MECHANISM OF RESISTANCE TO PARAQUAT IN THE WEEDY GRASSES *Hordeum leporinum* AND *H. glaucum*

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TABLE OF CONTENTS

Abstract

Paraquat is widely used in agriculture for weed control prior to crop sowing and to control weeds in perennial cropping systems. Resistance to this herbicide has appeared in several populations of both *Hordeum glaucum* Steud. and *H. leporinum* Link. in Australia following prolonged use of paraquat. All previous cases of paraquat resistance occurring in the field had been observed in perennial cropping operations where weeds have evolved resistance following a high selection pressure. In 1994, following 10 to 15 years of once annual applications of paraquat and diquat for weed control in zero-tilled cereals, failure of these herbicides to control *H. glaucum* in two separate fields occurred. Dose response experiments confirmed that both populations were more that 250-fold resistant to paraquat and were cross-resistant to diquat. This is the first example, world-wide, where paraquat resistance had evolved in weed species within zero-tillage annual cropping systems.

The mechanism of paraquat resistance in *Hordeum* spp. is unknown, but is correlated with reduced movement of paraquat within the plant. The mechanism is proposed to be sequestration of herbicide away from its site of action in the chloroplast either by enhanced binding to the cell wall or an alteration in a transporter responsible for allowing paraquat to cross plant membranes. Resistance to paraquat in all *Hordeum* spp. biotypes, including those from zero-tillage cropping systems, is effective only at low temperatures. Therefore, it is likely that all resistant biotypes have the same mechanism of resistance.

Polyamines are proposed to play an important role in the accumulation of paraquat within cells. The measurement of free polyamines in resistant and susceptible biotypes showed that, generally, constitutive putrescine and spermidine contents were higher in leaves of paraquat-resistant biotypes. However, these increased polyamine contents were poorly correlated with the

level of resistance within these biotypes. Polyamine contents increased in both resistant and susceptible seedlings following treatment of plants with paraquat and these increases were usually greater in the resistant biotypes. The increased putrescine content of resistant plants does not appear to be a primary mechanism of resistance in *Hordeum* spp. as there was a poor correlation between degree of resistance and putrescine content. The increased inducible putrescine contents in resistant biotypes are most likely a stress response as putrescine contents also increased following treatment of plants with oxyfluorfen, a herbicide to which the paraquat-resistant plants are susceptible.

Increased binding of paraquat to cell walls of the resistant biotype of *H. glaucum* as a possible mechanism of resistance was examined. Paraquat was found to bind strongly to the cell wall of both resistant and susceptible plants and did not readily exchange with the surrounding solution in the absence of divalent cations such as putrescine, benzyl viologen or paraquat. From binding experiments, it appears there are two types of binding site with different affinities for paraquat in cell walls of both resistant and susceptible biotypes. There were no obvious differences between paraquat binding characteristics to cell walls of resistant and susceptible biotypes of *H. glaucum*. Therefore, increased binding of paraquat to the cell wall appears not to be a mechanism of resistance.

Using measurement of photosynthetic activity, as a method for estimating paraquat appearance at the active site, protoplasts of *H. glaucum* were much more resistant to paraquat than were chloroplasts or leaf slices. Paraquat was also more effective at inhibiting O_2 evolution when the calcium concentration in the medium was reduced. Accumulation of paraquat by protoplasts of both biotypes occurred with apparently saturable kinetics; however, there were no differences between resistant and susceptible biotypes in paraquat accumulation by protoplasts.

From this study, it can be concluded that the mechanism of paraquat resistance in *Hordeum* spp. is not due to enhanced binding of paraquat to the cell wall or to increased putrescine contents. Resistance appears to be due to either sequestration of paraquat in the apoplast or in the vacuoles. On present evidence, it is likely that resistance is the result of sequestering of paraquat away from its active site by preventing the herbicide from entering the cell.

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I dedicate this thesis to my wife and daughter who have given me all the support and encouragement I have needed.

Declaration

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

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Chapter 1

Literature review

1.1. Introduction

Herbicide resistance is a widespread problem in monoculture and/or mono-herbicide cropping systems throughout the world. Resistance is defined as the inherited ability of a weed population to tolerate doses of herbicide that would be lethal to the great majority of individuals in a normal population of that species (Powles *et al.*, 1997). Resistance occurs because a herbicide kills susceptible individuals and reduces the population of susceptible plants dramatically. Weeds usually become resistant to herbicides following several years of application of a herbicide, or herbicides of the same chemical family.

Harper (1956) predicted that the repeated use of the same herbicide would be almost inevitably followed by development of resistant biotypes of a weed, as had happened with fungicides and insecticides. In 1968, herbicide resistance was first identified in a biotype of *Senecio vulgaris* resistant to simazine in a nursery in Washington State, U.S.A (Ryan, 1970), where atrazine and simazine had been used once or twice annually since 1958. Since then, there has been a rapid increase in the incidence of herbicide resistance worldwide (LeBaron and McFarland, 1990; Warwick, 1991; Holt *et al.*, 1993; Powles and Holtum, 1994; Powles *et al.*, 1997). About 65 weed species have evolved resistance to triazine herbicides and, in addition, at least 126 other species have biotypes resistant to one or more herbicides from 15 other herbicide classes (Heap, 1997). There are 16 different classes of herbicides for which resistance has been reported. The number of species and the years when resistance was first detected are listed in Table 1.1. The triazine, bipyridylium, ALS-inhibiting and ACCase-inhibiting herbicides are some of the most widely used herbicides in agriculture and it is these herbicides to which most resistance has been reported.

Table 1.1: Number of species having developed resistance to different herbicide classes.

Herbicide or class	Year first detected	Number of species with resistant biotypes
ACCase inhibitors	1982	14
ALS inhibitors	1986	50
Aminotriazoles	1986	2
Arsenicals	1984	1
Benzonitriles	1988	1
Bipyridyliums	1976	27
Carbamates	1988	2
Dinitroanilines	1973	7
Phenoxyacetic acids	1962	17
Picloram	1988	1
Pyridazinones	1978	1
Substituted ureas	1983	7
Triazines	1968	65
Uracils	1988	2
Urea/Amides	1986	17
Glyphosate	1996	1

From: Heap (1997)

In Australia, herbicide resistance was first identified in a population of *Lolium rigidum* Gaudin, which had become resistant to diclofop-methyl (Heap and Knight, 1982). Currently, there are many biotypes of *L. rigidum* and 20 other annual weed species which have been confirmed to be resistant to herbicides in this country (Bishop *et al.*, 1996). Resistance in Australia has been reported to a wide range of chemicals including the bipyridyliums (paraquat and diquat), ACCase-inhibitors, ALS-inhibitors and others.

1.2. **Paraquat resistance**

Paraquat resistance was first reported in a biotype of *Conyza bonariensis* (L.) Cronq. in the early 1980's in vineyard and citrus plantations in Egypt (Youngman and Dodge, 1981). To date, resistance to the bipyridylium herbicides, paraquat and diquat, has been reported in at least 7 grass species and 20 broadleaf weeds in 12 countries (Table 1.2; Heap 1997). Resistance to paraquat has also been successfully selected for in trials designed to select *Lolium perenne* L. which could survive paraquat treatment (Faulkner, 1975). Paraquat resistance in a number of species has also been obtained by *in vitro* selection. Hughes (1979) selected a soybean (*Glycine max* L.) cell line whose growth was not inhibited by 1 mM paraquat. Paraquat resistance cell lines of tobacco (*Nicotiana tabacum* L.) (Hughes *et al.,* 1984), tomato (*Lycopersicon esculentum* L.) (Thomas and Pratt, 1982), *Chenopodium rubum* (Bhargara, 1993), the unicellular green alga *Chlamydomonas reinhardtii* Dangeard (Kitayama and Togasaki, 1992), and the fern *Ceratopteris richardii* Brogn. (Carroll *et al.*, 1988) have also been selected in tissue culture.

All cases of paraquat resistance occurring in the field have been observed in perennial cropping operations. Weeds have evolved resistance following a high selection pressure of 5 to 10 applications of herbicide each year for 5 or more

years (Watanabe *et al.,* 1982; Fuerst and Vaughn, 1990), or after annual applications for 12 to 15 years (Powles, 1986). In Australia, paraquat resistance was first reported in *Hordeum glaucum* Steud. (Powles, 1986) and so far paraquat resistance has been confirmed in three other species, *Arctotheca calendula* (L.) Levyns (Powles *et al.,* 1989), *Hordeum leporinum* Link. (Tucker and Powles, 1991) and *Vulpia bromoides* (L.) S.F.Gray (Purba *et al.*, 1993a) (Table 1.2). Paraquat-resistant biotypes of these four species have been found in a single lucerne field with long history of paraquat use. Additional paraquat resistant biotypes of these species have been observed in several other lucerne (*Medicago sativa* L.) fields across southern Australia (Purba, 1993).

1.3. **Paraquat resistance in barley grasses (*H. glaucum* and *H. leporinum*)**

H. glaucum and *H. leporinum* (both known as barley grass) are annual grass weeds which infest lucerne crops of South Australia, Tasmania, and Victoria during the winter. Paraquat and diquat were used for many years as the sole weed control method in lucerne growing operations in Australia. In 1982, following once annual applications of paraquat for about 12-15 years, the first sign of failure to control this weed was observed in a mixed cereal cropping and sheep grazing enterprise at Willaura near Ararat, Victoria (Fig. 1.1). It was later confirmed that this biotype is 250 times more resistant to paraquat than the susceptible biotype (Powles, 1986). The mechanism of resistance in *H. glaucum* was reported to be reduced translocation and /or sequestration of herbicide (Bishop *et al.*, 1987; Preston *et al.*, 1992b). In *H. leporinum,* the mechanism of resistance is also shown to be a result of reduced paraquat translocation in the resistant biotype compared to the susceptible biotype (Preston *et al.*, 1992b).

Table 1.2: Weed species that have developed resistance to bipyridyl herbicides.

Species	First occurrence
Amaranthus lividus L.	Malaysia
Arctotheca calendula (L.) Levyns	Australia
Bidens pilosa L.	Kenya
Crassocephalum crepidioides (Benth.) S.Moore	Malaysia
Commelia benghalensis L.	Malaysia
Conyza bonariensis (L.) Cronq.	Egypt
Conyza canadensis (L.) Cronq.	Japan
Conyza linifolia	Egypt
Conyza sumatrensis (Retz.) E.H.Walker	Japan
Cuphea carthagenenis (Jacq.) Macbride	Fiji
Eleusine indica (L.) Gaertn.	Malaysia
Epilobium adenocaulon	Belgium
Epilobium ciliatum Rafn	Belgium
Erigeron philadelphicus L.	Japan
Hemistepta lyrata	Korea
Hordeum glaucum Steud.	Australia
Hordeum leporinum Link	Australia
Ischaemum rugosum	Malaysia
Lepidium virginicum L.	Canada
Mosla dianthera	Korea
Parthenium hysterophorus L.	Kenya
Paspalum thunbergii	Korea
Poa annua L.	U.K.
Solanum americanum Mill.	U.S.A.
Solanum nigrum L.	Malaysia
Vulpia bromoides (L.) S.F.Gray	Australia
Youngia japonica (L.) DC.	Japan

Source: Preston (1994); Heap (1997)

Figure 1.1: Resistance to paraquat in barley grass (*H. glaucum*) from a lucerne field with long history of paraquat and diquat use.

Resistance in both *Hordeum* species is controlled by a single incompletely-dominant nuclear gene (Islam and Powles, 1988; Purba *et al.,* 1993b).

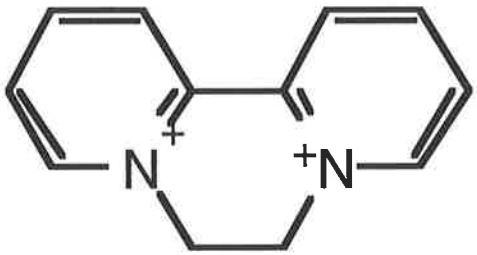
1.4. **Paraquat**

Paraquat (1,1'-dimethyl-4,4'-bipyridylium ion) and diquat (6,7-dihydrodipyrido[1,2-α:2′,1′-*c*]pyrazinediium ion) are two commercial members of the herbicide group known as the bipyridyliums. Paraquat was known in the 1930s as methyl viologen and used as an oxidation-reduction indicator for many years; however, its herbicidal properties were first discovered in the mid 1950s (Dodge, 1971). Paraquat and diquat are available commercially as the dichloride and dibromide salts, respectively. The structure of paraquat and diquat are shown in Fig. 1.2. The bipyridyl herbicides, paraquat and diquat, are commonly used to control a wide range of economically important weeds in Australia, including the barley grasses, *H. glaucum* and *H. leporinum.*

1.5. **Herbicidal properties**

Paraquat and diquat are non-selective, contact, foliar applied herbicides with rapid desiccant action. Paraquat binds to soil colloids irreversibly and is considered to be biologically inactive in most soils (Calderbank and Slade, 1976). Therefore, this herbicide is widely used to control annual weeds in perennial crops such as vineyards, orchards, and lucerne and for weed control before crop seeding in minimum-till agriculture. Diquat, on the other hand, is mainly used for pre-harvest desiccation or aquatic weed control. Paraquat and diquat kill all exposed green tissue of annual plants, but new foliage of perennial plants will usually appear after an initial application. Paraquat and diquat are light-dependent, act rapidly in the presence of light, and lead to bleaching and wilting of green tissue, often visible within a few hours (Summers, 1980).

Paraquat (E'_o = - 446 mV)



Diquat (E'_o = - 349 mV)

Figure 1.2: Chemical structure and midpoint redox potentials of paraquat (1,1'-dimethyl-4,4'-bipyridylium) and diquat (6,7-dihydrodipyrido[1,2-α:2´,1´-*c*]pyrazinediium ion).

1.6. Degradation

Paraquat is chemically stable in soils at temperatures up to 107°C and purely chemical breakdown of paraquat in soil is unlikely to be an important pathway of its degradation (Summer, 1981). Decomposition of paraquat and diquat from soil by volatilisation can also be discounted because of the very low vapour pressure of the salts (Calderbank, 1968).

Paraquat and diquat can be degraded photochemically by sunlight when they are absorbed on surface of plants and on dead plant material (Calderbank, 1968). Calderbank (1968) also showed that paraquat and diquat absorbed on films of soil can be degraded by ultraviolet light, but to a lesser degree than when films of the chemicals themselves were exposed. Slade (1966a) showed that ultraviolet light can induce photochemical degradation of paraquat. He identified two degradation products as 1-methyl-4-carboxypyridinium ion and methylamine hydrochloride and proposed a pathway for degradation of paraquat (Slade, 1966a), (Fig. 1.3). Very little paraquat remains on plants after a week of sunny conditions (Summers, 1980).

Plants do not metabolically degrade paraquat. There are no reports which show paraquat can be metabolised in any higher plants which have been investigated so far (Funderburk & Lawrence, 1964; Harvey *et al.*, 1978, Harvey and Harper, 1982, Norman *et al.*, 1993). Funderburk and Lawrence (1964) reported that neither ^{14}C-diquat nor ^{14}C-paraquat were degraded by beans (*Phaseolus vulgaris* L.) or alligator weed, *Alternanthera philoxeroids* (Martius) Griseb. Microorganisms, however, can degrade paraquat and diquat. Metabolism of paraquat has been observed in a few isolates of bacteria and fungi (Imai and Kuwatsuka, 1989). The main products of metabolism by microorganisms are

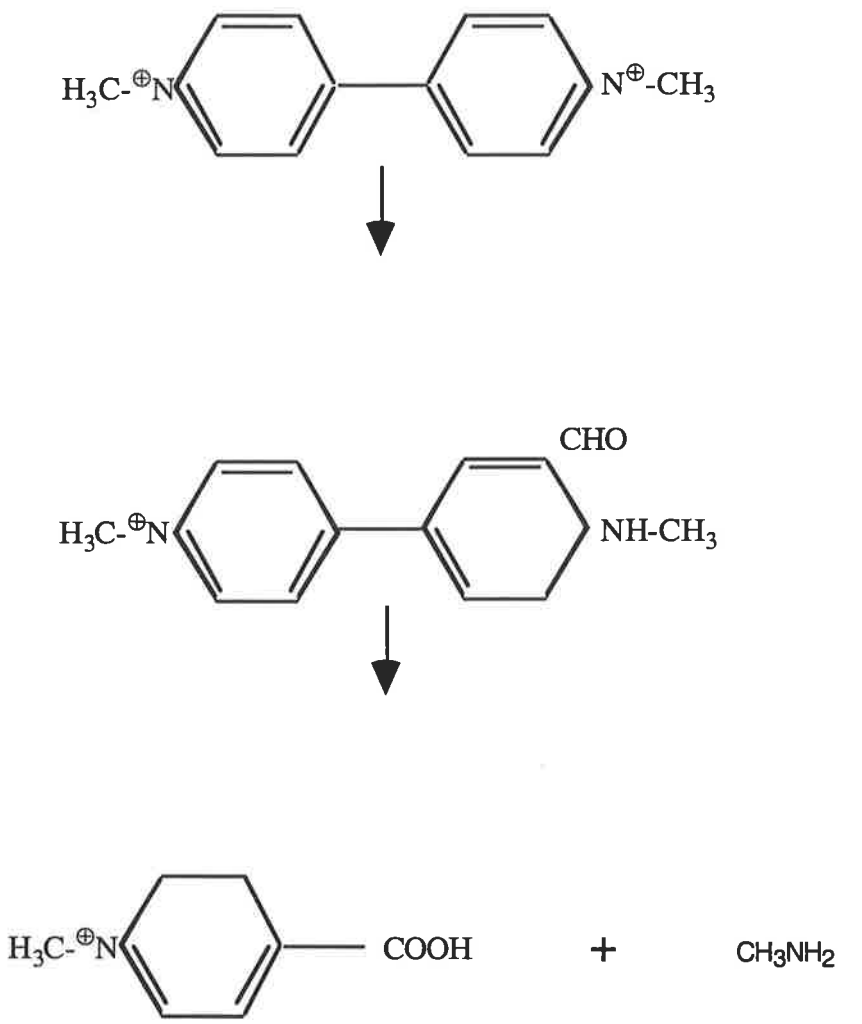


Figure 1.3: The photochemical breakdown of paraquat on the leaf surface. From: Funderburk and Bonzarth (1967).

monoquat and N-methyl isonicotinic acid or 4-carboxy-1-methylpridinium (Calderbank and Slade, 1976).

