (ABT), was able to intensify the symptoms of metribuzin toxicity in the resistant biotypes (Figure 5.4). ABT (70 μM) had no effect on plant growth when applied alone, both in this case and those previously

Table 5.1. The amount of metribuzin taken up after 4 hours exposure to nutrient solution containing 50 μM [^{14}C]metribuzin (25 MBq mmol^{-1}). Values are the means of 4 replicates recorded with the standard error.

Biotype	Metribuzin taken up * (nmol g FW^{-1})	Proportion in leaf tissue* (%)
VLR1	1.67 a ± 0.25	98.3 a ± 0.4
WLR2	1.50 a ± 0.11	98.7 a ± 0.6
VLR69	1.69 a ± 0.26	97.0 a ± 1.0

*Figures followed by the same letter are not significantly different at the 0.95 level of significance according to a t-test.

Figure 5.6. The effect of metribuzin alone (O) and in combination with ABT (●) on dry weight of hydroponically grown WLR2 (A) and VLR69 (B) plants. Each point is the mean of three replicates. Data is representative of one of two experiments.

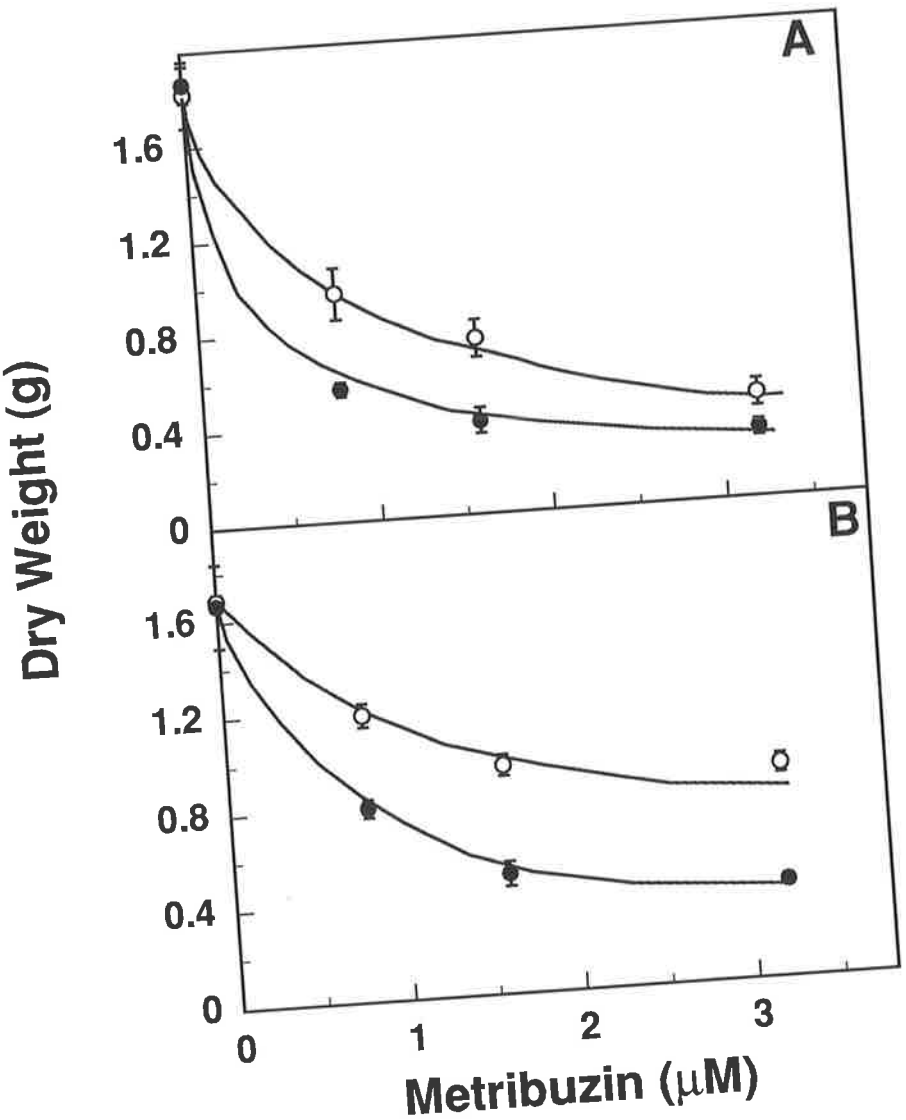
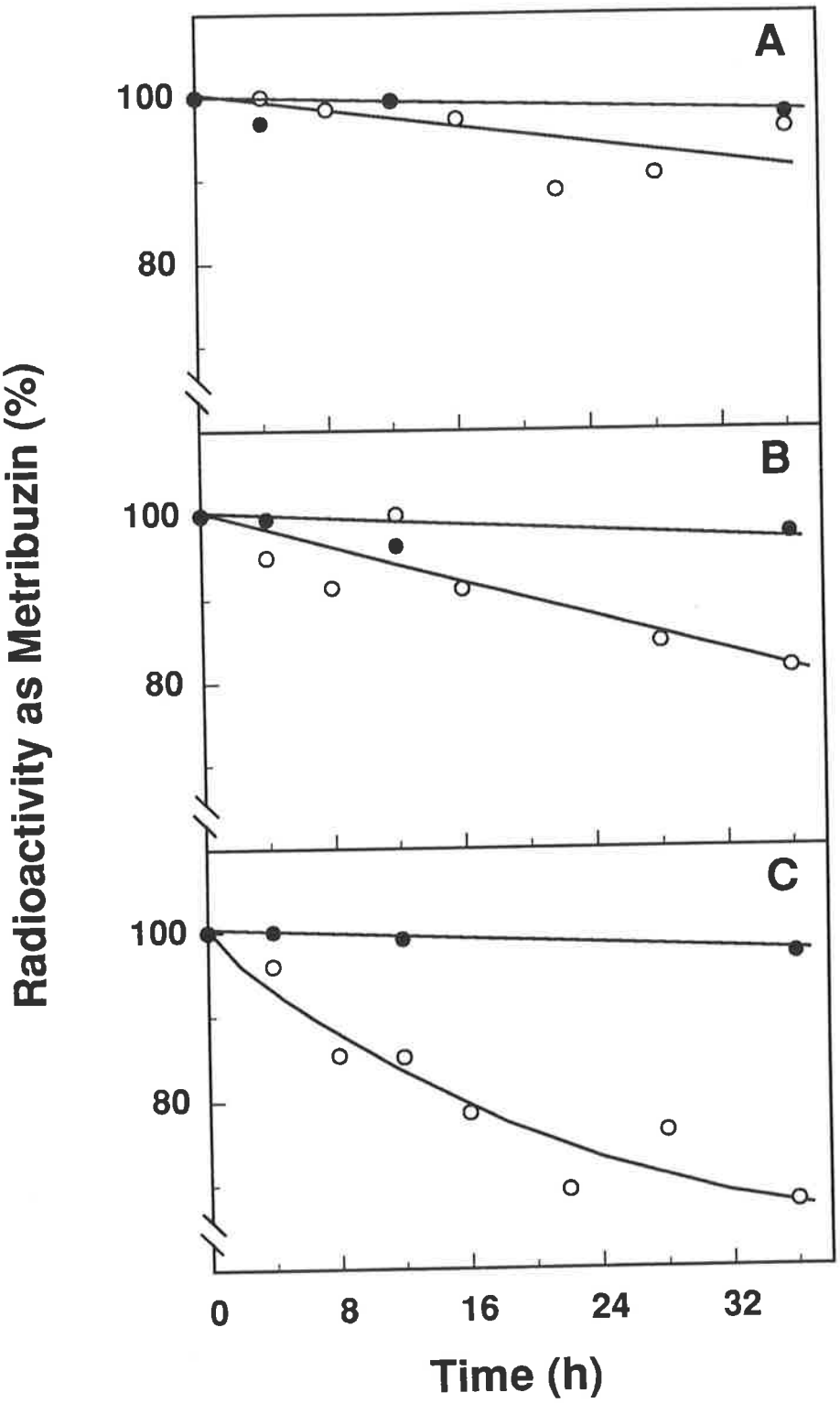

A
B
Dry Weight (g)
1.6
1.2
0.8
0.4
0
0
1
2
3
Metribuzin (μM)

Figure 5.7. The metabolism of [^{14}C]metribuzin in the presence (●) and absence (O) of ABT for VLR1 (A), WLR2 (B) and VLR69 (C). Metribuzin, was applied to the roots of plants for 4 h after which there was no further input of either material. Data is from one of three experiments and is representative of these.


A
B
C
100
80
100
80
100
80
0
8
16
24
32
Time (h)
Radioactivity as Metribuzin (%)

reported (Chapters three and four). When applied in combination with metribuzin, the dry weight of the shoot tissue of resistant plants was reduced compared with metribuzin alone (Figure 5.6). Given that ABT can inhibit the metabolism of metribuzin (see later sections) and that the symptoms observed were those associated with photooxidation, these data suggest that detoxification of metribuzin plays a role in the mechanism of resistance to the herbicide.

Metribuzin detoxification

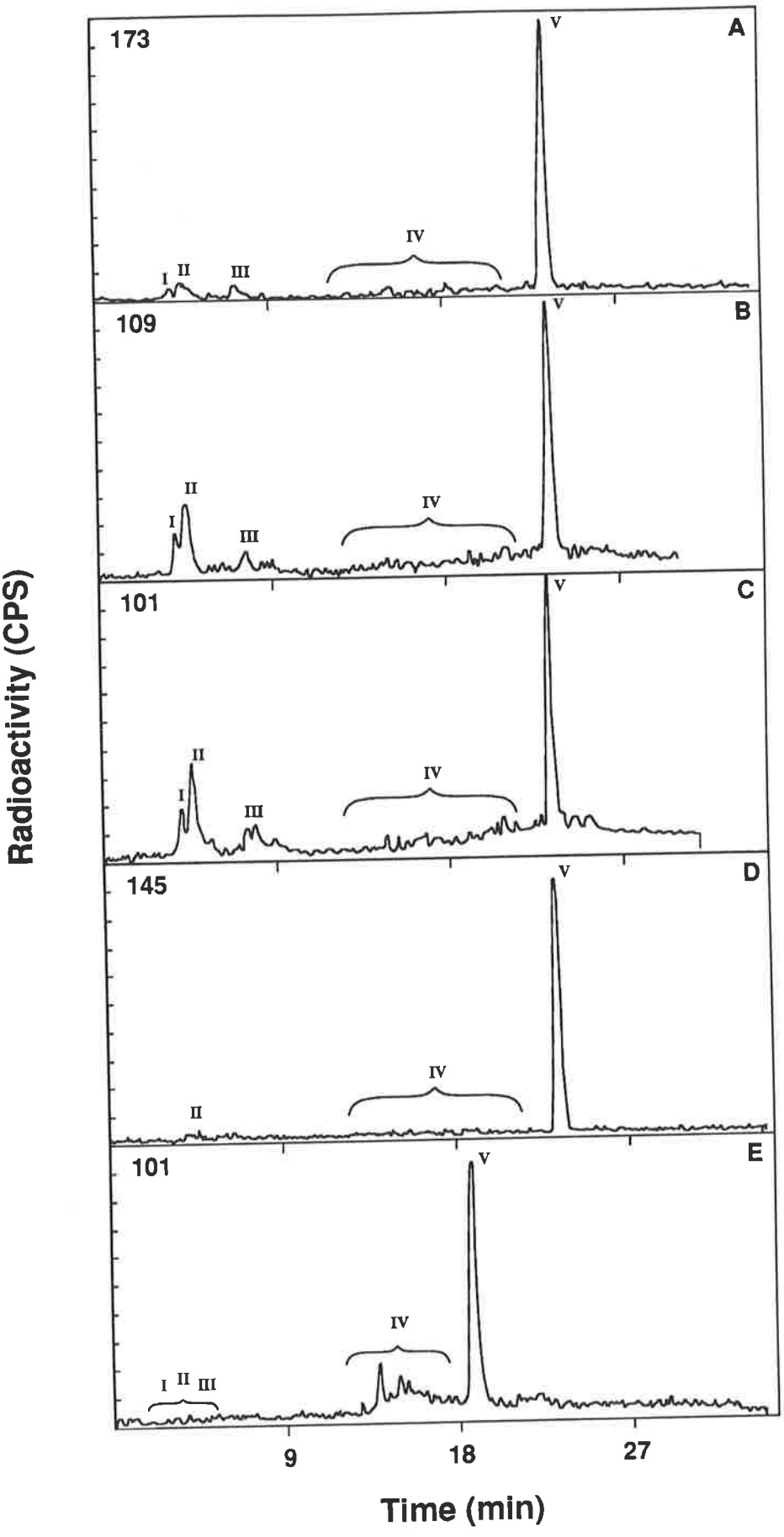
Metribuzin was metabolised to a limited extent in all *L. rigidum* biotypes. The resistant biotypes, VLR69 and WLR2, metabolised metribuzin to a greater extent than the susceptible, VLR1, however, none of the biotypes detoxified more than 35% of the herbicide dose by the end of the treatment period (Figure 5.7, Figure 5.8). While there exists a difference in the rate and extent of metabolism between the resistant and susceptible biotypes there also remains a large amount of extractable unmetabolised metribuzin in the shoot tissue of all biotypes. Binding of metribuzin in an unmetabolised form to insoluble plant components has been observed in other graminaceous species including wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and *Bromus tectorum* L (Gawronski *et al.*, 1987; Devlin 1987b). While the metribuzin remains methanol extractable in *L. rigidum* it is possible that it may be less available for metabolism, either through adsorption to the cell wall or sequestration to the vacuole, leading to the high levels of metribuzin remaining in the tissue.

The metabolites of metribuzin in *L. rigidum* extracts were not identified. Comparison of the chromatograms of extracts from each *L. rigidum* biotype indicate that the metabolites being produced had similar retention times (Figure 5.8A, B and C). The metabolites could be broadly categorised into the polar and the non-polar metabolites. There were three major polar metabolites in *L. rigidum* (I,II and III) and a number of poorly resolved minor peaks that are collectively labelled "IV" in Figure 5.8. Metribuzin metabolites extracted from wheat (cv. Blade) fell into the same two groups as those extracted from *L. rigidum*, however, there was a greater proportion of non-polar (IV) metabolites in the wheat extract (Figure 5.8E).

Figure 5.8A, B, C. Chromatograms of metribuzin and metabolites extracted from VLR1 (A), WLR2 (B) and VLR69 (C) plants exposed to 0.4 μM metribuzin for 168 h under the same conditions as those reported for Figure 5.9. The polar metabolites are indicated by the roman numerals: I, II, and III, the non-polar metabolites are collectively indicated by the numeral IV while metribuzin is indicated by the numeral V.

Figure 5.8D. Chromatogram of metribuzin and metabolites extracted from VLR69 plants exposed to 0.4 μM metribuzin for 168 h in combination with 70 μM ABT. This chromatogram provides part of the data for Figure 5.9D.

Figure 5.8E. Chromatogram of metribuzin and metabolites extracted from cut leaves of wheat cv. Blade, exposed to 400 μM metribuzin (spec. act. 40 MBq $mmol^{-1}$) for 3 h and harvested 33 h later. The extract was eluted with a gradient from 5 to 100 % acetonitrile in 20 minutes and was 4 min shorter than that used in the previous four chromatograms, hence the shortened retention times of metribuzin and metabolites. Allowing for the shorter retention times, metabolites broadly corresponding to those of *L. rigidum* are indicated with the same numerals.


173
A
109
B
101
C
145
D
101
E
I
II
III
IV
V
Radioactivity (CPS)
9
18
27
Time (min)

The reactions that occur in wheat are indicated in Figure 5.1. These include; deamination and conjugation with glucose or sulfoxidation and subsequent deamination. The polarity of the metabolites observed in *L. rigidum* may suggest that they are conjugates but this has not been investigated.

The addition of ABT (320 μM for 4 h) to the dosing solution inhibited the metabolism of metribuzin in all three biotypes of *L. rigidum* (Figure 5.7, see also, Figure 5.8D, Figure 5.9B, C and D). The sensitivity of metribuzin metabolism to ABT was sufficient to prevent detoxification in all biotypes for the duration of the experimental period. The effect of ABT on metribuzin metabolism suggests that the mechanism of resistance may be based on an oxidative reaction. The only such reaction with metribuzin that has been clearly demonstrated to occur in plants is sulfoxidation (Frear *et al.*, 1985). Thus sulfoxidation may be an important reaction in *L. rigidum*. This reaction has been shown to occur in wheat which also appears to have some metabolites in common with *L. rigidum*, thus supporting this proposition.

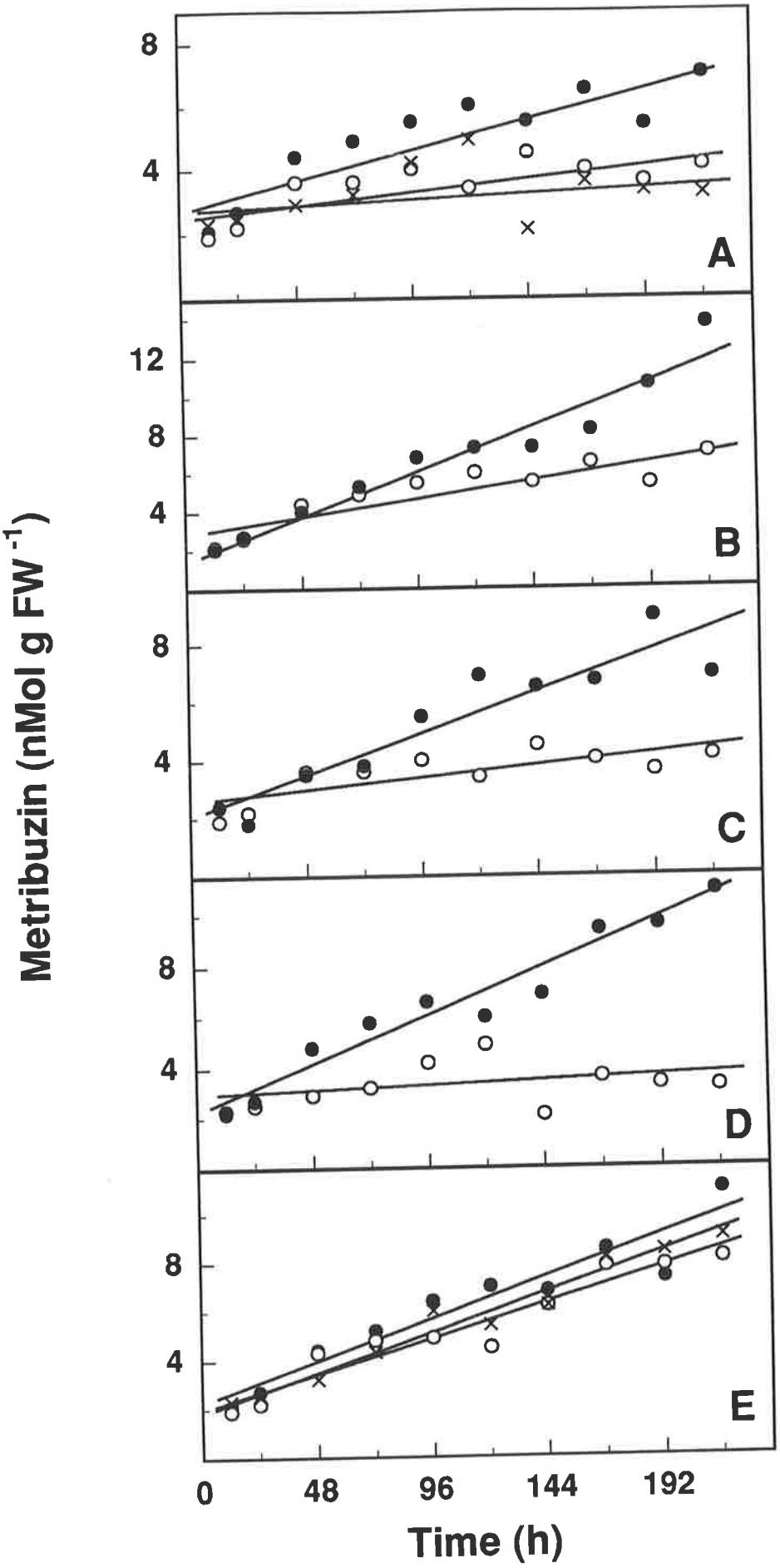
Metabolism of metribuzin under continuous exposure.

Given the low rate of metribuzin metabolism in the pulse chase experiments, metribuzin metabolism was examined using conditions similar to those used to test the interaction between ABT and metribuzin. Over nine days continuous exposure to 0.4 μM [^{14}C]metribuzin the susceptible biotype, VLR1, accumulated a greater amount of metribuzin than the resistant plants (Figure 5.9A). Uptake of metribuzin over this period was linear with slightly higher uptake in the susceptible plants (Figure 5.9E). If ABT (70 μM) was included in the nutrient solution all biotypes accumulated greater amounts of metribuzin while taking up the same amounts of radioactivity (Figure 5.9B, C, D). With the inclusion of ABT the resistant plants accumulated a similar amount of metribuzin to the susceptible plants treated with metribuzin alone. Given that ABT intensifies the effect of metribuzin in the resistant plants, this observation strengthens the connection between greater metribuzin metabolism and the metribuzin resistant phenotype. Similar conclusions were drawn from

Figure 5.9A. The amount of metribuzin in the shoot tissue of VLR1 (●), VLR69 (✕) and WLR2 (○) plants during 216 h of exposure to 0.4 μM [^{14}C]metribuzin.

Figure 5.9B, C and D. The accumulation of metribuzin in the tissues of the susceptible VLR1 (B), and the resistant biotypes WLR2 (C) and VLR69 (D) in the presence (●) and absence (○) of ABT.

Figure 5.9E: The cumulative uptake of metribuzin during 216 h exposure to 0.4 μM [^{14}C]metribuzin based on total extractable radioactivity for VLR1 (●), WLR2 (○) and VLR69 (✕). Data is from one of two experiments and is representative of these


Metribuzin (nMol g FW^{-1})
8
4
A
12
8
4
B
8
4
C
8
4
D
8
4
E
0
48
96
144
192
Time (h)

observations of the interaction between ABT simazine and chlorotoluron in these biotypes.

5.5 Conclusions

The resistant biotypes WLR2 and VLR69 have different histories of selection pressure but they are both resistant to triazines, phenylureas and metribuzin. In neither case were they exposed to metribuzin yet both exhibit cross-resistance to this herbicide. The degree of resistance to metribuzin is similar to the degree of resistance to simazine and chlorotoluron in these biotypes, and, in common with the other herbicide classes, metribuzin resistance was not conferred by changes at the target site or by differences in herbicide uptake. Like resistance to simazine and chlorotoluron, however, there is enhanced metabolism of the herbicide. In all cases this metabolism may be inhibited by the mixed-function-oxidase inhibitor ABT. ABT is thought to inhibit many different types of oxidative enzymes so its effect cannot be considered specific. ABT also intensifies the symptoms of all of these herbicides such that resistant plants respond in a similar manner to susceptibles treated with metribuzin alone. Under these conditions resistant plants treated with both ABT and metribuzin also contain similar amounts of the herbicide to the susceptibles treated with metribuzin alone. This suggests that the major difference between the resistant and susceptible plants is their ability to metabolise the herbicide.

Cross-resistance in these biotypes of *L. rigidum*, therefore, appears to be due to an increase in the ability of the plants to detoxify metribuzin. This capacity was probably conferred as a consequence of the selection pressure of atrazine or atrazine and diuron in the case of VLR69. The observation that adaptation to triazine herbicides through increased metabolism also resulted in an increase in metabolism of metribuzin suggests that there may be enzymes in common detoxifying both of these classes of herbicides. Alternatively changes in the regulation and expression of oxidative enzyme systems may also create these very broad changes in the potential for oxidative detoxification of herbicides. The implications of this are that the development of resistance to one herbicide may affect the

response to other unrelated herbicides through a general mechanism such as enhanced herbicide metabolism. Thus different chemical classes of herbicide which are vulnerable to similar oxidative reactions may be associated through cross-resistance.

Chapter Six

Amitrole Resistance in *L. rigidum*

6.1 Introduction

The following chapters describe experiments to elucidate the mechanisms of resistance to herbicides to which only one of the biotypes is resistant. This chapter will describe amitrole resistance in WLR2 and the following chapter will describe sulfonylurea resistance in VLR69.

History and applications

The synthesis of amitrole (3-amino-1,2,4-triazole, amino triazole) was first described in 1888 by Curtius and Lang (Sutherland, 1964). It was patented as a herbicide in 1954 by Amchem Products and it has been widely used in both industrial and agricultural applications. In particular, amitrole was found to be effective as a defoliant and regrowth inhibitor of cotton (Miller *et al.*, 1961). The extent of amitrole use has been limited by concerns over its effects on human health. In 1959 the New York Times published claims that amitrole caused thyroid tumours in rats leading to widespread public concern and the eventual deregistration of amitrole for use in food crops in the United States (Carter, 1975). The basis of this scandal, namely the supposedly carcinogenic properties of amitrole, was largely unfounded. Toxicological data for amitrole describe only to "thyroid symptoms" or goitre and make no mention of thyroid tumours. The enlargement of the thyroid is thought to be caused by the inhibition of a catalase-like enzyme, thyroid peroxidase, involved in the synthesis of thyroid hormone (Anderson, 1959; Castelfranco 1960). The sensitivity of this enzyme is such that although the acute LD_{50} for rats is 25000 mg kg^{-1}, dietary levels of only 1 mg kg^{-1} day^{-1} can be tolerated before thyroid symptoms are observed (Agrochemicals Handbook 1983).

Amitrole is registered in Australia for use on non-cropland, banana plantations, stone fruit orchards, vineyards, *Pinus radiata* plantations, and preplanting to wheat and barley. It is

commonly formulated with ammonium thiocyanate or with a residual herbicide like atrazine. The relative popularity of amitrole/atrazine mixtures in industrial applications is due to low cost. Where used in food crops a 56 day withholding period is required (Australian Weed Control Handbook 1987).

Chemistry

Amitrole is synthesised when equimolar quantities of aminoguanidine bicarbonate and formic acid are reacted at 100° to 120° C (Sjostedt et al 1955). The initial product is aminoguanidine formate, which condenses to amitrole with heating. The formic acid provides the 5-carbon and the ^{14}C label in the synthesis of [^{14}C]amitrole (See Figure 6.2 for structure of amitrole). The position of the carbon atom is important because the 5-carbon is lost during ring cleavage and thus label could be lost in some reactions. Amitrole is soluble in water, methanol, ethanol and chloroform, and insoluble in acetone and ether (Carter 1975). The polarity of amitrole allows convenient formulation as a solution in water.

Mode of action

The mode of action of amitrole, although extensively studied, has not been fully resolved. Amitrole has many sites of action. The relative importance of these differs in bacteria, fungi, algae, and higher plants. The most obvious symptom of amitrole toxicity in higher plants is the chlorosis of tissue initiated after application. This is referred to as "bleaching" because the tissue often turns white. Tissue that is already formed remains green following exposure to relatively low levels of amitrole, but at higher levels, the existing tissue is desiccated and the plant dies. Light plays a major role in the development of symptoms observed in higher plants and the inadequate control of light as an experimental variable has led to disagreement over possible modes of action. Given the importance of light, it is also likely that modes of action postulated from experiments on heterotrophs will not necessarily be important in autotrophs. An exception to this is the algae *Scenedesmus* which displays

normal chloroplast development even when grown heterotrophically in the absence of light. Such a system is perhaps the optimum means of separating the effects of light and enzyme inhibition (Sandmann and Albrecht, 1990).

Inhibition of Histidine Biosynthesis

Amitrole inhibits the growth of *Salmonella typhimurium, Escherichia coli,* and *Saccharomyces cerevisiae* (Hilton *et al.,* 1965). Growth inhibition of *S. cerevisiae* is reversed by the addition of histidine to the medium. A combination of adenine and histidine was required to restore growth in *S. typhimurium* and *E. coli.* A precursor to histidine, imidazole glycerol phosphate (IGP), accumulates in *S. cerevisiae* cells inhibited by amitrole and it was suggested that this resulted from the inhibition of IGP dehydratase (E.C. 4.2.1.19) (Klopotowski and Wiater, 1965). This inhibition was confirmed by Hilton *et al.* (1965) who demonstrated that amitrole has a ten times greater affinity for the enzyme than does IGP. Although amitrole inhibits IGP dehydratases from higher plants (Hilton, 1969) there is conflicting evidence for the role of this inhibition as a mechanism of toxicity. As found for microorganisms, exogenous histidine and adenine can overcome amitrole toxicity in cell cultures of *Cirsium arvense* (Hilton 1969). In contrast, exogenous histidine prevents the buildup of IGP in rose cell cultures without reducing amitrole toxicity, suggesting that inhibition of histidine biosynthesis was not the major cause of toxicity in this system (Hilton 1969). Similarly, exogenous histidine does not reduce amitrole toxicity to *Arabidopsis thaliana* seedlings (Heim and Larrinua, 1989). These and other results provide some indication that the mechanism of action of amitrole may differ between species and systems and generalisations about the mechanisms must, therefore, be limited.

Effects on purine metabolism

There is also evidence that adenine, riboflavin, serine, and methionine can influence the toxicity of amitrole to various species. These interactions have been reviewed by Hilton (1969), largely in the context of microorganisms. Hilton concluded that there are probably two sites of action in purine biosynthesis but these may be interrelated with the other effects

of amitrole. The effects of histidine and adenine on amitrole toxicity to algae depend on whether the algae are grown auxotrophically or heterotrophically (Carter 1975). In higher plants, amitrole had no significant effect on nucleic acid levels or the chloroplastic ribosomes when the plants were grown in the dark (Bartels *et al.*, 1967). In the presence of light these systems are disrupted and it is not clear whether amitrole is acting to "inhibit the production or promote the destruction" of these structures (Carter 1975). The latter possibility is considered most likely given their normal development in the absence of light. Thus, the effects on structures such as ribosomes are likely to be secondary effects.

Inhibition of catalase and other metalloproteins

The inhibition of catalase was one of the earliest reported mechanisms of amitrole toxicity (Margoliash et al 1959). This observation explained why catalase activities in the livers and kidneys of rats declined after injection of amitrole. Although this early work was conducted with mammalian catalase its applicability to higher plants has been confirmed by Feierabend and Kemmerich (1983) who demonstrated the inhibition of catalase in rye leaves by amitrole. Further support for the inhibition of catalase *in vivo* is found in the induction of glutathione production in amitrole treated barley. This effect is ascribed to the increase in H_2O_2 levels resulting from catalase inhibition by amitrole (Smith *et al.*, 1984) Inhibition of catalase may also explain the mild synergy evident between amitrole and atrazine (Figure 6.4A). Any disabling of a plants defences against free radical generation would intensify the effects of a herbicide blocking photosynthetic electron transport (Feierabend, 1984). Thus in mature leaves exposed to amitrole and a PS II inhibitor simultaneously, photooxidation may proceed more rapidly.

The mechanism by which amitrole inhibits catalase was further elucidated by Margoliash et al (1970). Amitrole is an irreversible inhibitor of the activated enzyme. The catalase-H_2O_2 complex I reacts with amitrole presumably forming a reactive amitrole-intermediate which then binds the imidazole nitrogen of the histidyl residue (position 74) of the protein, and not the prosthetic protohaem. In some respects this inhibition is similar to that observed for

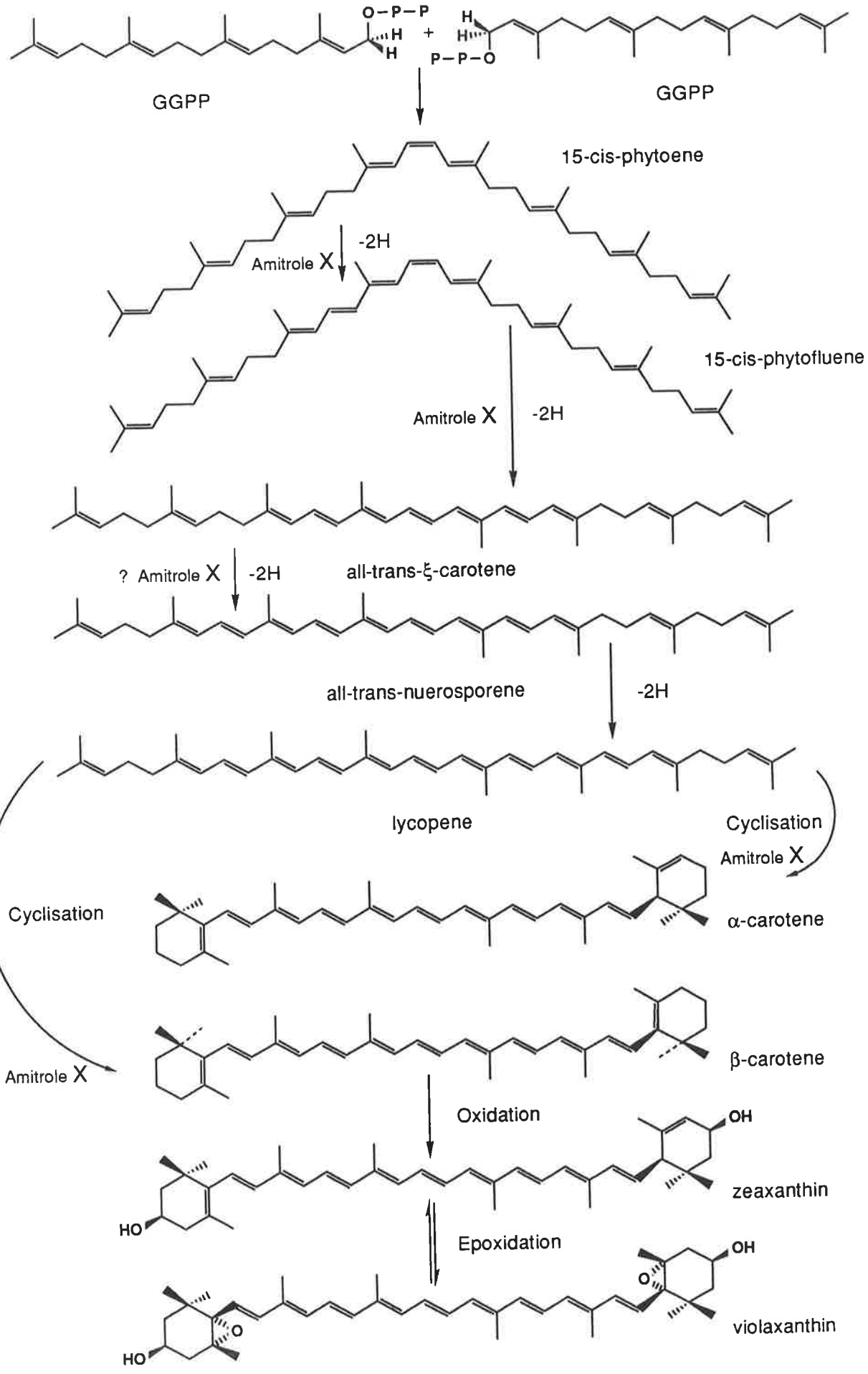
suicide substrates of cytochrome P-450 monooxygenase enzymes in which the activated substrate reacts with the protein causing irreversible inhibition (Ortiz de Montellano and Reich, 1986)

Amitrole also inhibits other metalloprotein enzymes such as tyrosinase, fatty acid peroxidase, thyroid peroxidase, superoxide dismutase; glutathione peroxidase and lactoperoxidase (Castelfranco 1960; Anderson, 1959; Simmons and Jamall, 1988). Hepatic cytochrome P-450 mixed function oxidase enzymes are also inhibited by amitrole *in vivo* and *in vitro*. Inhibition of the enzyme P_{450}IIE1 *in vitro* is time and NADPH dependent indicating that only the activated enzyme is susceptible to inhibition (Koop, 1990). Despite the activation requirement, radioactive amitrole is not incorporated into the protein or haem. This suggests that either the ^{14}C label at the 5-position is lost in the reaction or that amitrole causes irreversible binding between the haem and its own protein. Thus, amitrole appears to share some inhibitory properties with the 1,2,4-triazole analogues discussed in chapter one (Lenton, 1987). Compounds such as paclobutrazole (plant growth regulator) and triadimenol (fungicide) contain 1,2,4-triazole rings as in amitrole and inhibit cytochrome P-450 dependent mono-oxygenases. Inhibition by these compounds is due to the direct interaction between the azole nitrogen and the haem of the cytochrome P-450 (Lenton, 1987). Unlike the triazole fungicides, amitrole lacks the hydrophobic carbon backbone which determines specificity and affinity for particular enzymes. In comparison, amitrole consists only of the active triazole moiety. The simplicity of this structure may decrease its specificity but broaden its inhibitory potential through lack of steric hindrance.