1.7. **The site of action of paraquat at PSI**

Following application and uptake of paraquat into plants, the reduction of paraquat to the cation radical takes place by donation of an electron from the terminal end of photosystem I (Dodge, 1971). Photosystem I (PSI) is a membrane bound protein complex, in the thylakoid membranes of chloroplasts, which catalyses the light-driven oxidation of plastocyanin (PC) and reduction of ferredoxin (Fd) (Preston, 1994). PSI consists of a series of redox components with mid-potentials ranging from about -880 to -320 mV. The photoactive components include a primary electron donor, P700, which is considered to be a chlorophyll a dimer, and a primary electron acceptor, A_o, an intermediate phylloquinone acceptor or Vitamine K molecule, A_1, and three iron-sulfur centres, F_X, F_B, and F_A (Fig. 1.4). A photon of light excites an electron in the pigment bed and the excitation energy is then passed to the primary electron donor, P700. This excited electron is then transferred to an intermediate electron acceptor called A_o, which is probably a specialised chlorophyll monomer. The electron on A_o then traverses through a series of acceptors, with less negative redox potentials, A_1, (mid-potential about -730 mv) and F_X to the terminal electron acceptor(s) F_A/F_B with mid-point potentials of -590 and -530 mV, respectively (Golbeck, 1992, Golbeck and Bryant, 1991).

The low redox potential of the reducing side of PSI means that few chemicals are able to interact here. Paraquat is one chemical that is able to interfere with the PSI electron transport system (Calderbank and Slade, 1976). Paraquat (Fig. 1.2) has a highly negative redox potential (E'_o = -446 mV) which enables it to compete for electron flow with the primary electron acceptor of PSI ($NADP^+$).

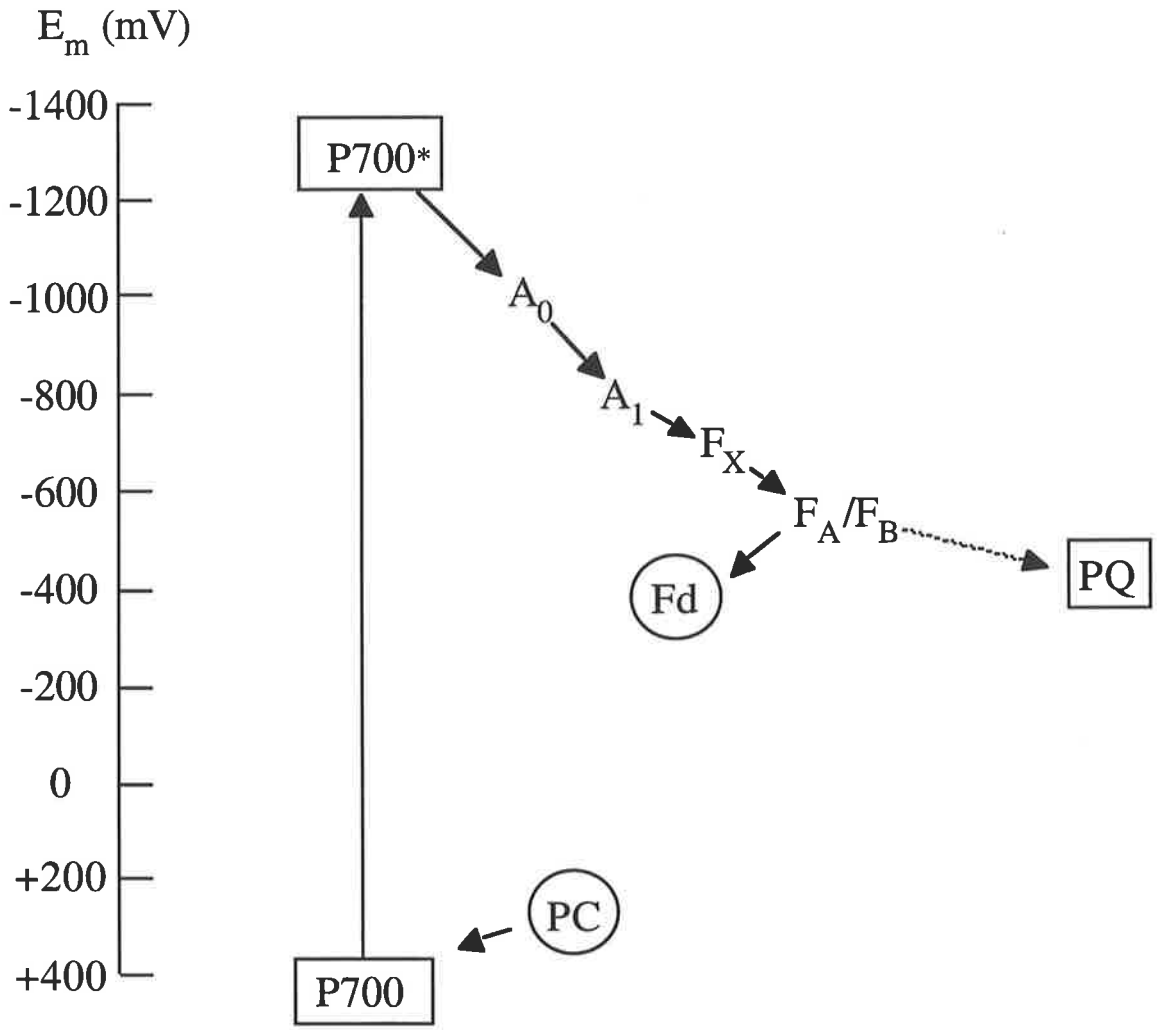


Figure 1.4: The energetics of different photosystem I electron carriers.

The redox potential of paraquat approximates that of the natural electron acceptor ferredoxin (-420 mv). The immediate consequence of electron diversion from ferredoxin is the inhibition of $NADP^+$ reduction, which reduces the yield of NADPH and carbon dioxide fixation (Kunert and Dodge, 1989).

1.8. **Mode of action of paraquat**

The primary toxic action of paraquat is a result of an interaction with the photosynthetic electron transport system within the thylakoid membrane in the chloroplast (Harris and Dodge, 1972; Calderbank, 1968). This herbicide can accept electrons from the reducing side of PS I, probably at one of the iron-sulfur complexes (Fuji *et al.*, 1990). This forms a paraquat cation radical (equation 1), which is unstable and is rapidly reoxidised by oxygen. The reoxidation of the paraquat free radical ($PQ^{+\bullet}$) results in the recycling of the herbicide ion and also the formation of the superoxide radical ($O_2^{-\bullet}$) (equation 2). Thus, only catalytic quantities of PQ^{2+} need to be associated with PS I to be phytotoxic. Although superoxide can act as either a base (proton acceptor) or an oxidising agent (electron acceptor), its reactivity in biological systems appears to involve primarily reactions that catalyse the generation of the highly reactive hydroxyl free radical ($OH^\bullet$) (Kunert and Dodge, 1989). In plants, superoxide is converted to oxygen and hydrogen peroxide (a reaction catalysed by superoxide dismutase, SOD) (equation 3). Hydrogen peroxide (H_2O_2) is toxic and can react with paraquat radicals and produce hydroxyl radicals (equation 4). The hydroxyl radical ($OH^\bullet$) can also be produced by an Fe^{2+} catalysed reaction with H^+ and H_2O_2 as reactants, the Fenton reaction (equation 5). The hydroxy radicals, rather than superoxide, are probably the damaging species. Babbs *et al.* (1989) reported that plants produced considerable quantities of hydroxyl radicals when exposed to paraquat. These oxygen radicals and hydroxyl radicals cause peroxidation of unsaturated fatty acids within the cell membranes, which results

in loss of membrane integrity, bleaching of chlorophyll, desiccation, and finally death of the plant.

$$PQ^{2+} + e^{-} \longrightarrow PQ^{+\bullet} \quad (1)$$

$$PQ^{+\bullet} + O_2 \longrightarrow PQ^{2+} + O_2^{-\bullet} \quad (2)$$

$$2\ O_2^{-\bullet} + 2\ H^{+} \xrightarrow{SOD} H_2O_2 + O_2 \quad (3)$$

$$H_2O_2 + PQ^{+\bullet} \longrightarrow PQ^{2+} + OH^{\bullet} + OH^{-} \quad (4)$$

$$H^{+} + Fe^{2+} + H_2O_2 \longrightarrow OH^{\bullet} + Fe^{3+} + H_2O \quad (5)$$

Despite the major mode of action of paraquat being the interaction with PS I in the light, low-potential electrons from other cellular sources can reduce paraquat and initiate the same sequences of oxidation and peroxidations. Paraquat has been shown to inhibit germination of seeds in the dark (Appleby and Brenchley, 1968; Stecko, 1974; Faulkner and Harvey, 1981). Also paraquat resistance is evident in germinating seedlings as well as in the photosynthetic tissue of plants. The resistance to foliar applied paraquat has also been shown to operate in germinating seeds of *L. perenne* (Faulkner and Harvey, 1981), *H. glaucum* (Powles, 1986), and *E. canadensis* (L.) and *E. sumatrensis* (Yamasue *et al.*, 1992). In fungi and animals, paraquat interacts with the NADPH reductase/ cytochrome P_{450} electron transport system (Edlich and Lyr, 1987; Bus *et al.*, 1974). This has also been suggested to be the site of action of paraquat in the dark in plants (Preston, 1994). Normal flow of electrons is from NADPH to NADPH reductase to cytochrome P_{450}, however, paraquat interferes with, and uncouples, electron flow by accepting electrons from the reductase. Upon

reduction of paraquat in this system, paraquat is reoxidised by O_2 to produce superoxide, and this superoxide can then cause lipid peroxidation (Fig. 1.5).

1.9. **Uptake and translocation of paraquat**

Brian (1967) reported that after paraquat application the herbicide is rapidly adsorbed on to leaves and uptake into the leaf is very fast. Entry into intact leaves is very rapid and takes place within 5 minutes (Summer, 1980). Uptake is largely complete within a few hours after application. Another report suggested that the uptake of paraquat is much faster in darkness than in light and therefore showed the paraquat uptake is cuticular rather than stomatal (Brian, 1967). Paraquat can move within plants; however, there is little movement from the shoot to the roots. In contrast, movement of paraquat from roots to shoots has been reported in some plants (Summer, 1980). Thrower *et al.* (1965) reported that movement of paraquat is primarily in the xylem and therefore, translocation of herbicide will normally be in acropetal direction from the point of application. However, reverse xylem flow may occur when water relations in the xylem break down following paraquat damage. That is, the rapid bleaching and wilting caused by paraquat damage results in an area of high water potential at the site of damage. This water will tend to flow down the xylem in response to lower water potentials in the rest of the leaf, carrying paraquat with it. In tomato, Baldwin (1963) demonstrated that long distance translocation of diquat and paraquat from the point of application does not occur until treated plants were placed in the light following a period of darkness. In the dark the herbicide had not moved out of the treated leaf to any extent. After being brought into the light; however, the herbicide was distributed almost completely through every part of the plant within 5 hours. This may be due to the fact that damage occurring in the light caused reverse xylem movement of herbicide to the rest of the plant.

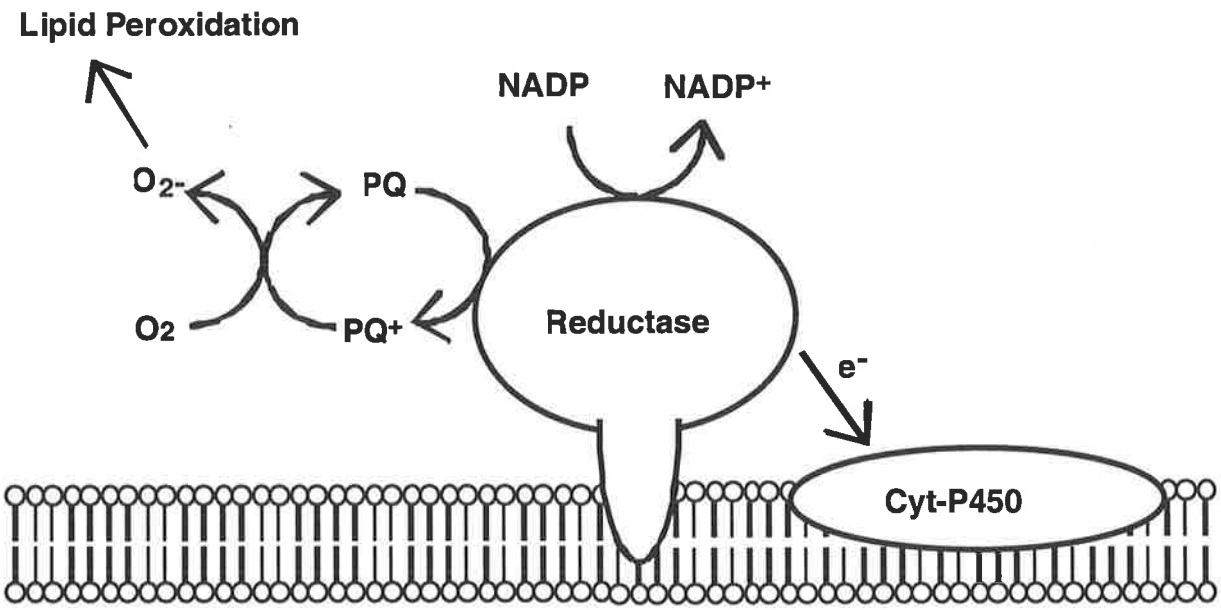


Figure 1.5: Electron flow from microsomal NADPH reductase to paraquat which is proposed to be the site of action of paraquat in dark.

Uptake and translocation of paraquat and diquat can also be influenced by environmental conditions such as high humidity, which increases uptake and translocation (Brian and Ward, 1967). Low humidity, particularly accompanied by high temperature, promotes rapid drying of treated leaves and prevents movement of the herbicide to untreated parts of plants. Since paraquat and diquat are highly water-soluble and are moved in the xylem, water stress in the plant plays an important part in the translocation of these herbicides (Summers, 1980).

1.10. **Movement of herbicides into plants**

Herbicide action requires the movement of the herbicide molecule from point of entry into the plant to a site of action. Since most herbicide target sites are in the cytoplasm, nucleus, or chloroplasts, almost all herbicides must enter into plant cells in order to be phytotoxic. Therefore, the herbicide must cross the cell wall and cell membranes to reach the site of action where accumulation of herbicide causes phytotoxicity.

1.10.1 *Plant cell membranes*

In higher plants, membranes define the outer boundary of the living protoplast and its internal compartmentation into organelles, which include the central vacuole, endoplasmic reticulum, mitochondria, plastids, and the nucleus. Membranes have the important role of regulating the internal chemical environment of the cytoplasm and organelles for metabolic events to take place (Baker and Hall, 1988). For example, membranes act as permeability barriers regulating solute movement and are involved in energy production. Membranes also play an important role in regulating herbicide behaviour in plants. For many

herbicides, the plasma membrane serves as a barrier restricting movement into plant cells. Phytotoxicity of certain herbicide classes such as bipyridyliums is due to the destruction of membrane integrity and cellular compartmentation (Dodge *et al.*, 1971; Calderbank, 1968). For these herbicides, a consequence of the action of the herbicide at the primary target site in the thylakoid membrane of the chloroplast is the destruction of membranes.

Solutes cross plant membranes from one compartment to another by one of the following pathways (Fig. 1.6):

1- Direct movement through the lipid bilayer of the membranes by diffusion. Certain compounds are able to partition into the lipid bilayer and diffuse in response to a concentration gradient from areas of higher concentration to lower concentration. This concentration gradient represents the driving force for movement across membranes (Stein, 1986). The presence of an electrical and/or pH difference across plant membranes can also serve as the driving force for solute movement across the lipid bilayer of membrane.

2- Solute movement by transport systems. Transmembrane movement of ions and most metabolites that contain polar groups involves the activity of a transport system. There are selective transport systems for specific solutes (Baker and Hall, 1988). The membrane associated transport system of plant cells can be classified either as a pump, a channel or a carrier (Stein, 1990) (Fig. 1.7). Pumps are transport systems that couple an energy-releasing chemical reaction such as ATP hydrolysis to the transmembrane movement of a solute. Transport pumps are and often called primary transport systems since linkage of transport to a metabolic reaction is direct (Briskin, 1994). In a channel transport system, transmembrane movement occurs through a proteinaceous pore whose opening and closing are closely regulated in response to chemical and/or environmental

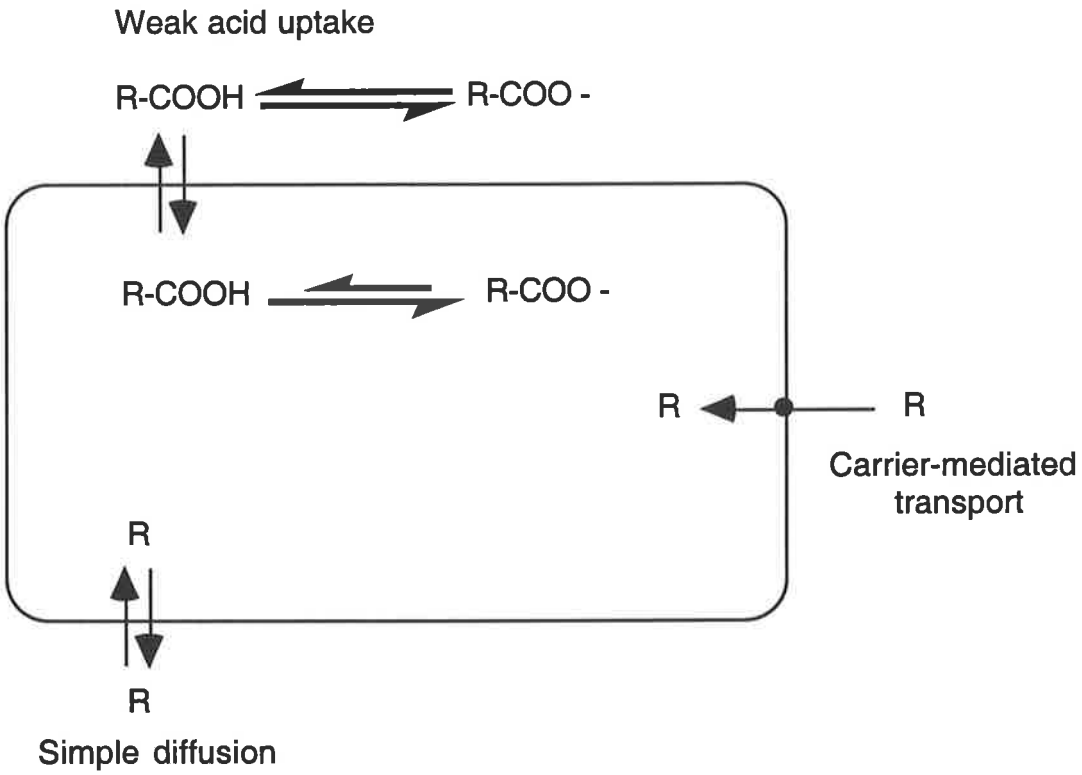


Figure 1.6: Possible mechanisms of herbicide movement into plant cells.

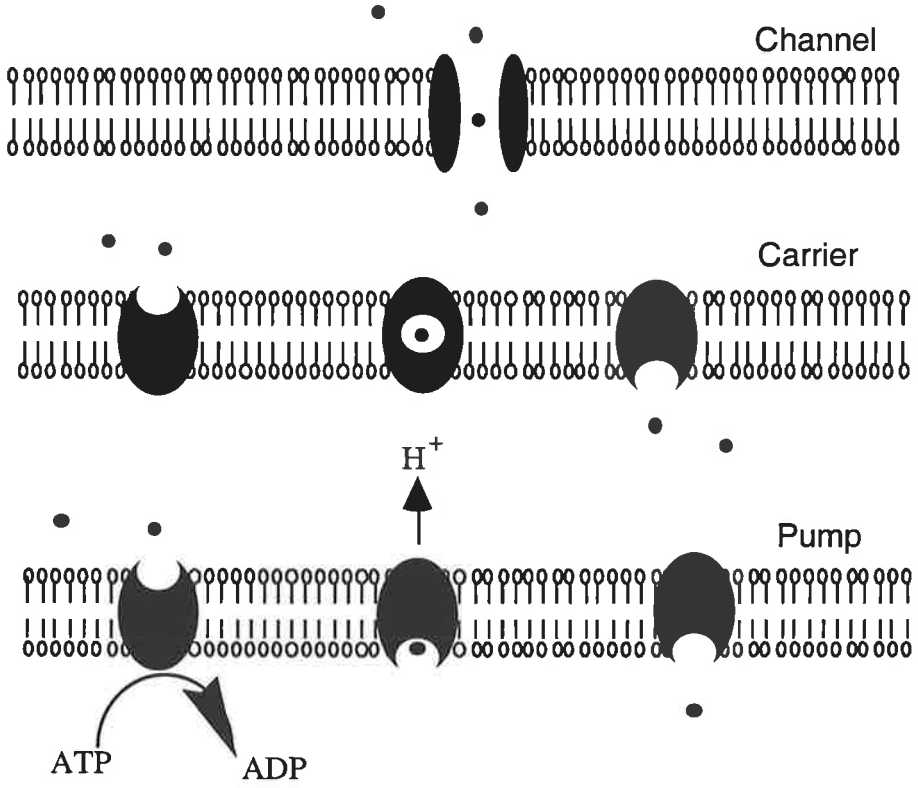


Figure 1.7: Methods of absorption of solutes across cell membranes

signals and are typically involved only in ion transport (Stein, 1990). Carrier transport systems mediate the movement of one or more solutes across membranes without direct coupling to a chemical reaction. Therefore, carriers generally do not have an associated ATPase activity like pumps. Carriers can mediate movement of a single solute alone or movement of one solute may be coupled to movement of another solute either in the same direction or the opposite direction (Briskin, 1994).

3- Ion trapping. Lipophilic, ionic molecules reach cellular concentrations greater than the external concentration due to ion trapping. This process involves the less lipophilic anion accumulating in alkaline compartments of the plant cell (Sterling, 1994). Accumulation of weak acid herbicides, such as bentazon, 2,4-D, chlorsulfuron, imidazolinones, and sethoxydim, in plant cells can be reduced by metabolic inhibitors or anoxia and enhanced by acidic pH external to the cell, suggesting accumulation is energy dependent and is mediated by ion trapping (Sterling, 1994). These herbicides exist in either the dissociated (anionic) form or the undissociated (neutral) form. They enter cells predominantly in the undissociated form and, once inside, ionize according to the pH of the compartment (Bromilow *et al.*, 1990). Uptake of weak acids across the plasmalemma is strongly related to solution pH, with greater uptake at lower pH. At low pH, more herbicide molecules will be in the undissociated form. The membrane permeability of an undissociated molecule is usually greater than that of its dissociated ion, therefore, movement of herbicides across plasmalemma is enhanced by low pH (Briggs *et al.*, 1987).

Ion trapping is unlikely to be the driving force behind movement of paraquat across cell membrane since paraquat is a positively charged herbicide. Charged herbicide molecules would be taken up mainly by a carrier-mediated system (Hart *et al.*, 1992a).

1.10.2 *Movement through the cell wall*

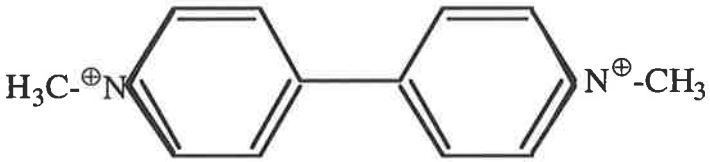
The cell wall is a highly ordered arrangement of cellulose microfibrils that gives cells mechanical strength that results in their three-dimensional form. The cell wall can be regarded as a very polar and relatively porous medium surrounding the cell. In general, the cell wall offers little resistance to the passage of herbicides into cells. However, there are several reports of herbicides being bound to constituents of cell walls. The herbicide 2,6-dichlorobenzonitrile binds to a protein in cotton cellulose fibres which results in reduced entry of the herbicide into the cell proper (Delmer et al., 1987). Strange and Rogers (1971) reported adsorption of trifluralin to components of cell wall. However, the component to which the herbicide was bound and the significance of this binding were not identified. There are also reports suggesting that paraquat is bound by a cell wall constituent in a paraquat resistant biotype of *Conyza canadensis* (Fuerst *et al.*, 1985) and a fern *Ceratopteris richardii* (Carroll *et al.*, 1988). This binding may prevent phytotoxic quantities of paraquat from reaching the site of action in the chloroplast.

1.10.3 *Paraquat movement across plant membranes*

The absorption across plant membranes and accumulation of herbicide in the plant cell is controlled by the physicochemical characteristics of the herbicide molecule including lipophilicity and acidity, and the electrochemical potential in the plant cell. Plant cells absorb herbicides mostly via non-facilitated diffusion (Hess, 1985). Lipophilic neutral herbicide molecules rapidly diffuse across the lipid bilayer of plant membranes down their concentration gradient to reach concentrations in the cell equal to concentrations in the external medium (Nobel, 1991). This pathway appears to be involved in the transport of amitrole, monuron,

norflurazon, oryzalin and triazine herbicides (Sterling, 1994). Charged herbicide molecules would have a low permeability across the lipid bilayer and would be taken up by a carrier-mediated system (Hart *et al.*, 1992a). This was observed in the case of 2,4-D, which is absorbed into the cell by carrier-mediated transport as well as by simple, nonionic diffusion (Kasai and Bayer, 1991).

Paraquat, which exist as a divalent cation, is actively transported across plant membranes (Hart *et al.,* 1992a). Active transport requires energy from metabolic processes to move a molecule across a membrane and usually involves a carrier on the membrane which transports the molecule into the plant cell (Nobel, 1991). In a study with animal cells, it was shown that accumulation of paraquat in lung tissue is energy dependent and has saturable kinetics (Rose *et al.*, 1974). Paraquat has also been shown to inhibit the uptake of the polyamines, such as putrescine, into animal cells (Smith and Wyatt, 1981). A carrier system is probably required to transport the polar paraquat molecules across the hydrophobic lipid bilayer even though paraquat is moving down its electrical potential to the interior of the plant cell (Sterling, 1994). From the saturable kinetics of paraquat uptake across the plasmalemma it was suggested that paraquat enters the symplast of plant cells via a protein-mediated transport system. Paraquat can also move in either direction across both plasmalemma and tonoplast (Hart *et al.*, 1992a). Since there is a structural similarity with putrescine, a divalent polyamine with charge distribution similar to paraquat (Fig. 1.8), and because of competitive inhibition of paraquat by putrescine, paraquat uptake across the plasmalemma is proposed to occur on a transport system which is normally responsible for transporting putrescine (Hart *et al.*, 1992a). Uptake kinetics of putrescine are similar to the kinetics of paraquat absorption. Kinetics for both have been resolved into a linear component, which is proposed to be adsorption due to binding to the cell wall, and a saturable component proposed to be absorption across the plasma membranes (Hart *et al.*, 1992b).



(PARAQUAT)

$H_3N^{\oplus}$-CH_2-CH_2-CH_2-CH_2-$^{\oplus}NH_3$ (PUTRESCINE)

$H_3N^{\oplus}$-CH_2-CH_2-CH_2-CH_2-CH_2-$^{\oplus}NH_3$ (CADAVERINE)

$H_3N^{\oplus}$ -CH_2-CH_2-CH_2-$^{\oplus}NH_2$-CH_2-CH_2-CH_2-CH_2-$^{\oplus}NH_2$-CH_2-CH_2-CH_2-$^{\oplus}NH_3$

(SPERMINE)

Figure 1.8: Molecular structure of paraquat and three polyamines, putrescine, cadaverine, and spermine.

1.11. Effect of paraquat analogues on resistance to paraquat

From studies with bacteria and algae there were reasons to expect that pre-treatment with paraquat analogues might confer tolerance against subsequent paraquat treatments. This was a result of an induction in the level of SOD after treatment of *Escherichia coli* with paraquat (Hassan and Fridovic, 1977) or was due to acidification of medium in the case of uric acid (Licochev and Fridovich, 1993). A slight increase in paraquat tolerance in the green alga *Chlorella sorkiniana* was also correlated to increased SOD activity after exposure to low levels of paraquat (Rabinowitch *et al.*, 1982). Lewinsohn and Gressel (1984) reported that treatment with benzyl viologen (10 μM), an analogue of paraquat, protected slightly against paraquat. This was demonstrated when the green alga *Spriodela oligorrhiza* was treated simultaneously with paraquat and its analogues, benzyl viologen, triquat, tetraquat, neutral red, and some other related compounds. They found no protection by other paraquat analogues except benzyl viologen when they measured CO_2 fixation. However, in a study with *H. glaucum*, there was no protective effect of benzyl viologen when the plant seedlings were exposed simultaneously to paraquat (Alizadeh, 1993). The effect of paraquat on chlorophyll bleaching of *Scenedemus obliquus* was inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). This protective effect was due to DCMU inhibiting photosynthesis and hence production of the paraquat cation was reduced (Bowyer *et al.*, 1989). The protective effect of desferrioxamine, a highly specific iron chelator, on paraquat action has been demonstrated in mice (Kohen, 1985), *Pisum sativum* L. (Zer *et al.*, 1994), and *E. coli* (Korbashi *et al.*, 1986). This protective effect is due to chelation of Fe^{2+}, which would reduce the Fenton reaction, but may also influence other mechanisms such as direct scavenging of free radicals, or blockage of paraquat uptake pathways. Such protective effects from paraquat injury have also been reported for several other substances such as magnesium deficiency (Cakmak and Marchner, 1992),

paclobutrazol (Kraus and Fletcher, 1994), vasoactive intestinal peptide (VIP) and helodermine (Pakbaz *et al.*, 1993). The protective effects of these substances are thought to be due to increases in activity of active O_2 detoxification afforded by enzymes and antioxidants.

1.12. **Mechanisms of herbicide resistance**

Several mechanisms can give herbicide resistance. Herbicide resistance could result from biochemical/ physiological changes, morphological alterations or phenological changes in weeds (Moss and Rubin, 1993). In weeds, biochemical herbicide resistance is the result of the changes at the target site of herbicide action, or non-target site changes such as reduced uptake or translocation, enhanced herbicide detoxification, or sequestration of herbicide away from the site of action. Examples of each of these types of mechanisms will be given below. In each case this will be followed by a discussion of the mechanisms that have been proposed to operate in paraquat-resistant biotypes.

1.12.1 *Target site changes*

Alteration of the target site of action to reduce effective herbicide binding is one of the more effective resistance mechanisms. Resistance is conferred by mutation of target site protein structures. Theoretically, these mutations exists in most or all plant populations, although the frequency of occurrence is unknown (Warwick, 1991). Variability in the functional qualities of these target site mutants exists and they may be equally (sulfonylurea resistance) or less competitive (s-triazine resistance) than their wild types (Beversdorf *et al.*, 1988). Resistance to triazine (Fuerst and Norman, 1991), ALS inhibiting (Hall and Devine, 1990; Saari *et al.*, 1990; Christopher *et al.*, 1992), dinitroaniline (Anthony *et al.*, 1998) and ACCase

inhibiting herbicides (Tardif *et al.,* 1993) are some important examples caused by modification of the herbicides site of action. Target site based resistance to triazines is due to a point mutation in psbA, the chloroplast gene encoding the D1 protein, which leads to a loss of herbicide binding. Mutations have been observed in at least five sites which can lead to amino acid substitutions in D1 which confer resistance (Trebst, 1991). Target site resistance normally results in cross-resistance to herbicides of the same chemical class and may result in cross-resistance to herbicides of different chemical classes that inhibit the same target enzyme. For example, weed biotypes resistant to ALS-inhibitor herbicides can be cross-resistant to other ALS-inhibiting herbicides from different chemical classes (Christopher *et al.*, 1992; Hall and Devine, 1990).