Pigment Synthesis

Before discussing the effects of amitrole on pigment synthesis it is necessary to digress and describe the processes of carotenoid biosynthesis in plants. To assist in the discussion, the biosynthetic pathways are illustrated (Figure 6.1). The production of carotenoids begins with the linkage of two molecules of geranylgeranylpyrophosphate (GGPP) to form phytoene. Phytoene is then subject to a series of desaturation reactions forming

Figure 6.1. A summarised pathway of carotenoid biosynthesis in plants. Adapted from Ridley (1982), Goodwin (1980) and Goodwin and Mercer (1985).


O–P–P
H
H
+
H
H
P–P–O
GGPP
GGPP
15-cis-phytoene
Amitrole X
-2H
15-cis-phytofluene
Amitrole X
-2H
? Amitrole X
-2H
all-trans-ξ-carotene
all-trans-nuerosporene
-2H
lycopene
Cyclisation
Amitrole X
α-carotene
Cyclisation
Amitrole X
β-carotene
Oxidation
OH
zeaxanthin
HO
Epoxidation
OH
O
violaxanthin
O
HO

phytofluene, ξ-carotene, nuerosporene and finally lycopene. At each step two molecules of hydrogen are eliminated with the formation of double bonds (hence desaturation) (Goodwin and Mercer, 1985). Both the formation of phytoene and its subsequent desaturation are thought to be mediated by enzymes on the chloroplast envelope (Lütke *et al.*, 1982). Phytoene desaturase catalyses both the desaturation of phytoene and phytofluene which explains the susceptibility of both of these steps to the same inhibitors (Sandmann and Böger, 1989, cited in Chollet *et al.*, 1990). Desaturation is dependent on NADP and oxygen for activity indicating that it is mediated by an oxidative enzyme(s) (Clarke *et al.*, 1982). Lycopene is converted to specific carotenoids via cyclisation of the end groups. Three different end groups can be formed depending on the position of the double bond in the ring and these determine the nature of the carotenoid pigment. Xanthophylls are produced by hydroxylation of the carotene rings (Goodwin, 1980). Two xanthophylls, violoxanthin and zeoxanthin undergo a cyclic, light dependent interconversion across the thylakoid membrane known as the xanthophyll cycle (Goodwin, 1980). The reaction is summarised in Figure 6.1. It is driven by the proton gradient established during photosynthesis and requires NADPH and O_2 for epoxide formation. This cycle, therefore, represents another oxidative reaction in pigment synthesis.

While this explanation of carotenoid synthesis is simplified, it is apparent that the sequential nature of the reactions will mean that inhibition at one point will affect the levels of many other pigments further down the pathway. Inhibition of pigment synthesis may also reduce the production of abscisic acid (ABA) which is derived from carotenoids (Parry and Horgon, 1992). Specific inhibitors of carotenoid synthesis, such as norflurazon, cause the accumulation of phytoene only, due to the complete inhibition of phytoene desaturase. This prevents the formation of any other pigments from this pathway leading to bleaching and growth responses associated with reduction in ABA synthesis (Schiff *et al.*, 1982). Unlike norflurazon, amitrole appears to be less specific and less potent, only partially inhibiting many of these enzymes. For this reason, its mode of action has been more difficult to define. The operating hypothesis in this review is that amitrole is a non-specific inhibitor of

certain metalloprotein enzymes such as catalases and mono-oxygenase and that the effects on pigment synthesis are a consequence of its inhibitory effects on oxidative metalloproteins in the pigment synthesis pathway. The many different effects of amitrole are considered to be a consequence of the diversity in oxidative and metallo-proteins which would have a range in sensitivity to amitrole. While amitrole may not be a particularly potent inhibitor of reactions in carotenoid synthesis it is proposed that the cumulative effect of inhibition at many different sites such as catalase and pigment synthesis would result in light induced toxicity of the type observed in higher plants.

Inhibition of pigment synthesis.

In higher plants amitrole causes chlorosis of tissue which emerges following amitrole treatment. Attempts to identify the cause of this chlorosis originally concentrated on chlorophyll synthesis but it was established that amitrole did not directly affect this process in wheat (Burns *et al.*, 1971). Observations that amitrole-affected leaves had highly disorganised membrane structures and lacked chloroplast ribosomes prompted ultrastructural examinations to attempt to resolve the effects of amitrole and light. Proplastids in wheat treated with amitrole developed normally if plants are kept in the dark but they are unable to form functional chloroplasts when transferred to the light (Bartels and Weier, 1969). Dark-grown amitrole treated plants possess chloroplastic ribosomes whereas light grown plants lack these (Bartels *et al.*, 1967; Feierabend and Schubert, 1978). These observations suggest that effects of amitrole on plastid structure and organisation are secondary effects of amitrole potentiated photooxidation.

Burns *et al.* (1971) examined the effects of "bleaching" herbicides on the levels of carotenoids and their precursors in amitrole treated wheat seedlings and found that following amitrole treatment, x-carotene, phytoene, and phytofluene accumulated. Under high light intensities treated plants were devoid of chlorophyll while at low light intensities some chlorophyll developed. When plants were transferred from low to high light, chlorophyll

was destroyed. Xanthophyll concentrations are also greatly reduced in amitrole treated plants. Lower xanthophyll levels may be a consequence of the reduction in synthesis of carotenoid precursors, a secondary effect of membrane destruction or an inhibition of carotenoid oxidation. These observations of reductions in carotenoid biosynthesis have been confirmed to varying extents by other workers (Grumbach 1984, Feierabend and Schubert 1978, and Vivekanandan and Gnanam 1975; Young *et al.*, 1989). An exception is that of the accumulation of ξ-carotene. While this compound accumulates *in vivo*, amitrole does not inhibit ξ-carotene desaturase *in vitro* raising questions about the nature of the inhibition causing this accumulation (Sandmann and Böger 1987).

It is generally accepted that carotenoids quench triplet chlorophyll and limit photooxidation of the photosynthetic apparatus (Britton, 1979; Ridley 1982). Amitrole prevents the synthesis of these pigments in dark grown wheat plants at concentrations which do not cause alterations to protoplastid structures (Carter, 1975). The proposed mechanism is, therefore, one in which the absence of carotenoids and xanthophylls, as well as the inhibition of catalase, allows photooxidation of chlorophyll, plastidic ribosomes, and proteins in developing leaves leading to chlorosis and eventual death.

Inhibition of carotenoid biosynthesis, therefore, appears to be a major mode of action of amitrole. There remain, however, some results which are not explained by this model. Counter arguments to the proposed mechansim include the observation that chlorophyll fragments extracted from amitrole treated wheat are not consistent with photooxidation as a cause of chlorophyll loss (Rudiger and Benz, 1979). This is supported by other reports of amitrole inhibiting the conversion of protochlorophyllide to chlorophyll in cucumber cotyledons (Ashtakala *et al.*, 1989). While there may be some effect on chlorophyll synthesis in some systems this does not appear to be the most consistent or sensitive effect of amitrole (Carter, 1975). Another important question concerns the mechanism by which amitrole exerts its effect in new tissue while not bleaching pre-existing tissue. Presumably mature leaves are less sensitive to its effects but it is not understood why this is so. One

explanation may be that established tissues have a lower requirement for carotenoid synthesis than newly synthesised tissue or that they are better able to withstand photooxidative stress. Finally, Heim and Larrinua (1989) have reported that root growth in *A. thaliana* seedlings is more sensitive to amitrole than pigment biosynthesis. These observations do not apply to *L. rigidum* for which shoot bleaching occurs at lower concentrations of amitrole than any detectable effects on root growth (authors observations). This may again reflect the importance of different mechanisms in different systems.

This general reactivity of triazoles with oxidative enzyme systems may explain the fundamental mode of action of amitrole. The inhibitory effects of amitrole on the biosynthesis of carotenoids and xanthophylls may be due to inhibition of oxidative reactions in their synthesis. Desaturation cyclisation and hydroxylation of carotenoids and their precursors involves mixed function oxidase reactions (Britton, 1979; Clarke *et al.*, 1982). Thus their inhibition by amitrole could be a consequence of the general affinity of amitrole for certain metallo-proteins.

Attempting to ascribe a principle mode of action to amitrole may not be a productive exercise. The many different sites of action elucidated in different organisms probably reflect the fact that amitrole is a small reactive molecule that interacts with a variety of biological systems. The reported inhibition of oxidative enzyme systems of diverse origin suggest that amitrole may have considerable non-specific activity against metallo proteins which will make generalisations about its specific effects difficult to sustain.

Uptake, Translocation, and Metabolism of Amitrole

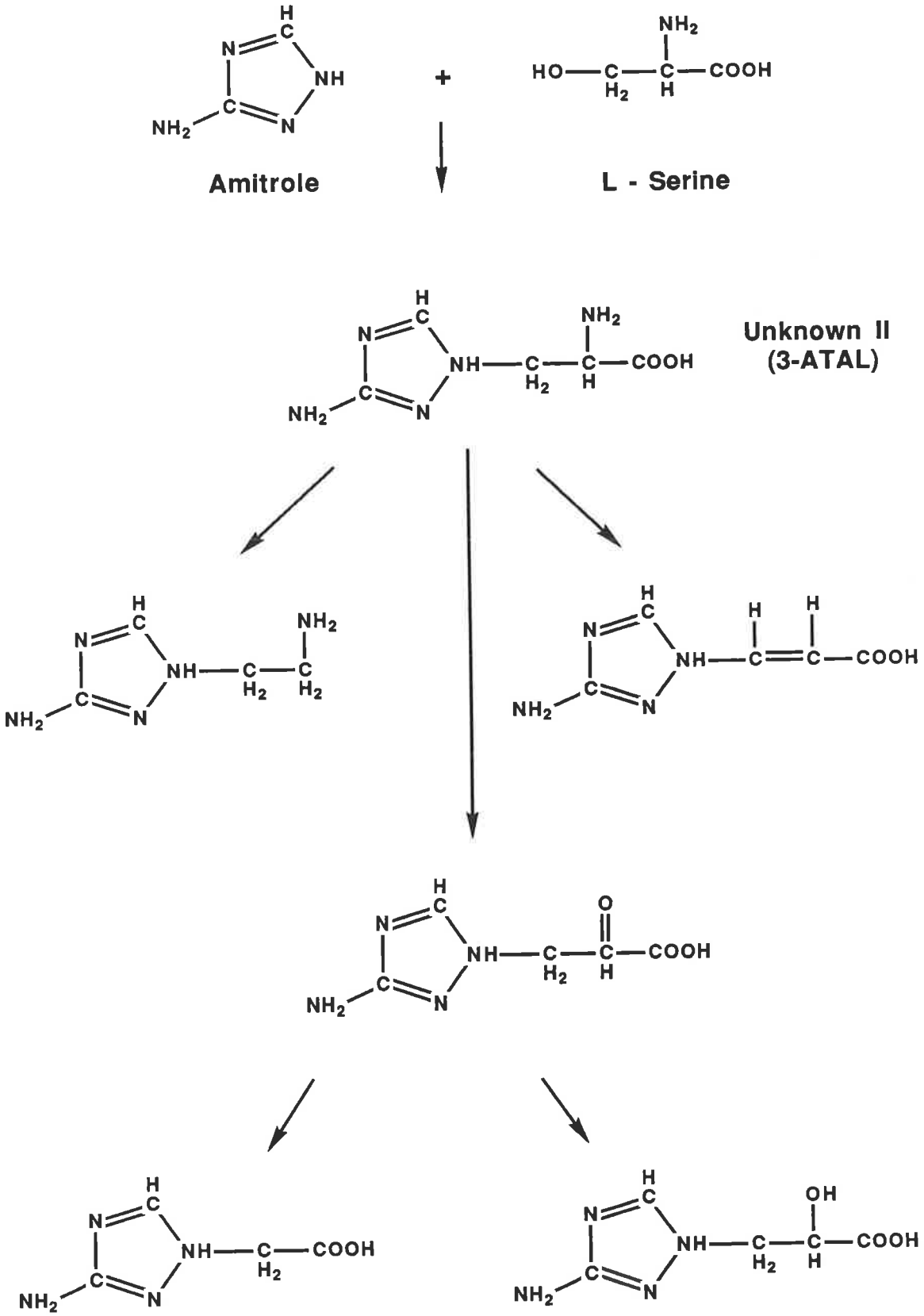
Amitrole is readily taken up via the foliage and the roots of plants and is translocated throughout the plant in both the symplast and the apoplast (Ashton and Crafts 1981). Translocation of amitrole is essential to ensure its presence at the meristematic regions of the plant. Metabolites of amitrole have different mobility (Carter 1975) thus in experiments using radio-labelled herbicide the distribution of label may not fully reflect the distribution of

amitrole. The symptoms of amitrole toxicity are, however, very distinctive and a simple means of observing the translocation of amitrole is to note the distribution of chlorosis in treated plants following a point application. An example of this can be demonstrated in ryegrass. When amitrole is placed on one tiller chlorotic symptoms appear in all tillers (Figure 6.9).

The metabolism of amitrole has been subject to extensive debate regarding the identity of the metabolites, their toxicity and their mobility. The debate has been further complicated by the use of different names for metabolites isolated in different laboratories (Ashton and Crafts 1981). Study of amitrole metabolism is also complicated by the possibility of loss of the 5-Carbon as CO_2. As already mentioned, ^{14}C is incorporated into amitrole at the 5-position. Thus ring cleavage and refixation of $^{14}CO_2$ will result in anomalous labelled materials (Carter, 1975).

The most commonly reported metabolite of amitrole is 3-amino-1,2,4-triazoylalanine (3-ATAL), a condensation product of amitrole and serine (Figure 6.2). This compound has been isolated by a number of laboratories (Smith 1967; Miller and Hall 1961; Lund-Hoie 1970; Herrett and Bagley 1964; Marshall et al, 1987) and identified using x-ray crystallography (Massini 1963). The best understood example of amitrole metabolism is that of *Cirsium arvense*. In this species amitrole is firstly condensed with serine to form 3-ATAL which is further metabolised into two different products, possibly by deamination or decarboxylation (Figure 6.2) (Smith and Chang 1973). Amitrole is also metabolised to similar products by extracts from *Pisum sativum* seedlings *in vitro*. These extracts contain an active tryptophan synthase which is inhibited by amitrole. In the absence of amitrole the enzyme normally converts indole and serine to tryptophan. In the presence of amitrole and serine, 3-ATAL is formed by the enzyme leading to the conclusion that amitrole metabolism may have a similar mechanism to tryptophan synthesis in peas. The primary product, 3-ATAL is not phytotoxic, is stable and its formation is inhibited by ammonium thiocyanate (Carter, 1975; Smith, 1967). The mechanism by which ammonium thiocyanate exerts this

Figure 6.2. The proposed pathway of metabolism for amitrole in *C. arvense* (redrawn from Smith and Chang, 1973).



Redrawn from Smith and Chang (1973).

effect is unknown but it is formulated with amitrole as a synergist.

While there is some consensus about the structure of 3-ATAL there is very little consensus about the identity and structure of other amitrole metabolites. Radioactivity derived from amitrole accumulates in bean fruits in a form other than 3-ATAL and possibly as sugars (Shimabukuro and Linck, 1965). Metabolism to this extent would suggest complete detoxification. Putatively novel metabolites have also been observed but not identified in other species and thus generalisation about amitrole metabolism is not possible (Miller and Hall, 1961). Herrett and Link (1961) and Lund-Hoie (1970) have both reported phytocidal, highly-mobile metabolites of amitrole, however Carter (1975) suggests that these are artifacts. The pathways of metabolism of amitrole are not clearly defined. The only commonly observed and conclusively identified metabolite is 3-ATAL. Given that this appears to be a primary metabolite and one which is not phytotoxic, production of this metabolite in vivo appears to provide a mechanism of tolerance to the herbicide.

Resistance to Amitrole

Aside from *L. rigidum* biotype WLR2 the only other report of resistance to amitrole is that of *Poa annua* in Belgium (Le Baron, 1991), however, there are some examples of differential tolerance between weed species. Ecotypes of *C. arvense* exhibit differential tolerance to amitrole (Smith, 1967) which is explained in part by greater amitrole metabolism in tolerant ecotypes. Differential amitrole tolerance is also linked to morphological differences between the ecotypes which may also contribute to resistance (Smith 1967). Differential tolerance to amitrole may also be conferred by differences in foliar uptake of the herbicide (Carter 1975). The amitrole tolerance of *Convolvus arvensis* compared with *C. arvense* is attributed to both differences in foliar uptake and differences in metabolism (Herrett and Linck, 1961).

Amitrole resistance in grass species has been generated by deliberate selection for this character in turf breeding programs. Following 3 cycles of selection with amitrole, tolerance in *Festuca rubra* and *Agrostus tenuis* increased five- and nineteen-fold respectively

(Johnston and Faulkner, 1991). These changes in sensitivity are of a similar order to that observed in *L. rigidum* (Figure 6.3). Resistance to amitrole is also reported to have arisen in tissue cultures of *Nicotiana sp.*. Deliberate selection for amitrole resistance yielded amitrole resistant cells which were up to 100 times less sensitive to amitrole in cell culture (Swartzberg *et al.*, 1985). No indication of the mechanism was given but the trait was not cytoplasmically inherited. Singer and McDaniel (1985) also obtained amitrole cross-resistant calli after selection for glyphosate tolerance. This resistance was not caused by differences between the cell lines in the uptake of amitrole. This was not surprising given that transport of amitrole into cultured tobacco cells is by passive diffusion. Amitrole uptake is increased, however, if cells are also treated with high concentrations of ammonium thiocyanate suggesting the possibility of uptake contributing to the synergy observed with this chemical (Singer and McDaniel, 1982). The level of tolerance observed by Singer and McDaniel (1985) was an order of magnitude lower than that obtained by Swartzberg et al (1985) who selected specifically for amitrole resistance. These examples of selection in cell culture demonstrate that variation with respect to amitrole resistance does exist and that it can be selected *in vitro*. This, the tolerance of some weed species, and the selection for resistance in turf grasses all suggest that amitrole resistance may occur in weeds following sufficient selection pressure.

Conclusions

The multiple sites of action of amitrole have made it a very difficult herbicide to study. It now appears that carotenoid biosynthesis is one of the most sensitive sites in higher plants (Burns *et al.*, 1971, Ridley, 1982). Catalase activity is also reduced at doses which inhibit carotenoid production (Feierabend and Kemmerich, 1983). Other proposed targets may be of more importance in established leaves or at higher doses. Many of the effects of amitrole could be explained by the affinity of its 1,2,4,-triazole heterocycle for metalloenzymes. The diversity of such proteins may explain why many different inhibitory effects of amitrole are observed. Amitrole resistance is not common but differences in tolerance due to differential uptake and metabolism have been described. Amitrole metabolism is best understood in *C.*

arvense and *P. sativum* but the various reactions are not well characterised. The primary metabolite in *C. arvense*, 3-ATAL, is found in many species. Catalysis of this reaction by tryptophan synthase may explain the ubiquity of this metabolite. Thus amitrole is a poorly understood herbicide whose simplicity of structure belies the complexity in its mode of action and metabolism.

6.2 Amitrole Resistance in *L. rigidum*

The only case of amitrole resistance identified in *L. rigidum* is that of biotype WLR2 which was described in Chapter two. Amitrole resistance developed after selection pressure from a mixture of amitrole and atrazine but resistance is expressed to the herbicides either alone or in combination. Given the very different nature of the two herbicide classes, resistance to each has been considered separately. In this Chapter experiments to elucidate the mechanism of amitrole tolerance in *L. rigidum* are described.

6.3 Materials and Methods

Plant material

The susceptible biotype used in this study was VLR1. The resistant biotype was WLR2 which is described in Chapter two.

Response to herbicides

Dose response to herbicides was performed according to the method in Chapter two.

Germination experiments

Amitrole was dissolved at various concentrations in 0.6% agar (w/v) and placed in 1 cm inverted polypropylene caps (1 ml/cap) to solidify. Seeds (15) were placed evenly over the agar surface and the caps enclosed in a loosely closed transparent polycarbonate 75 mL sample vial to maintain humidity while still transmitting light. Dishes were placed in an

incubator illuminated with fluorescent lights (photoperiod 12h, light intensity 50 μmol photons m^{-2} s^{-1}, temperature 20°C) in a randomised block design with four replicates. Shoot fresh weight and the proportion of seedlings which were bleached was determined 8 d after initiation.

Uptake and metabolism by seedlings

Seedlings were germinated on agar (1 mL) containing various concentrations of [^{14}C]amitrole (spec. act.100 or 200 MBq mmol) for 8 days before harvest of the first leaf (conditions as above). Leaves (usually 12 in number) were weighed and then homogenised in 1 mL of water:ethanol (1:1 v/v). The extract was centrifuged at 10,000 x g for five minutes and the pellet washed once with 0.5 mL of the same extractant. Radioactivity in the combined supernatant was quantified before the supernatant was evaporated to dryness and resuspended in water:ethanol (1:1, v/v) (400 μL). Amitrole and metabolites were separated using thin layer chromatography according to the method of Marshall *et al.* (1987 b). Extracts (20 μL) were applied to silica gel 60G plates (Merck art. 5748) and eluted with *iso*propyl alcohol:ammonia solution (15M):water (6:1:3 v/v/v). Rf. values for amitrole and the major metabolites extracted from *L. rigidum* were 0.83, 0.67, 0.59 and 0.36. These compare well with reported Rf. values in the same system of 0.89, 0.67 and 0.38 for amitrole and the major metabolites extracted from *Equisetum arvense* L. (Marshall *et al.*, 1987 b) (Figure 6.12).

Extraction and separation of carotenoids

Seeds were germinated and allowed to grow on medium containing various concentrations of amitrole for eight days, after which the first leaf was removed from the seed without the coleoptile. Carotenoids were extracted according to Young *et al.* (1989). Briefly, leaves were weighed, homogenised in ethanol buffered with 0.1 % triethylamine and then centrifuged at 10,000 x g for two minutes. The supernatant was evaporated under dry nitrogen, resuspended in diethyl ether, centrifuged as above and the supernatant again dried under nitrogen. Samples were then resuspended in acetonitrile:water:TEA (9:1:0.1 v/v).

Volumes of 100 µL were injected onto reverse phase HPLC (Brownlee Labs ODS-5A, 250 x 4.6 mm column). Samples were eluted using a gradient from acetonitrile:water:TEA (9:1:0.1 v/v) to ethylacetate over 24 minutes at a flow rate of 1mL min^{-1} and column temperature of 30°C. Eluting carotenoids were detected at 287 and 447 nm using two UV detectors in series. All solvents were degassed and, with the exception of the HPLC eluents, contained the antioxidant (10 mg L^{-1}) butylated hydroxy toluene (BHT).

Carotenoids and other pigments were tentatively identified on the basis of UV absorbtion spectrum and relative retention time. Fractions of HPLC eluate were collected every 30 s, dried under nitrogen and resuspended in ethanol before obtaining UV absorbtion spectra. Peak I had an absorbtion maximum at 286 nm with subsidiary peaks at 276 and 299 nm and was identified as a phytoene like compound. Peak II had maxima at 331, 346, and 368 nm and is likely to be phytofluene. Peak III had maxima at 442, 471, and 503 nm and was tentatively identified as lycopene after comparison with published chromatographs (Young, 1989). Peak IV had maxima at 431, 617, and 664 nm indicating that it was likely to be chlorophyll a. Peak V had maxima at 642 and 463 nm suggesting that it was likely to be chlorophyll b. Peak VI was not identified. Reference maxima were obtained from Goodwin (1980).

Uptake by whole plants.

Plants were grown to the four-leaf stage in hydroponic media and transferred to vials as described in Chapter three. Amitrole was applied to the axils of the youngest fully expanded leaves as a 2µL droplet of 35 mM amitrole (spec. act. 1.9 MBq $mmol^{-1}$). Plants were harvested at 24 h intervals over seven days and the leaves washed with two changes of water:ethanol (1:1 v/v) (20 mL). Root tissue was washed (2 x 10 mL H_2O) and the amount of radioactivity in the roots and nutrient solution was determined. Shoot material was macerated using liquid nitrogen and sand in a mortar and pestle and extracted with water:methanol (1:4 v/v) four times. Radioactivity in the pooled supernatants was quantified as the amount taken up by the tissue.

Autoradiography

Plants were grown to the early three leaf stage in a hydroponic system (as above). They were then transferred to vials and allowed to acclimate for 48 h. Amitrole (100 µM, spec. act. 200 MBq mmol) was applied to the roots for 1 h and the roots were then washed and transferred to new vials. The plants were then incubated in a growth room for a further 23 or 71 h after which the roots were washed and blotted dry. Two seedlings of each biotype were lightly pressed onto cardboard and covered with polyethylene film. A sheet of x-ray film was placed over the polyethylene, the assembly was covered and allowed to develop at -20°C for nine weeks.

Inheritance of resistance

Resistant WLR2 plants were selected as parents for use in crossing experiments by exposing them to a selection pressure of 2 kg ha^{-1} amitrole applied at the seedling stage. The selection pressure was applied because some variation in amitrole resistance was noted in the WLR2 population and it was reasoned that there was a greater likelihood of homozygousity in the most resistant individuals. Seven surviving WLR2 plants were selected from a sample of over 100 individuals and hybridised with susceptible WLR96 plants. The susceptible plants were a biotype of *L. rigidum* collected from a region near to the origin of WLR2. This was done to minimise ecotypic differences in flowering time and to provide a genetic marker for the other half of the cross. WLR96, although susceptible to amitrole, is resistant to the aryloxyphenoxypropionate herbicide haloxyfop (Table 1.1, Table 1.2). WLR2 is susceptible to haloxyfop. Thus each parent line is marked with a different resistance characteristic.

L. rigidum is a self-incompatible wind-pollinated plant (Kloot, 1983). Hybridisation was achieved by placing a pot containing an amitrole resistant WLR2 plant and an amitrole susceptible WLR96 plant within a sleeve of clear polythene prior to flowering (single plants

isolated in this system set zero or negligible amounts of seed) (Figure 6.16). The sleeve was 1.2 m high and open at both ends to reduce humidity (which is thought to reduce pollen viability) (Figure 6.17). Seeds resulting from crosses were collected separately from each female parent. Plants were screened for amitrole resistance at 0.25, 0.5, 1.0 and 2.0 kg ha^{-1} amitrole and compared with both the WLR2 and WLR96 parent populations. Screening was performed according to the section "dose response to herbicides". F_1 plants resistant to both amitrole and haloxyfop (definite hybrids) were either crossed to a susceptible VLR1 biotype or crossed to other plants derived from the same female parent to produce F_2 seed. Seeds from each female parent were always collected and assessed separately to allow for the possibility of maternal inheritance effects. In the absence of such effects, results from progeny of both parents were combined for analysis.

6.4 Results and Discussion

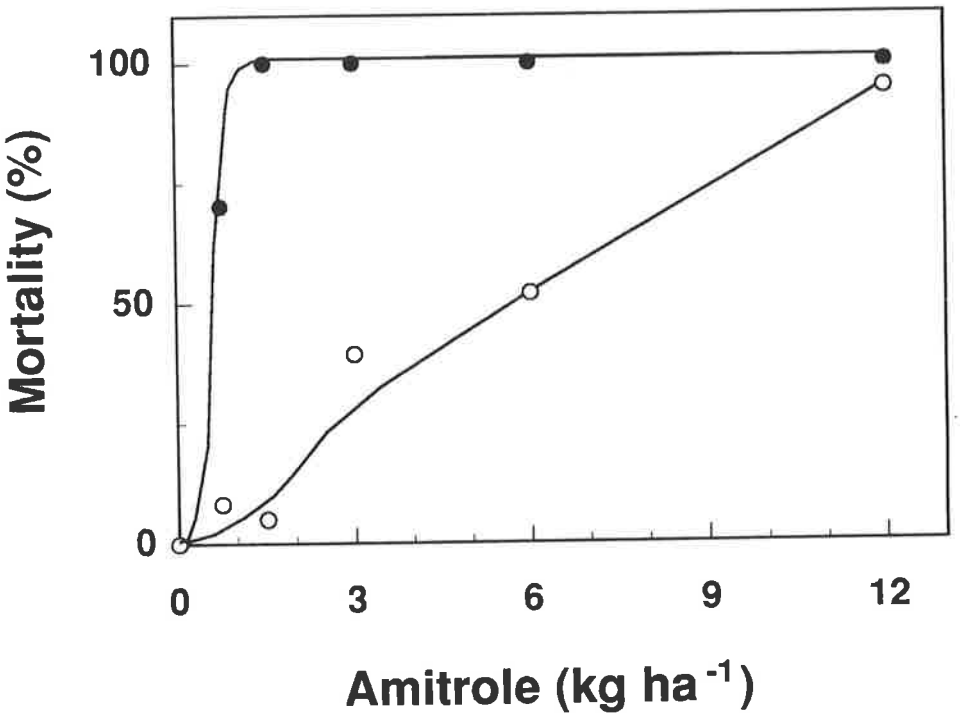
Dose response to amitrole

The resistant WLR2 plants are eleven times less sensitive to amitrole when it is applied as the formulated herbicide to three-leaf seedlings (Figure 6.3). The symptom of amitrole toxicity at low doses (0.75 kg ha^{-1}) in the susceptible plants is bleaching of tissue produced after application of amitrole (Figure 6.6). Viable tissue present prior to application of low doses remains green and presumably provides the photosynthate that allows the continued production of the bleached tissue (Figure 6.6). Resistant plants display only a brief period of toxicity at low doses which is manifested as a 2 to 3 cm band of bleaching on the leaf emerging at the time of spraying (Figure 6.6). The shorter period of toxicity, as indicated by the narrow bleached band on the leaf lamina, may suggest that the resistant plants have toxic levels of amitrole present at the active site for a shorter period than the susceptible plants.

At higher doses (1.5 kg ha^{-1}) the susceptible plants show marked reduction in growth while resistant plants display a greater extent of bleaching and mild growth reduction (Figure 6.6). At doses of 3.0 kg ha^{-1} and above susceptible plants suffer desiccation of established green tissue leading to rapid death while WLR2 plants exhibit large reductions in growth and extensive bleaching. The different symptoms of amitrole at different doses and particularly its effect on green tissue at higher doses suggests that amitrole may be acting at different targets sites within the plant as doses increase.

Amitrole is commonly formulated with a photosystem II inhibitor, atrazine, for enhanced weed control in the field. Amitrole enhances the toxicity of atrazine and simazine in the susceptible plants (Figure 6.4A, Figure 6.7), however, this effect is not apparent for atrazine

Figure 6.3. The response of the resistant WLR2 and susceptible VLR1 biotypes to amitrole (commercially formulated with NH_4SCN) sprayed onto seedlings at the three leaf stage. Each point is calculated from a sample of 63 plants.