Comprehensive investigations to determine the potential role of an altered site of action have been performed in paraquat resistant and susceptible biotypes of *H. glaucum* (Powles and Cornic, 1987), *H. leporinum* (Purba *et al.*, 1995), *C. bonariensis* (Fuerst *et al.*, 1985), *A. calendula,* (Preston *et al.,* 1994), and *L. perenne* (Harvey *et al.*, 1978). To date, a change in the target site has not been demonstrated as a mechanism of resistance to paraquat. There were no differences in the interaction of paraquat with the active site in the paraquat resistant and susceptible biotypes where the rate of O_2 consumption in isolated thylakoid membranes, prepared from both biotypes, was measured.

Due to the redox nature of paraquat interaction with PSI, resistance to paraquat resulting from a change in the active site is unlikely to occur. Mutations deficient in PSI will be lethal and, therefore, unlikely to survive in the field (Preston, 1994).

1.12.2 *Non target site changes*

1.12.2.1 Reduced uptake or translocation

Herbicides must pass the nonliving portion (apoplast) to the living tissue (symplast) of plants to have an effect. Herbicide resistance could be conferred in weeds by reduced uptake or restricted movement of herbicide to the site of action. Reduced translocation of paraquat has been reported for several paraquat-resistant biotypes. Quantitative measurement of paraquat translocation through excised leaves of resistant and susceptible biotypes of *H. glaucum* showed a significant difference between the two biotypes (Bishop *et al.,* 1987). In this experiment, ^{14}C paraquat was restricted to vascular tissue of resistant leaves following 60-min exposure, while susceptible leaves showed uniform distribution of paraquat. These authors suggested that paraquat movement was restricted to the apoplast in the resistant biotype whereas paraquat could easily enter the symplast in the susceptible biotype. Preston *et al.* (1992b), also measured the symplastic movement of paraquat in resistance and susceptible biotypes of *H. glaucum.* After spraying the plants, paraquat content and O_2 evolution of unsprayed young leaves of both biotypes was measured. A greater than 50% reduction in translocation of paraquat from sprayed leaves to young unsprayed leaves was detected in the resistant biotype compared with the susceptible biotype. This was coupled with a small reduction in O_2 evolution in the resistant biotype compared to a 62% reduction in O_2 evolution in the susceptible biotype. From the studies with *H. glaucum* it seems that the reduced translocation observed is primarily a result of reduced symplastic movement of paraquat in the resistant biotype (Bishop *et al.,* 1987; Preston *et al.,* 1992b). The biochemical mechanism responsible for this reduced movement of paraquat in resistant biotypes has not yet been determined.

Paraquat translocation has been also examined in resistant and susceptible biotypes of *E. philadelphicus*, *E. canadensis* (Tanaka *et al.,* 1986), *A. calendula* (Preston *et al.,* 1994), and *H. leporinum* (Preston *et al.*, 1992b). Tanaka *et al.* (1986) reported that the movement of paraquat in the leaves of the resistant biotypes of *E. philadelphicus* and *E. canadensis* was restricted compared with susceptible biotypes. ^{14}C-paraquat taken up through the cut ends of petioles was rapidly distributed through the leaves of the susceptible biotypes, but was hardly translocated in the leaves of the resistant biotypes. They concluded that paraquat must be excluded from the active site.

1.12.2.2 Detoxification of oxygen radicals

Enzymatic detoxification of oxygen radicals, which converts active oxygen species such as superoxide and hydrogen peroxide to non toxic metabolites, has been reported to be a possible mechanism of resistance in some paraquat resistant biotypes. In *C. bonariensis*, enhanced activities of superoxide dismutase (SOD), ascorbate peroxidase, and glutathione reductase in the stromal portion of chloroplasts isolated from the resistant biotype have been reported to be the mechanism for resistance (Shaaltiel and Gressel, 1986). Protective enzymes have also been reported to protect leaves of the resistant biotype from other factors that cause oxidative stress (Shaaltiel *et al.,* 1988), and to preserve integrity of chloroplasts isolated from the resistant biotype treated with paraquat (Shaaltiel and Gressel, 1987). However, there are contradicting reports about the role of protective enzymes in conferring resistance in this biotype (Norman, *et al.,* 1993; Vaughan *et al.,* 1989). Like *C. bonariensis*, paraquat resistance in *L. perenne* was also correlated with increased constitutive levels of SOD, catalase, and peroxidase (Harper and Harvey, 1978). These authors demonstrated a 42% increase in SOD levels in chloroplasts from paraquat resistant compared with susceptible plants. No enhanced activity of

detoxification enzymes has been reported in paraquat resistant *C. canadensis* (Polos *et al.,* 1988), *Ceratopteris richardii* (Carroll *et al.,* 1988), *E. canadensis* (Itoh and Matsunaka, 1990), and *H. glaucum* (Powles and Cornic, 1987). Jansen *et al.* (1990) reported a correlation between paraquat resistance and increased tolerance to photoinhibition and postulated that rapid detoxification or removal of oxygen radicals from chloroplasts mediated both resistances. Preston *et al.* (1991) could find no increase in tolerance to photoinhibition for paraquat resistant biotypes of *H. glaucum*, *H. leporinum*, *A. calendula,* and *C. bonariensis* when compared with susceptible biotypes. They concluded that there is no correlation between resistance to paraquat and increased tolerance to photoinhibition for these biotypes.

In *H. glaucum* the mechanism of resistance does not appear to be associated with enhanced activity of protective enzymes. Powles and Cornic (1987) examined the activity of protective enzymes extracted from leaves of resistant and susceptible biotypes. There was no difference in activities of oxygen radical detoxification enzymes, SOD, peroxidase, and catalase in the leaf tissue of resistant and susceptible biotypes. In another experiment they measured the activities of these enzymes, before spraying with paraquat, in F_3 plants of crosses between R and S and concluded that there was no correlation between activity of SOD, peroxidase or catalase and survival of the plant after paraquat application. Therefore, it is unlikely that resistance to paraquat in *H. glaucum* is due to increased ability to detoxify active oxygen species.

1.12.2.3 Enhanced metabolism

Herbicide detoxification is the major mechanism of crop selectivity to herbicides. It is also responsible for herbicide resistance in several weed biotypes that have evolved the capacity to rapidly degrade and/or conjugate the herbicide into less

or non-toxic compounds. For example, *Abutilon theophrasti* Medikus is resistant to the triazine herbicide atrazine due to an enhanced ability to detoxify the herbicide via glutathione conjugation, as occurs in maize (*Zea mays* L.) (Gronwald *et al.*, 1989). Maize and sorghum (*Sorghum bicolor* (L.) Moench) plants are resistant to atrazine primarily because they contain isozymes of glutathione-*S*-transferase (GSH) that rapidly detoxify the herbicide by conjugation. Enhanced metabolism was reported to be one of the mechanisms of resistance to ALS inhibiting herbicides in *L. rigidum* (Christopher *et al.*, 1991, 1992). The detoxification of herbicide in this biotype is probably mediated by cytochrome P450-dependent microsomal oxidases (Christopher *et al.*, 1991; 1992; 1994).

Paraquat can not be metabolised by higher plants (Slade, 1966a; Summers, 1980) and there have been no reports of paraquat resistance due to enhanced metabolism. For example, Harvey *et al.* (1978) could not detect evolution of $^{14}CO_2$ or ^{14}C-labelled breakdown products of paraquat in resistant *L. perenne* that had been treated with labelled herbicide. Thus, paraquat resistance is unlikely to be the result of metabolism of paraquat to an inactive form.

1.12.2.4 Sequestration

It is possible for herbicides to be sequestered in several plant locations before they reach their site of action. Herbicide resistance may be achieved by storage of the herbicide or its toxic metabolites in the cell vacuole, or their sequestration in cells or tissues remote from the site of action (Coupland, 1991). Sequestration of the herbicide has been suggested as one mechanism of resistance to diclofop-methyl in *Lolium rigidum* (Holtum *et al.*, 1991).

Sequestration of paraquat from its site of action in the chloroplast, as a mechanism for resistance, has been suggested for some resistant biotypes. This is supported by data showing decreased translocation of paraquat, in the resistant biotypes, and lack of paraquat injury in plant systems which do not have increased levels of protective enzymes (Preston *et al.*, 1992b). In *C. canadensis*, the excised leaves of resistant and susceptible biotypes were treated with a range of paraquat doses and placed in darkness for 4 hours prior to measurement of variable chlorophyll fluorescence. The resistant biotype required much higher (150-fold) concentrations of paraquat than the susceptible biotype to exhibit 50% quenching of chlorophyll fluorescence (Fuerst *et al.,* 1985). This implies a sequestration mechanism that prevents paraquat from diffusing into chloroplasts of the resistant biotype. Additional support for sequestration is based on chlorophyll loss by toxic oxygen generators unrelated to paraquat in *C. bonariensis* (Vaughan *et al.*, 1989). The loss of chlorophyll was not significantly greater in the susceptible biotype than resistant biotype after treatment with compounds that accept electrons from PSI or produce toxic oxygen species such as HEP II (5H,7H-2,3-dioxa-2a,6-dithia(2e-s^{IV})-1,4diazacyclopent-[cd]indene), BTD (2,1,3-benzothiadiazole-4,7-dicarbonitrile) and morfamquat. These authors concluded that differences between resistant and susceptible biotypes of *C. bonariensis* could only be due to differences in ability of paraquat to reach the active site.

From studies with protoplasts it appears that sequestration of paraquat in resistant biotypes of *H. glaucum* and *C. canadensis* requires either entirely, or partially, a structurally intact cell wall to operate. For example, isolated mesophyll protoplasts isolated from resistant plants were not more resistant to paraquat compared to protoplasts isolated from the susceptible biotype (Powles and Cornic, 1987; Norman *et al.,* 1993). In studies with *H. glaucum* (Powles and Cornic, 1987), the plasmalemma of isolated protoplasts exhibits very low

permeability to paraquat relative to whole plant systems. Paraquat concentrations up to 4 mM inhibited CO_2 fixation in protoplasts isolated from resistant and susceptible biotypes by only about 40%. However, photosynthesis was completely inhibited when the paraquat concentration inside the leaf was about 0.8 mM. (Bishop *et al.*, 1987). These authors concluded that isolated protoplasts from *H. glaucum* were much less sensitive to paraquat than whole leaves.

1.13. **Objectives of this project**

Resistance to paraquat has been reported in 16 weed species world-wide. There are four species with paraquat resistant populations in Australia. All of the reported paraquat resistant biotypes come from perennial cropping systems where extensive selection pressure was applied. A new development is the possibility of paraquat resistance appearing in annual cropping systems. An investigation on possible resistance to paraquat in two new biotypes from zero-tillage cropping systems was commenced to confirm their resistance status.

In addition to the above, the mechanism of resistance to paraquat has been investigated in biotypes of two species of barley grasses (*H. glaucum* and *H. leporinum*) to establish:

1-The movement of paraquat across the plasmalemma in protoplasts isolated from resistant and susceptible plants;

2- Possible sequestration of paraquat in the cell wall by measuring the binding and cation exchange capacity of cell walls of resistant and susceptible biotypes; and

3-The polyamine content of 9 resistant and 4 susceptible biotypes of *H. glaucum* and *H. leporinum* to determine a possible correlation between resistance to paraquat and changes in polyamine, especially putrescine, content of leaves.

Chapter 2

Paraquat-resistant biotypes of *Hordeum glaucum* from zero-till continuous cereals

2.1. Introduction

Minimum-tillage cropping systems (including zero tillage) are being promoted and adopted world-wide (Kapusta and Krausz, 1993; Moyer *et al.*, 1994). For Australian agriculture, major benefits of minimum-tillage cropping systems include reduced soil erosion (Hamblin, 1987), improved timeliness of seeding, and lower preparation costs (Pratley, 1995). Throughout much of southern Australia, which has a relatively short growing season and fragile soils, minimum tillage has become widely adopted. A major effect of the widespread adoption of minimum tillage has been a concomitant increase in herbicide use as the absence of soil cultivation means that weeds must be controlled by herbicides. In Australia, as elsewhere in the world, minimum tillage is almost universally facilitated by use of the non-selective herbicides glyphosate or paraquat and diquat to control weeds before crop seeding.

Paraquat and diquat are in widespread commercial usage in world agriculture as non-selective, rapid acting, non-residual herbicides ideally suited for weed control before crop seeding in minimum-till agriculture. Despite successful use world-wide for more than 40 years, there have been only isolated cases of target weed species demonstrating resistance to paraquat and diquat. Preston (1994) reported that biotypes of 16 weedy plant species have acquired paraquat and diquat resistance but only in situations where herbicide selection pressure was intense. This number has now increased to 27 (Chapter 1). Resistance to paraquat has been reported following 5 to 10 applications each year for 5 or more years (Watanabe *et al.*, 1982; Fuerst and Vaughn, 1990) or once annual applications for many years (Powles, 1986). In Australia, all previously reported cases of resistance to bipyridyl herbicides have occurred in lucerne fields where paraquat and diquat were used once annually as the only method of weed control for more than 12 years (Preston, 1994).

Temperature has been shown to influence the degree of resistance to paraquat. Purba *et al.* (1995) established a seasonal variation in the level of resistance in some paraquat resistant biotypes of *H. glaucum* and *H. leporinum*. The resistant biotypes showed a high level of resistance in winter but a much reduced level of resistance in summer. They concluded that the mechanism of resistance to paraquat in these biotypes (reduced translocation of paraquat to the site of action) is temperature sensitive and breaks down at higher temperature. A similar observation was also reported by Lasat *et al.* (1996) for the *H. glaucum* biotype. They reported that a resistant biotype of *H. glaucum* was nearly 7-fold more resistant when grown at 15°C than when grown at 30°C.

Until now there have been no cases worldwide of weed species developing paraquat resistance from use in minimum-tillage agriculture. However, a failure of paraquat to control *H. glaucum* was reported in 1994 in two zero-till continuous cereal fields in which paraquat had been used once annually for 10 to 15 years for knockdown weed control prior to crop seeding.

This chapter describes experiments that demonstrate these populations are highly paraquat resistant and demonstrate a change in the degree of resistance to paraquat when they were treated at high temperatures.

2.2. **Material and Methods**

2.2.1 Plant material

Seeds of the suspected resistant biotypes of *H. glaucum* (SHG3 and SHG4) were collected from two different South Australian fields devoted to continuous

cereal cropping for the past 10 to 15 years (Table 2.1). These two fields are close to each other and the farming practices were similar. Paraquat and diquat were applied once annually in these fields prior to seeding of direct-drilled cereal crops. Crops were seeded using a direct drill seeder with narrow point seeding tines of 1 cm width at 15 cm spacing. Therefore, there was no cultivation of the fields and very little soil disturbance associated with seeding. About 50 kg ha^{-1} of super phosphate fertiliser was drilled with 50 kg ha^{-1} of seeds at a seeding depth of about 5 cm.

The suspected resistant biotypes were compared with a known paraquat and diquat susceptible biotype and with a known paraquat and diquat resistant biotype of *H. glaucum*. The known resistant biotype was obtained from a mixed cereal cropping and sheep grazing enterprise at Willaura near Ararat, Victoria, with a long history of paraquat use and displays 250-fold resistance to paraquat (Powles, 1986). The susceptible biotype was obtained from a nearby field with no history of paraquat use. Seeds of all biotypes were germinated on agar (0.6% w/v) for 4 days in a seed incubator (light period 21^{o}C, 14h, 20-30 μmol photons m^{-2} s^{-1}; dark period 15^{o}C, 10h) and seedlings transplanted at the 1-leaf stage into 18-cm pots containing potting soil (5 plants per pot). The potted seedlings were then maintained outdoors during winter, the normal growing season for this species in southern Australia.

Table 2.1: Crop and paraquat/diquat herbicide history for continuous cereal zero-till fields in which *H. glaucum* populations SHG3 and SHG4 occurred.

Year	Biotype SHG3			Biotype SHG4		
	Crop	Herbicide (g a.i. ha^{-1})		Crop	Herbicide (g a.i. ha^{-1})	
		Paraquat[a]	Diquat[a]		Paraquat	Diquat
1980				Wheat	375	225
1981				Wheat	375	225
1982				Wheat	375	225
1983				Wheat	250	150
1984	Wheat	120-135	0-45	Wheat	500	300
1985	Wheat	120-135	0-45	Wheat	563	338
1986	Barley	120-135	0-45	Wheat	500	300
1987	Pasture	0	0	Wheat	1000	600
1988	Wheat	120-135	0-45	Wheat	438	263
1989	Wheat	120-135	0-45	Wheat	625	375
1990	Wheat	120-135	0-45	Wheat	500	300
1991	Pasture	0	0	Barley & Vetch	438	263
1992	Wheat	120-135	0-45	Wheat	613	188
1993	Triticale	120-135	0-45	Wheat	0	0
1994	Wheat	120-135	0-45	Wheat	875	525

[a] Exact amounts of herbicide used were not recorded, but fell within the limits given.

2.2.2 *Herbicide application*

Seedlings, when at the 2-3 tiller stage, were treated with a commercial paraquat formulation (Gramoxone, 200 g a.i. L^{-1}, Crop Care Australia) plus 0.2% (v/v) non-ionic surfactant. Paraquat was applied in a laboratory spray cabinet with an output of 130 L ha^{-1} at 1 m s^{-1} and a pressure of 250 kPa at rates ranging from 0 to 3200 g a.i. ha^{-1}. The plants were sprayed in the late afternoon, kept indoors overnight and returned outdoors on the following morning to maximise the effects of paraquat (Brian and Headford, 1968). The treated plants were monitored daily and finally assessed for mortality or survival at four weeks after spraying. To determine the response of these biotypes to other herbicides, plants were treated with the normal use rates of diquat (Reglone, 200 g a.i. L^{-1}, Crop Care), oxyfluorfen (Goal, 240 g a.i. L^{-1}, Monsanto), fluazifop-p-butyl (Fusilade, 212 g a.i. L^{-1}, Crop Care) and glyphosate (Roundup CT, 450 g a.i. L^{-1}, Monsanto). There were 4 replicates in each experiment and each experiment was conducted three times.

2.2.3 *Temperature experiment*

Seeds of the two putative paraquat resistant biotypes (SHG3 and SHG4) as well known as a resistant (VHG1) and susceptible (VHG2) biotype of *H. glaucum* were germinated on 0.6% (w/v) agar as described earlier. After 3-4 days, seedlings were transplanted into 10 cm diameter pots (5 plants per pot) filled with potting soil. Plants were grown in a growth room with a 12 h/12 h, 15°C/10°C, light/dark regime with an irradiance of 500 µmol photons m^{-2} s^{-1}. Since the temperature sensitive mechanism of resistance has been shown to operate after treatment with paraquat (Purba *et al.*, 1995), the plants were grown at 15°C to the 2-3 tiller stage, sprayed with different rates of a commercial formulation of paraquat, and transferred to either 15°C or 30°C growth rooms at the start of the dark period.

The pots were repositioned every few days to minimise variation in light intensity. The relative humidity of the two growth rooms was about 75%. The plants were visually monitored every day for paraquat effects. After two weeks, surviving plants were scored and harvested. There were 4 replicates in each experiment and each experiment was conducted twice.

2.3. **Results**

2.3.1 *Response of biotypes of* H. glaucum *to paraquat*

The response to paraquat of the two biotypes of *H. glaucum* from fields devoted to continuous cereal cropping and use of paraquat and diquat in zero tillage were compared to the response of a known susceptible and a known paraquat resistant biotype of *H. glaucum* (Fig. 2.1). Fig. 2.2A demonstrates that the susceptible population was controlled by very low rates of paraquat whereas, as expected, the known resistant biotype was highly paraquat resistant. The two putative resistant populations (SHG3 and SHG4) from zero tillage cereal fields were found to be highly paraquat resistant (Fig. 2.2B, C). Based on a comparison of LD_{50} values, these resistant populations demonstrated >250-fold resistance to paraquat. It should be noted that all resistant plants showed herbicide effects at the higher rates of paraquat (3200 g a.i. ha^{-1}) but the majority of plants recovered.

2.3.2 *Effect of other herbicides*

Farmers have a limited choice of pre-sowing contact herbicides in zero- and minimum-tillage cropping systems. To determine the susceptibility of the paraquat resistant biotypes to other herbicides, the resistant and susceptible

Figure 2.1: The response to paraquat of the two biotypes of *H. glaucum* (SHG3 and SHG4) from continuous cereal cropping and a known resistant (R) and susceptible biotype.

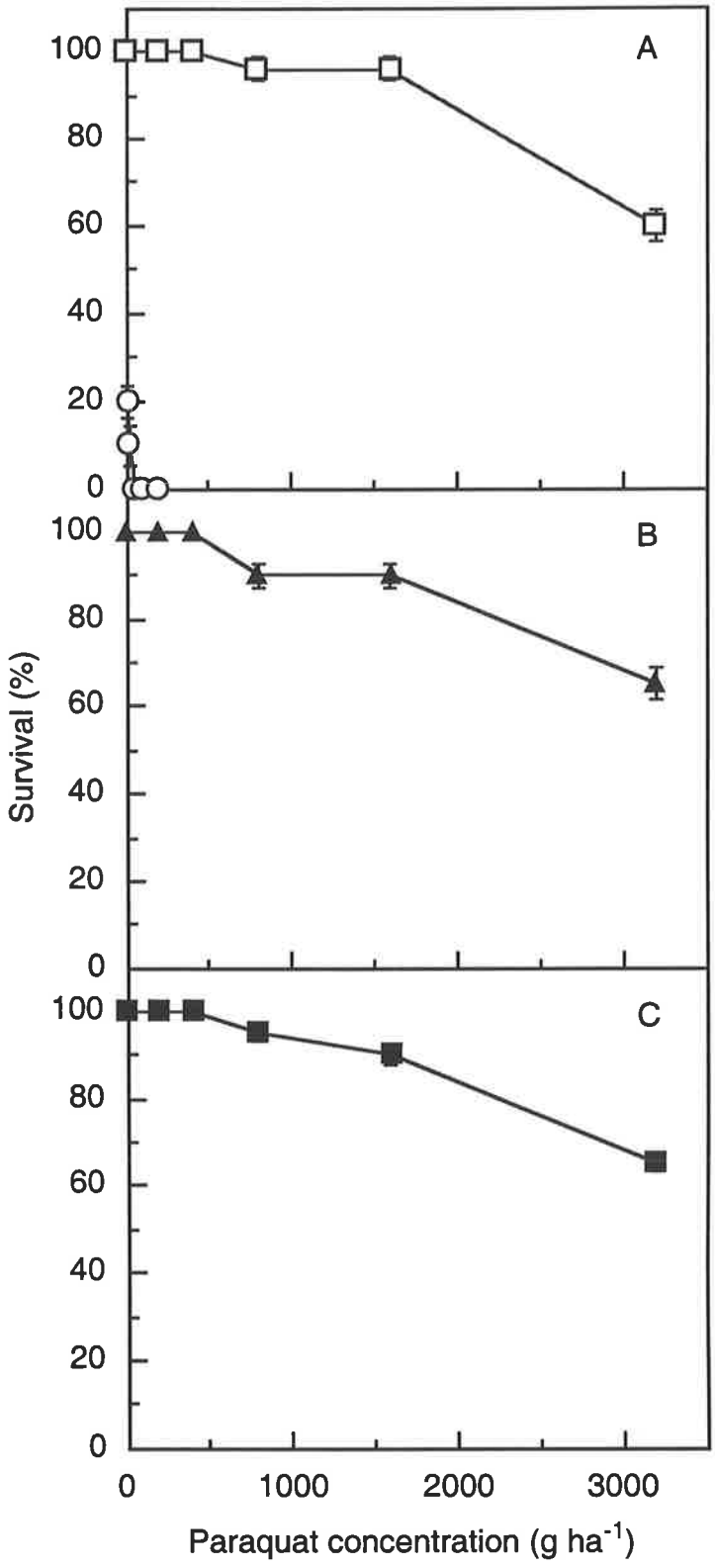


Figure 2.2: Effect of paraquat on survival of (A) known resistant (□) and susceptible (O) biotypes of *H. glaucum*; (B) biotype SHG3 (▲); and (C) biotype SHG4 (■). Data are means ± SE of 3 replicate experiments.

plants were sprayed at the normal use rates of alternative herbicides. Table 2.2 shows that the resistant and susceptible biotypes are equally susceptible to fluazifop-p-butyl or oxyfluorfen. These biotypes are also susceptible to glyphosate (Table 2.2) and the growers are now using glyphosate for control of these biotypes. Therefore, the paraquat resistant biotypes of *H. glaucum* are normally controlled by herbicides with different modes of action. However, the paraquat resistant biotypes were found to be cross-resistant to diquat (Table 2.2). This cross-resistance is expected in that paraquat and diquat are almost identical herbicides and most paraquat resistant weed biotypes also have some degree of resistance to diquat (Preston, 1994).

2.3.3 *Temperature effect*

The mechanism of resistance to paraquat in *H. leporinum* was shown to be temperature dependent and to operate to a higher degree at lower temperatures (Purba *et al.*, 1995). The paraquat resistance and susceptible biotypes alike are highly susceptible to paraquat when treated with more than 100 g a.i. ha^{-1} paraquat and placed at 30°C (Purba *et al.*, 1995). This temperature dependence provides a useful test to determine whether the resistance mechanisms in any other biotypes are similar. The two new paraquat resistance biotypes as well as the previously known paraquat resistance biotype (VHG1) were compared with a paraquat susceptible biotype (VHG2) to establish the effect of temperature on the degree of resistance to paraquat in these biotypes and to see if the mechanism of resistance in these biotypes is temperature dependent.

The susceptible and resistant biotypes were grown at 15°C and treated with different concentrations of paraquat and kept at either 15°C or 30°C. Fig. 2.3

Table 2.2: Survival of a susceptible and three paraquat-resistant *H. glaucum* populations after treatment with diquat, fluazifop-p-butyl, oxyfluorfen or glyphosate.

Herbicide	Susceptible	Known resistant	SHG3	SHG4
		Survival (%)		
Control	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Diquat (400 g a.i. ha^{-1})	0	90 ± 0.7	80 ± 0.8	90 ± 0.5
Fluazifop-p-butyl (106 g a.i. ha^{-1})	0	0	0	0
Oxyfluorfen (720g a.i. ha^{-1})	0	0	0	0
Glyphosate (450 g a.i. ha^{-1})	0	0	0	0

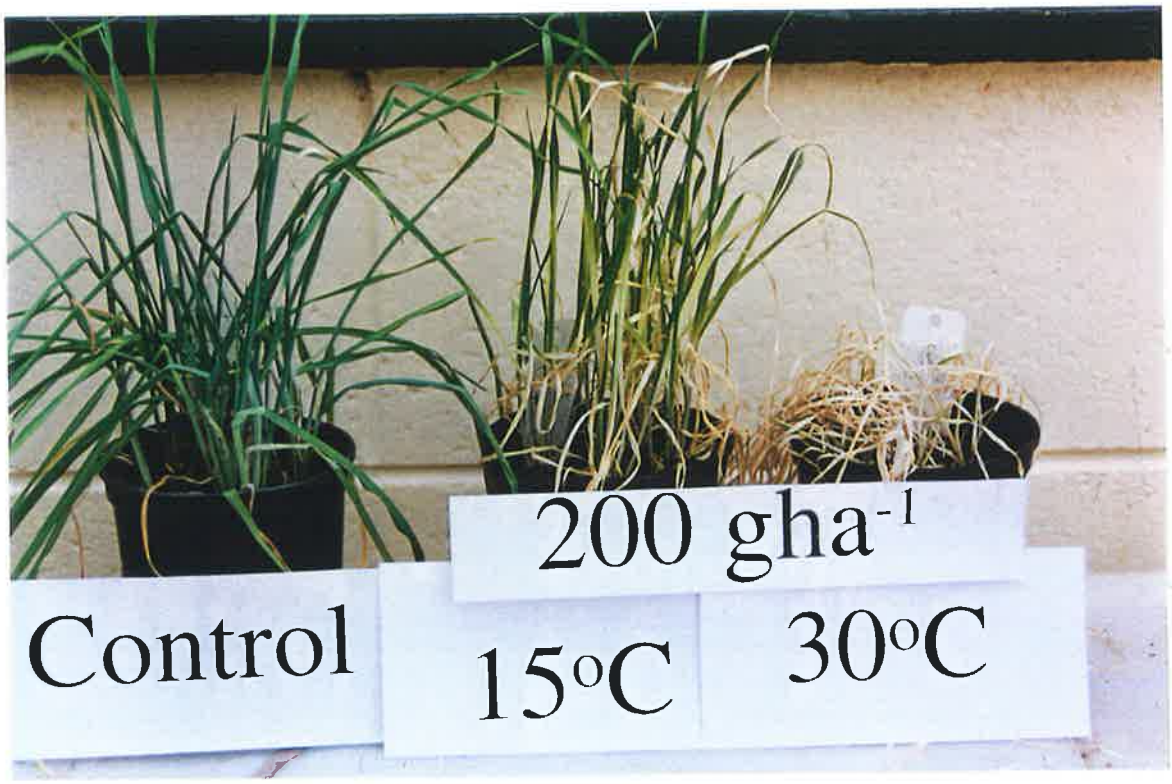


Figure 2.3: Effect of low (15°C) and High (30°C) temperatures on survival of a known resistant biotype of *H. glaucum* (VHG1) treated with 200 g a.i. ha^{-1} paraquat.

shows the effect of 200 g a.i. ha^{-1} paraquat on the growth of a resistant biotype at 15°C or 30°C. The susceptible biotype was very susceptible to paraquat and was killed at both 15°C or 30°C when treated with more than 100 g paraquat. There was, however, a small increase in survival of the susceptible biotype at 30°C compared with 15°C when treated with paraquat concentrations lower than 100 g a.i. ha^{-1} (Fig. 2.4A, B). In contrast, the resistant biotypes showed a high level of resistance to paraquat when the plants were kept at 15°C following treatment with paraquat. However, when the same biotypes were kept at 30°C following treatment with paraquat, the resistant biotypes showed a dramatic decrease in the level of resistance (Fig. 2.4).

2.4 **Discussion**

This study is the first report, world-wide, of paraquat resistance in a weed species from usage of paraquat in zero-tillage annual cropping. Given the dramatic increase in zero- and minimum-tillage farming systems throughout much of the world, and the reliance on contact herbicides to control weeds in these systems, this is an important development. Although weed species have developed paraquat resistance from intensive herbicide selection, all previously-reported cases of paraquat resistance have been observed in perennial cropping operations where weeds have evolved resistance following intense selection pressure (reviewed by Preston, 1994). This is the first case in which resistance has occurred in the most common usage of paraquat, zero- and minimum-tillage annual cropping. That this phenomena has been documented from two separate fields provides further cause for concern in that this event is not extremely rare. It is clear that a persistent selection pressure from paraquat in zero-tillage agriculture can result in paraquat resistant weeds.

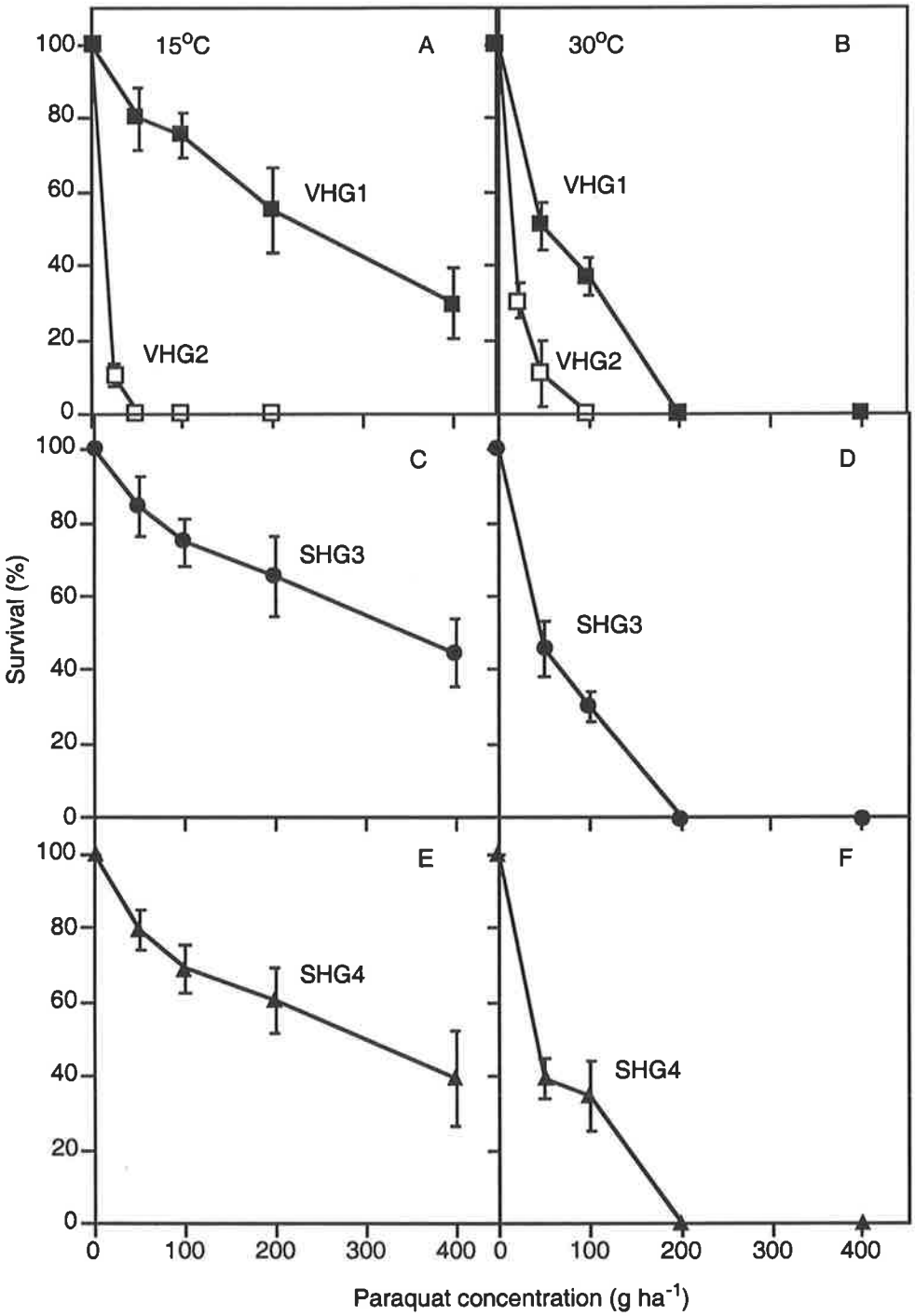


Figure 2.4: Effect of temperature on survival of a susceptible (□), a known paraquat resistant biotype VHG1 (■), and paraquat resistant biotypes from continuous cropping SHG3 (●) and SHG4 (▲) following paraquat treatment. Plants were grown in a growth room at 15°C/10°C and transferred to either 15°C (A,C,E) or 30°C (B,D,F). Data are means ±SE of 2 replicate experiments with 4 replicates in each experiment.