Mortality (%)
100
50
0
0
3
6
9
12
Amitrole (kg ha -1)

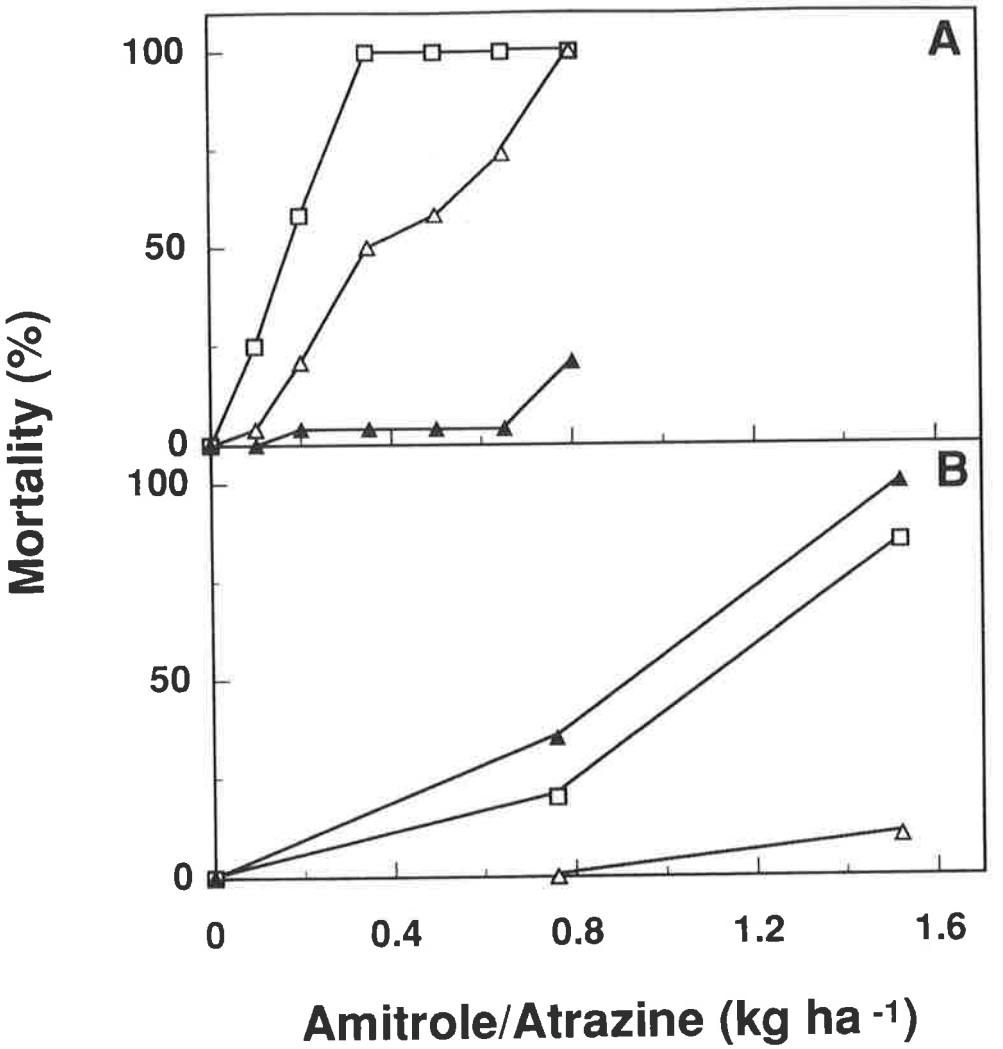
in the resistant plants (Figure 6.4B, see also Figure 2.1). The reason for the lack of interaction between amitrole and atrazine in WLR2 is not known. The susceptible plants display greatly intensified symptoms associated with the PS II inhibitor when treated with both amitrole and a triazine such as simazine (Figure 6.7). There is an immediate cessation of growth and leaf tissue is rapidly desiccated. Amitrole does not normally exert an obvious toxic effect in green tissue at low doses although, at higher doses green tissue is affected (Figure 6.7). In contrast, PS II inhibitors exert their toxic effect in functional leaves. A possible explanation for the interaction of amitrole and the PS II inhibitors is the inhibition by amitrole of catalase and similar enzymes. If these enzymes moderate the effect of radicals generated as a consequence of the blocked photosystem, their inhibition would intensify the effect of the PS II inhibitor. WLR2 plants are, however, susceptible to another PS II inhibitor, ioxynil (data not shown). In combination with ioxynil, amitrole does intensify the effect of the PS II inhibitor in WLR2 (Figure 6.4C). These data suggest that the ability of amitrole to intensify the effects of a PS II inhibitor is a general property, but that in the case of WLR2 and atrazine the synergy has been reversed by the selection process.

Amitrole is formulated with ammonium thiocyanate (NH_4SCN) because the latter compound is thought to inhibit the metabolism of amitrole in some species (Smith, 1967). To determine whether the response of *L. rigidum* to amitrole could be influenced by NH_4SCN, the two were applied using a factorial design. The dose response to amitrole in combination with 2 and 4 kg ha^{-1} NH_4SCN is illustrated in Figure 6.5. NH_4SCN is usually formulated approximately 1:1 by weight with amitrole. At NH_4SCN application rates above this ratio, NH_4SCN did not increase the toxicity of amitrole in either biotype. Thus at rates well above those applied in the commercial product NH_4SCN had no influence on the toxicity of amitrole to *L. rigidum*. The absence of any interaction in VLR1 further suggests that amitrole metabolism is insensitive to NH_4SCN *in vivo*.

Figure 6.4A. The response of the susceptible biotype VLR1 to atrazine (▲), amitrole (Δ) and the two in combination (□). Each point is the mean of three pots of 12 plants.

Figure 6.4B. The response of the resistant biotype WLR2 to atrazine (▲), amitrole (Δ) and the two in combination (□). Each point is calculated from a total of 20 plants.

Figure 6.4C. The response of the resistant biotype WLR2 to ioxynil alone (▲), and in combination with 0.75 kg ha-1 amitrole (□). Each point is calculated from 24 treated plants.


A
B
100
50
0
100
50
0
Mortality (%)
0
0.4
0.8
1.2
1.6
Amitrole/Atrazine (kg ha -1)

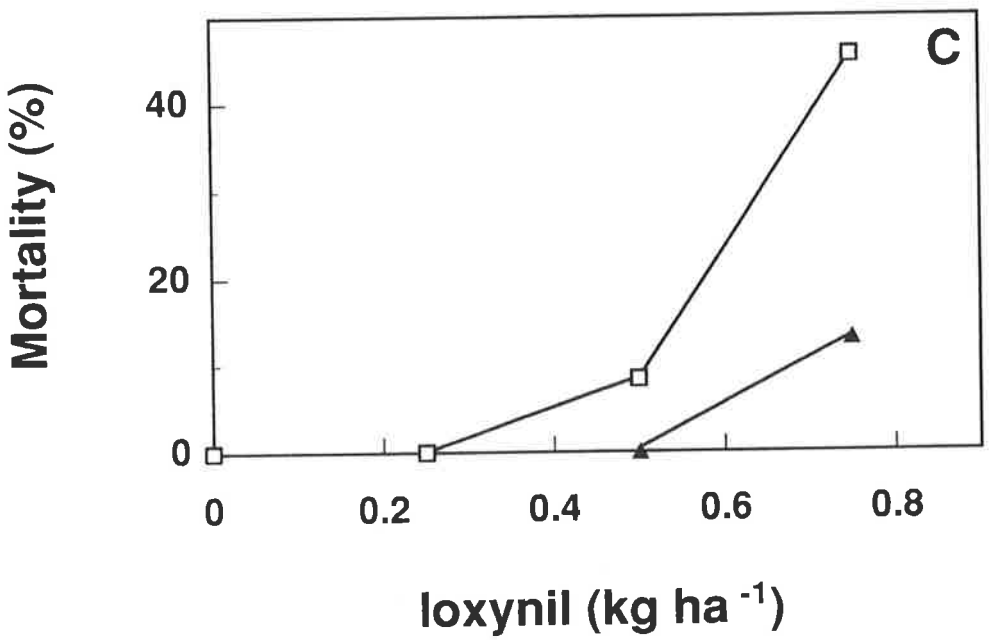

C
40
20
0
Mortality (%)
0
0.2
0.4
0.6
0.8
Ioxynil (kg ha -1)

Figure 6.5. The response of the resistant WLR2 (O, Δ) and susceptible VLR1 (●, ▲) plants to amitrole alone (O, ●) or in combination (Δ, ▲) with 2 kg ha^{-1} NH_4SCN (A and B) or 4 kg ha^{-1} NH_4SCN (C and D) compared with control plants not treated with either chemical.

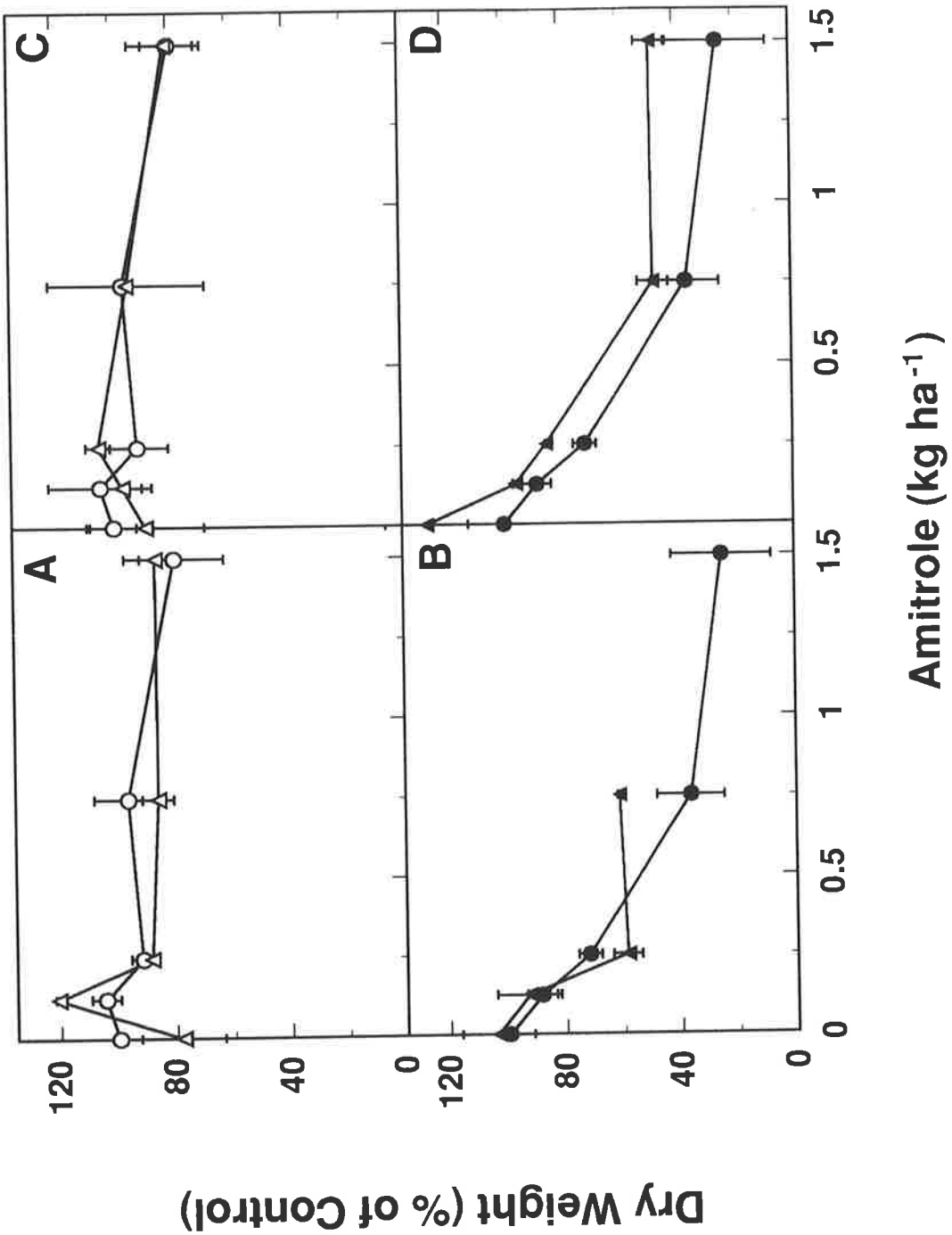

A
B
C
D
120
80
40
0
0
0.5
1
1.5
Dry Weight (% of Control)
Amitrole (kg ha-1)

Figure 6.6. The response of the resistant WLR2 (bottom) and susceptible VLR1 (top) seedlings to 0.75 and 1.5 kg ha^{-1} amitrole sprayed at the three leaf stage.

Figure 6.7 The response of susceptible VLR1 seedlings to: 1 kg ha^{-1} simazine alone (right), 1 kg ha^{-1} simazine and 1 kg ha^{-1} amitrole (second from right), 1 kg ha^{-1} amitrole alone (second from left) compared with the control (left).


CONTROL
SUSCEPTIBLE
750 g a.i. ha AMITROLE
SUSCEPTIBLE
1500 g a.i. ha AMITROLE
SUSCEPTIBLE
CONTROL
RESISTANT
750 g a.i. ha AMITROL
RESISTANT
1500 g a.i. ha AMITROL
RESISTANT

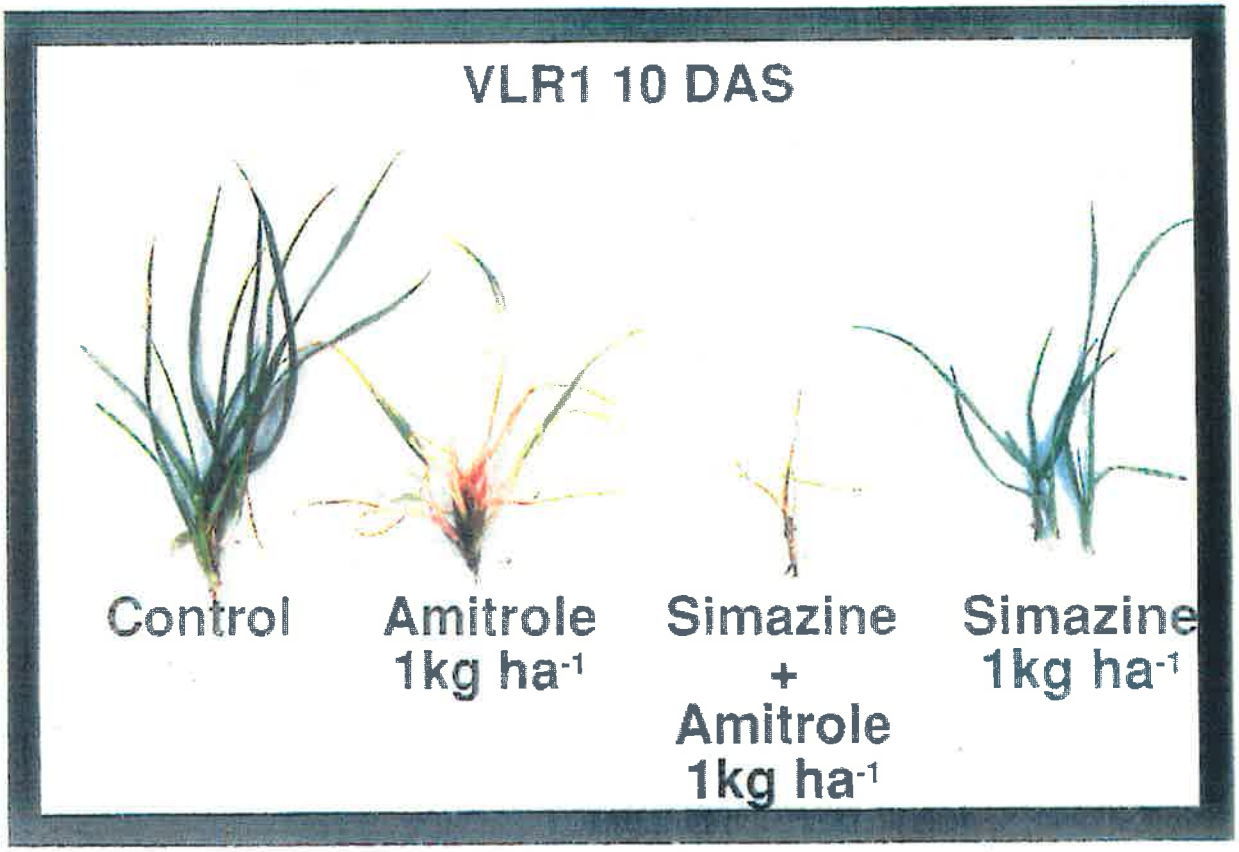
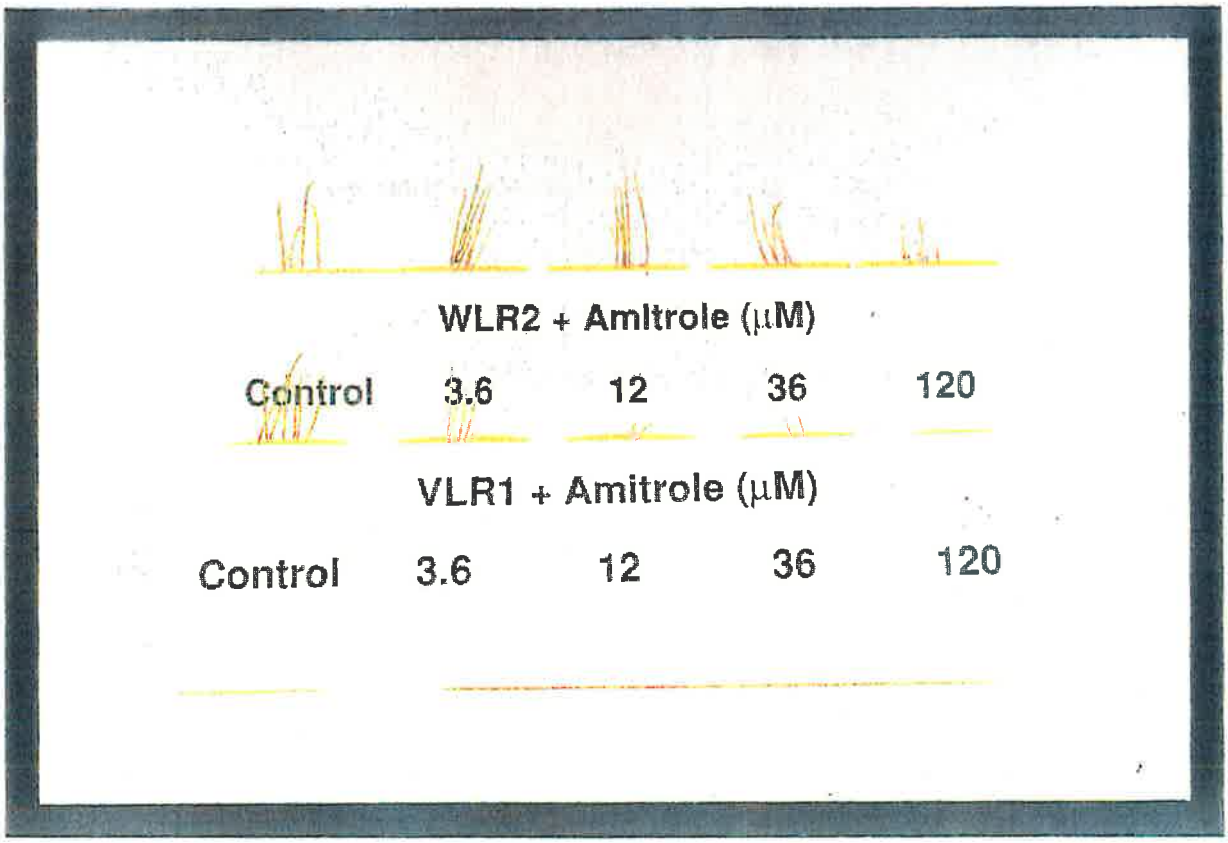

VLR1 10 DAS
Control
Amitrole
1kg ha-1
Simazine
+
Amitrole
1kg ha-1
Simazine
1kg ha-1

Figure 6.8. The response of the resistant WLR2 (rear) and susceptible VLR1 (front) seedlings to various concentrations of amitrole supplied in the germination medium.

Figure 6.9 The pattern of chlorosis exhibited in a resistant WLR2 plant treated with a point application of 1 μL of 150 mM amitrole to one tiller. Symptoms appear in leaves growing on both tillers. The subject was one plant of a number treated with a range of concentrations and this individual is representative of the results obtained in the experiment. The experiment was conducted in a growth room where symptoms are generally less extreme, hence the relatively high dose. Susceptible plants required approximately ten times less amitrole to display similar symptoms in this system.


WLR2 + Amitrole (μM)
Control
3.6
12
36
120
VLR1 + Amitrole (μM)
Control
3.6
12
36
120


1 μl of 0.15 M amitrole
applied to one tiller
RESISTANT

Uptake of amitrole by whole plants.

Amitrole may be taken up via both the foliage and the root system (Ashton and Crafts, 1981). The uptake and distribution of amitrole following an axillar dose can be observed in the distribution of symptoms over the whole plant (Figure 6.9). The bleaching evident on both tillers demonstrates that amitrole may be taken up through the foliage of *L. rigidum* and that it translocates between different tillers. Lower doses were required to have the same effect in susceptible VLR1 plants. These observations do not, however, provide quantitative estimates of amitrole uptake, thus uptake of ^{14}C labelled amitrole was examined. WLR2 and VLR1 plants treated with a 2μL axillar dose of 35 mM amitrole had leaf amitrole contents of 143 (±36) and 148 (±16) nmol g^{-1} (±se, N = 7). While it appears that uptake is similar, recovery of radioactivity was only 50% for both biotypes. Low recovery of radioactivity precludes firm conclusions on the uptake and metabolism of the herbicide in this system. Part of the balance of radioactivity may have been held in insoluble residues, however, these could not be accurately quantified in the absence of oxidation equipment. Given the poor recoveries obtained in this system, metabolism was studied using the germination system.

Uptake via the roots and translocation to the foliage appeared to be similar in both biotypes as indicated by the distribution of radioactivity in foliar tissue following the application of amitrole to plant roots (Figure 6.10). There is some indication that for each biotype, the bulk of the foliar radioactivity appears to have been translocated to the tips of the leaves. Interpretation of this data must be qualified, however, because the intensity of the signal detected will depend in part on the proximity of the film to the tissue. Leaf tips and vascular tissue running in surface ridges will be most closely and evenly presented to the film and thus may give even more intense exposure. Nevertheless, despite these qualifications, the similarity of the images obtained with both biotypes suggests that there are no major qualitative differences between the two in the distribution of amitrole within the plant.

Uptake and metabolism of amitrole from germinating seedlings.

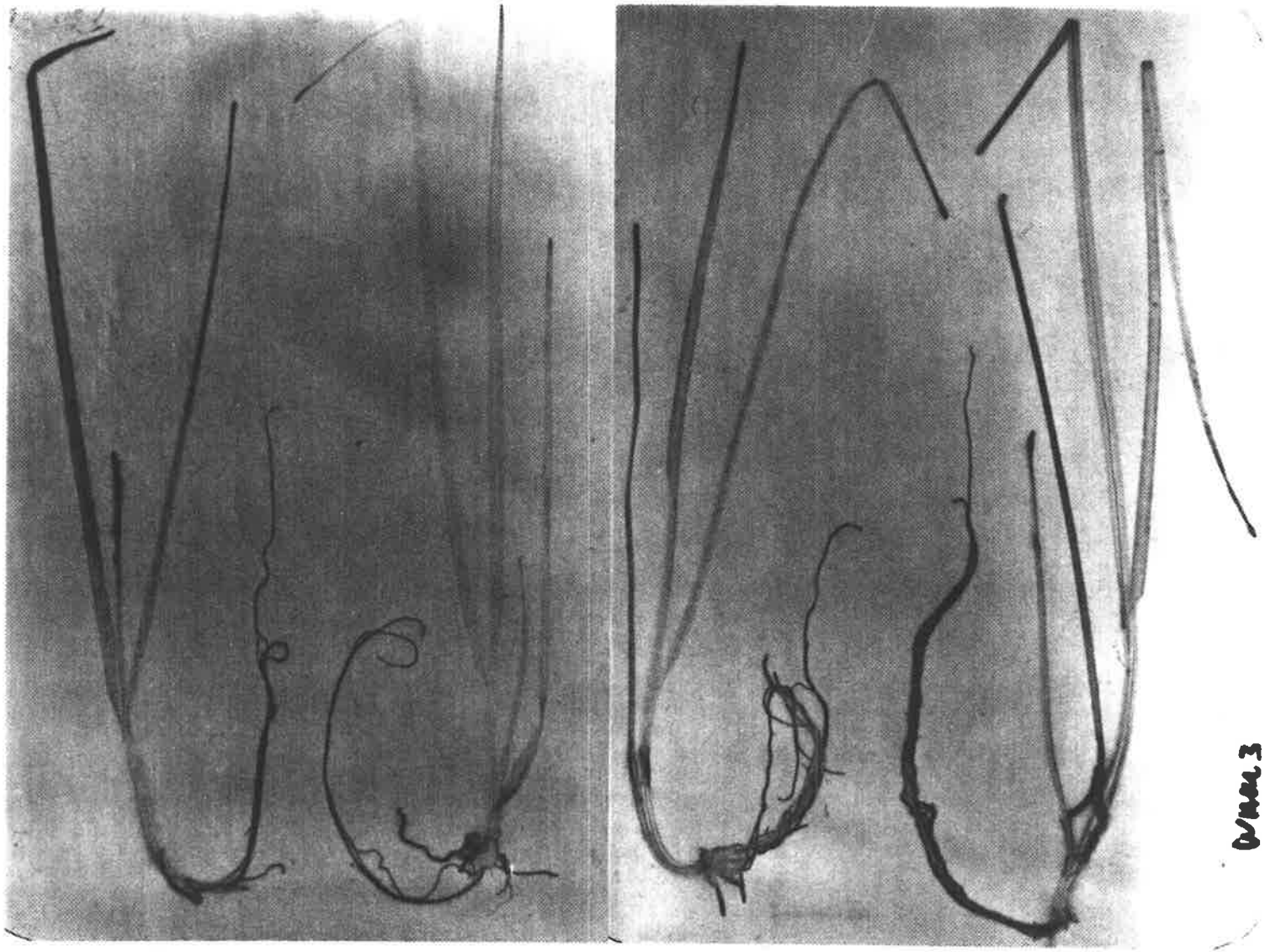
Seedlings germinated on medium containing amitrole grow and display symptoms similar to those observed in plants treated at a more advanced growth stage (Figure 6.8). Symptoms include bleaching and reduction in growth which is measured by fresh weight (Figure 6.8, Figure 6.11A). The degree of resistance in the germination system as determined by leaf fresh weight or carotenoid content is approximately ten-fold (Figure 6.8, Figure 6.11A, Figure 6.14). This is similar to the degree of resistance observed in pot experiments (Figure 6.3) indicating that the germination system is a good model for studying amitrole resistance.

Resistant and susceptible seeds germinated on agar containing [^{14}C]amitrole take up similar quantities of radioactivity into the first leaf (Figure 6.11C). The proportion of the radioactivity present as amitrole and its various metabolites is very similar in extracts made from the leaves of both the resistant and susceptible biotypes (Figure 6.12). Therefore, there appears to be no difference in metabolism of the herbicide in the leaf. Consequently, both biotypes have similar concentrations of amitrole in their leaf tissue (Figure 6.11B). Despite this, they exhibit very different leaf symptoms (Figure 6.8, Figure 6.11A). The resistant plants require at least a ten times higher tissue concentration of the herbicide for them to display symptoms approaching those exhibited by the susceptible (Figure 6.11B). The difference in symptoms in the presence of similar amounts of the herbicide indicates that either the herbicide is being sequestered or that unknown target sites are less sensitive.

Effect of amitrole on shoot carotenoid content

As previously discussed, amitrole is thought to have many different effects including inhibition of carotenoid production. To determine the effect of amitrole on carotenoid production *in vivo*, carotenoids were extracted from seedlings germinated on agar containing various concentrations of the herbicide. A number of pigments varied in response to increasing doses of amitrole and, of these, the increase in the amount of phytoene (I) was most obvious (Figure 6.13, Figure 6.14A).

Figure 6.10. Autoradiographs of resistant WLR2 (right) and susceptible VLR1 (left) plants after application of amitrole to the roots in nutrient solution. 21 d old plants were treated for 1 h with 100 μM amitrole (200 MBq $mmol^{-1}$).



Susceptible (VLR1)

Resistant (WLR2)

Figure 6.11A. The leaf fresh weight relative to untreated controls (fresh weight: WLR2, 3.62 mg leaf^{-1}; VLR1, 3.29 mg leaf^{-1}) of resistant WLR2 (O) and susceptible (●) seedlings germinating on agar containing various concentrations of amitrole. Each point is the mean of three replicates and is plotted with the standard error. The first leaves of seedlings were harvested eight days after initiating germination. This data is from one of three experiments and is representative of these.

Figure 6.11B. The total uptake of radioactivity (calculated as amitrole) by resistant WLR2 (O) and susceptible (●) seedlings germinating on agar containing various concentrations of amitrole. Each point is the mean of three replicates and is plotted with the standard error. Data is derived from the same plants as those described in 6.11A.

Figure 6.11C. The amount of amitrole in the first leaf of the resistant WLR2 (O) and susceptible (●) seedlings germinating on agar containing various concentrations of amitrole. Data is based on the total extractable radioactivity adjusted for the proportion found as metabolites. Each point is the mean of three replicates and is plotted with the standard error. Data is derived from the same plants as those described in 6.11A.

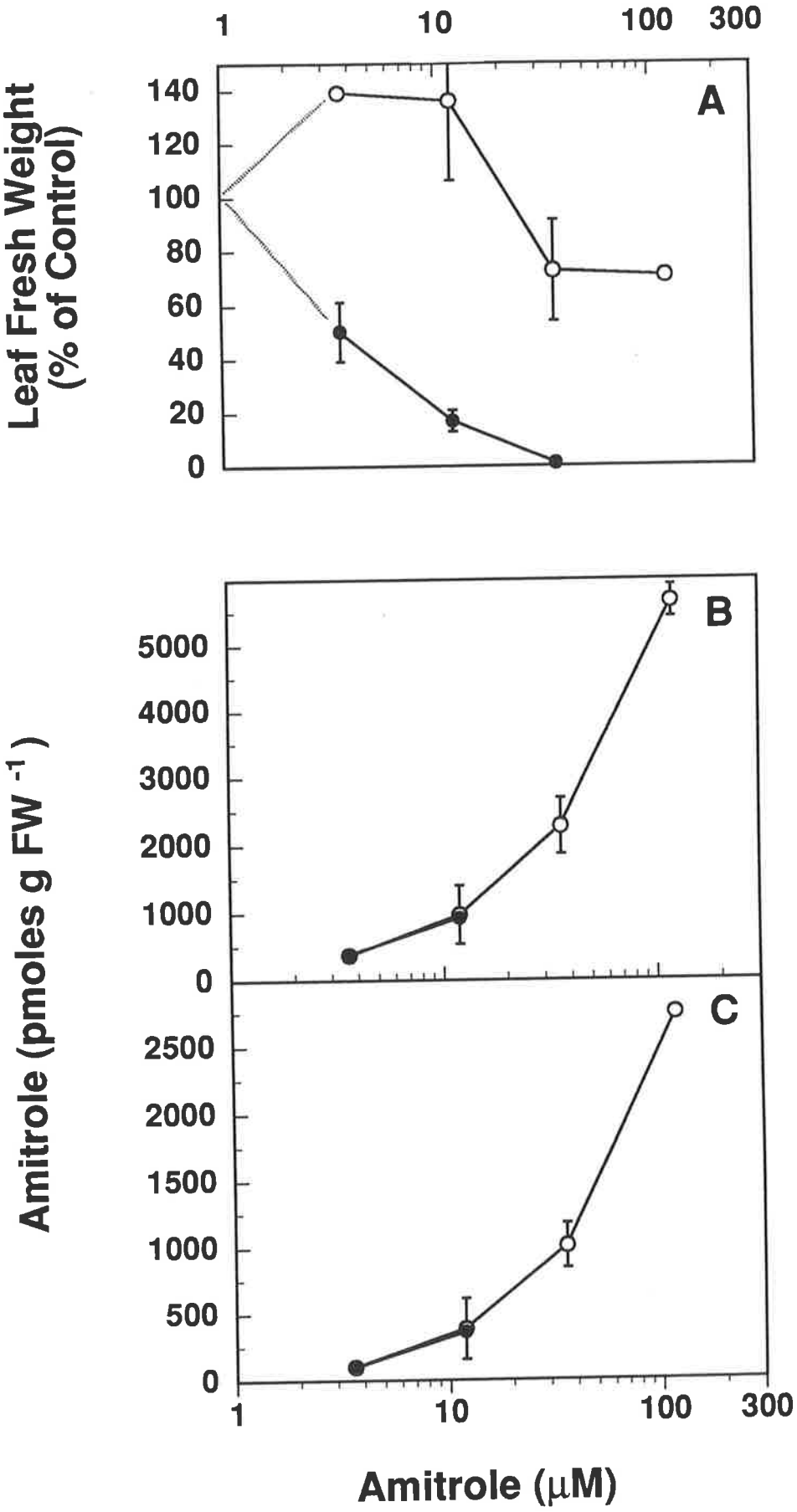
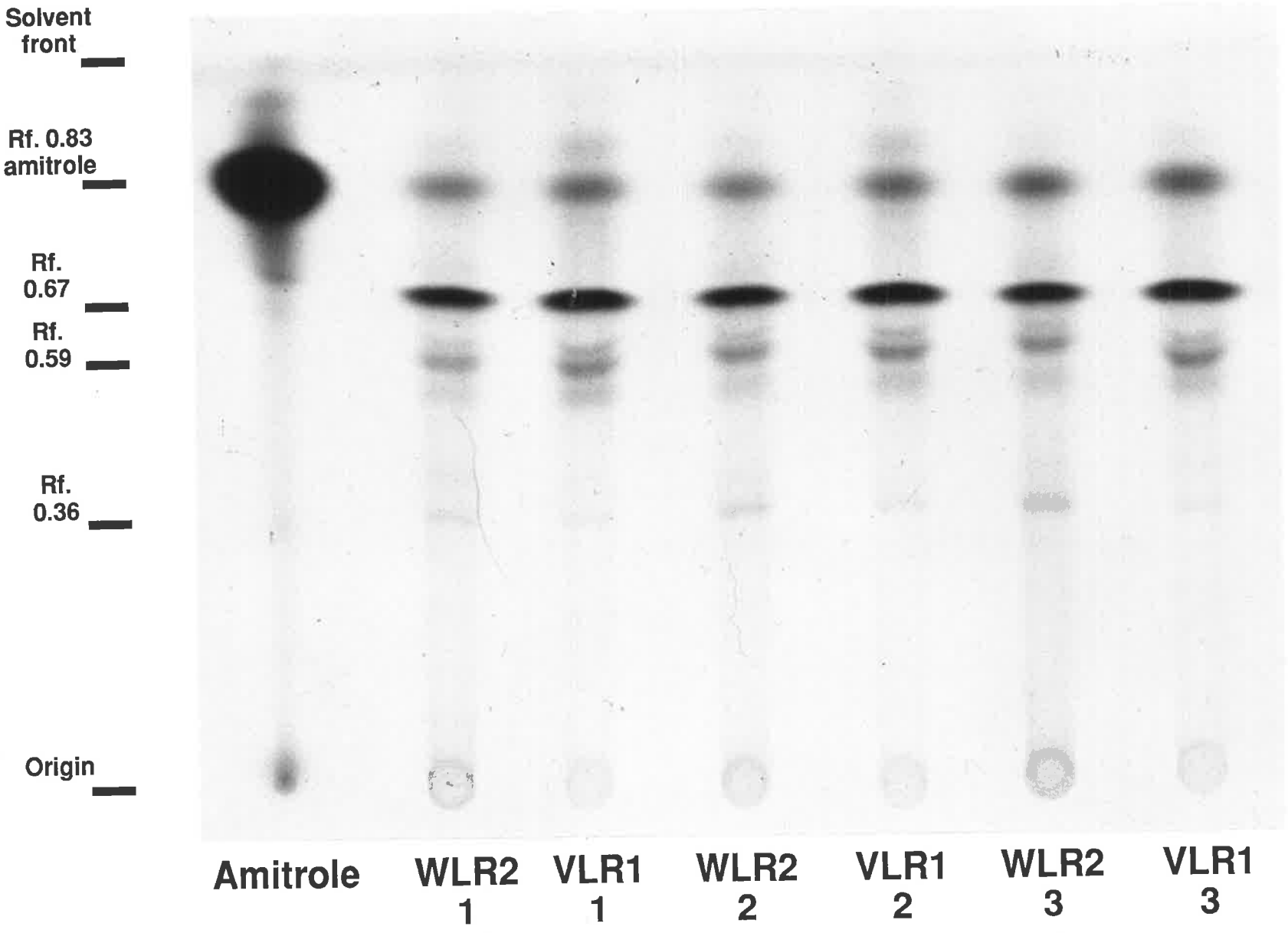

Leaf Fresh Weight
(% of Control)
A
B
C
Amitrole (pmoles g FW -1)
Amitrole (μM)
1
10
100
300
140
120
100
80
60
40
20
0
5000
4000
3000
2000
1000
2500
1500
500

Figure 6.12. Autoradiograph of TLC separation of amitrole and metabolites extracted from resistant WLR2 and susceptible VLR1 seedlings treated under the same conditions as those used to generate the data in Figure 6.11. The lane on the left is radio labelled amitrole which co-migrates with technical grade amitrole in the next lane (visualised under UV illumination and therefore, not visible on this film). The following six lanes are extracts from resistant WLR2 (lanes 1, 3 and 5) and susceptible VLR1 (lanes 2, 4 and 6) seedlings germinated on 50 μM [^{14}C]amitrole (spec. act. 200 MBq mmol^{-1}). Each of the lanes is a replicate of the same treatment. This data is one of three separate experiments and is representative of these.