High temperature after paraquat treatment decreased resistance in these biotypes of *H. glaucum* (Fig. 2.4). Purba et al. (1995) and Lasat et al. (1996) reported a similar response to paraquat. They reported that the difference in response to paraquat between resistant and susceptible biotypes of *H. glaucum*, grown at 15°C and 30°C, reduced from >40-fold to 3-fold.

The mechanism of resistance to paraquat in biotypes SHG3 and SHG4, as well as the previously known paraquat resistant biotype of *H. glaucum* and the closely related *H. leporinum*, are likely to be the same as all resistant biotypes of these species showed the same sensitivity to paraquat at higher temperature. The mechanism of resistance to paraquat in *H. glaucum* and *H. leporinum* has been shown to correlate with reduced translocation of paraquat to its site of action in chloroplasts (Bishop *et al.*, 1987; Preston *et al.*, 1992b). Purba *et al.* (1995) suggest that temperature has an effect on the mechanism of this reduced translocation of paraquat to its site of action. They postulated that at high temperature this mechanism breaks down resulting in increased translocation of paraquat and more herbicide reaching the active site in other parts of the plant. Lasat *et al.* (1996) also suggested that the mechanism of resistance to paraquat in *H. glaucum* might be a temperature-dependent alteration in paraquat transport across the plasma membrane. Such alteration may result in herbicide sequestration within the symplasm. As a small amount of herbicide is sufficient to kill the plant, a small increase in paraquat translocation or sequestration may lead to a significant shift in the level of resistance.

In conclusion, the paraquat resistant biotypes, SHG3 and SHG4 from continuous cereal cropping, are the first examples showing paraquat resistance can develop in weed species within the zero-tillage farming sytems that are being adopted around the world. This is a direct consequence of farmers being strongly dependant on contact herbicides for weed management in zero- and minimum

tillage system. Since these paraquat resistant biotypes have the same degree of paraquat resistance as a known resistant biotype and also show the same reduction in paraquat resistance at high temperature, the mechanism of resistance is likely similar to that observed in other paraquat-resistant biotypes of *H. glaucum*.

Chapter 3

Constitutive and induced leaf putrescine content in paraquat resistant *Hordeum glaucum* and *H. leporinum*

3.1. Introduction

Polyamines are found in all organisms including bacteria, mammals and plants (Oshima, 1983; Smith, 1985a). In plants, they are essential for cell viability and are correlated with a variety of physiological events including cell division, embryogenesis, flowering, and pollen tube growth (Evans and Malmberg, 1989). In addition, polyamines have an ill-defined role in protecting against a variety of stress conditions. Polyamine levels, particularly putrescine, have been shown to rise after imposition of many different stress factors. Increases in leaf putrescine content have been observed under conditions of high salinity (Smith, 1983), low pH (Bagni and Pistocchi, 1985; Young and Galston, 1983), ammonium stress (Smith, 1973), water stress (Turner and Stewart, 1988; Flores and Galston, 1982a), chilling (Lee *et al.*, 1995; McDonald and Kushed, 1986), heat shock (Roy and Ghosh, 1996), and ozone treatment (Langebartels *et al.*, 1991). Putrescine and spermidine also provided protection against ozone injury in oxidant resistance in tomato plants (Ormrod and Beckerson, 1986). Herbicides have also been shown to increase polyamine contents of plants. DiTomaso *et al.* (1988) demonstrated that putrescine and cadaverine levels increased in pea (*Pisum sativum* L.) roots following treatment with the herbicide napropamide. Similar results were obtained by Zhelera *et al.* (1993) when they examined the effects of atrazine on polyamine contents of pea leaves.

Szigeti *et al.* (1996) demonstrated that constitutive leaf putrescine content was higher in a paraquat-resistant biotype of *Conyza canadensis* compared to a susceptible biotype. Further, the addition of exogenous putrescine had a protective effect against paraquat in the susceptible biotype but not in paraquat-resistant biotypes of *C. canadensis*. These authors speculated that paraquat resistance may be due, at least in part, to a higher constitutive putrescine content of leaves of resistant compared to susceptible biotypes. Ye *et al.* (1997) recently

reported that external oxidant stresses can increase the level of toxic oxygen detoxification enzymes as well as polyamines, especially putrescine, in oxidant stress resistant wheat and *Conyza bonariensis*. Putrescine was constitutively elevated (2.5-5.7-fold) in resistant wheat and *Conyza* biotypes. The authors concluded that putrescine has an antioxidant defence function in these biotypes. Preston *et al.* (1992a) reported that putrescine did not alleviate paraquat toxicity in paraquat resistant and susceptible biotypes of *H. glaucum* whereas cadaverine (200 μM) protected the susceptible, but not resistant biotype, against paraquat. They suggested that there may be two components of paraquat uptake into cells. One a high affinity component, which can be competitively inhibited by polyamines, is only present in susceptible biotype and a second component is present in both biotypes (Preston *et al.*, 1992a).

Paraquat uptake into animal cells can be inhibited by polyamines such as putrescine as both molecules likely share the same, as yet unidentified, membrane transport pathway (Smith and Wyatt, 1981). Putrescine also competitively inhibits paraquat accumulation by maize roots (Hart *et al.*, 1992a), therefore, these compounds may also play a role in paraquat resistance in *Hordeum* spp.

Putrescine is degraded by a diamine oxidase located in the cell wall (Federico and Angelini, 1986). It is proposed that this enzyme functions as part of the peroxidative lignification process in the cell wall (Smith and Barker, 1988). Diamine oxidase may also have a role in regulating polyamine content of tissues, by increased or decreased rates of oxidation (Torigiani *et al.*, 1989). Therefore, increased polyamine content in the apoplast may result from reduced diamine oxidase activity as well as from increased biosynthetic activity.

The aim of these experiments was to evaluate the effect of paraquat on levels of free polyamines (putrescine, cadaverine, spermidine, spermine) in leaves of paraquat resistant and susceptible biotypes of *H. glaucum* and *H. leporinum*. In addition, activity of diamine oxidase activity was measured to determine what effect, if any, this enzyme was having on polyamine accumulation. It was intended that these experiments might determine whether paraquat-induced polyamine accumulation, especially putrescine, might be involved in resistance of these biotypes to paraquat.

3.2. **Materials and methods**

3.2.1 Plant material

Seeds of four resistant biotypes of *Hordeum glaucum* Steud. and five resistant biotypes of *H. leporinum* Link. were collected from lucerne fields at different locations in southern Australia with a history of at least 15 years of once annual applications of paraquat and diquat for weed control (Powles, 1986; Purba *et al.*, 1995; Tucker and Powles, 1991; Chapter 2). Seeds of susceptible biotypes from each species were obtained from adjacent fields with no history of paraquat use. Seeds were germinated on agar (0.6% w/v) in an incubator (light period 21°C, 14h, 20-30 μmol photons m^{-2} s^{-1}; dark period 15°C, 10h). After 4 days, seedlings were transplanted at the 1-leaf stage into plastic trays (20 × 40 cm) containing sterile potting soil and transferred to a controlled environment growth room. Growth conditions were 15°C, 12h, 330 μmol m^{-2} sec^{-1} light period/10°C, 12h dark period.

3.2.2 *Herbicide application*

Seedlings were treated at the 3 to 4-leaf stage with a commercial paraquat formulation plus 0.2% (v/v) non-ionic surfactant. Paraquat was applied at a rate of 100 g ha^{-1} using a belt-driven laboratory sprayer with an output of 130 L ha^{-1} at a pressure of 250 kPa and a speed of 1 m s^{-1}. This rate of herbicide kills the susceptible biotypes but not the resistant biotypes under growth room conditions (Purba *et al.*, 1995; Chapter 2). After herbicide treatment, plants were returned to the growth room in the light. Leaves were harvested 1 h after herbicide application, except for the time course experiments where leaves were harvested 0, 1, 2, 4, 8, and 24 hours after paraquat treatment. To determine the effect of other herbicides on polyamine accumulation, plants were treated with 100 g ha^{-1} diquat or 400 g ha^{-1} oxyfluorfen. The level of paraquat resistance in both *H. glaucum* and *H. leporinum* is strongly temperature dependent (Purba, *et al.*, 1995; Chapter 2). Therefore, experiments to examine the effect of temperature on putrescine levels were conducted with plants grown at 15°C and treated with 100 g ha^{-1} paraquat before transferring to either 15°C or 30°C.

Harvested leaves were weighed, frozen in liquid N_2 and stored at -20°C prior to extraction of polyamines. There were 4 replicates in each experiment and each experiment was conducted twice unless stated otherwise.

3.2.3 *Extraction and determination of free polyamines*

Because free polyamines are the form suggested to be active in the regulation of plant physiological processes (Smith, 1985b), only free polyamines were analysed in this experiment. Leaf tissue (0.2 to 0.5 g) was homogenised in 3 to 4 ml of cold methanol:chloroform:water (12:5:3) with a cold mortar and pestle. Homogenised tissue samples were extracted in the methanol:chloroform:water

mixture for at least 20 min in an ice bath. Samples were then centrifuged at 24,000 ×g for 15 min at 4°C, and the supernatant, containing the free polyamines, was removed and used for benzolyation following the method of Flores and Galston (1982b) with some modification. One ml of 2 M NaOH was mixed with either 1 ml of extract or 100 µL of 1 mM polyamine standards in 0.01 M HCl. After addition of 15 µl benzoyl chloride, the mixtures were vortexed for 10 s and incubated at room temperature for 20 min. Two ml of saturated NaCl and 2.5 ml of anhydrous diethyl ether were added to each sample and the tubes were gently mixed. After centrifugation at 15,000 ×g for 5 min at 4°C, 1.5 ml of the ether phase was collected, evaporated to dryness under vacuum and redissolved in 600 µL methanol. Standards for putrescine, spermidine, spermine, and cadaverine were treated similarly to tissue extracts. The benzoylated polyamines were separated by HPLC equipped with a reverse-phase C18 column (250 mm × 4.6 mm i.d.; Brownlee Labs ODS-5 Spherisorb; Applied Biosystems, Lincoln City, CA). Samples were eluted from the column at room temperature with a flow rate of 1.2 ml min^{-1}. Solvents used were 10% (v/v) acetonitrile:89% (v/v) H_2O:1% (v/v) acetic acid (solvent A) and 90% (v/v) acetonitrile:9% (v/v) H_2O:1% (v/v) acetic acid (solvent B). Separation was achieved with a linear gradient from 30% B to 60% B in 20 min, followed by a linear gradient from 60% B to 100% B in 10 min. The column was washed with solution B for 5 minutes and re-equilibrated with the starting conditions prior to injection of the next sample. Polyamine peaks were detected by absorbance at 254 nm and peak areas corrected to nmol polyamine by comparison to known standards (Fig. 3.1).

3.2.4 *Preparation of diamine oxidase*

All extraction procedures were carried out in a 4-5°C cold room. About 20 g leaf tissue of R and S biotypes of *H. glaucum* were homogenized with 60 ml of chilled 0.1 M potassium phosphate buffer, pH 7, by a pre chilled polytron (Ultra-Turrax

Tp18/10). The homogenates were filtered through four layers of miracloth and centrifuged for 20 min at 27,000 ×g at 4°C. The precipitates were discarded, the supernatant brought to 70% saturation with $(NH_4)_2SO_4$ while stirring for about 2 hours, followed by centrifugation for 20 min at 27,000 ×g at 4°C. The supernatants were discarded and the pellets were dissolved in 2.7 ml 0.1 M potassium phosphate buffer and 2.5 ml was loaded onto a column containing Sephadex G-25 (PD-10 column, Pharmacia, Uppsala, Sweden) previously equilibrated with the same buffer. The void volume was eluted with 2.7 ml of 0.1 M potassium phosphate pH 7.0 buffer. The eluate was stored on ice and assayed immediately for DAO activity. Protein was determined according to method of Bradford (1976).

DAO activity was assayed according to a modified method of Rinaldi *et al.* (1982) using a Clark-type oxygen electrode. The reaction was carried out in a continuously stirred reaction vessel at 30°C using air as the gaseous phase. The standard reaction mixture (1 ml) contained 50 µg catalase and the required amount of enzyme in 0.1 M potassium phosphate buffer pH 7.0. The reaction was started by addition of a small volume of substrate (putrescine) solution after a 10 min pre-incubation. Reaction rates were calculated from the putrescine-induced increase in oxygen consumption.

3.3. **Results**

3.3.1 *Constitutive leaf putrescine content of resistant and susceptible populations*

The leaf polyamine content of nine resistant and four susceptible biotypes of *H. glaucum* and *H. leporinum* was determined. Putrescine was the major polyamine detected in leaf tissue of both species. The absolute level of paraquat resistance

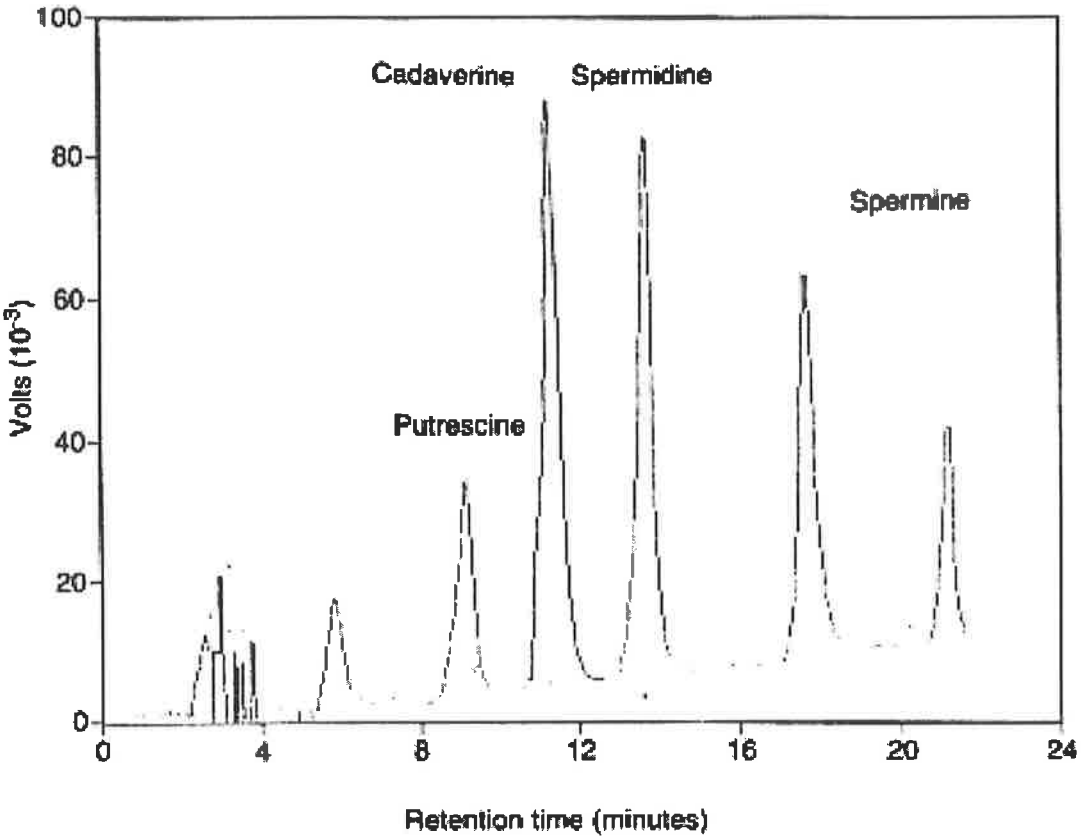


Figure 3.1: HPLC of benzolyated polyamine standards, putrescine, cadaverine, spermidine, spermine. Standard peaks represent 16.67 nmoles of benzoylated polyamine.

varies across the nine resistant biotypes, whereas the four susceptible biotypes are uniformly susceptible to paraquat (Powles, 1986; Tucker and Powles, 1991; Purba, 1993; Purba *et al.*, 1995). The constitutive leaf putrescine content of these biotypes is plotted in Figs. 3.2, and 3.3. The results show that, for most resistant biotypes, the constitutive leaf putrescine content is higher than in the susceptible biotypes (Fig. 3.2); however, for some resistant biotypes this difference is not significant. Fig. 3.3 compares the leaf putrescine content compared to the GR_{50} for paraquat for each biotype. The GR_{50} is the concentration of paraquat required to inhibit growth by 50% and is a quantitative determination of the level of paraquat resistance. There is considerable variation in the constitutive leaf putrescine level across biotypes and there is a poor correlation (r^2=0.31, Fig. 3.3) between leaf putrescine content and paraquat GR_{50} for the resistant biotypes. Individual resistant biotypes can be strongly paraquat resistant (high GR_{50}) and yet have lower levels of putrescine than biotypes with a lower level of resistance (Fig. 3.3).