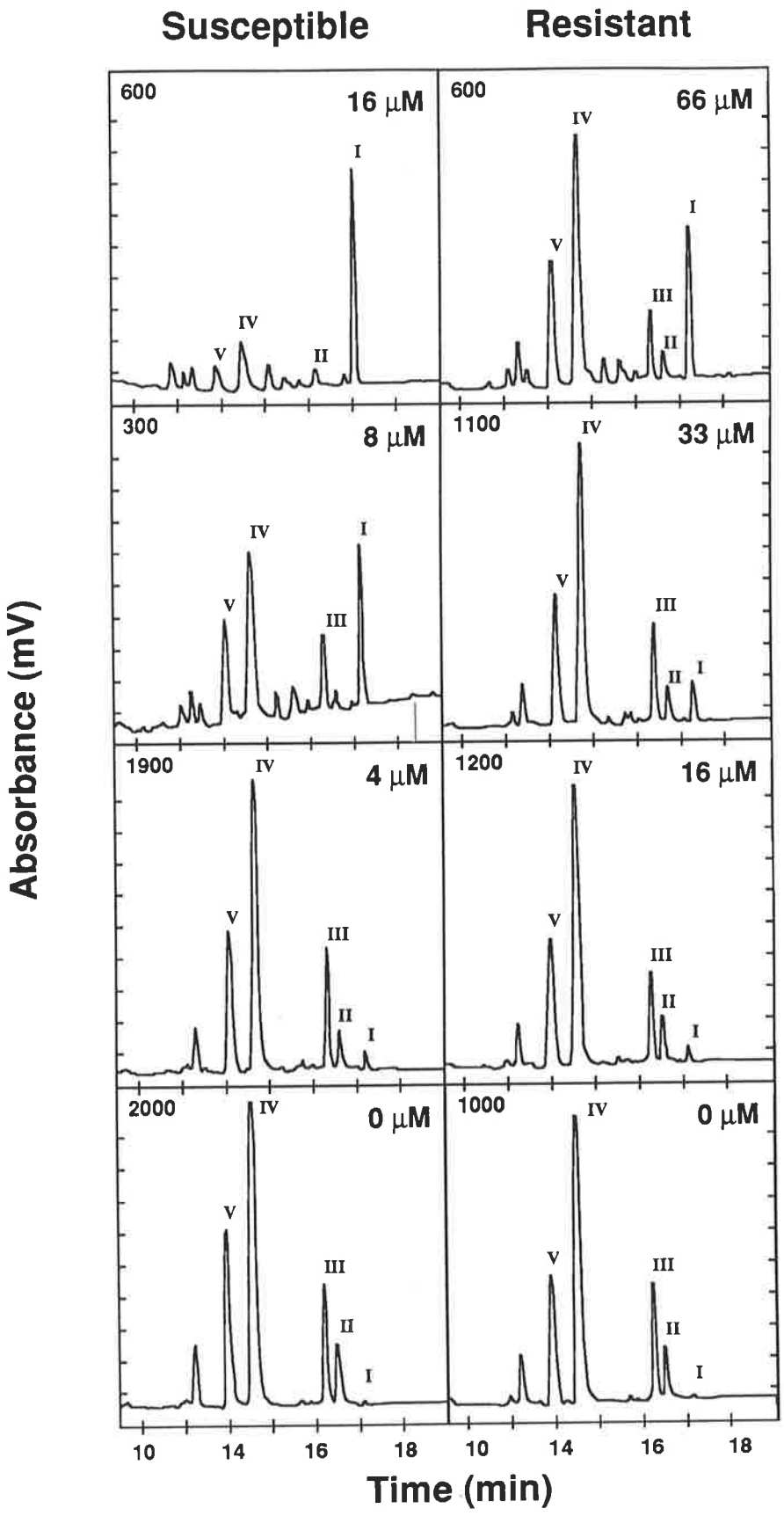

Solvent front
Rf. 0.83 amitrole
Rf. 0.67
Rf. 0.59
Rf. 0.36
Origin
Amitrole
WLR2 1
VLR1 1
WLR2 2
VLR1 2
WLR2 3
VLR1 3

The general effect of increasing doses of amitrole on plant pigments is reflected in the chromatograms of extracted pigments monitored at 287 nm (Figure 6.13). At 16 μM amitrole (Figure 6.13G) the chromatogram of extracts from resistant plants shows only a slight change in the relative peak area due to phytoene (I) when compared with the control (Figure 6.13H). However, at the same dose the susceptible biotype exhibits a dramatic reduction in all pigments with a large increase in the relative amount of phytoene (I) (Figure 6.13A and D). Chromatograms of resistant extracts comparable to those derived from susceptible plants were the result of an eight to ten times greater dose of amitrole (Figure 6.13). This indicates that amitrole still effects the pigment composition of the resistant plants but that greater concentrations of the herbicide are required to have the same effect.

The proportion of chlorophyll a (IV) and b (V) relative to other pigments declined in response to increasing amitrole doses in both biotypes although the concentration required to cause this differed (Figure 6.13). The reduction in chlorophyll levels following amitrole treatment is thought to be a secondary effect produced by light in the absence of protective carotenoids. These experiments were conducted in the presence of 12 h continuous light (50 $\mu E\ m^{-2}\ s^{-1}$) over seven days so the effect on chlorophyll in this instance is considered a secondary effect.

Data from the chromatograms illustrated (Figure 6.13) can also be expressed as the relative peak area. The proportion of total absorbance at 287 nm attributable to phytoene increases with amitrole concentration suggesting the inhibition of phytoene desaturase (Figure 6.14A). There is at least a ten-fold difference in the external concentration of amitrole required to have the same effect on both biotypes. While absorbance due to phytoene increases with amitrole concentration, absorbance due to the unidentified peak VI declines as amitrole increases indicating that it is likely to be a product of the carotenoid synthesis pathway. The same observations can be made for peaks monitored at 447 nm. Two unidentified peaks respond differently to amitrole. Again one increases as a proportion of total absorbance

Figure 6.13. Chromatograms of extracts from the leaves of seedlings which had germinated and grown on media containing various concentrations of amitrole. Figures A, B, C and D are from susceptible VLR1 seedlings. Figures E, F, G and H are from resistant WLR2 seedlings. Peaks marked I to V are tentatively identified as phytoene, phytofluene, lycopene, chlorophyll a and chlorophyll b respectively while peak VI was not identified. Data is from one of three experiments and is representative of these. Data is not intended to be quantitative but is intended to permit comparison of relative peak area.


Susceptible
Resistant
600
16 µM
600
66 µM
300
8 µM
1100
33 µM
1900
4 µM
1200
16 µM
2000
0 µM
1000
0 µM
I
II
III
IV
V
Absorbance (mV)
10
14
16
18
Time (min)

(Figure 6.15A) while the other declines relative to the other peaks (Figure 6,15B). In all cases the difference in sensitivity to amitrole as determined by the response of the various pigments was in the order of ten-fold. This difference in sensitivity is consistent with observations from the field and suggests that a similar mechanism of resistance operates at both stages of plant growth.

When assessing such data, two effects can be expected. The first is the inhibition of certain steps in the synthesis of carotenoids leading to the buildup of precursors and intermediates. The second is the degradation of pigments due to an impaired light interception system. The effects of paraquat, for example, can be observed by the presence of β-carotene-5,6-epoxide and degradation products of chlorophyll (Young *et al.,* 1989). Amitrole is likely to cause both effects. Amitrole inhibits desaturation reactions, thus preventing the conversion of phytoene to phytofluene and causing phytoene to accumulate (Burns *et al.,* 1971; Young *et al.,* 1989). The absence of carotenoids results in the photo-oxidation of pigments leading to the loss of chlorophyll amongst other pigments (Carter, 1975). To control for the effects of pigment destruction, low or zero light should be used (Sandmann and Albrecht, 1990). In these experiments differentiation of symptoms was essential so illumination was maintained.

The nature of the chromatogram obtained following treatment with inhibitors such as amitrole will depend on the wavelength at which the eluate is monitored. By choosing the absorbtion maximum for the pigment of interest the sensitivity of detection for that pigment will be maximised. Thus, to detect phytoene, absorbtion at 287 nm was monitored. To detect phytofluene, ξ carotene, and lycopene efficiently, the optimum wavelengths would be 350, 401 and 475nm respectively (Young *et al.,* 1989). Simultaneous monitoring of all of these wavelengths is possible with the use of a diode array HPLC detector. In the absence of such equipment two variable wavelength detectors in series were used at 287 and 447 nm.

Figure 6.14. The effect of different amitrole concentrations at germination on the relative peak area of phytoene (A) and the unknown VI (B) from chromatograms of resistant WLR2 (O) and susceptible VLR1 (●) extracts monitored at 287 nm. Each point is the mean of three replicates and is plotted with the standard error. Data is from one of three experiments and is representative of these.

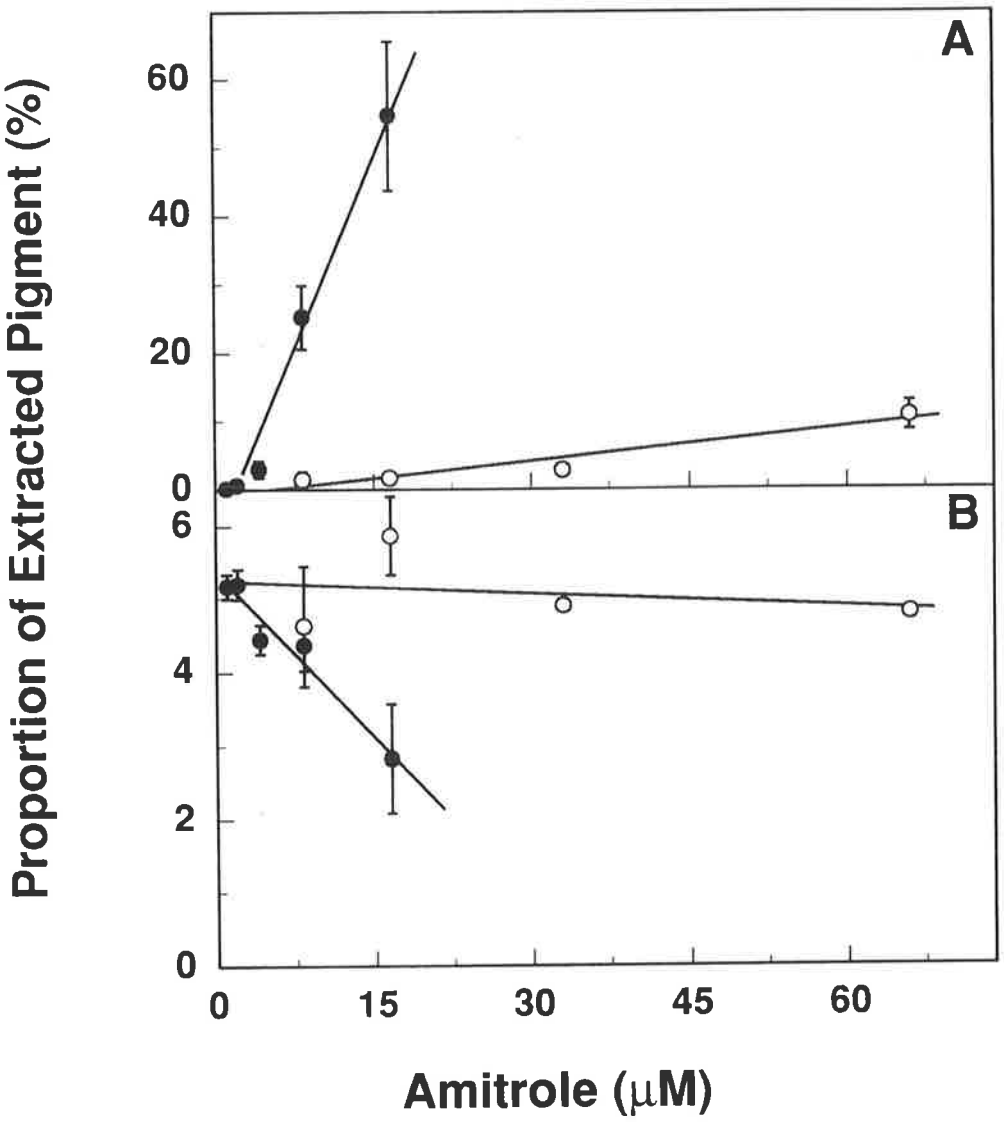
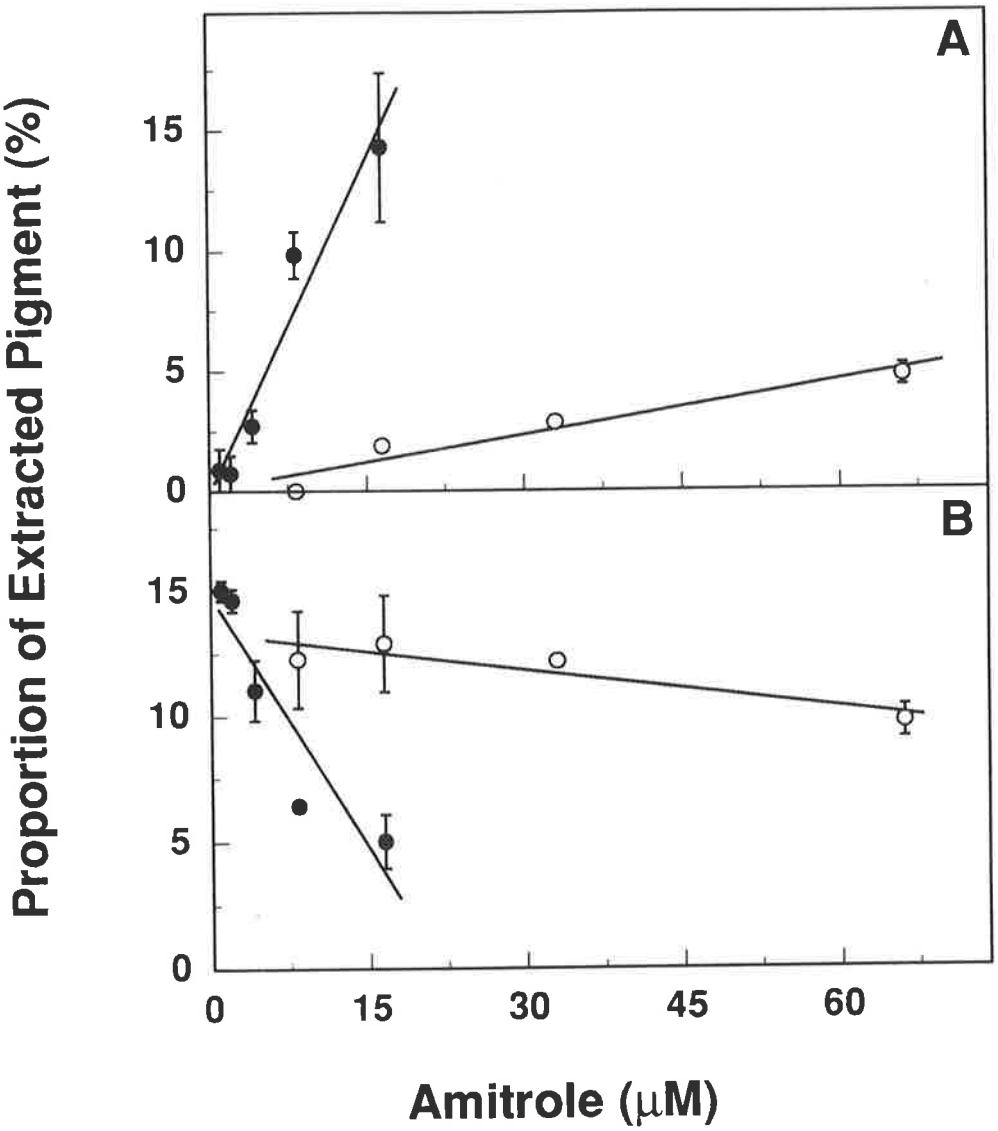

A
B
60
40
20
0
6
4
2
0
0
15
30
45
60
Proportion of Extracted Pigment (%)
Amitrole (μM)

Figure 6.15. The effect of different amitrole concentrations at germination on the relative peak area of two unknown pigments from chromatograms of resistant WLR2 (O) and susceptible VLR1 (●) extracts monitored at 447 nm. Part A describes the response of a peak with a retention time of 12.5 minutes and part B describes the response of a peak with a retention time of 18.7 minutes. Each point is the mean of three replicates and is plotted with the standard error. Data is from one of three experiments and is representative of these.


A
B
15
10
5
0
15
10
5
0
0
15
30
45
60
Proportion of Extracted Pigment (%)
Amitrole (μM)

Amitrole inhibits many different steps in carotenoid biosynthesis including carotenoid cyclisation and desaturation in the production of lycopene, phytofluene, and ξ carotene (Ridley, 1982). One objective of this study was to determine whether there were any particular pigments in the resistant extracts that appeared to be insensitive to amitrole relative to the response of other pigments when compared with the susceptible. If, for example, there had been no accumulation of phytoene in the resistant biotype it may have been possible to conclude that the resistant biotype possessed a mutant phytoene desaturase. Such a mutation would have lead to the accumulation of lycopene. This is not observed, instead the effect of amitrole on pigment composition appeared to be similar in both biotypes but with an eight to ten-fold shift in the amitrole concentration required to have the same effect. This suggests that there is no particular enzyme in carotenoid biosynthesis which is dramatically less sensitive in the resistant biotype. This was not unexpected for two reasons. Firstly, amitrole produces a similar intensity of bleaching (but of different duration) in both resistant and susceptible plants and this may reflect a similarity in the sensitivity of targets sites. Secondly, amitrole is thought to inhibit a number of different steps in the synthesis of carotenoids. Unless these are mediated by the same enzyme, regulated in the same way or the activity of the enzyme is limiting, a change in just one of the enzymes would seem insufficient to increase carotenoid production in the presence of amitrole. Given that there are no qualitative differences in the relative response of the pigments extracted from the two biotypes it is proposed that a factor other than the sensitivity of particular enzymes in the pigment synthesis pathway is responsible for resistance. However, this cannot be firmly concluded until the inhibition of each reaction is assayed *in vitro*.

Inheritance of amitrole resistance

While many aspects of the study of amitrole resistance are relatively complex, the strong visual symptoms of amitrole toxicity simplify studies on the inheritance of resistance. *L. rigidum* is an out crossing species, thus hybridisation is readily achieved by isolating two plants in a plastic sleeve (Figure 6.17). *L. rigidum* pollen is thought to be short lived and sensitive to heat and humidity (Heap, 1988). To minimise the effects of these variables, the sleeves were open at both ends and the pots were maintained in a shade house under 50 % shade cloth (Figure 6.16). To control for the effects of self pollination or pollen transfer between sleeves, single plants in pots are maintained alongside the crossing tubes. These plants set negligible amounts of seed due to the high level of self incompatibility in this species (Kloot, 1983). This system is, therefore, an efficient way to perform many different crosses. F_1 plants derived from a cross between WLR2, an amitrole resistant/haloxyfop susceptible biotype, and WLR96, an amitrole susceptible/haloxyfop resistant biotype, were in the order of 90 to 95 % resistant to both herbicides (Table 6.1, Appendix 1). This indicated that the large majority of F_1 seed was a product of a hybridisation between the two parent biotypes confirming the low rate of self fertilisation in *L. rigidum*. F_1 plants that survived applications of 1.5 kg ha^{-1} amitrole and 0.2 kg ha^{-1} haloxyfop were allowed to produce F_2 seed or were hybridised with a susceptible plant (VLR1) to produce a "pseudo-back cross" generation which could be assessed for segregation for resistance to both herbicides. The absence of segregation in the F_1 indicates that the parent lines were homozygous for resistance genes.

F_2 and backcross populations were screened with 0.25, 1.0 and 2.0 kg ha^{-1} amitrole. The rationale for this screening procedure is as follows. A low rate is used to positively identify fully susceptible individuals. An intermediate rate is used to positively identify individuals expressing resistance and to eliminate susceptibles. A high dose is chosen in an attempt to differentiate heterozygote and homozygote individuals. These doses were selected on the basis of the dose response of the WLR2 population (Figure 6.3).

Figure 6.16. Arrangement of plants maintained for crossing within a shade house at the Waite Institute during the summer of 1991.

Figure 6.17. View of two plants confined within a plastic sleeve to ensure hybridisation between specific parents.

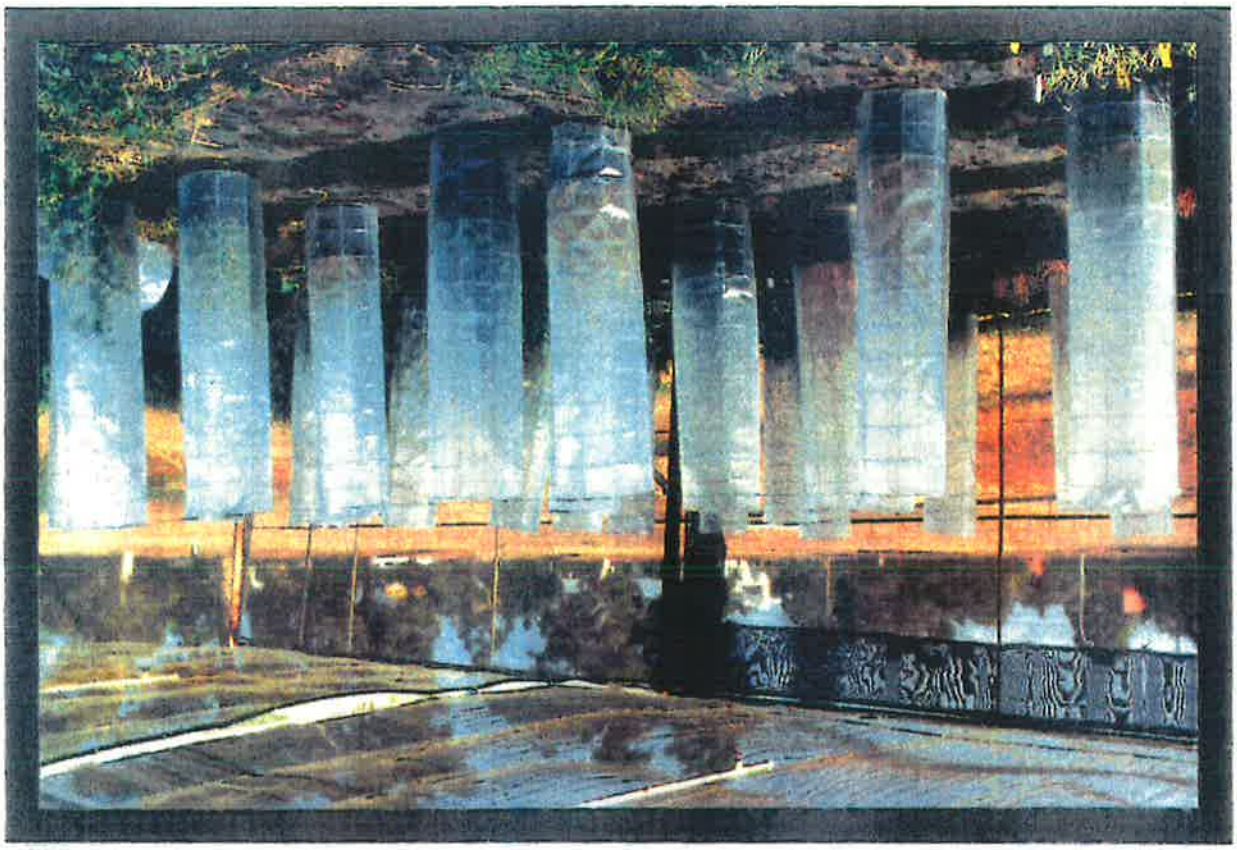


Table 6.1. Segregation for resistance to amitrole in crosses between WLR2 an amitrole resistant/ haloxyfop susceptible biotype and WLR96 an amitrole susceptible/haloxyfop resistant biotype. The amitrole susceptible/haloxyfop susceptible biotype VLR1 is included for comparison. "BC" is the abbreviation for the backcross between the F_1 and the susceptible biotype and "P" is the abbreviation for the parent population.

Cross		250 g ha $^{-1}$ Amitrole				1000 g ha $^{-1}$ Amitrole			
		total	bleached	exp. 1 gene dom.	chi ^{2}	total	bleached	exp. 1 gene dom.	chi ^{2}
WLR96	P	133	129	133	0.120	65	65	65	0.000
WLR2	P	39	1	0	0.026	71	0	0	0.000
VLR1	P	58	58	58	0.000	40	40	40	0.000
1	F1	81	13	0	2.086	35	3	0	0.257
	F2	34	6	9	0.735	56	22	14	4.571*
	BC	65	28	33	0.623	117	61	59	0.107
2	F1	83	1	0	0.012	27	2	0	0.148
	F2	44	15	11	1.455	77	18	19	0.081
	BC - 1	18	8	9	0.111	52	28	26	0.154
	BC - 2					35	10	18	3.214
	BC - 3	64	21	32	3.781	70	27	35	1.829
3	F1	75	2	0	0.053	75	4	0	0.213
	F2	31	11	8	1.363				
	BC - 1	72	33	36	0.250	87	44	44	0.006
	BC - 2	40	13	20	2.450	114	41	57	4.491*
	BC - 3	35	9	18	4.129*	59	27	30	0.212
4	F1	54	2	0	0.074	42	4	0	0.381
	F2	71	17	18	0.032	98	28	25	0.500
	BC - 1	71	35	36	0.007	156	76	78	0.051

* Value of chi square exceeds the critical value (3.841, a = 0.05) indicating poor fit of a single locus model.

Figure 6.18 Segregation for resistance to amitrole amongst plants derived from a backcross screened with 0.25 kg amitrole ha^{-1}. The segregation ratio is 1:1. The plant on the left displays symptoms which indicate that it is susceptible while the plant on the right exhibits no symptoms indicating that it is resistant.

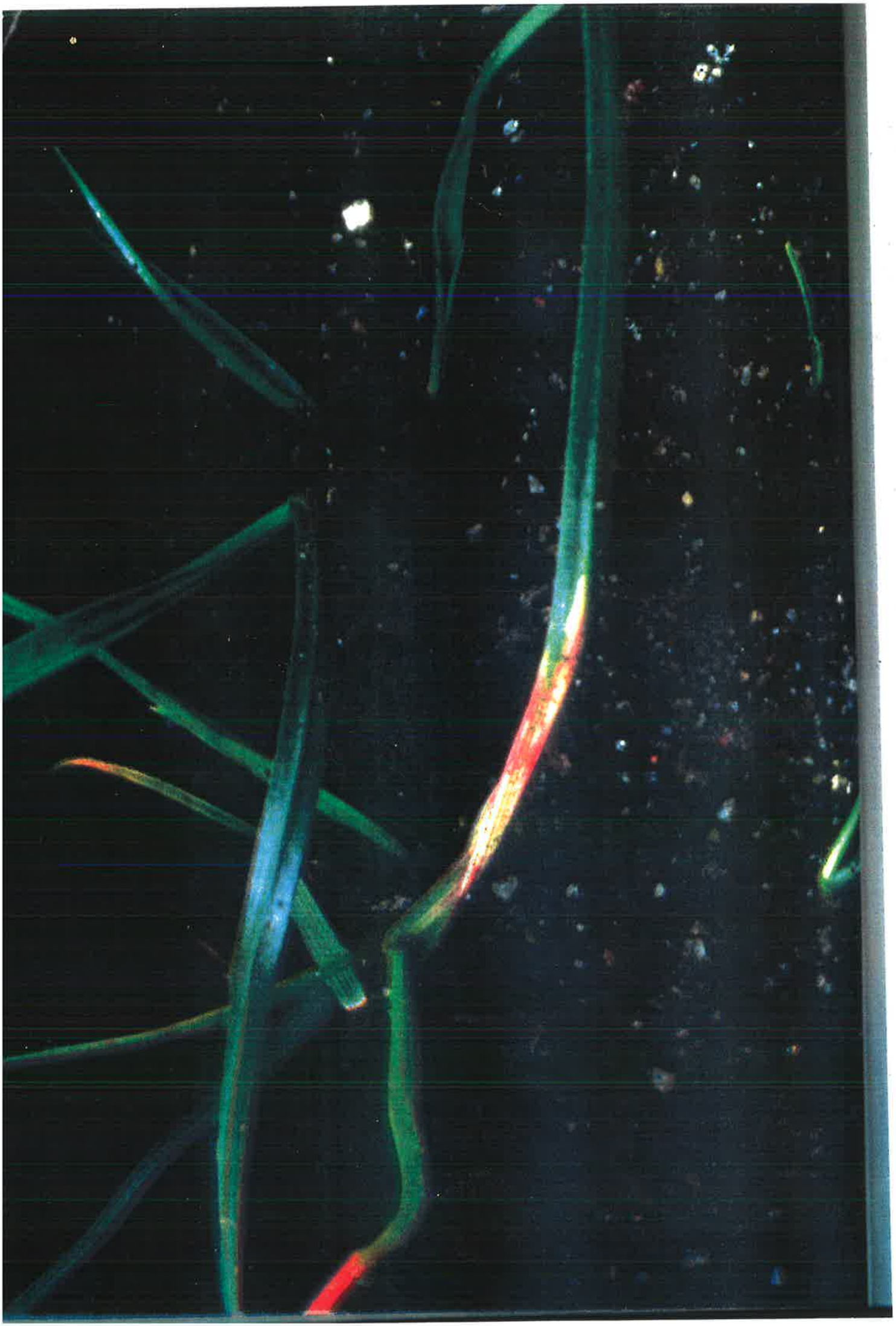


Table 6.2. Segregation for resistance to amitrole by F_2 plants derived from a cross between WLR2 an amitrole resistant/haloxyfop susceptible biotype and WLR96 an amitrole susceptible/ haloxyfop resistant biotype. The effect of the herbicide is recorded as the number of plants bleached by amitrole. "P" is the abbreviation for the parent population.

Cross		Number of plants					
		total	bleached	expected (1 gene) dominant	chi ^{2}	expected (1 gene) semi-dominant	chi ^{2}
		Amitrole 0.5 kg ha^{-1}					
WLR96	P	32	28	32	0.500	32	0.500
2	F2	126	43	31	1.399	31	1.399
4	F2	47	18	12	1.108	12	1.108
		Amitrole 1.0 kg ha^{-1}					
WLR96	P	65	65	65	0.000	65	0.000
WLR2	P	30	1	0	0.000	0	0.000
2	F2	77	18	19	0.081	19	0.081
4	F2	98	28	25	0.500	25	0.500
		Amitrole 2.0 kg ha^{-1}					
WLR2	P	33	6	0	1.091	0	1.091
2	F2	45	32	11	40.091*	34	0.272
4	F2	86	63	22	76.041*	64	0.105

* Value of chi square exceeds the critical value (3.841, a = 0.05) indicating poor fit of a single fully dominant locus model.

At 0.25 kg ha^{-1} amitrole susceptible plants can be identified by a 2 cm bleached region on the leaf emerging at the time of spraying (Figure 6.18). At this dose resistant plants do not show any symptoms. At 1.0 kg ha^{-1} amitrole, susceptible plants show complete bleaching of all new tissue and are killed. Resistant plants exhibit only a 3 cm zone of bleaching on the emerging leaf (see Figure 6.6 for an indication of this effect). There was no indication of segregation between intermediate (heterozygotes) and highly resistant plants (homozygotes) at 1.0 kg ha^{-1} amitrole. At 2.0 kg ha^{-1} amitrole only highly resistant plants survive and these plants display full bleaching of three or more leaves. It was reasoned that if the parent generation had survived 2 kg ha^{-1} amitrole a proportion of F_2 progeny would do likewise. Therefore, 2 kg ha^{-1} amitrole was chosen as the highest rate for examination of segregation.

Segregation for amitrole resistance within both the back-cross and F_2 populations is consistent with a single semi-dominant locus conferring resistance. The amitrole resistant parent has a high level of resistance up to doses of 2 kg ha^{-1} (Table, 6.2, Figure 6.3), however, beyond this dose resistance begins to break down. In F_2 plants derived from the two most fecund crosses (#s 2 and 4), 75% of the population dies at 2 kg ha^{-1} amitrole (table 6.2) while the WLR2 suffers only 20% mortality which is consistent with the dose response recorded in Figure 6.3. At lower doses of amitrole, approximately 25% of F_2 plants were classified as susceptible. This indicates that resistance is inherited as a single semi-dominant locus. This conclusion is supported by the observation that amongst the backcross populations, 50 % of individuals were classified as susceptible at a dose of 0.25 kg ha^{-1} amitrole. This was also observed at 1.0 kg ha^{-1} amitrole. The same results were obtained from all four amitrole resistant parents that were successfully crossed, suggesting that a single major gene is responsible for amitrole resistance in the WLR2 population.

6.5 Conclusions

Amitrole inhibits many different processes in a variety of organisms Whilst some of these have been identified and characterised there is not yet a full explanation of the mechanism by which amitrole causes bleaching in newly formed tissue. The very broad effects of amitrole may be due to the affinity of its 1,2,4-triazole structure for metal containing enzymes. The wide variety of sensitive target sites in plants suggests that it is unlikely that a mutation of only one target protein would confer resistance. The myriad of possible targets also complicates attempts to assay their activity and sensitivity *in vitro*. Species that have exhibited tolerance to amitrole have employed differential metabolism and translocation of the herbicide to reduce its effect. Mechanisms such as these may be more effective for herbicides with multiple sites of action.

Amitrole resistance in *L. rigidum* is at a moderate to low level. Resistant plants display transient symptoms at low doses of the herbicide whereas susceptible plants exhibit similar bleaching for a greater time after treatment (Figure 6.6). This is not due to changes in the uptake or metabolism of amitrole (Figure 6.11). Resistant and susceptible plants contain similar amounts of amitrole, however, WLR2 plants exhibit fewer symptoms, suggesting either changes to a target site, metabolism or sequestration of the herbicide confers resistance. The similarity in the proportion of radioactivity as amitrole and metabolites in both biotypes indicates that differential metabolism is not the mechanism of resistance. Target sites of amitrole in the carotenoid biosynthesis pathway respond to amitrole in the same way in both resistant and susceptible plants, however, resistant plants require a greater concentration of amitrole in the germination medium to have the same effect (Figure 6.13). Differences of the type observed could be explained by differential sequestration of the herbicide within the cell although this has not been demonstrated. Amitrole resistance is inherited as a single semi-dominant gene (Table 6.1, Table 6.2). If this gene encoded a membrane bound transport protein, changes in its expression or affinity for amitrole would alter amitrole distribution in the cell with the potential to confer resistance. It is proposed that amitrole resistance in *L. rigidum* is due to a mechanism such as differential transport.

This could be tested by examining uptake and efflux of amitrole in protoplasts made from each biotype. If there is differential transport into or within WLR2 cells this should be apparent in the rates of uptake or efflux. Other methods designed for the study of amino acid transport may also be suitable. However, given that amitrole uptake by tissue cultured tobacco cells is passive (Singer and McDaniel, 1982), it would seem that even differential intracellular transport may not be involved. Thus, the mechanism of resistance to amitrole in WLR2 is not known but it is unlikely to be due to differences in uptake into the plant or metabolism of the herbicide.

Chapter Seven

Sulfonylurea Resistance in VLR69

7.1 Introduction

Resistance to the sulfonylurea herbicides has become increasingly frequent since they were released for use in agriculture in 1982. As noted in Chapter one, the sulfonylurea and imidazolinone herbicides inhibit the enzyme, acetolactate synthase (ALS), a key enzyme in the synthesis of the branched chain amino acids (La Rossa and Schloss, 1984; Ray, 1984; Shaner *et al.,* 1984). Changes in the sensitivity of this enzyme has caused resistance in many weed species including *L. rigidum* and *L. perenne* (Saari *et al.,* 1989; Mallory-Smith *et al.,* 1990; Primiani et al, 1990; Saari *et al.,* 1992; Christopher *et al.,* 1992).

In Australia two types of sulfonylurea resistance have been reported. *L. rigidum* can exhibit cross-resistance to certain sulfonylurea and imidazolinone herbicides following selection for resistance to other herbicides (Christopher *et al.,* 1991, Cotterman and Saari 1992; Heap and Knight, 1986; Heap and Knight, 1990). The mechanism of resistance to chlorsulfuron in SLR31 involves enhanced detoxification without any change to ALS (Christopher *et al.,* 1991, Cotterman and Saari 1992). This type of resistance is very similar to that in wheat which also detoxifies some ALS inhibiting herbicides such as chlorsulfuron, triasulfuron and metsulfuron to which it is resistant despite having a sensitive ALS (Anderson *et al.,* 1989; Meyer and Muller, 1989; Sweetser, 1982). Both SLR31 and wheat are susceptible to sulfometuron which is not rapidly metabolised by wheat (Sweetser, 1985). The similarity in the patterns of resistance in wheat and ryegrass suggests that the detoxification systems are similar (Christopher *et al.,* 1991). In contrast, a recently identified resistant *L. rigidum* biotype (WLR1) is resistant to a wide range of ALS inhibiting herbicides including sulfometuron and imazapyr. The basis for sulfometuron resistance in WLR1 is a less sensitive target enzyme (Christopher *et al.,* 1992). These studies suggest that the response of a biotype to sulfometuron provides an indication of the sulfonylurea resistance mechanism expressed in the biotype.

As noted in Chapter two, biotype VLR69 is resistant to the sulfonylurea and imidazolinone herbicides including sulfometuron. This resistance developed following six seasons of selection pressure by chlorsulfuron, with the latter three of these being less effective in the control of *L. rigidum*. VLR69 is, therefore, only the second *L. rigidum* biotype to develop sulfonylurea resistance following selection pressure by this herbicide (the first was WLR1). Given that chlorsulfuron was one of the major selective pressures on VLR69 and that such cases were relatively few, it was of interest to elucidate the mechanism of resistance to the sulfonylureas. Furthermore, the dose response of VLR69 to different sulfonylureas and imidazolinones revealed anomalies which suggested that there may have been more than one mechanism of resistance operating in the biotype. The objective of this chapter is to examine the mechanism of resistance to ALS inhibitors in VLR69 and to describe techniques for the detection of multiple mechanisms of resistance.

7.2 Materials and Methods

Plant Material.

Two biotypes of *L. rigidum*, VLR1 and VLR69, were used in this study and they are fully described in Chapter two. In addition, a subset of the VLR69 that had survived 90 g ha^{-1} sulfometuron were retained and allowed to inter-cross in isolation, producing seeds that are termed "R_1". Greater than 95% of R_1 plants are resistant to 90 g ha^{-1} sulfometuron. Plant material from this generation used in ALS assays survived two (pre-emergent and at the two leaf stage) applications of sulfometuron (100 g ha^{-1}) 14 d apart with minimal growth retardation.

Dose Response to Herbicides.

Plants were maintained and herbicides applied under the same conditions as reported in Chapter two.

Dose response in agar. Sulfometuron formulated as the commercial product (750 g kg^{-1}) was dissolved in 0.6% agar at concentrations between 0.09 and 27 nM. Agar was placed in 9 cm petri dishes (20 mL/dish) and allowed to solidify before seeds (0.13 g) were distributed evenly over the agar surface. Four replicates of each treatment were made for each biotype. The dishes were placed in an incubator illuminated with fluorescent lights (photoperiod 12 h, 50 to 60 µmol photons m^{-2} s^{-1} 20 C day/night) in a randomised block design. Each block of dishes was sealed with polyethylene film to prevent moisture loss. Five days after initiation the seedlings were counted to determine the total number of seeds, the proportion in which the coleoptile had emerged and the proportion in which the first leaf had emerged over 1 cm from the coleoptile. The latter category are considered to be the resistant plants.

Enrichment procedure. To enrich for putative ALS mutants, VLR69 seeds were germinated on 27 nM sulfometuron in agar for 5 d (as described above). Vigorously growing seedlings that had green, fully extended primary leaves similar in appearance to seedlings germinating in untreated agar, were classified as sulfometuron resistant (SR) and were transferred to separate containers for further growth. Seedlings that were retarded in their growth were classified as sulfometuron susceptible (SS) and also retained separately as they remain viable (although slightly stunted) once transferred to soil. VLR69 and VLR1 seedlings growing on untreated agar were retained as controls. The advantage of this selection technique is that susceptible plants can be recovered in a viable state for further analysis.

ALS Enzyme Studies. Seedlings were grown in a growth cabinet as previously described (Christopher *et al.*, 1991). Growth room conditions were 22°C 14 h, 280 to 320 µmol photons m^{-2} s^{-1} light period/ 15°C, 10 h dark period. Shoot material (4-5 leaf stage) was harvested at soil level, frozen in liquid nitrogen and stored at -80°C until extraction. The method of enzyme extraction was based on that of Ray (1984) and Huppatz and Casida

(1985) included a precipitation using 50% saturation $(NH_4)SO_4$ which was the only purification step. ALS assays were as described by Ray (1984) except that 139 mM pyruvate was used. Acetolactate was determined by the method of Westerfield (1945) and LaRossa and Schloss (1984).

Metabolism studies. Ryegrass seedlings were treated with [phenyl-U-^{14}C]chlorsulfuron and extracted as previously described (Christopher *et al.*, 1991; 1992) except that the leaf lamina were separated from the lower portion of the shoots (culms) for separate extraction and HPLC analysis (Christopher *et al.*, 1992). The culms of these two leaf seedlings excised at soil level consisted of the sheath of the first leaf and the section of the second leaf enclosed therein. At the end of the incubation period, plant material was harvested and ground in methanol/water (4:1) to extract chlorsulfuron and its metabolites. The tissue was extracted three times with the supernatants being pooled and concentrated *in vacuo* prior to analysis by HPLC.

HPLC analysis was carried out using an ODS 250 X 4 6 mm 5 µm C18 Brownlee Labs column with a flow rate of 1.5 ml/min. Separation was achieved using two phases A= 99% H_2O 1% v/v acetic acid, B= 99.8% acetonitrile 0.2% acetic acid v/v with a gradient from 15% B to 40% B in 23 min followed by a gradient from 40% B to 100% B in 10 min. [^{14}C]Chlorsulfuron and metabolites were detected using a Radiomatic series A100 radiation monitor fitted with a yttrium silicate solid scintillant cell of 250 µL void volume.

7.3 Results and Discussion

Dose response to herbicides.

The resistant biotype VLR69 is more than 20 times less sensitive to chlorsulfuron (Figure 7.1A) and 25 times less sensitive to triasulfuron (Figure 7.1B) based on a comparison of LD_{50}. Resistance is also evident to the non-selective herbicide sulfometuron (7.5 fold) (Figure 7.1C). At high rates of sulfometuron most of the population were killed although approximately 4% survived 30 to 90 g ha^{-1} sulfometuron with only minor effects (Figure 7.1C, Figure 7.2B). This subset of the population maintain a high dry weight when treated with 90 g ha^{-1} sulfometuron whereas the majority of the population do not (Figure 7.2B). Thus, the majority of the population is highly resistant to chlorsulfuron and triasulfuron whereas only a minority are highly resistant to sulfometuron.

VLR69 also exhibits resistance to the imidazolinone herbicides but to a lesser extent than for the sulfonylureas. VLR69 was seven times less sensitive to imazaquin, a legume-selective (Shaner and Robson, 1985) imidazolinone herbicide, compared with the susceptible control VLR1 (Figure 7.3A). VLR69 exhibited relatively mild resistance to the non-selective imidazolinone, imazapyr (Figure 7.3B), however, there was a small component of the population markedly resistant to imazapyr up to a dose of 25 g ha^{-1}. Again the presence of a small number of plants with a high dry weight demonstrates that the VLR69 population is skewed with respect to its response to non-selective ALS inhibitors (Figure 7.4B). Thus, the response of the VLR69 population to imazapyr is similar to the response to sulfometuron.

Figure 7.1. The response of the susceptible biotype, VLR1 (●), and the resistant biotype VLR69 (O) to: chlorsulfuron (A), triasulfuron (B) and sulfometuron (C) applied at the two leaf stage to plants growing in pots. Each point is the mean of three replicates of 17 plants and is plotted with the standard error. Data is from one of at least two experiments of this type.

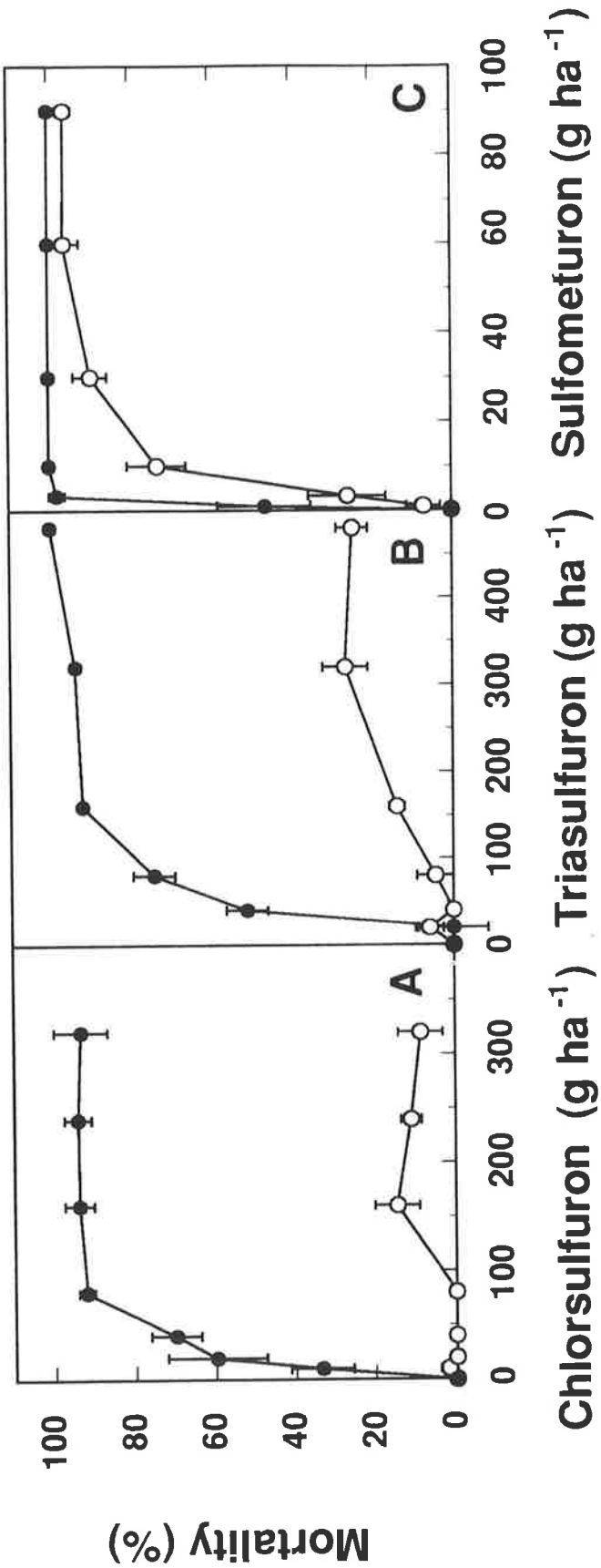

Mortality (%)
0
20
40
60
80
100
A
B
C
0
100
200
300
0
100
200
300
400
0
20
40
60
80
100
Chlorsulfuron (g ha-1)
Triasulfuron (g ha-1)
Sulfometuron (g ha-1)

Figure 7.2. Dry weight distribution for plants treated with 90 g ha^{-1} sulfometuron and harvested 21 d after spraying. The plant populations were:

A. The susceptible biotype VLR1 germinated on water agar.

B. The resistant biotype VLR69 germinated on water agar and, therefore, unselected.

C. The sulfometuron susceptible component of the VLR69 population as determined by germination on 27 nM sulfometuron.

D. The sulfometuron resistant component of VLR69 as determined by selection on 27 nM sulfometuron agar.

Each distribution is based on at least 34 plants.

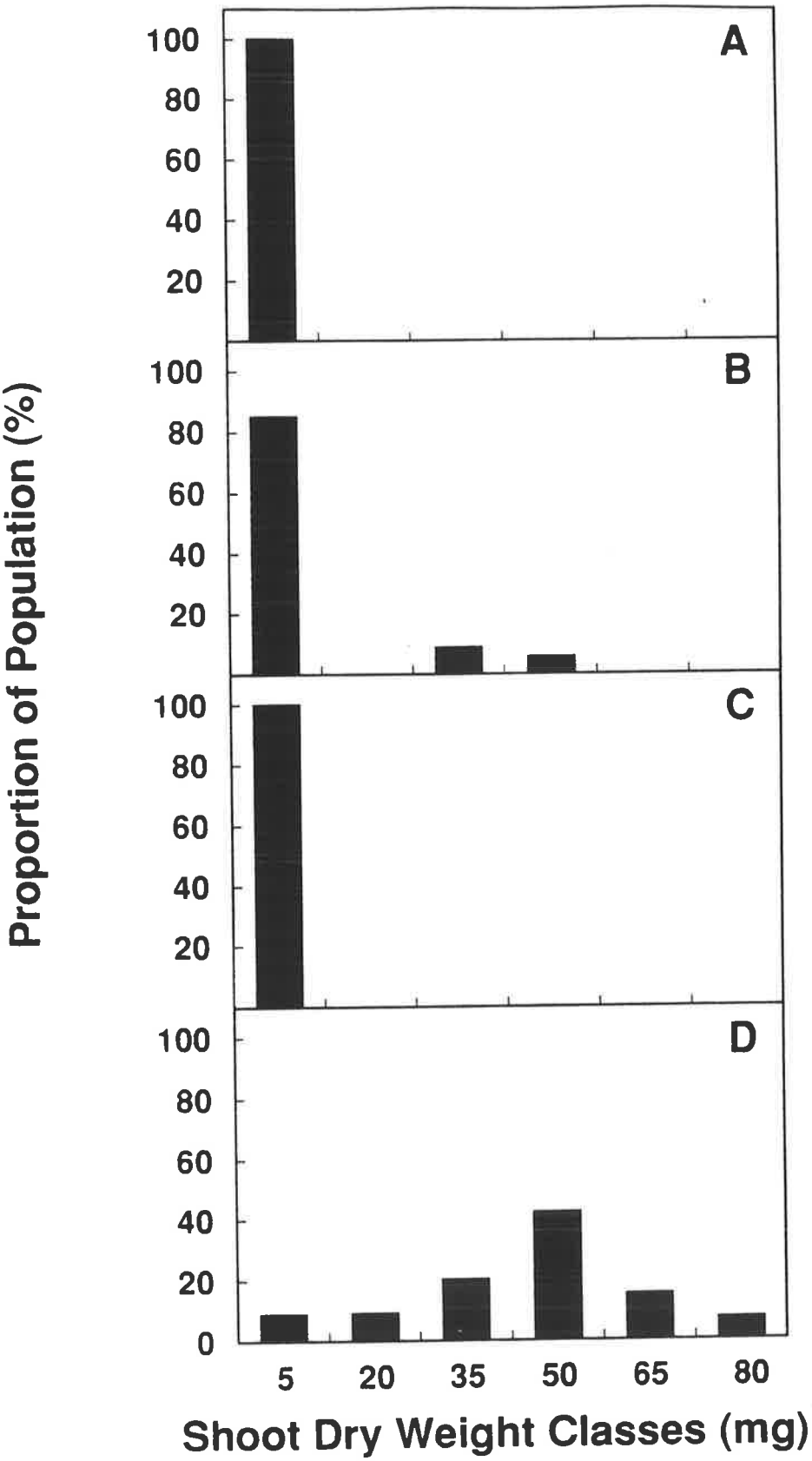

A
B
C
D
100
80
60
40
20
0
Proportion of Population (%)
5
20
35
50
65
80
Shoot Dry Weight Classes (mg)

Figure 7.3. The response of the susceptible biotype, VLR1 (●), and the resistant biotype VLR69 (O) to imazaquin (A) and imazapyr (B) applied at the two leaf stage to plants growing in pots. Each point is the mean of three replicates of 17 plants and is plotted with the standard error.

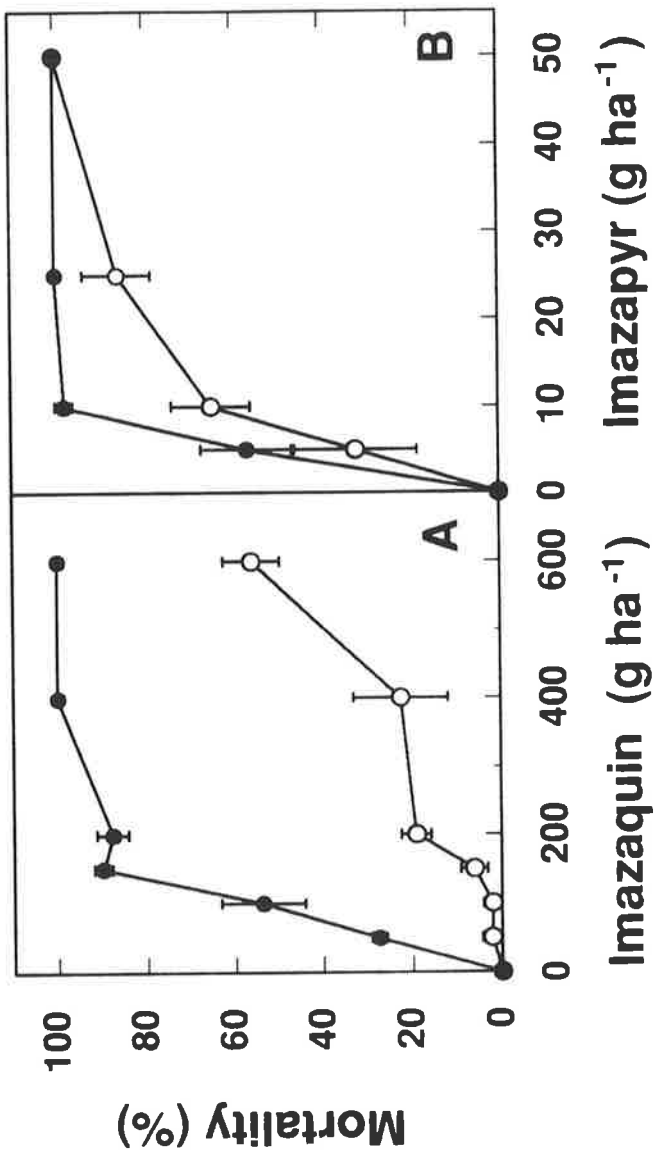
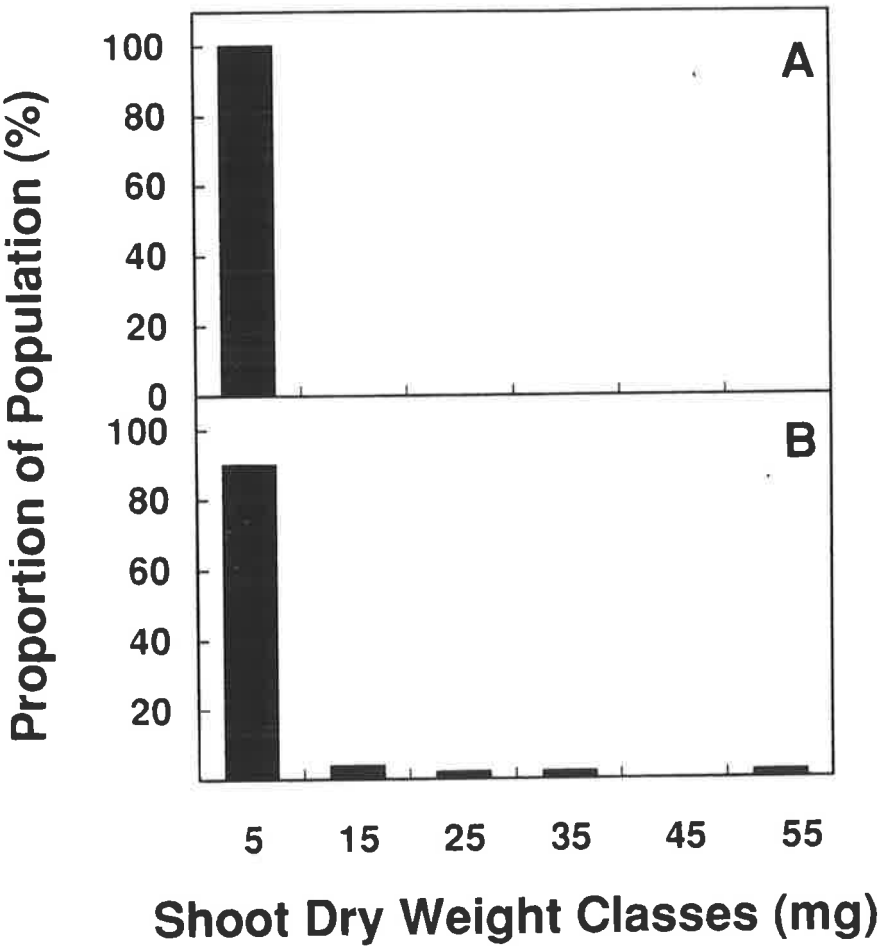

Mortality (%)
0
20
40
60
80
100
A
B
0
200
400
600
0
10
20
30
40
50
Imazaquin (g ha-1)
Imazapyr (g ha-1)

Figure 7.4. Dry weight distribution for plants treated with 25 g ha^{-1} imazapyr and harvested 21 d after treatment. The plant populations were: the resistant biotype VLR69 (A) and the susceptible biotype VLR1 (B), both germinated on water agar and, therefore, unselected. These data were obtained from the same experiment as that recorded in Figure 7.3.


Proportion of Population (%)
100
80
60
40
20
0
A
100
80
60
40
20
B
5
15
25
35
45
55
Shoot Dry Weight Classes (mg)

Dose response in agar.

Germination of VLR69 seedlings on agar medium containing various concentrations of sulfometuron further revealed that there was a small proportion of the population that grew normally at doses of sulfometuron sufficient to inhibit the majority of the VLR69 population and the susceptible VLR1 plants (Figure 7.5). The similarity in the proportion that withstands high doses of sulfometuron both in pots (Figure 7.1C) and agar (Figure 7.5) suggested that they may be the same component of the population. To test this hypothesis a larger number of such seedlings were screened from the rest of the population using sulfometuron treated agar as the selective medium.

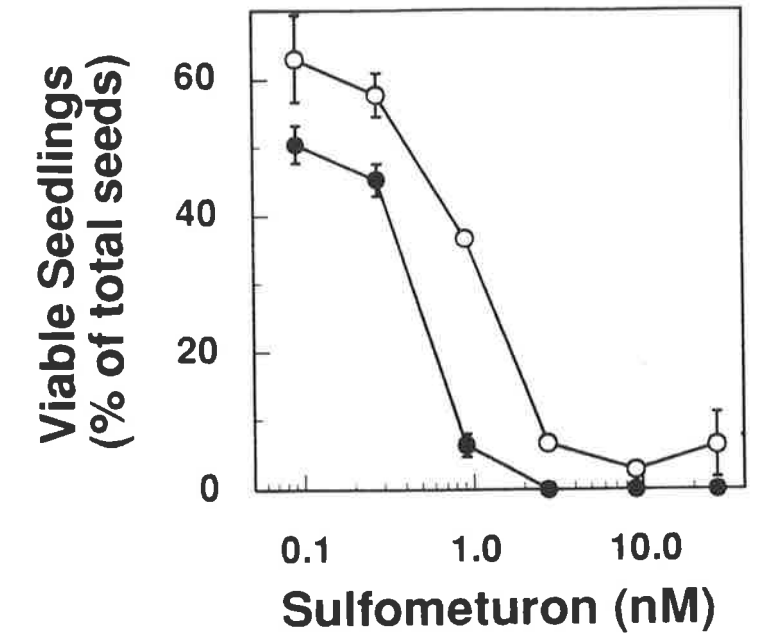
Enrichment for sulfometuron resistance.

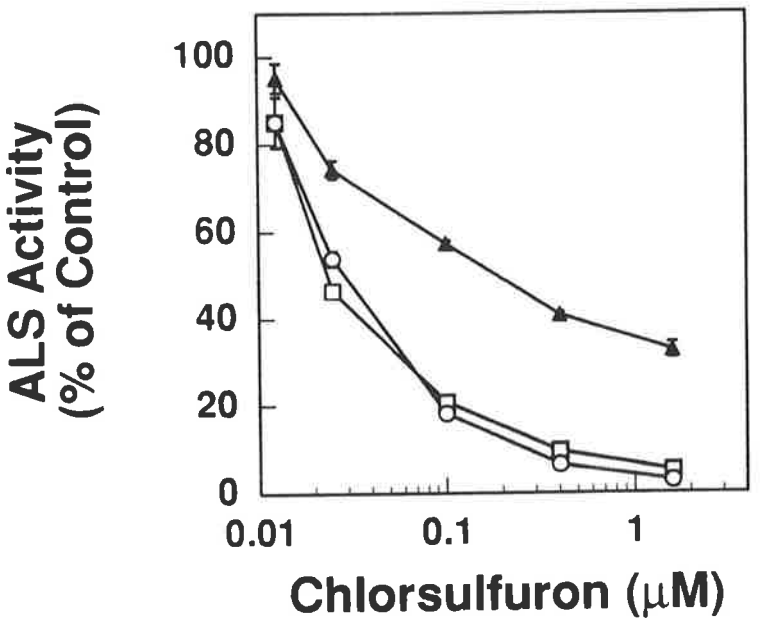
Putative sulfometuron susceptible (SS) or resistant (SR) seedlings from the VLR69 population were selected on the basis of their growth on medium containing 27 nM sulfometuron. These seedlings were transplanted to pots, grown outdoors and sprayed with 90 g ha^{-1} sulfometuron. All susceptible VLR1 and VLR69 SS plants were killed at this dose of sulfometuron (Figure 7.2A, C). The majority of unselected VLR69 plants were also killed by this treatment, however, a small proportion survived (Figure 7.2B). In contrast, 90 % of SR plants survived treatment with 90 g ha^{-1} sulfometuron, demonstrating that the SR sub-population is enriched for sulfometuron resistant plants (Figure 7.2D) (the 10 % of plants that died represent false positives from the agar selection procedure). The efficiency of this preselection on agar in enriching for sulfometuron resistant plants is reflected in the shift in the dry weight mean in the plants selected as sulfometuron resistant versus the unselected VLR69 population (Figure 7.2B, D). Therefore, the agar selection procedure is able to discriminate successfully between individual plants within a population that are sulfometuron resistant or susceptible. This procedure allows a distinct component of the population to be characterised separately from the bulk of the population.

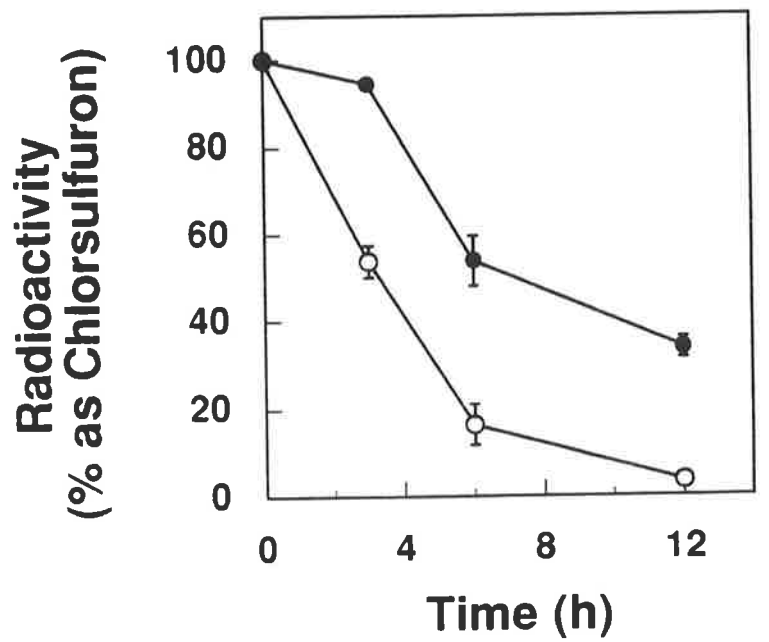
Figure 7.5. The proportion of seedlings with a fully extended green first leaf when the susceptible biotype, VLR1 (●), and the resistant biotype ,VLR69 (○), are germinated on a medium containing sulfometuron. Each point is the mean of four replicates and is plotted with the standard error. Data is from one of three experiments of this type.

Figure 7.6. Inhibition of ALS activity extracted from: the unselected VLR69 population (○), the sulfometuron susceptible component (SS) of the VLR69 population as determined by germination on 27 nM sulfometuron agar (□) and the sulfometuron resistant component (SR) of VLR69 as determined by selection on 27 nM sulfometuron agar (▲). Each point is the mean of three measurements and is plotted with the standard error. Data is from one of three experiments and is representative of these. See Materials and Methods for explanation of selection procedure.

Figure 7.7. Detoxification of [^{14}C]chlorsulfuron by the susceptible biotype, VLR1 (●), and the resistant biotype VLR69 (○) in the culms of excised seedlings. Each point is the mean of four replicates and is plotted with the standard error. This data was obtained with the direct involvement of Jack Christopher, Dept. of Crop Protection, Waite Institute.


Viable Seedlings
(% of total seeds)
60
40
20
0
0.1
1.0
10.0
Sulfometuron (nM)


ALS Activity
(% of Control)
100
80
60
40
20
0
0.01
0.1
1
Chlorsulfuron (μM)


Radioactivity
(% as Chlorsulfuron)
100
80
60
40
20
0
0
4
8
12
Time (h)

Inhibition of ALS.

The ALS activity extracted from the sulfometuron resistant component (SR) of the population is less sensitive to inhibition by chlorsulfuron (I_{50} = 260 nM) than is the ALS extracted from the sulfometuron susceptible component (SS) of VLR69 (I_{50} = 35 nM) (Figure Figure 7.6). The I_{50} for the unselected VLR69 population was 46 nM. These observations are supported by similar observations on the effect of sulfometuron and imazapyr on ALS activity extracted from SR plants. ALS activity extracted from the progeny of SR plants, the R_1, was also less sensitive to inhibition by these herbicides (data not shown). The insensitivity of ALS extracted from sulfometuron resistant (SR) plants to inhibition by a sulfonylurea herbicide explains why these plants survive treatment at relatively high rates of sulfometuron (Figure 7.1C, 7.2D).

Chlorsulfuron metabolism.

Given that only 4% of the VLR69 population express an insensitive ALS (Figure 7.6) an alternative mechanism must confer resistance in the bulk of the population. Seedlings of the unselected VLR69 population exhibited a greater capacity to detoxify chlorsulfuron than the susceptible VLR1 population (Figure 7.7). Uptake of chlorsulfuron into the cut shoots as indicated by the amount of radioactivity recovered was similar for both biotypes. These data indicate that enhanced detoxification contributes to the mechanism of resistance to chlorsulfuron in VLR69. The metabolites observed in VLR69 have a similar HPLC elution profile to those of *L. rigidum* biotype SLR31 (Christopher *et al.*, 1991) and VLR1 indicating that the enhanced detoxification is not due to the production of novel metabolites.

7.4 Conclusions

It is apparent that there are at least two mechanisms of resistance to ALS inhibiting herbicides in the VLR69 population. Plants that do not possess an altered ALS are resistant to chlorsulfuron due to enhanced rates of detoxification (Figure 7.7). Individuals possessing this mechanism are not resistant to sulfometuron presumably because they cannot metabolise the herbicide. However, some individuals in the VLR69 population possess a

less sensitive ALS (Figure 7.6) which endows resistance to ALS inhibitors including sulfometuron. The reason for the different frequency of the two resistance mechanisms is unknown. The relative fitness of plants expressing an altered ALS versus those with enhanced detoxification in the presence and absence of chlorsulfuron is also unknown. The high frequency of plants in the population employing the enhanced detoxification resistance mechanism may be due to the history of herbicide use in the field which first selected plants able to detoxify triazine and phenylurea herbicides (Chapters three and four). The subsequent use of chlorsulfuron may have favoured plants with some degree of cross-resistance through metabolism. If enhanced metabolism was fully adequate in endowing resistance there would appear to be no selective advantage for individuals possessing altered ALS enzymes. However, the rate of herbicide metabolism varies with changes in the environment and stress may reduce the rate of detoxification (Beyer *et al.*, 1988). It is possible that under stress conditions the altered ALS mechanism may have been favoured and these plants may have become more prevalent in the population.