3.3.2 Time course of leaf putrescine accumulation following paraquat application

To determine whether paraquat treatment induces putrescine synthesis, the leaf putrescine content was measured before and after paraquat application to intact plants. Paraquat treatment was found to result in a rise in putrescine levels in leaves. Fig. 3.4 shows the time course of the increase in leaf putrescine content following treatment with paraquat in a single paraquat-resistant and a single paraquat-susceptible biotype of *H. glaucum* and *H. leporinum*. The resistant biotypes for this experiment were selected based on their higher GR_{50} compared with other resistant biotypes. Constitutive leaf putrescine content of the particular resistant biotypes of both *H. glaucum* and *H. leporinum* used for this study were at least double that of the susceptible biotypes. Following paraquat treatment, the

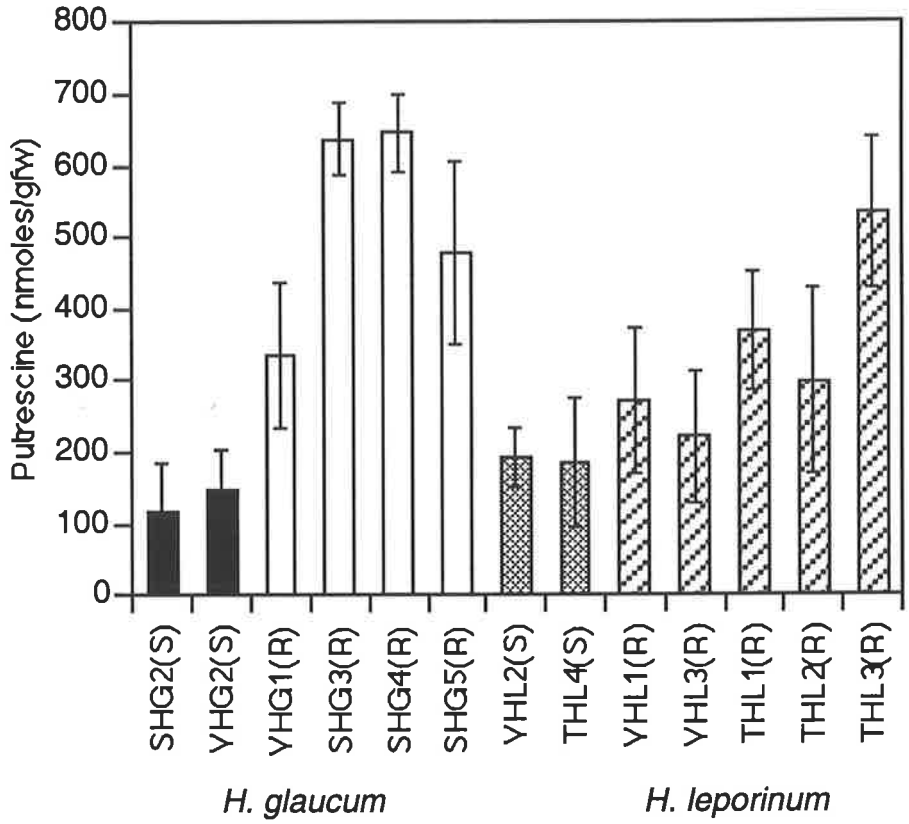


Figure 3.2: Constitutive putrescine content in leaf tissue of susceptible (■, ▩) and resistant (□, ▨) biotypes of *H. glaucum* and *H. leporinum*. All plants were grown on a growth room at 15°C/10°C till 3-4-leaf stage. Bars are means ± SE of three replicates.

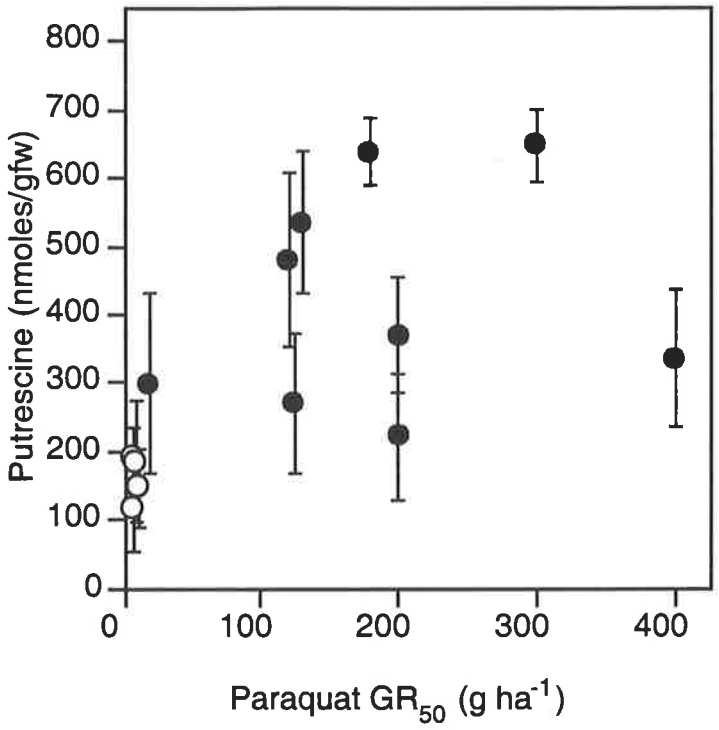


Figure 3.3: Constitutive putrescine content in leaf tissue (3 to 4-leaf stage) of nine paraquat-resistant (●) and four paraquat-susceptible (O) biotypes of *H. glaucum* and *H. leporinum* compared to the GR_{50} for paraquat for each of these biotypes. All plants were grown under the same conditions. Each data point represents the mean ± SE of two independent experiments with 4 replicates.

putrescine content increased for 4 to 8 hours after treatment in both resistant and susceptible plants. However, these increases were larger for the resistant compared to the susceptible biotypes (Fig. 3.4). Susceptible biotypes treated with paraquat showed symptoms of leaf injury one hour after treatment, whereas such symptoms were not evident in resistant biotypes until much later. Therefore, we selected one hour after paraquat treatment as the best time to harvest plants, as minimum damage was evident at this time.

When examined across a range of resistant biotypes of *H. glaucum* and *H. leporinum*, the leaf putrescine content was observed to increase in both resistant and susceptible biotypes following treatment of plants with paraquat (Fig. 3.5). While the resistant biotypes generally had higher inducible putrescine levels than susceptible biotypes, there was a poor correlation (r^2=0.41) between induced putrescine content and the degree of resistance to paraquat as measured by GR_{50}. Thus, some highly-resistant biotypes displayed a smaller induction of putrescine following paraquat application whereas other biotypes with lower paraquat resistance had a larger induction of putrescine.

Therefore, while leaves of the paraquat resistant biotypes in general had higher constitutive putrescine contents (Figs. 3.2, and 3.3) and the leaf putrescine content further increased following application of paraquat (Figs. 3.4, and 3.5), leaf putrescine content of individual resistant biotypes did not strongly correlate with their level of paraquat resistance. Some of the paraquat resistant biotypes used in this study have been shown to be up to 250 times more resistant than susceptible biotypes (Powles, 1986; Tucker and Powles, 1991; Purba, 1993; Purba *et al.*, 1995; Chapter 2). However, putrescine contents in resistant biotypes were only a maximum of 6-fold greater than for the susceptibles. Therefore, the elevated constitutive putrescine content in resistant biotypes of *H. glaucum* and

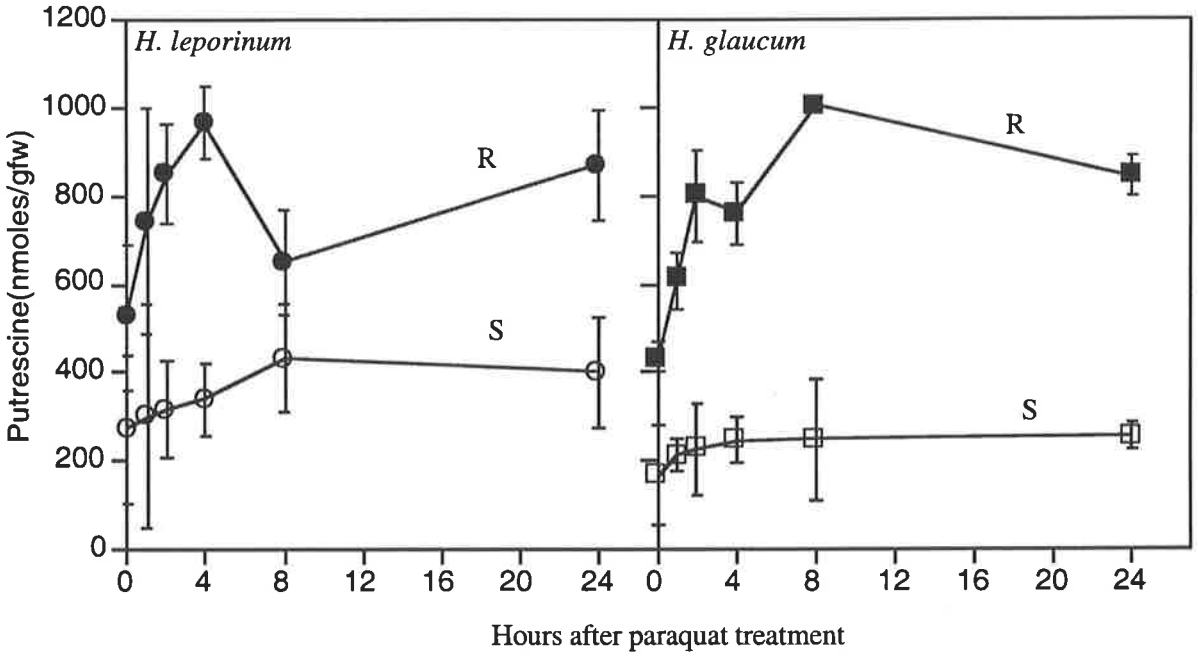


Figure 3.4: Time course of induction of putrescine in intact leaf tissue following application of 100 g ha^{-1} paraquat in paraquat-resistant (■, ●) and -susceptible (□, O) biotypes of *H. glaucum* (A) and *H. leporinum* (B). Each data point represents the mean ± SE of four replicates.

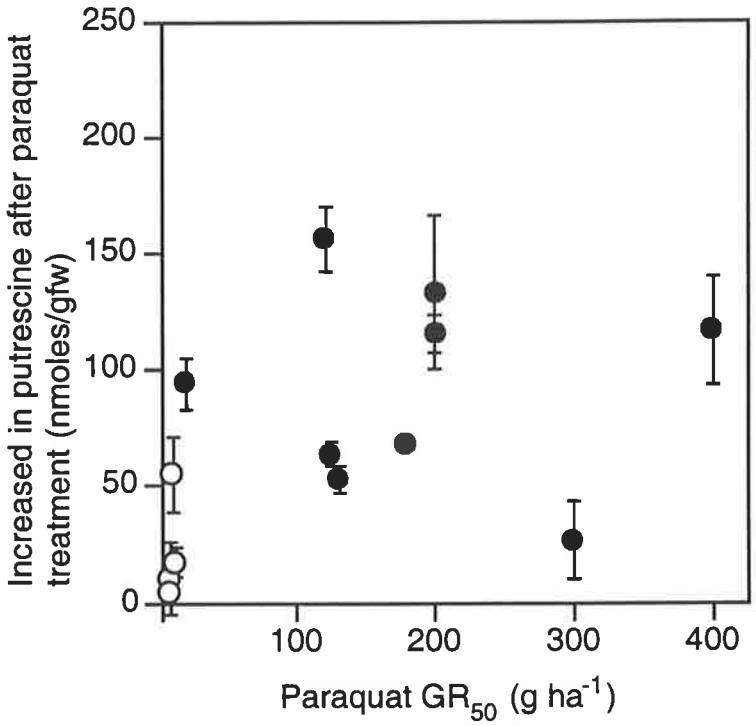


Figure 3.5: Paraquat induction of putrescine in leaf tissue of nine paraquat-resistant (●) and four paraquat-susceptible (O) biotypes of *H. glaucum* and *H. leporinum* compared to the GR_{50} for paraquat for each of these biotypes. Values plotted are the mean increase (± SE) in leaf putrescine level above the constitutive level one hour after application of 100 g ha^{-1} paraquat in two independent experiments with 4 replicates.

H. leporinum compared with susceptible biotypes may not be a primary mechanism of resistance.

3.3.3 *Accumulation of other polyamines*

Spermidine was the only other polyamine detected in any concentration in leaf tissue of all populations tested (Fig. 3.6). There was considerable variation among paraquat-resistant and -susceptible biotypes in initial spermidine content with little correlation to paraquat resistance (Fig. 3.6A). As for putrescine (Fig. 3.5), the content of spermidine also increased in leaves of most resistant and susceptible biotypes one hour after application of paraquat (Fig. 3.6B). Whilst, in general, paraquat treatment resulted in spermidine induction to a greater extent in resistant biotypes, there was no apparent relationship between paraquat-induced spermidine content and resistance to paraquat in these biotypes (Fig. 3.6B).

3.3.4 *Effect of temperature on leaf putrescine content*

Resistance to paraquat in *H. glaucum* and *H. leporinum* is strongly temperature dependent (Purba *et al.*, 1995; Chapter 2). Resistant plants grown at low (15°C) and immediately transferred to higher temperature (30°C) following paraquat treatment are considerably less resistant to paraquat compared to those maintained at 15°C (Purba *et al.*, 1995; Chapter 2). In contrast, temperature has a small effect following application of paraquat to susceptible biotypes. Therefore, the paraquat resistance mechanism in *H. glaucum* and *H. leporinum* is much less efficient at higher temperatures (Purba *et al.*, 1995). The temperature dependence of the paraquat resistance mechanism in *Hordeum* provides an experimental system to test for any correlation between the polyamine contents of leaves of resistant and susceptible biotypes and the level

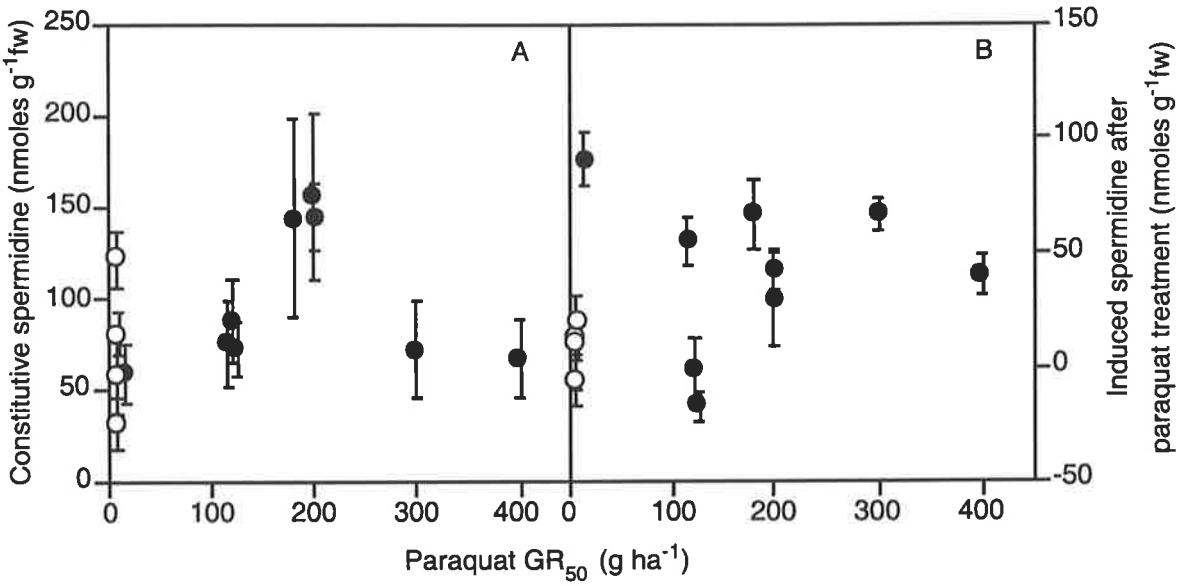


Figure 3.6: A) Constitutive spermidine content of leaves of nine paraquat-resistant (●) and four paraquat-susceptible (O) biotypes of *H. glaucum* and *H. leporinum.* B) Induction of spermidine in leaves of paraquat-resistant and susceptible biotypes one hour after application of 100 g ha^{-1} paraquat. Points are means ± SE of two independent experiments with 4 replicates.

of resistance at different temperatures. To examine this, resistant and susceptible biotypes were grown at 15°C, and sprayed with 100 g ha^{-1} paraquat at the 3 to 4-leaf stage. The plants were then either maintained at 15°C or moved to 30°C immediately after treatment. One hour later, the leaves were harvested and leaf polyamine content measured. The constitutive putrescine content in both species was found to be higher in resistant biotypes compared to susceptible biotypes and further increased following paraquat treatment (Fig. 3.7). This increase was greater in resistant biotypes compared with susceptible. The constitutive putrescine content was slightly higher in leaves of plants maintained at 30°C after treatment compared with 15°C in both species. There was no correlation between paraquat induction of putrescine and the temperature at which plants were maintained after paraquat treatment. Therefore, although the paraquat resistance mechanism functions much more efficiently at 15°C than at 30°C in *Hordeum* (Purba *et al.*, 1995; Chapter 2) there are no differences in the level of inducible leaf putrescine in treated plants maintained at 15°C compared to 30°C.