Resistance to ALS inhibiting herbicides in biotype VLR69 demonstrates that more than one mechanism of herbicide resistance may be selected in one weed population of a weed species at one site. It is now evident that large populations of out-crossing species such as *L. rigidum* exhibit considerable variation for resistance mechanisms. Physiological studies on a sample of the whole population may reveal broad trends but mechanisms at lower frequencies may be obscured by individuals with different resistance mechanisms. The differential effect of chlorsulfuron and sulfometuron *in vivo* (Figure 7.1) allow the identification of individuals (Figure 7.2) that possess different mechanisms of resistance which would not otherwise be apparent. Thus, the fact that sulfometuron is less amenable to metabolism allows its use to detect ALS mutants in a background of chlorsulfuron metabolising resistant plants. The same techniques could also be applied to other herbicide systems. Studies of herbicide resistance mechanisms in species such as *L. rigidum* may, therefore, be more conclusive if a population is screened to discriminate individuals with a different complement of resistance mechanisms. In conclusion, it is proposed that studies

on the physiological basis of resistance may be simplified if mechanisms are first elucidated in plant sub-populations that are highly enriched, or homogeneous, for the particular mechanism of interest. This may be especially important in cases such as *L. rigidum* biotype VLR69 where there has been selection pressure from many different types of herbicides applied to a genetically variable out-crossing species.

Chapter Eight

General Discussion and Conclusions

8.1 Resistance to Photosystem II Inhibitors

It is apparent from Chapters, three four and five that the *Lolium rigidum* biotypes WLR2 and VLR69 have evolved resistance to the triazines, phenylureas and metribuzin. Resistance is not due to changes in target site sensitivity or changes in uptake of the herbicide. The only consistent difference between the resistant biotypes and the susceptible is in the rate of detoxification of these herbicides. While there appear to be no major barriers to herbicide transport into the leaves it is not known whether transport into the cells or chloroplasts of the resistant biotypes is the same as in the susceptible biotype. To determine whether detoxification alone could account for the resistant phenotype the effects of the mixed function oxidase inhibitor, ABT, were examined. In combination with all three classes of herbicide, ABT caused reductions in fresh and dry weight such that resistant plants resemble susceptibles treated with the herbicide alone. When applied in combination with ^{14}C labelled herbicides under conditions similar to those causing reductions in growth, ABT had the effect of increasing the amount of herbicide accumulated by resistant plants to near levels accumulated by susceptibles treated with the herbicide alone. Thus inhibition of detoxification returned the resistant plants to a near susceptible phenotype indicating that differential metabolism is the major difference between the biotypes in this system.

The relatively low level of resistance evident in these biotypes is consistent with detoxification being the primary mechanism of resistance. Similarly, the observation that VLR69 is slightly more resistant than WLR2 for all herbicides is consistent with the slightly greater rate of detoxification exhibited by VLR69 in all experiments. Lastly, cross-resistance in these biotypes is explained by the differential metabolism of each class of herbicide. If, for example, there had been no evidence of differential metabolism of chlorotoluron in WLR2, then there would have been cause to invoke other mechanisms of

resistance to account for the cross-resistance phenomena. The consistency across all three classes, and particularly between chlorotoluron and simazine, creates no such need.

Given that enhanced detoxification can explain the resistant phenotype it is of interest to speculate as to how resistance arose and by what means detoxification has been increased. In Chapter two it was noted that WLR2 and VLR69 are derived from very different systems of selection pressure. Given these differences it is important to consider how these distinct origins resulted in biotypes expressing similar mechanisms of resistance.

WLR2 was selected with high rates of amitrole and atrazine. These rates were well in excess of those which would cause complete mortality of the susceptible biotype under optimal circumstances (Figure 2.1). It was proposed in Chapter two, that the nature of the sprayed area with its long narrow geometry lent itself to selection pressure at, and invasion by, species growing at the margin. These margins are an area where the herbicide is diluted through drift and runoff, where there is better soil and in which there is sufficient vegetation to provide large numbers of seed for invasion of the sprayed zone. Under these circumstances it is hypothesised that directional selection for increases in quantitative resistance may have occurred.

The development of resistance in VLR69 differed in that it was exposed to more herbicides over a greater period in an agricultural system. The first decade of control was achieved with diuron, with timing and placement of the herbicide providing selectivity (Table 1.1). The rates of diuron employed were initially low, probably due to the narrow margin of safety between the *L. perenne* crop and *L. rigidum*. This less extreme selection pressure may have allowed the development of a metabolism based resistance mechanism which confers a low level of resistance. The other major selective agents were atrazine and chlorsulfuron. Atrazine appears to have mechanisms of detoxification in common with diuron as indicated by cross-resistance in WLR2 and the similarity of resistance mechanisms elucidated in Chapter three and four. Thus, atrazine may have selected diuron resistant

plants whose detoxification enzymes were also active against the triazine herbicides. Chlorsulfuron has not been implicated in cross-resistance phenomena with the triazines or the phenylureas and it is assumed that the increase in chlorsulfuron detoxification in this population is a separate, independently selected phenomenon, although the population may have been predisposed to enhancement of detoxification capacity. Diclofop resistance has been associated with chlorsulfuron resistance in some biotypes (SLR31, NLR12, SLR10) but like chlorsulfuron, has not been associated with cross-resistance to the phenylureas or triazines in other biotypes. No genetic studies were conducted on VLR69 so the inheritance of resistance in this biotype is not known. However, like WLR2, VLR69 exhibits variation in response to the triazines and phenylureas suggesting the contribution of quantitative traits to resistance. The development of quantitative resistance traits in VLR69 cannot be ascribed to the effects of the margin, as these are reduced in large agricultural fields. Rather, it is proposed that the use of relatively low rates in the initial stages of selection allowed the accumulation of genes with limited effect, which in combination provide the plant with a low level of resistance.

Given that in both cases the resistance to PS II inhibiting herbicides appears to be a low level quantitative phenomenon, are there models which would explain the mechanistic basis of such resistance and cross-resistance? This thesis has established that there is increased metabolism of all three herbicide classes. In each case this may be inhibited by ABT but there are differences in the magnitude of the synergy obtained with ABT for each class, with chlorotoluron metabolism appearing most sensitive. The most simple model to explain this is that there is a change in a single enzyme, most likely a mixed function oxidase, that uses all three herbicides as a substrate. The nature of this change is unknown but it could include a mutation of the gene leading to a changed amino acid sequence and altered affinity for the substrates. Alterations to the level of expression might also have the same effect. Altered expression could result either from amplification or changes in regulation. Amplification of single genes encoding target sites have been demonstrated *in vitro* using cell suspension cultures and various herbicides (Donn *et al.* 1984; Deak *et al.*, 1988). While cell cultures

subjected to selection pressure may amplify target site genes there are no reports of the amplification of genes for detoxification enzymes in plants or plant cell cultures. However, atrazine resistance in *A. theophrasti* may be due to changes in the level of expression of specific GST enzymes. This could be a consequence of either amplification or altered regulation (Anderson & Gronwald, 1991). Insecticide resistance may be conferred by similar phenomena (Sundseth *et al.* 1989; Brown, 1990). Thus directional selection may result in amplification but whether this is a likely response in whole plants and in genes for herbicide detoxification is less clear.

8.2 A model to explain cross- and multiple-resistance due to metabolism

A model can be constructed in which there are increases in the activity of more than one enzyme again either through amplification or changes in regulation. The model is presented in a manner analogous to an elution profile and the 10 enzymes postulated in the model can be considered as near pure eluate fractions of equal total protein, which have been tested for activity with a range of substrates. The separation of GST isozymes using FPLC can provide similar data when fractions are tested against atrazine and CDNB (Anderson and Gronwald, 1991). Activity differs for each substrate within the same fraction leading to the style of presentation of Figure 8.1.

In this model (Figure 8.1) the various mixed function oxidases that increase in activity, range in their affinity for the selecting herbicide, however, each provides some protection (Figure 8.1A, enzymes 3, 4, 6, 9). In combination they provide a relatively higher level of resistance. For herbicides like simazine or chlorotoluron that are detoxified in two steps they may also differ in their ability to perform each of these steps thus making the combination more effective than either alone. This subset of mixed function oxidases will also vary in their affinity for other herbicides or their sensitivity to inhibitors. This variation in activity against the various herbicide substrates can be illustrated hypothetically using the model and comparing the height of the bars for one enzyme with different substrates (Figure 8.1). In this model a nominal figure of 10 enzymes is postulated (although there may be up

to 70 genes in mammalian cells, Nebert and Gonzalez, (1987) and an unknown number in plants).

Each of the 10 enzymes has various levels of activity against the herbicides listed and various degrees of sensitivity to inhibitors. The model is designed to illustrate how cross-resistance to metribuzin and the phenylureas developed in WLR2. In the model, enzymes 3, 4, 6 and 9 all have relatively high activity against simazine. Enzymes 3 and 9 have high activity against chlorotoluron while enzymes 3, 6 and to a lesser extent 9 are most active against metribuzin. Thus over expression of any of 3, 6 or 9 will lead to cross-resistance to metribuzin and chlorotoluron following selection by simazine while differences in the relative expression of each of these would alter the degree of cross-resistance (Figure 8.1A, B and C).

Cross-resistance to diclofop in WLR2 is not observed and the model therefore postulates that only enzymes 1 and 7 are active against diclofop (Figure 8.1D). Similarly, WLR2 displays no cross-resistance to chlorsulfuron and the model, therefore, postulates that chlorsulfuron is detoxified by enzymes 7 and 10 (Figure 8.1E). The association between enzymes 7 and 10 is made in consideration of a number of observations. Firstly, chlorsulfuron and diclofop are associated in cross-resistance relationships in a number of biotypes (SLR31, NLR12, SLR10) (Heap and Knight, 1990) with differential metabolism implicated in some cases (Christopher *et al.*, 1991; Holtum *et al.*, 1991) Therefore, chlorsulfuron and diclofop are shown having a detoxifying enzyme in common, 7. However, Zimmerlin and Durst (1991), report that a diclofop hydroxylase prepared from wheat induced with phenobarbital was not competitive with, or active against, chlorsulfuron or chlorotoluron, hence the specification of enzyme 1 which does not hydroxylate chlorsulfuron or chlorotoluron but is responsive to phenobarbital (Figure 8.1H). In contrast, McFadden et al (1989) demonstrated competition between chlorsulfuron and diclofop in microsomes that were not induced, thus the inclusion of the enzyme in common, 7 (which is less responsive to phenobarbital), to accommodate this observation. The model breaks down somewhat

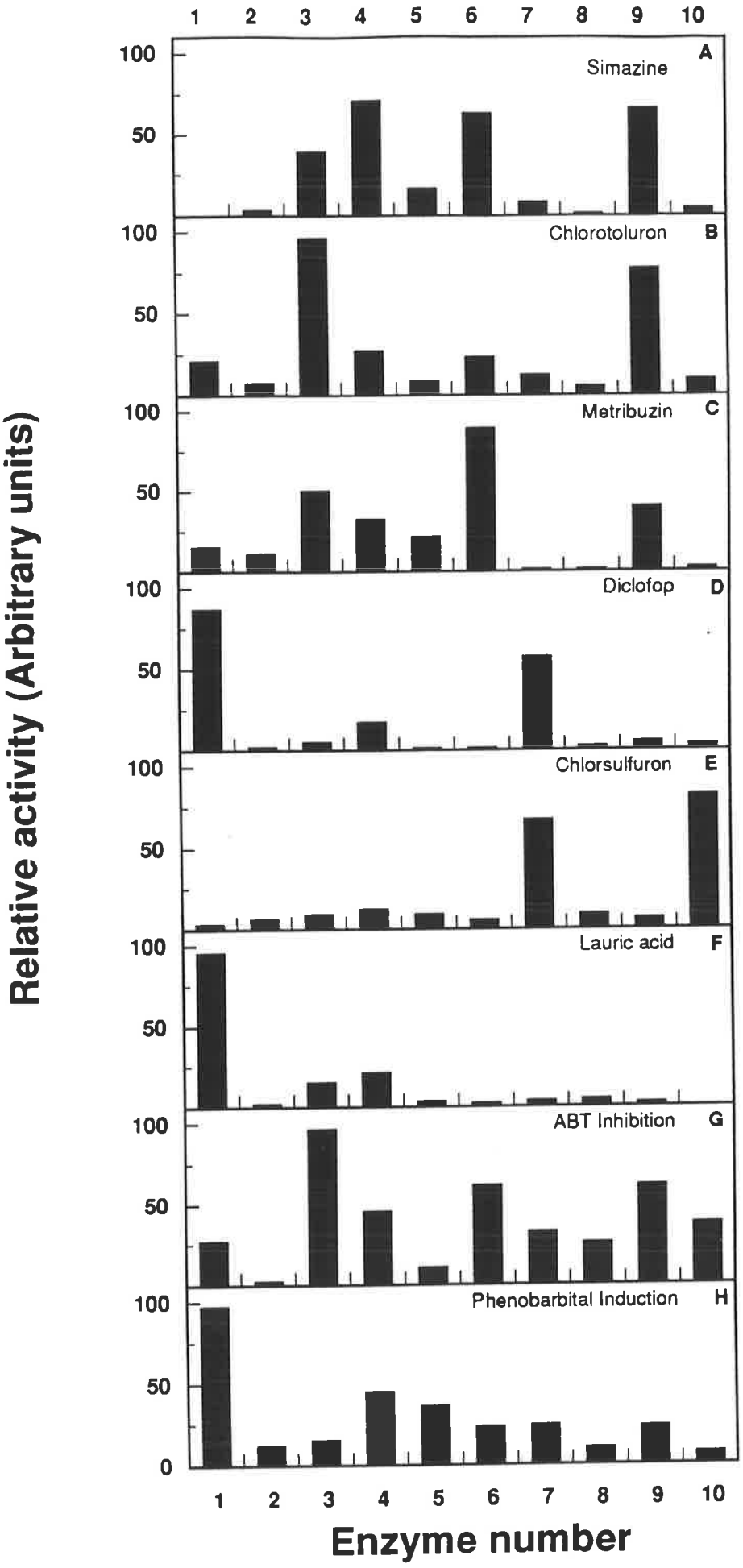
with the observation of Frear et al (1991) that chlorsulfuron hydroxylase is induced by phenobarbital in wheat (cv Olaf), however, these authors report only weak competition between triasulfuron and diclofop.

The enzymes can also have endogenous functions with some being isozymes performing the same reaction (Werck-Reichhart *et al.*, 1990). For the purpose of the model, lauric acid hydroxylation is used as an example (Figure 8.1F). This activity is induced by phenobarbital in *H. tuberosus* tubers and wheat seedlings (Salaün *et al.*, 1981; Zimmerlin and Durst, 1991). The activity also seems closely associated with diclofop hydroxylase activity (Zimmerlin and Durst, 1991). Given these two observations, the model accommodates all of this activity as enzyme 1 (Figure 8.1F and D). The association between diclofop and lauric acid hydroxylase can be tested by determining the competitive inhibition of lauric acid on diclofop hydroxylase. If lauric acid cannot suppress this activity it would seem unlikely that they are the same enzyme. In fact, each is a potent competitor with the other for metabolism *in vitro*, suggesting that they may be metabolised by enzymes in common (Zimmerlin, 1992).

The effect of an inhibitor may also be used to distinguish between isozymes. In this case the effects of ABT on various enzymes is postulated. ABT is a weak inhibitor of triasulfuron and diclofop hydroxylation in wheat microsomes so the inhibition of enzymes 1, 7 and 10 is shown as being low (Frear *et al.*, 1991; McFadden *et al.*, 1989) (Figure 8.1G). ABT has contrasting activity against the two major reactions of chlorotoluron detoxification in wheat (Canivenc *et al.*, 1989; Mougin *et al.*, 1990) and so ABT is assigned strong inhibition of enzyme 3 (ring-methyl hydroxylation) and lower inhibition of enzyme 9 (N-demethylation). ABT also inhibits simazine and metribuzin metabolism (Chapters three and five) and so enzymes 4 and 6 are also shown with moderate inhibition (Figure 8.1G).

The model is necessarily simplistic as its only purpose is to provide a conceptual framework for understanding the diversity of mixed function oxidase enzymes and how changes in

Figure 8.1. A model for the relative activity of ten hypothetical mixed function oxidase enzymes for various substrates including simazine (A), chlorotoluron (B), metribuzin (C), diclofop (D), chlorsulfuron (E), and lauric acid (F), compared with their response to ABT (G) and phenobarbital (H). The model is abstracted largely from data obtained with graminaceous systems (McFadden *et al.*, 1989; Frear *et al.*, 1991; Zimmerlin and Durst 1991; Mougin *et al.*, 1990; 1991; Canivenc *et al.*, 1989; Reichhart *et al.*, 1986; Reichhart *et al.*, 1982; Cabanne *et al.*, 1987; Werck-Reichhart *et al.*, 1990; Salaün *et al.*, 1981; Christopher *et al.*, 1991; Holtum *et al.*, 1991; Nebert and Gonzalez, 1987)


Relative activity (Arbitrary units)
1 2 3 4 5 6 7 8 9 10
100
50
Simazine A
Chlorotoluron B
Metribuzin C
Diclofop D
Chlorsulfuron E
Lauric acid F
ABT Inhibition G
Phenobarbital Induction H
0
Enzyme number

these enzymes may lead to the cross- and multiple-resistance phenomena observed in *L. rigidum*. The model was formulated from publications and the material presented is derived from many different species and cultivars. Given the apparent variation between these groups for characters such as herbicide sensitivity, the model cannot hope to be accurate on specific issues. Rather, the model is designed to indicate that changes in the level of expression of a range of enzymes will lead to cross-resistance due to metabolism. The resistance or cross-resistance status of the plant will depend on which suite of enzymes increase in activity following selection pressure. Similarly, exposure to a safener may induce a range of enzymes with the breadth of the safeners effect being determined by which suite is induced. This hypothetical model, therefore, provides an explanation for how cross-resistance might develop. Its major assumption is that increases in the expression of suites of oxidative enzymes may occur in plants following selection pressure from herbicides or induction by safeners. There are precedents for this in insecticide resistance. Insect biotypes with resistance due to enhanced detoxification may express a novel size class of cytochrome $P\text{-}450$ enzymes (Sundseth *et al.*, 1989). In this example, selection with DDT lead both to a 20 fold increase in a previously rare class of cytochrome $P\text{-}450$ enzymes and a concomitant increase in malathion resistance. Similarly, hepatic systems induced with phenobarbital express a number of cytochrome $P\text{-}450$ isoforms with a broad spectrum of activity against xenobiotics (Nebert and Gonzalez, 1987). Thus enhanced expression of a group of enzymes by various means can have the effect of increasing oxidation of many different compounds.

All good models should be able to make predictions which will either support or invalidate them. With respect to cross-resistance in WLR2 and VLR69 it is predicted that microsomes isolated from seedlings of these biotypes will exhibit oxidative activity against metribuzin, simazine and chlorotoluron. Furthermore, each of these substrates will be competitive with the other to an appreciable extent. If a range of inducers are tested, they may vary in the degree to which they induce each reaction and in their effect on competition between substrates, due to differences in the degree of induction of each enzyme by different

inducers. Finally, it is predicted that chlorotoluron and simazine will be better substrates than diuron and atrazine respectively.

Other herbicide resistant biotypes also display cross-resistance to different chemical classes of herbicides that inhibit the same target site. For the bulk of the VLR69 population, cross-resistance to imidazolinones is not due to changes in the sensitivity of ALS (Figure 7.3, 7.6). This majority of the VLR69 population exhibit an enhanced capacity to metabolise chlorsulfuron and display high resistance only to imidazolinones which are also susceptible to metabolism in plants (Chapter seven). Similar results have been found in biotype SLR31 (Christopher *et al.*, 1991). This association suggests that the same enzymes or enzyme systems mediate the detoxification of both classes of herbicides. The model, if extended to these herbicides, it would predict that imazamethabenz and chlorsulfuron may be competitive in microsomal preparations from *L. rigidum.*

Implicit in this speculation is that herbicides which bind to the same target may be subject to detoxification by the same enzymes. Thus, selection pressure from atrazine leads to a biotype which can detoxify simazine and chlorotoluron more readily. It is possible that their similar affinity for a target site provides them with sufficient similarity at the molecular level for them to be substrates of the same degradative enzyme. The cross-resistance to imidazolinones in chlorsulfuron resistant biotypes could be an example of this relationship. The proposition that herbicides of the same class may be detoxified by the same enzymes is supported by the observation of chlorsulfuron competition with triasulfuron metabolism (Frear *et al.*, 1991). Therefore resistance to many members of a herbicide class such as the resistance to most triazine analogues in WLR2 (Table 2.1) may be due to the effects of the same enzyme(s). Such assumptions need to be examined by isolating and testing the enzyme system *in vitro*.

The major conclusion that can be made from the study of triazine, phenylurea and metribuzin resistance in WLR2 and VLR69 is that the mechanism of resistance to each herbicide appears

similar. Given that two triazine/phenylurea resistant biotypes are cross-resistant to metribuzin, it would appear likely that the detoxification of metribuzin is catalysed by the same enzymes that detoxify the triazines and phenylureas.

8.3 Amitrole Resistance

The mechanism of amitrole resistance in WLR2 was not elucidated in this study, however, resistance is inherited as a single semi-dominant gene. There was no measurable difference in the metabolism of the herbicide in the leaves of germinating seedlings (Figure 6.12). The absence of symptoms at high internal amitrole concentrations (Figure 6.12) in WLR2 plants suggests that there is either a less sensitive target or enhanced sequestration. The many different potential targets of amitrole binding make it difficult to assess the sensitivity of these enzymes. If resistance did involve an altered target, single gene inheritance would suggest that only one target is involved. Alteration of a single target would only confer resistance if the next most sensitive target was at least tenfold less sensitive than the first. Given the variety of potential amitrole targets and the observation that amitrole resistance in WLR2 appears "shifted" rather than different in any particular character, it is considered unlikely that a target site alteration confers resistance in WLR2. Instead, by elimination, it is proposed that differences in the transport and sequestration of the herbicide at the cellular level may confer resistance. There is, however, no direct evidence for this.

8.4 Resistance to ALS Inhibitors

Plants in the VLR69 population exhibited two mechanisms of resistance to the sulfonylureas. The majority are resistant to chlorsulfuron and have a greater capacity to metabolise the herbicide (Figure 7.7). While ALS extracted from the whole population is sensitive to inhibition by chlorsulfuron, ALS extracted from a sulfometuron resistant subset of the population is less sensitive (Figure 7.6). This difference in the ALS expressed by different individuals in the VLR69 population manifests itself when the plants are sprayed

with high rates of sulfometuron. Only those with an altered ALS survive sulfometuron treatment. Given that sulfometuron is less readily metabolised in wheat (Sweetser, 1985) it is assumed that it is also poorly metabolised in *L. rigidum*, hence sulfometuron resistance mechanisms are confined to ALS mutations (in biotypes investigated thus far).

The dichotomy in the VLR69 population made apparent by the effects of sulfometuron illustrates a major theme emerging from studies of herbicide resistance in *L. rigidum*. This is the potential for a plant population to adapt by more than one mechansim to a herbicide selection pressure. At the relatively low rates of herbicide used in Australia it is likely that more than one type of mutation will allow a plant to survive and set seed following herbicide treatment. Furthermore, the seed populations of *L. rigidum* are high (circa 10^4 m^{-2}) (Rerkasem *et al.*, 1980a) and the areas under treatment are very large (circa 50 to 500 ha per farm, at least). Thus the potential for independent mutations would seem large given that 10^{10} *L. rigidum* seedlings may exist in one field of 100 ha. The outcrossing nature of *L. rigidum* means that plants derived from independent mutations must eventually cross which may result in more than one mechanism being maintained in a population. While continuous application of one herbicide has yielded target site mutations in biotypes such as WLR1 and WLR96 (Christopher *et al.*, 1992; Holtum and Powles, 1993), there are other biotypes such as VLR69 which have developed under variable selection pressure. Under such a regime, adaptation via specific target site mutation will not protect the plant from the different herbicides to which it may be exposed. In these circumstances it would appear that the optimum strategy is to accumulate (at the population and individual level) a variety of mechanisms which can protect against a greater variety of herbicides. If the degree of resistance conferred by differential metabolism is sufficient, the resistant biotype will compete successfully with a crop and there will be no selective advantage for target site mutants although they may be less affected by particular herbicides compared with the detoxifiers. Target site mutants may, however, be less sensitive to the herbicide in extreme environments or under strong competition. Interpollination between surviving resistant parent plants possessing different resistance mechanisms would, therefore ensure that

individuals soon possessed more than one resistance traits. Individuals with target site mutations alone would be removed by the occasional use of a herbicide with a different target site while those with multiple mutations would survive the treatment. The selective advantage of target site mutations in a background of enhanced detoxification would be slight except under situations where detoxification was inadequate, such as cold conditions (Beyer *et al.*, 1988). In such conditions the advantage may be sufficient to boost its frequency in the population. The use of herbicides with other sites of action ensures, however, that a general mechanism must also be present for the biotype to survive.

Examination of the history of herbicide application to VLR69 reveals how this might have applied to the relative frequency of detoxification and ALS mutations in this population. Chlorsulfuron was applied 6 times in seven seasons despite falling levels of control. During that time the biotype was exposed to five applications of diuron, three applications of atrazine and three applications of bypyridyls (Table 1.1). Enhancement of chlorsulfuron metabolism may have occurred given that the biotype had also adapted to triazines and phenylureas by metabolism. Control of *L. rigidum* was poor during this period as indicated by the increasing rates and number of herbicides being applied to the population (Table 1.1). Thus any plant surviving this period of selection would have required a general set of resistance mechanisms. This would occur most rapidly if new traits were added to the adaptations already in place and even more rapidly if the traits were derivations of existing mechanisms such as enhanced expression of detoxification enzymes.

Ryegrass biotype VLR69 illustrates the way a variety of selection pressures may select for multiple-resistance by more than one mechanism. The significance of this is that the presence of more than one alteration enhances the potential range of cross-resistance and increases its probability of occurring. Furthermore, alteration of general mechanisms such as the affinity or expression of detoxification enzymes may lead to slight changes in sensitivity to some other herbicides. These slight changes, such as those observed with VLR69 and metolachlor, lower the mean sensitivity of the population. The population may

not be explicitly cross-resistant to field use of the herbicide but there would be greater survival than expected and resistance would develop rapidly. Similarly, the presence of ALS mutants at a frequency of 4% in the VLR69 population would ensure that after only one "successful" season of sulfometuron use, the population would be fully resistant due to the strong selection for ALS mutation applied by this herbicide.

8.5 Final Conclusions

In seeking to establish mechanisms of cross-resistance in two resistant *L. rigidum* populations it was essential to first identify the mechanisms operating against each individual herbicide and then to establish whether they had any characteristics in common. This process is most complete for the three classes of photosystem II inhibitors and it appears that enhanced metabolism is the most likely explanation for cross-resistance to these chemically dissimilar herbicide groups. Thus, adaptation via mechanisms other than target site mutation has the potential to generate a phenotype with resistance to a variety of xenobiotics which may not succumb to the immediate alternatives. This type of resistance creates imperatives for integrated weed management just as resistance did for insect pest management two decades ago.

Appendices

Inheritance of Haloxyfop Resistance

A1.1 Introduction

To facilitate experiments on the inheritance of amitrole resistance, biotype WLR2 (amitrole resistant, haloxyfop susceptible) was crossed with another Western Australian biotype that was of interest to the group, WLR96 (amitrole susceptible, haloxyfop resistant). This was intended to improve synchronisation of flowering and allow the inheritance of two resistance traits to be studied within a single cross. In this section the results of the experiments on the inheritance of aryloxyphenoxypropionate resistance in WLR96 are described.

A1.2 Materials and Methods

Resistant WLR96 plants were selected as parents for use in crossing experiments by exposing them to a selection pressure of 1.7 kg ha^{-1} haloxyfop. Seven surviving WLR96 plants were selected from a sample of 64 individuals and hybridised with haloxyfop susceptible WLR2 and VLR1 plants. Hybridisation was achieved using the methods reported in Chapter seven. Seeds resulting from crosses were collected separately from each female parent and assessed separately. No maternal inheritance was detected so results from progeny of reciprocal crosses were combined. F_1, F_2 and back-cross plants were screened for response to haloxyfop at the rates of 26 and 208 g ha^{-1} and compared with both the resistant WLR96 and susceptible WLR2 and VLR1 parent populations. F_2 progeny were produced either by allowing a group of F_1 plants to pollinate and set seed in isolation, or by crossing within a sleeve as described above. F_2 generations that yielded large numbers of seeds were also screened against 208, 832 and 1664 g ha^{-1} haloxyfop and 1.5, 3.0 and 4.5 kg ha^{-1} diclofop. Screening was performed according to the section "dose response to herbicides". Two crosses provided sufficient seed for the latter assessment.

A1.3 Results and Discussion

F1 plants that were progeny of both WLR2 and WLR96 parent plants had a high level of resistance (90 to 95 %) to both herbicides. This indicates that the plants had a high level of cross-pollination in this system. Furthermore, only F1 plants resistant to both herbicides were used further in experiments because these were most likely to be heterozygous for the resistance traits.

Segregation for haloxyfop and diclofop resistance in the F2 and "backcross" generations indicates that resistance to the herbicides is conferred by a single major gene which exhibits dominance at the rates of herbicide applied in these experiments (Table A1.1). Segregation for resistance in the F2 plants derived from crosses 2 and 4 was not dose responsive to either diclofop or haloxyfop up to doses 4.5 and 1.7 kg ha^{-1} respectively (Table A1.2). This suggests there is no heterozygote effect at these screening rates.

The observation of only one major dominant gene conferring resistant in this biotype was surprising given that WLR96 has both membrane depolarisation and acetyl-CoA-carboxylase based resistance mechanisms (Häusler *et al.*, 1991; Holtum and Powles, 1992). Membrane repolarisation is very weak in WLR96 while the biotype is one of the most resistant phenotypes isolated thus far (Häusler *et al.*, 1991; Heap and Knight, 1990), suggesting that the membrane effect is a subsidiary mechanism in this biotype. Thus, the screening and crossing regime would have been unlikely to detect genes for a mechanism present only at a low frequency. However, the plants were screened at rates of the herbicide easily tolerated by the parent generation and the methods would therefore seem appropriate for the detection of the major mode of inheritance. These data are somewhat similar to those of Betts *et al.* (1992) who reported a single semi-dominant gene conferring resistance to diclofop in an *L. multiflorum* biotype which expresses an insensitive ACCase isozyme.

A1.4 Conclusions

Aryloxyphenoxypropionate resistance in WLR96 segregates as a single major locus with a

high level of dominance. Data from both F2 and backcross plants support this conclusion. The same pattern of segregation is observed for both diclofop and haloxyfop (Table A1.2) suggesting the mechanism is similarly efficient for both types of herbicide. Given the clear segregation, the high level of resistance to haloxyfop and the ACCase insensitivity data reported by Holtum and Powles (1992), it is considered likely that the major gene segregating in these crosses was one encoding an altered ACCase.

Table A1.1. Segregation for resistance to haloxyfop in crosses between WLR96 (haloxyfop resistant biotype) and WLR2 and VLR1 (haloxyfop susceptible biotypes). "BC" is the abbreviation for the backcross between the F1 and the susceptible biotype

Cross		26 g ha $^{-1}$ Haloxyfop				208 g ha $^{-1}$ Haloxyfop			
		Total	Alive	Exp.1 gene	chi ^{2}	Total	Alive	Exp.1 gene	chi ^{2}
WLR96	P	81	81	81	0.000	156	155	156	0.000
WLR2	P	18	0	0	-	15	0	0	-
VLR1	P	39	0	0	-	43	0	0	-
1	F1	39	39	39	0.000	53	53	53	0.000
	F2	44	38	33	0.658	8	6	6	0.000
	BC	118	62	59	0.145				
2	F1	60	60	60	0.000	76	76	76	0.000
	F2	45	42	34	1.621	194	166	146	2.506
	BC - 1	95	50	48	0.125				
	BC - 2	59	26	30	0.471	23	16	12	1.266
	BC - 3	10	7	5	0.571	62	24	31	2.042
3	F1	60	60	60	0.000	45	45	45	0.000
	F2					60	51	45	0.706
	BC - 1					145	72	73	0.003
	BC - 2	178	96	89	0.510	212	104	106	0.038
	BC - 3	46	26	23	0.346	196	114	98	2.246
4	F1	40	40	40	0.000	38	38	38	0.000
	F2	46	43	35	1.680	171	127	128	0.012
	F2					53	44	40	0.411
	BC - 1	141	68	71	0.092	131	77	66	1.718
6	F2	186	149	140	0.606	191	133	143	0.790
	F2	149	106	112	0.312	133	108	100	0.630

Table A1.2. Segregation for resistance to haloxyfop and diclofop by F_2 plants derived from a cross between WLR96 (haloxyfop resistant biotype) and WLR2 (haloxyfop susceptible biotype).

Cross		Diclofop				Haloxyfop			
		Total	Alive	Exp. 1 gene	chi ^{2}	Total	Alive	Exp.1 gene	chi ^{2}
		Diclofop 1.5 kg ha^{-1}				Haloxyfop 0.2 kg ha^{-1}			
WLR96	P	29	29	29	0.000	32	31	32	0.000
WLR2	P	9	1	0	1.000				
VLR1	P	5	0	0	-	5	0	0	-
2	F2	96	78	72	0.462	83	67	62	0.337
4	F2	33	25	25	0.003	58	43	44	0.006
		Diclofop 3.0 kg ha^{-1}				Haloxyfop 0.8 kg ha^{-1}			
WLR96	P	32	32	33	0.000	32	32	32	0.000
WLR2	P	10	0	0	-				
2	F2	86	62	65	0.101	92	63	69	0.571
4	F2	35	30	26	0.469	46	38	35	0.322
		Diclofop 4.5 kg ha^{-1}				Haloxyfop 1.7 kg ha^{-1}			
WLR96	P	33	33	33	0.000	6	6	6	0.000
2	F2	126	88	95	0.480	92	67	69	0.060
4	F2	60	42	45	0.214	53	36	40	0.391

Abbreviations

A2.1 Herbicide common and chemical names

Alachlor,	2-chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl)acetamide;
ABT,	1-aminobenzo-triazole;
Acifluorfen	5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid;
Ametryn,	N-ethyl-N'-(1-methylethyl)-6-(methylthio)-1,3,5-triazine-2,4-diamine;
Amitrole,	3-amino-1,2,4-triazole;
Atrazine,	6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine;
Carbetamide,	(R)-N-ethyl-2-[[(phenylamino)carbonyl)oxy)]proponamide;
Chlorsulfuron,	(2-chloro-N-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino] carbonyl]benzene-sulfonamide);
Chlorotoluron,	N'-(3-chloro-4-methylphenyl)-N,N-dimethylurea;
Cyanazine,	2-[[4-chloro-6-(ethylamino)-1,3,5-triazin-2-yl]amino]-2-methylpropane nitrile;
Diclofop,	((±)-2-[4-(2,4-dichlorophenoxy)phenoxy]propionic acid);
Diuron,	N'-(3,4-dichlorophenyl)-N,N-dimethylurea;
Diquat,	6,7-dihydrodipyrido[1,2-a:2',1'-c]pyrazinediium ion;
EPTC,	S-ethyl dipropyl carbamothioate;
Fluazifop,	(±)-2-[4-[[5-(triflurомethyl)-2-pyridinyl]oxyl]phenoxy] propionic acid);
Fluometuron,	N,N-dimethyl-N'-[3-(triflurомethyl)phenyl]urea;
Fluorodifen,	2,4'-Dinitro-4-trifluoromethyl diphenylether
Glyphosate,	N-(phosphonomethyl)glycine;
Isoproturon,	N,N-dimethyl-N'-[4-(1-methylethyl)phenyl]urea;
Haloxyfop, ;	
Linuron,	N'-(3,4-dichlorophenyl)-N-methoxy-N-methylurea;
Methabenzthiazuron,	N-2-benzothiazolyl-N,N-dimethylurea
Methazole,	2-(3,4-dichlorophenyl)-4-methyl-1,2,4-oxadiazolidine-3,5-dione;
Metolachlor,	2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide;
Metoxuron,	N'-(3-chloro-4-methoxyphenyl)-N,N-dimethylurea;
Metribuzin,	4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4H)-one;
Metsulfuron,	(2-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl] amino]sulfonyl]benzoicacid);
Oxyfluorfen,	2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(triflurомethyl) benzene;
Paraquat,	1,1'-dimethyl-4,4'-bypyridinium ion;
Pendimethalin,	N-(1-ethylpropyl)-3,4-dimethyl-2,6-dinitrobenzenamine;
Prometryn,	N,N'-bis(1-methylethyl)-6-(methylthio)-1,3,5-triazine-2,4-diamine;
Propachlor,	2-chloro-N-(1-methylethyl)-N-phenylacetamide;
Propazine,	6-chloro-N,N'-bis(1-methylethyl)-1,3,5-triazine-2,4-diamine;
Simazine,	6-chloro-N,N'-diethyl-1,3,5-triazine-2,4-diamine;
Sulfometuron,	(2-[[[[(4,6-dimethyl-2-pyrimidinyl)amino]carbonyl] amino] sulfonyl] benzoic acid);
Terbutryn,	(N-1,1-dimethylethyl)-N'-ethyl-6-(methylthio)-1,3,5-triazine-2,4-diamine)
Tridiphane,	2-(3,5-dichlorophenyl-2-(2,2,2-trichloroethyl) oxirane;
Trifluralin,	2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)benzenamine.

Bibliography

Agrawal B.B.L., Margoliash E., Levenberg M.I., Egan R.S., Studier M.H., 1970. Identification of a product of reaction of 3-amino-1,2,4-triazole with catalase-H_2O_2 complex 1. Federation Proceedings, 29, 732.

Alexander N.M., 1959.. Antithyroid action of 3-amino-1,2,4-triazole. Journal of Biological Chemistry, 234, 148-150.

Anderson J. J., Priester T. M., Shalaby L. M., 1989. Metabolism of metsulfuron-methyl in wheat and barley. Journal of Agricultural and Food Chemistry, 37,1429-1434.

Anderson M.P., Gronwald J.W., 1991. Atrazine resistance in a velvetleaf (*Abutilon theophrasti*) biotype due to enhanced glutathione S-transferase activity. Plant Physiology, 96,104-109.

Andersen R.N., Gronwald J.W., 1987. Noncytoplasmic inheritance of atrazine tolerance in velvetleaf (*Abutilon theophrasti*). Weed Science, 35, 496-498.

Anon. Australian Weed Control Handbook. Melbourne, Inkata Press, 1987.

Anon. Herbicide Handbook 5th ed. Champaign Illinois, Weed Science Society of America, 1983.

Arnon D. I., 1949. Copper enzymes in isolated chloroplasts, polyphenoloxidase in *Beta vulgaris*.. Plant Physiology, 24, 1-15.

Arntzen C.J., Pfister K., Steinback K.E., 1982. The mechanism of chloroplast triazine resistance, alterations in the site of herbicide action. In, Le Baron H., Gressel J., eds. Herbicide Resistance in Plants, pp 185-214. John Wiley and sons, New York.

Ashtakala S.S., Dodds G.T., Lamoureux S.W., 1989. Effect of 3-amino-1,2,4-triazole on the stimulation of chlorophyll and carotenoid biosynthesis in the presence of exogenous cytokinins and aminolevulinic acid. Journal of Plant Physiology, 135, 86-93.

Ashton F. M., Crafts A. S., 1981. Amitrole. In, Mode of Action of Herbicides. 2nd ed., pp 119-138. John Wiley &Sons, New York.

Bandeen J.R., Stephenson G.R., Cowett E.R., 1982. Discovery and distribution of herbicide resistant weeds in North America. In, Le Baron H., Gressel J., eds. Herbicide Resistance in Plants, pp 9-30. John Wiley and sons, New York.

Baron J.J., Monaco T.J., 1986. Uptake, translocation, and metabolism of hexazinone in blueberry (*Vaccinium sp.*) and Hollow Goldenrod (*Solidago fistulosa*). Weed Science, 34, 824-829.

Barry P., Young A.J., Britton G., 1990. Photodestruction of pigments in higher plants by herbicide action. I. The effect of DCMU (diuron) on isolated chloroplasts. Journal of Experimental Botany, 41, 123-129.

Barta I. Cs., Dutka F.,. 1991. Influence of 1-aminobenzotriazole (ABT) derivatives on the toxicity of EPTC to monocotyledonous plants. Weed Research, 31, 89-95.

Barta I. Cs., Dutka F., 1991. Interaction of maize cytochrome P_{450} with safeners and 1-aminobenzotriazole. In, Proceedings of the Brighton Crop Protection Conference -

Weeds, 1127-1132.

Bartels P.G., Matsuda K., Siegel A., Weier T.E., 1967. Chloroplastic ribosome formation, inhibition by 3-amino1,2,4-triazole. Plant Physiology, 42, 736-741.

Bartels P.G., Weier T.E., 1969. The effect of 3-amino-1,2,4-triazole on the ultrastructure of plastids of *Triticum vulgare* seedlings. American Journal of Botany, 56(1): 1-7.

Benveniste I., Gabriac B., Durst F., 1986. Purification and characterisation of the NADPH-cytochrome P-450 (cytochrome c) reductase from higher plant microsomal fraction. Biochemistry Journal, 235, 365-373.

Bettini P., McNally S., Sevignac M., Darmency H., Gasquez J., Dron M., 1987. Atrazine resistance in *Chenopodium album*.. Plant Physiology, 84, 1442-1446.

Betts K.J., Ehlke N.J., Wyse D.L., Gronwald J.W., Somers, D.A., 1992. Mechanism of inheritance of diclofop resistance in italian ryegrass (*Lolium multiflorum*). Weed Science, 40, 184-189.

Beyer E. M., Duffy M. J., Hay J.V., Schlueter D. D., 1988. Sulfonylureas. Chapter 13 In, Kearney P.C. and Kaufman D.D., eds., Herbicides. Vol 3, pp 118-169. Marcel Dekker, New York.

Bieringer H., Bauer K., Hacker E., Heubach G., Leist K.H., Ebert E., 1989. Hoe 70542 - a new molecule for use in combination with fenoxaprop-ethyl allowing selective post-emergence grass weed control in wheat. Proceedings of the Brighton Crop Protection Conference - Weeds, 77-82.

Blee E., Durst F., 1987 Hydroperoxide-dependent sulfoxidation catalyzed by soybean microsomes. Archives of Biochemistry and Biophysics, 254, 43, 52.

Bleeke M.S., Casida J.E., 1984. Metribuzin metabolites in mammals and liver microsomal oxidase systems: identification, syntheis and reaction. Journal of Agricultural and Food Chemistry, 32, 749-753.

Böger P., Kunert K.J., 1979. Differential effects of herbicides upon trypsin treated chloroplasts. Z. naturforsch, 34c, 1015-1025.

Brattsten L.B., Holyoke C.W., Leeper J.R., Raff K.F., 1986. Insecticide resistance: challenge to pest management and basic research. Science, 231, 1255-1260.

Britton G., 1979. Carotenoid biosynthesis - a target for herbicide activity. Z. naturforsch, 34c, 979-985.

Brown M. A., Chui T.Y. Miller P., 1987. Hydrolytic activation versus oxidative degradation of Assert herbicide, an imidazolinone aryl-carboxylate, in susceptible wild oat versus tolerant corn and wheat. Pesticide Biochemistry and Physiology, 27, 24-27.

Brown H. M., Neighbours S.M., 1987. Soybean metabolism of chlorimuron ethyl: physiological basis for soybean selectivity. Pesticide Biochemistry and Physiology, 29, 112,120.

Brown T.M., 1990. Biochemical and genetic mechanisms of insecticide resistance. In, W.K. Moberg and H.M. Le Baron, eds. A.C.S. Symposium. Series. Fundamental and Practical Approaches to Combating Resistance, pp 61-76. American Chemicals Society, Washington .

Buman R.A., Gealy D.R. Fuerst E.P. 1992. Relationship between temperature and triazinone herbicide activity. I. Herbicide binding to the thylakoid membranes. Pesticide Biochemistry and Physiology, 43, 22-28.

Burke J.J., Wilson R.F., Swafford J.R., 1982. Characterization of chloroplasts isolated from triazine-susceptible and triazine-resistant biotypes of *Brassica campestris* L. Plant Physiology, 70, 24-29.

Burns E.R., Buchanan G.A., Carter M.C., 1971. Inhibition of carotenoid biosynthesis as a mechanism of action of amitrole, dichlormate and pyriclor. Plant Physiology, 47, 144-148.

Burnet M.W.M., Hildebrand O.B., Holtum J.A.M., Powles S.B., 1991. Amitrole, triazine, substituted urea and metribuzin resistance in a biotype of rigid ryegrass (Lolium rigidum). Weed Science, 39, 317-323.

Cabanne F., Gaillardon P., Scalla R., Durst F., 1985a. Aminobenzotriazole as a synergist of urea herbicides. Proceedings of the 1985 British Crop Protection Conference - Weeds, 1163-1170.

Cabanne F., Gaillardon P., Scalla R., 1985b. Phytotoxicity and metabolism of chlortoluron in two wheat varieties. Pesticide Biochemistry and Physiology, 23, 212-220.

Cabanne F., Huby D., Gaillardon P., Scalla R., Durst F., 1987. Effect of the cytochrome P-450 inactivator 1-aminobenzotriazole on the metabolism of chlorotoluron and isoproturon in Wheat. Pesticide Biochemistry and Physiology, 28, 371-380.

Canivenc M-C., Cagnac B., Cabanne F., Scalla R., 1989. Induced changes of chlorotoluron metabolism in wheat cell suspension cultures, 27,193-201.

Carter M.C., 1975. Amitrole. In, Kierney P.C., Kaufman D.D., eds. Herbicides, Their Chemistry, Degradation and Mode of Action. 2d ed. Marcel Dekker, New York.

Castelfranco P. Inhibition of some metalloprotein enzymes by 3-amino-1,2,4-triazole. Biochimica et Biophysica Acta, 1960, 41, 485-491.

Chollet R., Sandmann G., Diethelm R., Felix H., Milzner K., Böger P., 1990. ξ-Carotene accumulation and bleaching by new pyrimidine compounds. Pesticide Science, 30, 326-329.

Christopher J.T., Powles S.B., Holtum J.A.M., Liljegren D.R., 1991. Cross-resistance to herbicides in annual ryegrass (*Lolium rigidum*): II Chlorsulfuron resistance involves a wheat-like detoxification system. Plant Physiology, 95, 1036-1043.

Christopher J.T., Powles S.B., Holtum J.A.M., 1992. Resistance to acetolactate synthase inhibitors in annual ryegrass (*Lolium rigidum*) involves at least two mechanisms. Plant Physiology in press.

Clarke G.M., McKenzie J.A., 1987. Developmental stability of insecticide resistant phenotypes in blowfly; a result canalizing natural selection. Nature, 325, 345-346.

Clarke I.E., Sandmann G., Bramley P.M., Böger P., 1982. Carotene biosynthesis with isolated photosynthetic membranes. FEBS Letters, 140, 203-206.

Clay D.V., 1987. The response of simazine resistant and susceptible biotypes of *Chamomilla susveolans*, *Epilobioum cilatum* and *Senecio vulgaris* to other herbicides. Proceedings of the British Crop Protection Conference - Weeds, 925-932.

Cole D.J., Edwards R., Owen W.J., 1987. The role of metabolism in herbicide selectivity. In, Hutson D.H., Roberts T.R., eds. Herbicides, pp 57-103. John Wiley and Sons, London.

Cole D.J., Owen W. J., 1987. Influence of monooxygenase inhibitors on the metabolism of the herbicides chlortoluron and metolachlor in cell suspension cultures. Plant Science, 50, 13-20.

Conard S.G., Radosevich S.R., 1979. Ecological fitness of *Senecio vulgaris*, and *Amaranthus retroflexus* biotypes susceptible or resistant to atrazine. Journal of Applied Ecology, 16, 171-177.

Cotterman J.C., Saari L.L., 1992. Rapid metabolic inactivation is the basis for cross-resistance to chlorsulfuron in diclofop-methyl-resistant rigid ryegrass (*Lolium rigidum*) SR4/84. Pesticide Biochemistry and Physiology, 43, in press.

Darmency H., Gasquez J., 1981. Inheritance of triazine resistance in *Poa Annua*, consequences for population dynamics. New Phytologist, 89, 487-493.

Darmency H., Gasquez J., 1990. Fate of herbicide resistance genes in weeds. In, M.B.Green, W.K.Moberg and H.Le Baron, eds. Managing Resistance to Agrochemicals, From Fundamental Research to Practical Strategies, pp. 353-363, American Chemical Society.

Darr S., Machado V.S., Arntzen C.J., 1981. Uniparental inheritance of a chloroplast photosystem II polypeptide controlling herbicide binding. Biochimica et Biophysica acta, 634, 219-228.

Davis D.G., Olson P.A., Swanson H.R., Frear D.S., 1991. Metabolism of the herbicide metribuzin by an N-glucosyltransferase from tomato cell cultures. Plant Science, 74, 73-80.

Deak M., Donn G., Fehr A., Dudits D., 1988. Dominant expression of a gene amplification-related herbicide resistance in Medicago cell hybrids. Plant Cell Reports, 7, 158-161.

Devlin D.P., Gealy D.R., Morrow L.A., 1987a. Differential metabolism of metribuzin by downy brome (*Bromus tectorum*) and winter wheat (*Tritcum aestivum*). Weed Science, 35, 741-745.

Devlin D.P., Gealy D.R., Morrow L.A., 1987b. Retention, absorbtion, and loss of foliage applied metribuzin. Weed Science, 35, 775-779.

De Prado R., Scalla R., Gaillardon P., 1990. Differential toxicity of simazine and diuron to *Torilis arvensis* and *Lolium rigidum*.. Weed Research, 30, 213-221.

Donald C.M., 1951. Competition among pasture plants. I, Intra-specific competition among annual pasure plants. Australian Journal of Agricultural Research, 2(4), 355-377.

Donn G., Tischer E., Smith, J.A., Goodman H.M., 1984. Herbicide resistant alfalfa cells: an example of gene amplification in plants. Journal of Molecular and Applied Genetics, 2, 261-635.

Donnelly M.J., Hume D.J., 1984. Photosynthetic rates and growth analysis of triazine-tolerant and normal rapeseed. Canadian Journal of Plant Science, 64(2), 432.

Ducruet J-M., De Prado R., 1982. Comparison of inhibitory activity of amides derivitives in triazine-resistant and -susceptible chloroplasts from *Chenopodium album* and

Brassica campestris.. Pesticide Biochemistry and Physiology, 18, 253-261.

Ducruet J.M., Gaillardon P., Vienot J., 1984. Use of chlorophyll fluorescence induction kinetics to study translocation and detoxication of DCMU-type herbicides in plant leaves. Z. Naturforsch, 39 c, 1-5.

Ducruet J-M., Lemoine Y., 1985. Increased heat sensitivity of the photosynthetic apparatus in triazine-resistant biotypes from different plant species. Plant Cell Physiology, 26(3), 419-429.

Ducruet J-M., Miranda T., Creuzet S., 1992. Hydrogen bonding properties of several PS-II herbicides and their role in the distal influence on the primary acceptor Q_A^-. Proceedings of the "Molecular Regulation of Chloroplast Functions" meeting. Omiya, August, 1992.

Ducruet J-M., Ort D.R., 1988. Enhanced susceptibility of photosynthesis to high leaf temperature in triazine-resistant *Solanum nigrum* L. Evidence for photosystem II D1 protein site of action. Plant Science, 56, 39-48.

Elstner E.F., Pils I., 1979. Ethane formation and chlorophyll bleaching in DCMU-treated Eugleana gracilis cell and isolated spinach chloroplast lamellae. Z. naturforsch, 34c, 1040-1043.

Erickson J.M, Rahire M., Rochaix J-D., 1985. Herbicide resistance and cross-resistance, changes at three distinct sites in the herbicide-binding protein. Science, 228, 204-207.

Esser H.O., Dupious G., Ebert E., Vogel C., Marco G.J., 1975. s-Triazines. In, P. C. Kearney and D. D. Kaufman, eds. Herbicides: Their Chemistry, Degradation and Mode of Action.2nd ed. Marcel Dekker, New York.

Fedtke C., Strang, R.H., 1990. Synergistic activity of the herbicide safener dichlormid with herbicides affecting photosynthesis. Z. Naturforsch., 45c, 565-567.

Feierabend J., Kemmerich P., 1983. Mode of interference of chlorosis-inducing herbicides with peroxisomal enzyme activities. Physiologia Plantarum, 57, 346-351.

Feierabend J., Schubert B., 1978. Comparative investigation of the action of several chlorosis-inducing herbicides on the biogenesis of chloroplasts and leaf microbodies. Plant Physiology, 61, 1017-1022.

Feierabend J., 1984. Comparison of the Action of Bleaching Herbicides. Z. Naturforsch, 39 c, 450-454.

Fonné-Pfister R., Kreuz K., 1990. Ring-methyl hydroxylation of chlorotoluron by an inducible cytochrome $P\text{-}_{450}$ dependent enzyme from maize. Phytochemistry, 29, 2793-2796.

Forcella F., 1984. Wheat and ryegrass competition for pulses of mineral nitrogen. Australian Journal of Experimental Agriculture and Animal Husbandry, 24, 421-425.

Forcella F., 1987. Herbicide-resistant crops: yield penalties and weed thresholds for oilseed rape (*Brassica napus* L.). Weed Research, 27, 31-34.

Frear D.S., Swanson H.R., Tanaka F.S., 1969. N-demethylation of substituted 3-(phenyl)-1-methylureas, isolation and characterisation of a microsomal mixed function oxidase from cotton. Phytochemistry, 8, 2157-2169.

Frear D.S., Swanson H.R., 1970. Biosynthesis of S-(4-ethylamino-6-isopropylamino-2-s-

triazino) glutathione, partial purification and properties of a glutathione S-transferase from corn. Phytochemistry, 9, 2123-2132.

Frear D.S., Swanson H.R., 1973. Metabolism of substituted diphenyletherherbicides in plants I. Enzymatic cleavage of fluorodifen in peas (*Pisum sativum* L.). Pesticide Biochemistry and Physiology, 3, 473-482.

Frear D.S., Swanson H.R., 1974. Monuron metabolism in excised *Gossypium hirsutum* leaves, aryl hydroxylation and conjugation of 4-chlorophenylurea. Phytochemistry, 13, 357-360.

Frear D.S., Swanson H.R., Mansager E.R., 1983. Acifluorfen metabolism in soybean: diphenylether bond cleavage and the formation of homoglutathione, cystein, and glucose conjugates. Pesticide Biochemistry and Physiology, 20, 299-310.

Frear D.S., Swanson H.R., Mansager E.R., 1985. Alternate pathways of metribuzin metabolism in soybean: formation of N-glucoside and homoglutathione conjugates. Pesticide Biochemistry and Physiology, 23, 56-65.

Frear D.S., Swanson H.R., Thalacker F.W., 1991. Induced microsomal oxidation of diclofop, triasulfuron, chlorsulfuron, and linuron in wheat. Pesticide Biochemistry and Physiology, 41, 274-287.

Fuerst E.P., Gronwald J.W., 1986. Induction and rapid metabolism of metolachlor in sorghum (*Sorghum bicolor*) shoots by CGA 92194 and other anitdotes. Weed Science, 34, 354-361.

Fuerst E.P., 1987. Understanding the mode of action of the chloroacetamide and thiocarbamate herbicides. Weed Technology, 1, 270-277.

Feurst E. P. Vaughn K. C., 1990. Mechanisms of paraquat resistance. Weed Technology, 4, 150-156.

Gawronski S.W., Haderlie L.C., Stark J.C., 1986. Metribuzin absorbtion and translocation in two barley (*Hordeum vulgare*) cultivars. Weed Science, 34, 491-495.

Gawronski S.W., Haderlie L.C., Stark J.C., 1987. Metribuzin metabolism as the basis for tolerance in barley (*Hordeum vulgare* L.). Weed Research, 27, 49-55.

Gaillardon P., Cabanne F., Scalla R., Durst F., 1985. Effect of mixed function oxidase inhibitors on the toxicity of chlortoluron and isoproturon in wheat. Weed Research, 25, 397-402.

Gasser R., Hauri H.P., and Meyer U.A., 1982. The turnover of cytochrome $P_{450}b$. FEBS Letters, 147, 239-242.

Geissbuhler H., Martin H., Voss G., 1975. The Substituted Ureas. In, Kierney P. C., Kaufman D. D., eds. Herbicides, Their Chemistry, Degradation and Mode of Action. 2d ed., 209-291, Marcel Dekker, New York.

Georghiou G.P., 1990. Overview of insecticide resistance. In, W.K. Moberg and H.M. Le Baron, eds. A.C.S. Symposium Series. Fundamental and Practical Approaches to Combating Resistance, pp 18-41. Am. Chem Soc., Washington.

Gonneau M., Pasquette B., Cabanne F., Scalla R., 1988. Metabolism of chlortoluron in tolerant species, possible role of cytochrome $P\text{-}_{450}$ mono-oxygenases. Weed Research, 28, 19-25.

Goodwin T.W., 1980. The biochemistry of the carotenoids, Volume 1: Plants, 2d ed. Chapman and Hall, London.

Goodwin T.W., Mercer E.I., 1985. Introduction to plant biochemistry. 2d ed. Pergamon Press, Oxford.

Goren R., 1969. The effect of fluometuron on the behaviour of citrus leaves. Weed Research, 9, 121-135.

Gossett B.J., Murdock E.C., 1990. Palmer amaranth resistance to the dinitroaniline herbicides. Proceedings of the Southern Weed Science Society Meeting, 43.

Gramshaw D., Stern W.R., 1977a. Survival of annual ryegrass (*Lolium rigidum* Gaud.)seed in a mediterranean type environment. 1. Effect of summer grazing by sheep on seed numbers and seed germination in autumn. Australian Journal of Agricultural Research, 28, 81-91.

Gramshaw D., Stern W.R., 1977b. Survival of annual ryegrass (*Lolium rigidum* Gaud.) seed in a mediterranean type environment. 2. Effects of short term burial on persistance of viable seed. Australian Journal of Agricultural Research, 28, 93-101.

Gressel J., 1991. Why get resistance? It can be prevented or delayed. In, J.C.Caseley, G.W.Cussons and R.K.Atkin, eds. Herbicide Resistance in Weeds and Crops, pp 1-30, Butterworth-Heineman, Oxford.

Gressel J., 1985. Herbicide tolerance and resistance, alteration of site of activity. In, Duke S.O., ed. Weed Physiology, II, 159-189. CRC Press, Boca Raton.

Gressel J., Ammon H.U., Fogefors H., Gasquez J., Kay Q.O.N., Keees H., 1982. Discovery and distribution of herbicide resistant weeds outside North America. In, Le Baron H., Gressel J., eds. Herbicide Resistance in Plants, pp 32-56. John Wiley and sons, New York.

Gressel J., Shimabukuro R.H., Duysen M.E., 1983. N-Dealkylation of atrazine and simazine in *Senecio vulgaris* biotypes, a major degradation pathway. Pesticide Biochemistry and Physiology, 19, 361-370.

Gronwald J.W., Andersen R.N., Yee C., 1989. Atrazine resistance in velvetleaf (*Abutilon theophrasti*) due to enhanced atrazine detoxification. Pesticide Biochemistry and Physiology, 34,149-163.

Gronwald J.W., Connelly J.A., 1991. Effect of monooxygenase inhibitors on bentazon uptake and metabolism in maize cell suspension cultures. Pesticide Biochemistry and Physiology, 40, 284-294.

Gronwald J.W., Eberlein C.V., Betts K.J., Roscow K.M., Ehlke N.J., Wyse D.L., 1989. Diclofop resistance in a biotype of Italian ryegrass. Plant Physiology, 89, S-115.

Gronwald J.W., Plaisance K.L., Wyse D.L., 1992. Isolation and characterisation of glutathione-S-transferase isozymes in CGA-133205-treated sorghum. Proceedings of the Weed Science Society of America Conference, Abst. 180.

Grumbach K. H., 1984. Herbicides which interfere with the biosynthesis of carotenoids and their effect on pigment excitation, chlorophyll fluorescence and pigment composition of the thylakoid membrane. Z. Naturforsch, 39 c, 455-458.

Guttieri M.J., Eberlein C.V., Mallory-Smith C.A., Thill D.C., Hoffman D.L., 1992. DNA sequence variation in domain A of the acetolactate synthase genes of herbicide resistant

and susceptible weed biotypes. Weed Science, submitted.

Haack A.E., Balke, N.E., 1992. Comparitive aryl-hydroxylation of bentazon and cinnamic acid by microsomes isolated from several crop species treated with napthalic anhydride. Proceedings of the Weed Science Society of America Conference, Abst. 184.

Hagmann M.L., Heller, W., Grisebach, H., 1983. Induction and charactrisation of a microsomal flavinoid 3'hydroxylase from parsley cell culture. European Journal of Biochemistry, 134, 547-554.

Hall J.C., Romano M.L., Shimabukuro R.H., Devine M.D., 1992. Electrophysiological differences between diclofop methyl resistant and susceptible biotypes of wild oat (*Avena fatua* L.). Weed Science Society of America abstracts, 167.

Hamilton R.H., 1964. Tolerance to several grass species to 2-chloro-s-triazine herbicides in relation to degradation and content of benzoxazinone derivatives. Journal of Agricultural and Food Chemistry, 12(1), 14-17.

Hansch C., 1969. Theoretical considerations of the structure-activity relationship in photosynthesis inhibitors. Progress in photosynthesis research, III, 1685-1692.

Harper S.S., 1988. Sorption of metribuzin in surface and sub-surface soils of the mississippi delta region. Weed Science, 36, 84-89.

Hartley D., Kidd H., eds.,1983. The Agrochemicals Handbook. 1st ed. The Royal Society of Chemistry, Nottingham.

Hartwig E.E., Barantine W.L., Edwards C.J., 1980. Registration of Tracy-M soybean. Crop Science, 20, 825.

Hatzios K. K., Penner D.D., 1988. Metribuzin. In, P.C. Kearney and D.D. Kaufman, eds. Herbicides, Their Chemistry, Degradation and Mode of Action, 2nd ed. pp 191-243. Marcel Dekker, New York.

Häusler R.E., Holtum J.A.M. Powles S.B., 1991. Cross-resistance to herbicides in annual ryegrass (*Lolium rigidum*). IV. Correlation between membrane effects and resistance to graminicides. Plant Physiology, 97, 1035-1043.

Haughn G.W., Smith J., Mazur B., Somerville C.R., 1988. Transformation with a mutant *Arabidopsis* acetolactate synthase gene renders tobacco resistant to sulfonylurea herbicides. Molecular and General Genetics, 211, 266-271.

Heap I., 1988. Herbicide Resistance in Annual Ryegrass (*Lolium rigidum*). Ph. D. Dissertation, University of Adelaide.

Heap I., Knight R., 1986. The occurance of herbicide cross-resistance in a population of annual ryegrass, *Lolium rigidum*, resistant to diclofop-methyl. Australian Journal of Agricultural Research, 37, 149-156.

Heap I.M., Knight R., 1990. Variation in herbicide cross-resistance among populations of annual ryegrass (*Lolium rigidum*) resistant to diclofop-methyl. Australian Journal of Agricultural Research, 41, 121-128.

Heap J., Knight R., 1982. A population of ryegrass tolerant to the herbicide diclofop-methyl. The Journal of the Australian Institute of Agricultural Science, 12, 156-157.

Heim D.R., Larrinua I.M., 1989. Primary site of action of amitrole in *Aribidopsis thaliana*

involves inhibition of root elongation but not of histidine or pigment biosynthesis. Plant Physiology, 91, 1226-1231.

Hendry G.A.F., Jones O.T.G., 1984. Induction of cytochrome P-450 in intact mung beans. New Phytologist, 96, 153-159.

Herrett R.A., Bagley W.P., 1964. The metabolism and translocation of 3-amino-1,2,4-triazole by canada thistle. Journal of Agricultural and Food Chemistry, 12(1), 17-20.

Herrett R.A. Linck A.J., 1961. The metabolism of 3-Amino-1,2,4-triazole by canada thistle and field bindweed and the possible relation to its herbicidal action. Physiologia Plantarum, 14, 767-776.

Hilton J.H., 1969. Inhibition of growth and metabolism by 3-amino-1,2,4-triazole (amitrole). Journal of Agricultural and Food Chemistry, 17(2), 182-198.

Hilton J.L., Kearney P.C., Ames B.N., 1965. Mode of action of the herbicide, 3-Amino-1,2,4-triazole (Amitrole): Inhibition of an enzyme of histidine biosynthesis. Archives of Biochemistry and Biophysics, 112, 544-547.

Hirschberg J., McIntosh L., 1983. Molecular basis of herbicide resistance in *Amaranthus hybridus*. Science, 222, 1346-1348.

Holt J.S., 1988. Reduced growth, competitiveness, and photosynthetic efficiency of triazine-resistant *Senecio vulgaris* from California. Journal of Applied Ecology, 25, 307-318.

Holt J.S. 1990. Fitness and ecological adaptability of herbicide resistant biotypes. In, W.K.Moberg and H.M.Le Baron, eds. A.C.S. Symposium. Series. Fundamental and Practical Approaches to Combating Resistance. pp 419-429. American. Chemical Society, Washington.

Holt J.S., Radosevich S.R., Stemler A.J. Differential efficiency of photosynthetic oxygen evolution in flashing light in triazine-resistant and triazine-susceptible biotypes of *Senecio vulgaris* L. Biochimica et Biophysica acta, 1983, 722, 245-255.

Holt J.S., Stemler A.J., Radosevich S.R., 1981. Differential light response of photosynthesis by triazine-resistant and triazine-susceptible *Senecio vulgaris* biotypes. Plant Physiology, 67, 744-748.

Holtum J.A.M., Matthews J.M., Häusler R.E., Liljegren D.R., Powles S.B. 1991. Cross-resistance to herbicides in annual ryegrass (*Lolium rigidum*). III. On the mechanism of resistance to diclofop-methyl. Plant Physiology, 97, 1026-1034.

Holtum J.A.M., Powles, S.B., 1992. Haloxyfop resistance in *Lolium rigidum*. Plant Physiology, submitted.

Huppatz J. L., Casida J. E., 1985. Acetohydroxyacid synthase inhibitors: N-phthalayl-L-valine anilide and related compounds. Z. Naturforsch. 40:652-656.

Hutchison J.M., Shapiro R., Sweetser P.B., 1984. Metabolism of chlorsulfuron by tolerant broadleaves. Pesticide Biochemistry and Physiology, 22, 243-247.

Hutson D. H., 1987. The bioactivation of herbicides. Proceedings of the 1987 British Crop Protection Conference - Weeds.

Ireland C.R., Telfer A., Covello P.S., Baker N.R., Barber J., 1988. Studies on the limitations to photosynthesis in leaves of the atrazine-resistant mutant of *Senecio*

vulgaris L. Planta, 173, 459-467.

Irzyk, G.P., Fuerst, E.P., 1992. Isolation and Characterisation of a glutathione-S-transferase from benoxacor treated corn. Proceedings of the Weed Science Society of America Conference, Abst. 178.

Ishimaru A., Yamazaki I., 1977. Hydroperoxide-dependent hydroxylation involving "H_2O_2 reducible hemoprotein" in microsomes of pea seeds. The Journal of Biological Chemistry, 252, 6118-6124.

Ishizuka M., Kondo Y., Nagare T., Takeuchi Y., 1985. Metabolism of isouron [3-(5-tert-Butyl-3-isoxazolyl)-1, 1-dimethylurea], rapid conversion of the herbicide to less phytotoxic metabolites in a resistant plant, Sugarcane. Journal of Agricultural and Food Chemistry, 33, 1070-1075.

Islam A.K.M.R., Powles S.B., 1988. Inheritance of resistance to paraquat in barley grass *Hordeum glaucum* steud. Weed Research, 28, 1-5.

Itoh K., 1988. Paraquat resistance in *Erigeron philadelphicus* L. J.A.R.Q, 22(2), 85-90.

Ivie G.W., Dorough H.W., Cardona R.A., 1973. Photodecomposition of the herbicide methazole. Journal of Agricultural and Food Chemistry, 21, 386-391.

Jansen M.A.K., Hobe J.H., Wesselius J.C., van Rensen J.S., 1986. Comparison of photosynthetic activity and growth performance in triazine-resistant and susceptible biotypes of *Chenopodium album*. Physiol. Vegetale, 24(4), 475-484.

Jensen K.I.N., Stephenson G.R., Hunt L.A., 1977. Detoxification of atrazine in three gramineae subfamilies. Weed Science, 25(3), 212-220.

Jensen K.I.N., 1982. The roles of uptake, translocation and metabolism in the differential intraspecific response to herbicides. In, Le Baron H. and Gressel J., eds. Herbicide Resistance in Plants. John Wiley and sons, New York.

Johnston, D.L., and Faulkner, J.S., 1991. Herbicide resistance in the Graminaceae - a plant breeders view. In, J.C. Caseley, G.W. Cussons and R.K. Atkin, eds. Herbicide Resistance in Weeds and Crops, pp 319-330, Butterworth-Heineman, Oxford.

Jones O.T.G., 1991. Cytochrome $P\text{-}_{450}$ and herbicide resistance. In, J.C. Caseley, G.W. Cussons and R.K. Atkin, eds. Herbicide Resistance in Weeds and Crops, pp 213-226, Butterworth-Heineman, Oxford.

Kemp D.R., 1988. The effects of flowering and leaf area on sward growth in winter of temperate pasture grasses. Australian Journal of Agricultural Research, 39, 597 - 604.

Kemp D.R., Eagles C.F., Humphreys M.O., 1989. Leaf growth and apex development of perennial ryegrass during winter and spring. Annals of Botany, 63, 349 -355.

Kemp M.S., Casely J.C., 1987. Synergistic effects of 1-aminobenzotriazole on the phytotoxicity of chlortoluron and isoproturon in a resistant population of blackgrass (*Alopecurus myosuroides*). Proceedings of the 1987 British Crop Protection Conference - Weeds, 895-899.

Kemp M.S., Moss S.R., Thomas T.H., 1990. Herbicide resistance in *Alopecurus myosuroides*. In, M.B. Green, W.K. Moberg and H. Le Baron, eds. Managing Resistance to Agrochemicals, From Fundamental Research to Practical Strategies. pp. 376-393, American Chemical Society.

Khan S.U., Warwick S.I., Marriage P.B., 1985. Atrazine metabolism in resistant and susceptible biotypes of *Chenopodium album* L., *Chenopodium strictum* Roth., and *Amaranthus powellii* S. Wats. Weed Research, 25, 33-37.

Klamroth E.E., Fedtke C., Kubach W.C., 1989. Mechanisms of synergism between metribuzin and MZH 2091 on ivyleaf morning glory. Weed Science, 37, 517-520.

Kloot P.M., 1983. The Genus *Lolium* in Australia. Australian Journal of Botany, 31, 421-435.

Klopotowski T., Waiter A., 1965. Synergism of aminotriazole and phosphate on the inhibition of yeast imidazole glycerole phosphate dehydratase. Archives of Biochemistry and Biophysics, 112, 502-506.

Knight, R., 1990. A survey of annual ryegrass, *Lolium rigidum*, populations in South Australia and their resistance to herbicides. Proceedings of the Ninth Australian Weeds Conference.

Köller W., 1987. Isomers of sterol synthesis inhibitors: fungicidal effects and plant growth regulator activities. Pesticide Science, 18, 129-147.

Koop D.R., 1990. Inhibition of ethanol-inducible cytochrome P_{450}IIEI by 3-amino-1,2,4-triazole. Chemical Research and Toxicology. 3, 377-383.

Lamoureux G.L., Rusness D.G., 1986. Tridiphane [2-(3,5-Dichlorophenyl)-2-(2,2,2,-trichloroethyl)oxirane] an atrazine synergist, enzymatic conversion to a potent glutathione-S-transferase inhibitor. Pesticide Biochemistry and Physiology, 26, 323-342

Lamoureux G. L., Rusness D. G., 1989. The role of glutathione and glutathione-S-transferases in pesticide metabolism, selectivity, and mode of action in plants and insects. In, Dolphin D., Poulson R., Avramovic O. eds. Glutathione, Chemical, Biochemical and Medical Aspects, John Wiley and Sons, New York.

Lamoureux G.L., Shimabukuro R.H., Frear, D.S., 1991. Glutathione and glucoside conjugation in herbicide selectivity. In, J.C. Caseley, G.W. Cussons and R.K. Atkin, eds. Herbicide Resistance in Weeds and Crops, pp 226-261, Butterworth-Heineman, Oxford.

LaRossa R.A., Schloss J.V., 1984. The sulfonylurea herbicide sulfometuron methyl is an extremely potent and selective inhibitor of acetolactate synthase in *Salmonella typhimurium*. Journal of Biological Chemistry 259, 8753-8757.

Lay, M.M., Casida, J.E., 1976. Dichloroacetamide antidotes enhance thiocarbamate sulfoxide detoxification by elevating corn root glutathione content and glutathione-S-transferase activity. Pesticide Biochemistry and Physiology, 6, 442-456.

Leah J.M., Worrall T.L., Cobb A.H., 1989. Metabolism of bentazone in soybean and the influence of tetcyclasis and BAS110 and BAS111. Brighton Crop Protection Conference - Weeds, 433-440.

Le Baron H.M., McFarland J., 1990. Overview and prognosis of herbicide resistance in weeds and crops. In, W.K. Moberg and H.M. Le Baron, eds. A.C.S. Symposium Series. Fundamental and Practical Approaches to Combating Resistance. pp. 336-353. American Chemical Society, Washington

Le Baron H. M. and J. McFarland B.J. Simoneaux E. Ebert, 1975. Metolachlor pages 335-382. In, P.C. Kearney and D.D. Kaufman, eds. Herbicides, Their Chemistry,

Degradation and Mode of Action, 2nd ed. Marcel Dekker, New York.

Lee K.Y., Townsend, J., Tepperman J., Black M., Chui, C.F., Mazur B., Dunsmuir P., Bedbrook J., 1988. The molecular basis for sulfonylurea resistance in tobacco. EMBO Journal, 7, 1241-48.

Lenton J., 1987. Mode of action of triazole growth retardants and fungicides - a progress report. News Bulletin of the British Plant Growth Regulator Group, 9(1), 1-12.

Lichtenthaler H. K., 1984. Chloroplast biogenesis, its inhibition and modification by new herbicide compounds. Z. Naturforsch, 39 c, 492-499.

Lund-Hoie K., 1970. The Correlation of the phytocidal effect of 3-Amino-1,2,4-triazole with the growth stage of oat plants. Weed Research, 10, 367-377.

Lütke-Brinkhaus F., Liedvogel B., Kreuz K., Kleinig H., 1982. Phytoene synthase and phytoene dehydrogenase associated with envelope membranes from spinach chloroplasts. Planta, 156, 176-180.

Mallory-Smith, C. A., Thill D.C., Dial M.J., 1990. Identification of herbicide-resistant prickly lettuce (*Lactuca serriola*). Weed Technology 4, 163-168.

Marcar N.E., 1987. Salt tolerance in the genus *Lolium* (ryegrass) during germination and growth. Australian Journal of Agricultural Research, 38, 297-307.

Margoliash E., Novogrodsky A., Schejter A., 1960. Irreversible reaction of 3-Amino-1,2,4-triazole and related inhibitors with the protein of catalase. Biochemical Journal 74, 339-348.

Marshall G., Kirkwood R.C., Martin D.J., 1987. Studies on the mode of action of asulam, aminotriazole and glyphosate in *Equisetum arvense* L. (field horsetail). II: The metabolism of [^{14}C]asulam, [^{14}C]aminotriazole and [^{14}C]glyphosate. Pesticide Science, 18, 65-67.

Massini P., 1963. Aminotriazlylalanine, a metabolic product of aminotriazole from plants. Acta Botanica Neerlandica, 12, 64-72.

Matthews J.M., Holtum J.A.M., Liljegren D.R., Furness B., Powles S.B., 1990. Cross-resistance to herbicides in annual ryegrass (*Lolium rigidum*): I. ACC and ALS activities. Plant Physiology, 94, 1180-1186.

Mattoo A.K., St. John J.B., Wergin W.P., 1984. Adaptive reorganization of protein and lipid components in chloroplast membranes as associated with herbicide binding. Journal of Cellular Biochemistry, 24, 163-175.

Mazur B.J., Falco S. C., 1989. The development of herbicide resistant crops. Annual Review of Plant Physiology and Plant Molecular Biology, 40, 441-470.

McCarthy K., 1987. Inheritance of diclofop resistance in *L. rigidum*. Honours thesis, Department of Agronomy, The University of Adelaide.

McFadden J.J., Frear D.S. Mansager E.R., 1989. Aryl hydroxylation of diclofop by a cytochrome P_{450} dependent monooxygenase from wheat. Pesticide Biochemistry and Physiology, 34,92-100.

McKay A.C., Fisher J.M., Dube A.J., 1982. Control of the nematode associated with annual ryegrass toxicity. Australian Journal of Agricultural Research, 34, 403-413.

McKenzie, J.A., Game, A.Y., 1987. Diazinon resistance in *Lucilia cuprina*; mapping of a fitness modifier. Heredity, 59, 371-381.

Mc.Kinley, N., 1990. Sulfonylurea herbicide resistance in weeds in cereal and non-crop areas in the U.S. and Canada. In, Proceedings of the 9th Australian Weeds Conference. Adelaide, South Australia. August 6-10. pp 268-269.

McMurray, T.J., Groves, J.T., 1986. Metalloporphyrin models for cytochrome P-450. In, P.R. Ortiz de Montellano, ed. Cytochrome P-450. pp 1-28. Plenum Press, New York.

Meyer A.M., Muller F., 1989. Triasulfuron and its selective behaviour in wheat and *Lolium perenne*. Proceedings of the British Crop Protection Conference Weeds 1989, pp 441-443.

Medd R.W., Auld B.A., Kemp D.R., Murison R.D., 1985. The Influence of wheat density and spatial arrangement on annual ryegrass, *Lolium rigidum* Gaudin, competition. Australian Journal of Agricultural Research, 36, 361-371.

Michel H., Epp O., Deisenhofer J., 1986. Pigment-protein interactions in the photosynthetic reaction centre from *Rhodopseudomonas viridis*. EMBO Journal, 5, 2445-2451.

Miller C.S., Hall W.C., 1961. Absorbtion and Metabolism of Aminotriazole in Cotton. Journal of Agricultural and Food chemistry, 9(3), 210-212.

Miller K.D., Irzyk G.P., Fuerst E.P., McFarland J.E., 1992. Metabolism and induction of glutathione-S-transferase activity in suspension cultures of *Zea mays* by the herbicide safener benoxacor. Proceedings of the Weed Science Society of America Conference, Abst. 179.

Mitsutake K, Iwamura H, Shimizu R, Fujita T., 1986. Quantitative structure activity relationship of photosystem II inhibitors in chloroplasts and its link to herbicidal action. Journal of Agricultural and Food Chemistry 34, 725-732.

Monaghan N.M., 1980. The biology and control of *Lolium rigidum* as a weed of wheat. Weed Research, 20, 117-121.

Montgomery M.L., Botsford D.L., Freed V.H., 1969. Metabolism of hydroxysimazine by corn plants. Journal of Agricultural and Food Chemistry, 17(6), 1241-1243.

Montgomery M.L., Freed V.H., 1964. Metabolism of triazine herbicides by plants. Journal of Agricultural and Food Chemistry, 12(1), 11-14.

Moreland D.E., 1969. Inhibitors of chloroplast electron transport: structure-activity relations. Progress in photosynthesis research, III, 1693-1711.

Moreland D.E., Corbin F.T., Novitzky W.T., Parker C.E., Tomer K.B., 1990. Metabolism of metolachlor by a microsomal fraction isolated from grain sorghum (*Sorghum bicolor*) shoots. Z. Naturforsch, 45, 558-564.

Morrison I.N., Todd B.G., Nawolsky, K.M., 1989. Confirmation of trifluralin-resistant green foxtail (*Setaria viridis*) in Manitoba. Weed Technology, 3, 544-552.

Moss S.R., 1987. Herbicide resistance in black grass (*Alopecurus myosuroides*). Proceedings of the Brighton Crop Protection Conference-Weeds, 879-886.

Moss S.R., Cussans G.W., 1991. The development of herbicide resistant populations of

Alopecurus myosuroides (black-grass) in England. In, J.C. Casely, G.W. Cussans, R.K. Atkin eds. Herbicide Resistance in Weeds and Crops, pp 45-55, Butterworth Heinemann, Oxford.

Mougin C., Cabanne F., Canivenc M-C, Scalla R., 1990. Hydroxylation and N-demethylation of Chlorotolruon by Wheat Microsomal Enzymes. Plant Science, 66, 195-203.

Mougin C., Scalla R., Cabanne F., 1991. Occurrence of cytochrome P_{450} mono-oxygenases in the metabolism of chlorotoluron by wheat microsomes. In, J.C. Caseley, G.W. Cussons and R.K. Atkin, eds. Herbicide Resistance in Weeds and Crops, pp 458-459, Butterworth-Heineman, Oxford.

Mozer T.J., Tiemeier D. C., Jaworski E. G., 1983, Purification and characterisation of corn glutathione-S-transferase. Biochemistry, 22, 1068-1072.

Mudge L.C., Gossett B.J., Murphy T., 1984. Resistance of goosegrass (*Eleusine indica*) to dintroaniline herbicides. Weed Science, 32, 591-594.

Nebert D.W., Gonzalez F.J., 1987. P_{450} genes: structure, evolution and regulation. Annual Review of Biochemistry, 56, 945-993.

Oettmeier W., Masson K., Fedtke C., Konze J., Schmidt R., 1982. Effect of different photosystem II inhibitors on chloroplasts isolated from species either susceptible or resistant toward s-triazine herbicides. Pesticide Biochemistry and Physiology, 18, 357-367.

Oettmeier W., Stevens E., 1992. Orientation of herbicides within the photosystem II QB binding niche. Proceedings of the "Molecular Regulation of Chloroplast Functions" meeting. Omiya, August, 1992.

O'Keefe D.P., Leto K.J., 1989. Cytochrome P-$_{450}$ from the mesocarp of avocado (*Persea americana*). Plant Physiology, 89, 1141-1149.

Onley J.H., Yip G., Aldridge M. H., 1968. A metabolic study of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron) applied to corn seedlings. Journal of Agricultural and Food Chemistry, 16(3), 427-433.

Ortiz de Montellano P., ed., 1986. Cytochrome P-$_{450}$, Structure Mechanism and Biochemistry, pp. 273-314. New York, Plenum Press.

Ortiz de Montellano P.R., Reich N.O., 1986. Inhibition of cytochrome P-$_{450}$ enzymes. In, P. R. Ortiz de Montellano, ed. Cytochrome P-$_{450}$, pp 273-314. Plenum Press, New York.

Pallett K.E., Dodge A.D., 1979, The role of light and oxygen in the action of the photosynthetic inhibitor herbicide monouron.. Z. naturforsch, 34c, 1036-1039.

Parry A.D., Horgon R., 1992. Abscisic acid biosynthesis in roots. I. The identification of potential abscisic acid precursors and other carotenoids. Planta, 187, 185-191.

Pearce G.A., Holmes J.E., 1976. The control of annual ryegrass. Journal of Agriculture, Western Australia, 17(3), 77-81.

Petersen M., Seitz H.U., 1988. Reconstitution of cytochrome P-$_{450}$-dependent digitoxin 12b-hydrolase from cell cultures of foxglove (*Digitalis lanata* EHRH). Biochemistry Journal, 252, 537-543.

Pfister K., Steinback K.E., Gardner G., Arntzen C.J., 1981. Photoaffinity labeling of an herbicide receptor protein in chloroplast membranes. Proceedings of the National Academy of Science, 78(2), 981-985.

Pillai P., St. John J.B., 1981. Lipid composition of chloroplast membranes from weed biotypes differentially sensitive to triazine herbicides. Plant Physiology, 68, 585-587.

Polos E., Mikulàs J., Laskay G., Lehoczki E., 1985. Herbicide Resistance of Horseweed (*Conyza canadensis*) in Hungarian Vineyards. Proceedings of the 1985 British Crop Protection Conference - Weeds, 187 -195.

Polos E., Laskay G., Szigeti Z., Pataki Sz., Lehoczki E., 1987. Photosynthesis properties and cross-resistance to some urea herbicides of triazine-resistant *Conyza canadensis* Cronq. (L.). Z. Naturforsch, 42c, 783-793.

Powles S.B., Holtum J.A.M., Matthews J.M., Liljegren D.R., 1989. Multiple herbicide resistance in annual ryegrass (*Lolium rigidum*): The search for a mechanism. American Chemical Society Symposium. Fundamental and Practical Approaches to Combating Resistance, pp 394-406. American Chemical Society, Washington.

Powles S.B., Howat P.D., 1990. Herbicide-resistant weeds in Australia. Weed Technology, 4, 178-185.

Powles S.B., Matthews J.M., 1992. Multiple herbicide resistance in annual ryegrass (*Lolium rigidum*), the driving force for the adoption of integrated weed management. In, I Denholm, A Devonshire, and D Holloman, eds. Achievements and Developments in Combating Pest Resistance. Elsevier, London, in press.

Primiani M.M., Cotterman J.C., Saari L.L. 1990. Resistance of kochia (*Kochia scoparia*) to sulfonylurea and imidazolinone herbicides. Weed Technology, 4, 169-172.

Radosevich S.R., 1977. Mechanism of atrazine resistance in lambsquarters and pigweed. Weed Science, 25(4), 316-318.

Radosevich S.R., Steinback K.E., Arntzen C.J., 1979. Effect of photosystem II inhibitors on thylakoid membranes of two common groundsel (*Senecio vulgaris*) biotypes. Weed Science, 27(2), 216-218.

Ray T.B., 1984. Site of action of chlosulfuron: inhibition of valine and isoleucine synthesis in plants. Plant Physiology, 75, 827-831.

Reeves T.G., Smith I.S., 1975. Pasture management and cultural methods for the control of annual ryegrass (*Lolium rigidum*) in wheat. Australian Journal of Experimental Agriculture and Animal Husbandry, 15, 527-530.

Reeves T.G., 1976. Effect of annual ryegrass (*Lolium rigidum* Gaud.) on yield of wheat. Weed Research, 16, 57-63.

Reichhardt D., Simon A., Durst F., Matthews J.M., Ortiz de Montellano P., 1982. Autocatalytic inactivation of plant cytochrome P_{-450} enzymes: selective inactivation of cinnamic acid 4-hydrolase from *Helianthus tuberosus* by 1-aminobenzotriazole. Archives of Biochemistry and Biophysics, 216, 522-529.

Reichardt D., Salaün J-P., Benveniste I., Durst F., 1986. Time course of induction of cytochrome P_{-450}, NADPH-cytochrome c reductase and cinnamic acid hydroxylase by phenobarbital, ethanol herbicides and manganse in higher plant microsomes. Plant Physiology, 66, 600-604.

Rendina A., Felts J , Beaudoin J.D., Craig-Kinnard A.D., Look L.L., Paraskos S.L., Hagenah J.A., 1988. Kinetic characterisation, stereoselectivity and species selectivity of the inhibition of plant acetyl-CoA carboxylase by the aryloxyphenoxyproionic acid herbicides. Archives of Biochemistry and Biophysics, 265, 219-225.

Renger G., 1976. Studies on the structural and functional organization of system II of photosynthesis. The use of trypsin as a structurally selective inhibitor at the outer surface of the thylakoid membrane. Biochimica et Biophysica Acta, 440, 287-300.

Rerkasem K., Stern W.R., Goodchild N.A., 1980a. Associated growth of wheat and annual ryegrass. 1. The effect of varying total density and proportion in mixtures of wheat and annual ryegrass. Australian Journal of Agricultural Research, 31, 649-658.

Rerkasem K., Stern W.R., Goodchild N.A., 1980b. Associated growth of wheat and annual ryegrass. 2. Effect of varying time of ryegrass germination in stands of wheat. Australian Journal of Agricultural Research, 31, 659-672.

Ridley S.M., 1977. Interaction of chloroplasts with inhibitors. Plant Physiol., 59, 724-732.

Ridley S.M., 1982. Carotenoids and herbicide action. In, G. Britton, T.W. Godwin eds. Carotenoid Chemistry and Biochemistry. pp 353-368. Pergamon Press, London.

Roget D.K., Venn N.R., Rovira A.D., 1987. Reduction of rhizoctonia root rot of direct drilled wheat by short term chemical fallow. Australian Journal of Experimental Agriculture, 27, 425-430.

Roush R.T., McKenzie J.A., 1987. Ecological genetics of insecticide and acaricide resistance. Annual Review of Entomology, 32, 361-380.

Rowe L., Rossman E., Penner D., 1990. Differential response of corn hybrids and inbreds to metolachlor. Weed Science, 38, 563-566.

Roy D.N., Konar S.K., Charles D.A., Feng J.C., Prassad R., Campbell T.A., 1989. Determination of the persistance, movement, and degradation of hexazinone in selected canadian boreal forest soils. Journal of Agricultural and Food Chemistry, 37(2), 443-447.

Ryan G.F., 1970. Resistance of common groundsel to simazine and atrazine. Weed Science, 18, 614.

Ryan P.J., Owen W.J., 1982. The mechanism of selectivity of chlortoluron between cereals and grassweeds. Proceedings of the 1982 British Crop Protection Conference - Weeds, 317-324.

Saari, L.L.,. Cotterman J.C., Primiani M.M., 1989. Mechanism of sulfonylurea herbicide resistance in the broadleaf weed, *Kochia scoparia.* Plant Physiology, 93, 55-61.

Saari L.L., Cotterman J.C., Smith W.F., Primiani M.M., 1992. Sulfonylurea resistance in common chickweed, perennial ryegrass, and russian thistle. Pesticide Biochemistry and Physiology, 42, 110-118.

Salaün J.-P., Benveniste I., Reichhart D., Durst F., 1981. Induction and specificity of a cytochrome $P\text{-}450$ dependent laurate in-chain-hydroxylase from higher plant microsomes. European Journal of Biochemistry, 119, 651-655.

Salaün, J.-P., Simon, A., Durst, F., 1986. Specific induction of lauric acid hydroxylase by clofibrate, diethylhexyl-pthalate and 2,4-dichlorophenoxyacetic acid in higher plants.

Lipids, 21, 776-779.

Sandmann G., Böger P., 1987. Herbicides affecting plant pigments. Proceedings of the British Crop Protection Conference-Weeds, 139-148.

Sandmann G., Böger P., 1989. In, Böger P., Sandmann G., eds. Target Sites of Herbicide Action. pp. 25-44. CRC Press, Boca Raton, Florida.

Sandmann G., Albrecht M., 1990. Accumulation of colourless carotenes and derivatives during interaction of bleaching herbicides with phytoene desaturation. Z. Naturforsch, 45c, 487-491.

Scarponi L., Perucci P., 1986. Effect of Some s-triazine Herbicides on Phosphatases from Corn (*Zea mays*) Roots. Weed Science, 34, 807-810.

Schiff J.A., Cunningham F.X., Green M.S., 1982. Carotenoids in relation to chloroplasts and other organelles. In, G. Britton, T.W. Godwin eds. Carotenoid Chemistry and Biochemistry. pp 329-338, Pergamon Press, London.

Schonfeld M., Yaacoby T., Michael O., Rubin B., 1987. Triazine resistance without reduced vigour in *Phalaris paradoxa.* Plant Physiology, 83, 329-333.

Secor J., Cseke C. Owen W. J., 1989. The discovery of the selective inhibition of acetyl coenzyme A carboxylase activity by two classes of graminicides. Proceedings of the British Crop Protection Conference - Weeds, 3, 145-154.

Shaaltiel Y., Glazer A., Bocion P.F., Gressel J., 1988. Cross tolerance to herbicidal and environmental oxidants of plant biotypes tolerant to paraquat, sulfur dioxide, and ozone. Pesticide Biochemistry and Physiology, 31, 13-23.

Shaner D. L., Anderson P. C., Stidham M. A., 1984. Imidazolinones, potent inhibitors of acetohydroxyacid synthase. Plant Physiology, 76, 545-546.

Shaner D.L., Robson P.A., 1985. Absorbtion, translocation and metabolism of AC 252 214 in soybean (*Glycine max*), common cockleburr (*Xanthium strumarium*) and velvet leaf (*Abutilon theophrasti*). Weed Science, 33, 469-471.

Shaw S. L., Fang S. C., 1973. Metabolism of monuron in excised leaves of corn and bean plants. Weed Research, 13, 59-66.

Shimabukuro R.H., Linck A.J., 1965. The metabolism of 3-amino-1,2,4-triazole in the fruit of bean, *Phaseolus vulgaris*. Physiologia Plantarum, 18, 532-539.

Shimabukuro R.H., 1967. Atrazine Metabolism and Herbicidal Selectivity. Plant Physiology, 42, 1269-1276.

Shimabukuro R.H., 1968. Atrazine Metabolism in Resistant Corn and Sorghum. Plant Physiology, 43, 1925-1930.

Shimabukuro R.H., Swanson H.R., 1969. Atrazine metabolism, selectivity, and mode of action. Journal of Agricultural and food chemistry, 17, 199-205.

Shimabukuro R. H., Walsh W.C., Hoerauf R.A., 1979. Metabolism and selectivity of diclofop-methyl in wild oat and wheat. Journal of Agricultural and Food Chemistry, 27, 615-622.

Shimabukuro R.H., 1985. Detoxication of herbicides. In S.O. Duke ed. Weed Physiology Volume II. pp 215-240. CRC Press, Boca Raton.

Shimabukuro R. H., 1990. Selectivity and mode of action of action of a postemergence herbicide diclofop-methyl. Plant Growth Regulator Society of America Quarterly, 18, 37-54.

Shimabukuro R. H., Hoffer B.L., 1991. Metabolism of diclofop-methyl in susceptible and resistant biotypes of *Lolium rigidum*. Pesticide Biochemistry and Physiology, 98, 1415-1422.

Shimabukuro R. H., Hoffer B.L., 1992. Effect of diclofop on the membrane potentials of herbicide resistant and susceptible annual ryegrass root tips. Plant Physiology, 39, 251-260.

Shimizu R., Iwamura H., Fujita T., 1988. Quantitative structure-activity relationships of photosystem II inhibitory anilides and triazines. Topolagical aspects of their binding to the active site. Journal of Agricultural and Food Chemistry, 36, 1276-1283.

Sigematsu Y., Sato F., Yamada Y., 1989. The mechanism of herbicide resistance in tobacco cells with a new mutation in the Q_B protein. Plant Physiology, 89, 986-992.

Simmons T.W., Jamall I.S., 1988. Significance of alterations in hepatic antioxidant enzymes. Primacy of glutathione peroxidase. Biochemistry Journal, 251, 913-917.

Singer S.R., McDaniel C.N., 1982. Transport of the herbicide 3-amino-1,2,4-triazole by cultured tobacco cells and leaf protoplasts. Plant Physiology, 69, 1382-1386.

Singer S.R., McDaniel C.N., 1985. Selection of glyphosate-tolerant tobacco cali and the expression of this tolerance in regenerated plants. Plant Physiology, 78, 411-416.

Sinning I., 1992. Structural principles of herbicide resistance in *Rhodopsuedomonas viridis*. Proceedings of the "Molecular Regulation of Chloroplast Functions" meeting. Omiya, August, 1992.

Sjostedt G., Gringas L., 1955. 3-Amino-1,2,4-triazole. In, Horning E. C., ed. Organic Synthesis Collective Volume 3. pp. 95-96. John Wiley, New York.

Smith A.E., Phatek S.C., Emmatty D.A., 1989. Metribuzin metabolism by tomato cultivars with low, medium and high levels of tolerance to metribuzin. Pesticide Biochemistry and Physiology, 35, 284-290.

Smith I.K., Kendall A.C., Keys A.J., Turner J.C., Lea P.J., 1984. Increased levels of glutathione in a catalase-deficient mutant of barley (*Hordeum vulgare* L.). Plant Science Letters, 37, 29-33.

Smith J.W., Sheets T.J., 1967. Uptake, distribution, and metabolism of monuron and diuron by several plants. Journal of Agricultural and Food Chemistry, 15(4), 577-581.

Smith L.W., 1967. Metabolism of amitrole (3-amino-1,2, 4-triazole) in relation to the differential susceptibility of *Cirsium arvense* (L.) Scop. Ecotypes. Ph. D dissertation, University of California, Davis.

Smith L.W., Chang F-Y., 1973. Aminotriazole metabolism in *Cirsium arvense* (L.) Scop. and *Pisum sativum*. Weed Research, 13, 339-350.

Soliday C.L., Kolattukudy P.E., 1978. Midchain hydroxylation of 16-hydroxypalmitic acid by the endoplasmic reticulum fraction from germinating *Vicia faba*. Archives of Biochemistry and Biophysics, 188, 338-347.

Souza Machado V., 1982. Inheritance and breeding potential of triazine tolerance and resistance in plants, In, H. Le Baron and J. Gressel, eds. Herbicide Resistance in Plants. pp. 257-273, John Wiley and Sons, New York.

Steinback K.E., McIntosh L., Bogorad L., Arntzen C.J., 1981. Identification of the triazine receptor protein as a chloroplast gene product. Proceedings of the National Academy of Science, 78(12): 7463-7467.

Stowe A.E., Holt J.S., 1988. Comparison of triazine-resistant and -susceptible biotypes of *Senecio vulgaris* and their F1 hybrids. Plant Physiology, 87, 183-189.

Subramanian M.V., Loney-Gallant V., Dias J., Mireles L.C., 1991. Acetolactate Synthase inhibiting herbicides bind to the regulatory site. Plant Physiology, 96, 310-313.

Sundseth S.S., Kennel S,J., Waters L.C., 1989. Monoclonal antibodies to resistance related forms of cytochrome P-450 in *Drosophila melanogaster*. Pesticide Biochemistry and Physiology, 33, 176-188.

Sutherland G. L. 3-Amino-s-triazole. Zweig, IV, 17-26.

Suzuki T., Casida J.E., 1981. Metabolites of diuron, linuron, and methazole formed by liver microsomal enzymes and spinach plants. Journal of Agricultural and Food Chemistry, 29, 1027-1033.

Swartzberg D., Izhar S., Beckmann J.S., 1985. Tobacco callus line tolerant to amitrole, selection, regeneration of plants and genetic Analysis. Journal of Plant Physiology, 121, 29-35.

Sweetser P.B., Schow G.S., Hutchison J.M., 1982. Metabolism of chlorsulfuron by plants, biological basis for selectivity of a new herbicide for cereals. Pesticide Biochemistry and Physiology. 17, 18-23.

Sweetser, P.B. 1985. Safening of sulfonylurea herbicides to cereal crops: mode of herbicide antidote action. Proceedings of the British Crop Protection Conference - Weeds:1147-1154.

Tanaka F.S., Wien R.G., 1979. Radical oxidation of 3-(4-Chlorophenyl)-1,1-dimethylureain Aqueous Media. Journal of Agriculture and Food Chemistry, 27(2): 311-315.

Tardif F. and S.B. Powles. 1992. Sethoxydim resistance in annual ryegrass (*Lolium rigidum*). Planta. submitted.

Thill D.C., Mallory-Smith C.A., Saari L.L., Cotterman J.C., Primiani M.M., Saladini J.L. 1991. Sulfonylurea herbicide resistant weeds: Discovery distribution, biology, mechanism and management. *In* J.C. Caseley, G.W. Cussons and R.K. Atkin, eds. Herbicide Resistance in Weeds and Crops, pp 213-226, Butterworth-Heineman, Oxford.

Tischer W., Strotman H., 1977. Relationship between inhibitor binding by chloroplasts and of photosynthetic electron transport. Biochimica et Biophysica Acta, 113-125.

Trebst A., 1991. The molecular basis of resistance of phtosystem II herbicides. In J.C. Casely, G.W. Cussans, R.K. Atkin eds.*Herbicide Resistance in Weeds and Crops*, pp 145-164, Butterworth Heinemann, Oxford.

Tucker E.S., PowlesS.B., 1991. A biotype of hare barley (*Hordeum leporinum*) resistant to paraquat and diquat. Weed Science 39,159-162.

van Oorschot J.P.L., van Leeuwen P.H., 1984. Comparison of the photosynthetic capacity between intact leaves of triazine-resistant and -susceptible biotypes of six Weed Species. Z. Naturforsch, 39c, 440-442.

Vaughn K.C., Duke S.O., 1984. Ultrastructural alterations to chloroplasts in triazine-resistant weed biotypes. Physiologia Plantarum, 62, 510-520.

Vaughn K.C., Marks M.D., Weeks D.P., 1987. A dinitroaniline-resistant mutant of *Eleusine indica* exhibits cross-resistance and supersensitivity to antimicrotubule herbicides and drugs. Plant Physiology, 83, 956-964.

Vaughn K.C. and Vaughan M.A. 1990. Structural and biochemical characterization of dinitroaniline resistant *Eleusine*. In Managing Resistance to Agrochemicals: from Fundamental Research to Practical Strategies, pp 364 - 375. eds Green, M.B., LeBaron, H.H., Moberg, W.K. ACS Symposium Series 421.

Vencill W.K., Foy C.L., Orcutt D.M., 1987. Effects of temeperature on triazine resistant weed biotypes. Environmental and Experimental Botany. 27, 473-480.

Vivekanandan M., Gnanam A., 1975. Studies on the mode of action of aminotriazole in the induction of chlorosis. Plant Physiology, 55, 526-531.

Waldrop D.D., and Banks P.A. 1983. Interactions of herbicides with insecticides in soybeans (*Glycine max*). Weed Science, 31, 730-734.

Weber A., Fischer E., Schipp von Branitz H., Lüttge U., 1988. The effect of the herbicide sethoxydim on transport processes in sensitive and tolerant grass species. I. Effects on membrane potential and alinine uptake. Z. Naturforsch., 43, 249-256.

Weimer M.R., Swicher B.A., Vogel K.P., 1988. Metabolism as a basis for differential atrazine tolerance in warm-season forage grasses. Weed Science, 36, 436-440.

Werck-Reichardt D., Jones O.T.G., Durst F., 1988. Haeme synthesis during cytochrome $P\text{-}_{450}$ induction in higher plants. Biochemical Journal 249, 473-480.

Westerfeld W.W., 1945. A colorimetric determination of blood acetoin. Journal of Biological Chemistry, 161, 495-502.

Winder T., Spalding M.H., 1988. Imazaquin and chlorsulfuron resistance and cross-resistance in mutants of *Chlamydomonas reinhardtii*. Molecular and General Genetics, 213, 394-399.

Wright J.P., Shimabukuro R.H., 1987. Effect of diclofop and diclofop methyl on the membrane potentials in wheat and oat coleoptiles. Plant Physiology, 85, 188-193.

Young O., Beevers H., 1976. Mixed function oxidases from germinating castor bean endosperm. Phytochemistry, 15, 379-385.

Young A.J., Britton G., Musker D., 1989. A rapid method for the analysis of the mode of action of bleaching herbicides. Pesticide Biochemistry and Physiology, 35,244-250.

Zimmerlin A., Durst F. (1991). Xenobiotic metabolism in higher plants: aryl hydroxylation of diclofop by a cytochrome P_{450} enzyme from wheat microsomes. *In* J.C. Caseley, G.W. Cussons and R.K. Atkin, eds. Herbicide Resistance in Weeds and Crops, pp 491-492, Butterworth-Heineman, Oxford.

Zimmerlin A., 1992. Ph.D. dissertation, Laboratoire d'Enzymologie, Institut de Botanique, Strasbourg France.