3.3.5 *Effect of other herbicides*

The mechanism of action of paraquat and diquat involves the formation of toxic oxygen radicals (Chapter 1). These radicals result in peroxidation of lipids in membranes which leads to loss of membrane integrity, bleaching of chlorophyll and cell death (reviewed by Preston, 1994). Oxyfluorfen, a herbicide with a completely different mode of action, also results in the formation of O_2 radicals (Kunert and Dodge, 1989). Therefore, the effects of O_2 radical producing herbicides diquat and oxyfluorfen on polyamine levels of resistant and susceptible biotypes were measured to determine any correlation between herbicide mode of action and polyamine levels. The paraquat-resistant *Hordeum* populations are also resistant to diquat (Tucker and Powles, 1991; Preston, 1994) but not to oxyfluorfen (Chapter 2).

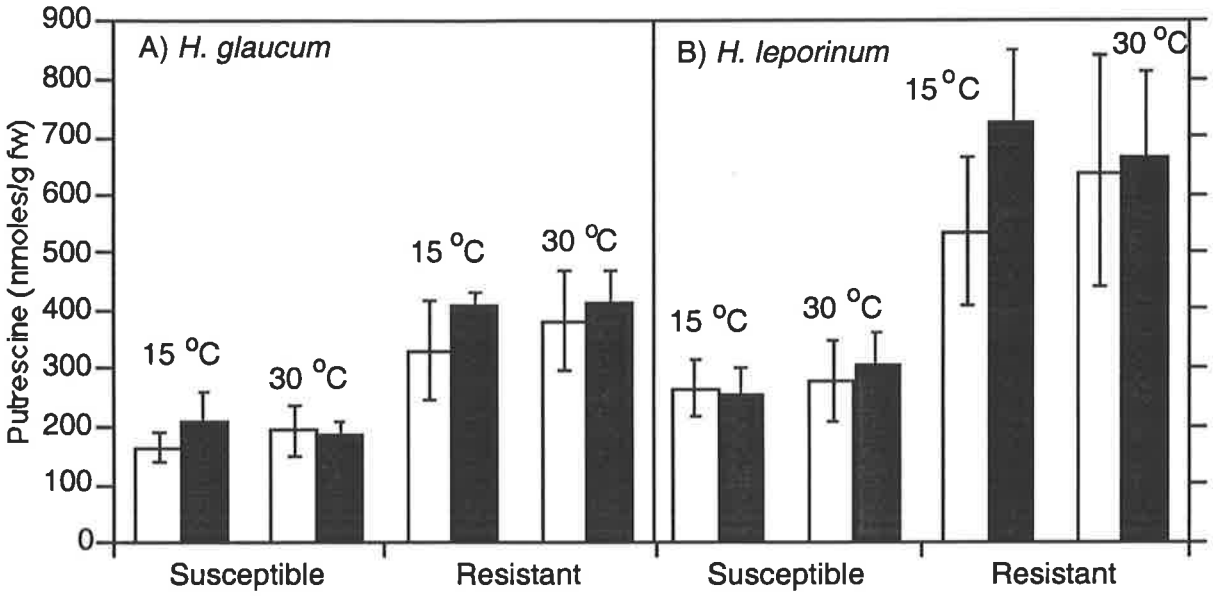


Figure 3.7: Putrescine content of leaves of resistant and susceptible biotypes of *H. glaucum* (A) and *H. leporinum* (B) one hour after application of paraquat at 15 °C or 30 °C. Control plants (open bars), paraquat treated plants (closed bars). Bars are means ± SE of two separate experiments with 4 replicates.

Leaf putrescine content increased in both resistant and susceptible biotypes following application of paraquat and oxyfluorfen but not diquat (Fig. 3.8). This increase was greater in resistant biotypes than susceptible biotypes. However, none of the treatments resulted in a significant difference in putrescine contents for any biotype. The fact that diquat had less effect on putrescine content than paraquat may be due to diquat being a less effective herbicide on grasses.

3.3.6 *Diamine oxidase activity measurement*

Diamine oxidase (DAO) activity of shoots of resistant and susceptible biotypes was measured using an oxygen electrode (Table 3.1). Using putrescine as substrate, DAO enzyme exhibited similar activity of 199 and 186 nmoles O_2 h^{-1} mg^{-1} protein for resistant and susceptible *H. glaucum*, respectively. However, no significant difference between resistant and susceptible plants in DAO activity was observed.

3.4. **Discussion**

Higher constitutive putrescine content in leaves of paraquat-resistant *Conyza canadensis* biotypes have been reported and suggested to play a role in paraquat resistance in this species (Szigeti *et al.*, 1996). To examine the role of polyamines in paraquat resistance in *Hordeum* species, the leaf putrescine content of nine paraquat-resistant and four susceptible biotypes of *H. glaucum* and *H. leporinum* was measured prior to and after application of paraquat. Constitutive putrescine contents were generally higher in resistant biotypes compared to susceptible biotypes (Figs. 3.2, and 3.3). Also, the putrescine content increased in all biotypes one hour after paraquat treatment, but increased to a greater extent in the resistant biotypes (Figs. 3.4, and 3.5). Increases in putrescine content, which have been often associated with plant

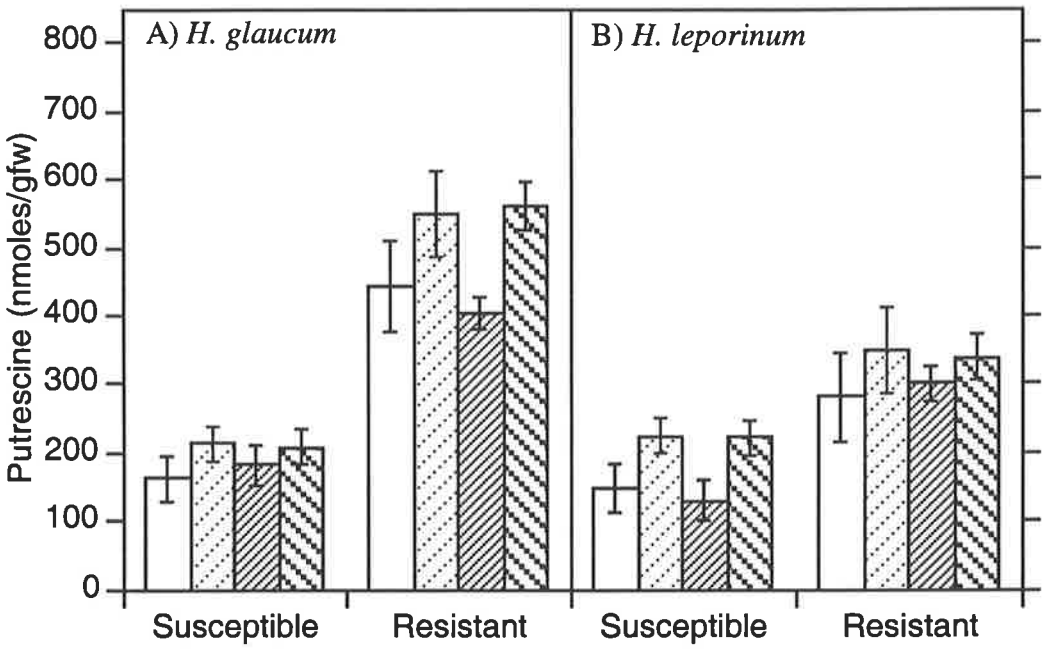


Figure 3.8: Putrescine content of leaves of untreated (□) and herbicide treated, paraquat (▨), diquat (▨), or oxyfluorfen (▨), resistant and susceptible biotypes of *H. glaucum* (A) and *H. leporinum* (B). Plants were treated with 100 g ha^{-1} paraquat, 100 g ha^{-1} diquat, or 400 g ha^{-1} oxyfluorfen and harvested one hour after treatment. Bars are means ± SE of three replicates.

Table 3.1: Diamine oxidase activity in shoots of paraquat resistant and susceptible biotypes of *H. glaucum*. DAO activity was measured using a oxygen electrode at 30°C. Each data represents the mean ± SE of three replicate experiments.

Biotype	DAO activity (nmoles O_2 h^{-1} mg^{-1} protein)
Resistant	199 ±10
Susceptible	186 ±13

stress, are rarely coupled to a simultaneous increase in spermidine or spermine (Smith, 1985b, Slocum *et al.*, 1984). However, these results demonstrate that paraquat treatment induces accumulation of both putrescine and, to a smaller extent, spermidine, with somewhat variable results across a range of biotypes (Fig. 3.6).

Increased polyamine content, especially putrescine, in the resistant biotypes appears not to be a primary paraquat resistance mechanism in *Hordeum* spp. While the resistant populations generally had higher constitutive leaf putrescine content than susceptibles (Figs. 3.2, and 3.3), there was a poor correlation between this and the degree of resistance to paraquat measured by GR_{50} (Fig. 3.3). Equally, there was a poor correlation between paraquat-inducible putrescine content and paraquat resistance (Fig. 3.5). In addition, induction of putrescine was also observed following application of oxyfluorfen, a herbicide of different mode of action that also imposes oxygen radical stress (Fig. 3.8). The paraquat resistant biotypes are not resistant to oxyfluorfen. Further evidence of no correlation between the level of resistance and leaf putrescine content was obtained with experiments conducted at 15°C compared to 30°C (Fig. 3.7). The mechanism of paraquat-resistance in these biotypes is strongly temperature dependent (Purba *et al.*, 1995; Chapter 2); however, accumulation of putrescine following paraquat treatment was independent of temperature (Fig. 3.7).

The higher constitutive leaf putrescine content in resistant compared with susceptible biotypes may be indirectly related to the mechanism of resistance. Since putrescine and paraquat share the same uptake pathway in animals (Smith and Wyatt, 1981) and possibly plants (Hart *et al.*, 1992a), it is possible that a change in the carrier responsible for influx of putrescine into the cell or vacuole, or efflux from the cell or vacuole, has occurred. This change might lead to increased accumulation of both paraquat and putrescine either in the vacuole or

outside the cell. Therefore, the higher constitutive leaf putrescine content measured may be a result of a change in activity of a carrier responsible for transporting putrescine, and paraquat, across membranes.

Increases in putrescine content are normally concomitant with a change in the activity of polyamine synthesis enzymes (Slocum *et al.*, 1984), which have been shown to increase in oat leaves in response to osmotic shock and acid or pH stress (Flores and Galston, 1982a; Young and Galston, 1983). Therefore, the higher constitutive leaf putrescine content in resistant biotypes may be the result of greater activity of polyamine biosynthetic enzymes, which result in production of more putrescine in resistant biotypes. Minton *et al.* (1990) reported that paraquat toxicity for *Eschericha coli* increased over 10-fold in mutants defective in the biosynthesis of polyamines compared to wild type cells. They postulated that polyamines, such as spermidine, might protect cellular components from damage by oxidation. Ye *et al.* (1997) recently reported an increase in putrescine biosynthesis enzymes in a paraquat resistant biotype of *C. bonariensis*. They reported a 2 to 3-fold increase in the activities of arginine decarboxylase and ornothine decarboxylase in 10-week old resistant *C. bonariensis* compared to susceptible plants.

Alternatively, a reduction in the activity of diamine oxidise, the enzyme responsible for degradation of putrescine in the cell wall (Smith, 1985b), may also result in higher putrescine content in resistant biotypes. However, the activity of diamine oxidase *in vitro* was not different between resistant and susceptible biotypes of *H. glaucum* (Table 3.1). This implies that the higher putrescine content of resistant biotypes cannot be attributed to reduced putrescine catabolism. In conclusion, the reason for the higher constitutive leaf putrescine content in paraquat-resistant biotypes of *H. glaucum* and *H. leporinum* is most

likely a consequence of these biotypes being resistant to paraquat rather than a cause of resistance.

Chapter 4

Binding of paraquat to cell walls of resistant and susceptible biotypes of *H. glaucum*

4.1. Introduction

Cell walls form the basic structural framework of the plant, defining the shape and size of plant cells and tissues. The cell wall contains regulatory molecules that control the growth and development of plants as well as presenting a physical barrier to micro-organisms and other agents harmful to the plant (York *et al.*, 1985). Plant cell walls are complex amalgams of carbohydrates, proteins, lignin, water, and other substances. The cellulose-pectin matrix of the cell wall contains numerous cation exchange sites with additional sites contributed by cell wall localised proteins and polyphenols (Price, 1979). Cation exchange properties of the cell wall are largely due to de-esterified galacturonans in the pectin fraction. Calcium, polyamines, and other cations bind non-covalently to pectins and other cell wall components (Baydoun and Brett, 1988; Pistocchi *et al.*, 1987).

The sites of action of most herbicides are located inside plant cells. Therefore, herbicides have to pass through the cell walls, the plasma membrane and organellar membranes of a plant cell to reach their site of action, where accumulation causes phytotoxicity (Sterling, 1994). One of the major factors preventing movement of herbicides within the cell wall is interaction of herbicides with cell wall structure or compounds leading to partitioning or binding of herbicides. Paraquat, which is a divalent cation, has been shown to bind to plant cell walls (Hart *et al.,* 1992b, Chun *et al.*, 1997a). Paraquat binds by ionic interactions due to the presence of large numbers of negatively charged binding sites within the cell wall matrix.

Resistance to paraquat has been suggested to be due to sequestration of herbicide outside the cells in resistant biotypes (Powles and Cornic, 1987; Preston *et al.*, 1992b; Fuerst *et al.*, 1985). One of the possible mechanisms of paraquat sequestration is increased paraquat binding to cell wall material. Chun

et al. (1997a) reported that paraquat binding to the cell wall in the paraquat tolerant *Rehmannia glutinosa* was almost two-fold greater than in corn and soybean resulting in the reduction of paraquat movement to its site of action. They suggested that this increased binding might play a part in tolerance of this species to paraquat. However, resistance to paraquat in *Conyza bonariensis* is unlikely to be due to increased binding of the herbicide to the cell wall. Fuerst *et al.* (1985) reported a strong binding of paraquat to a cell wall component in *C. bonariensis* but were not able to find any significant difference in paraquat binding between susceptible and resistant biotypes. These authors suggested that the loss of some binding components during the extraction procedure might have contributed to this result. In *A. calendula*, binding of diquat to cell walls of resistant and susceptible biotypes has been estimated by the efflux of herbicide from leaf tissue lacking cell contents (Preston *et al.*, 1994). They reported no differences in binding of diquat to cell walls of resistant and susceptible biotypes as measured by either absorption or efflux. In contrast, Powles and Cornic (1987) postulated that enhanced apoplastic binding might be involved in the mechanism of resistance in *H. glaucum*. This hypothesis was based on restricted movement of ^{14}C-paraquat fed to the resistant biotype (Bishop *et al.*, 1987) and the equal sensitivity to paraquat of protoplasts isolated from resistant and susceptible biotypes (Powles and Cornic, 1987). However, this was disputed by Lasat *et al.* (1996) who failed to find any significant differences in apoplastic binding of paraquat in roots of resistant and susceptible biotypes of *H. glaucum*.

The aim of the experiments conducted here was to characterise paraquat binding capacity as well as cation exchange capacity in leaves of resistant and susceptible biotypes of *H. glaucum*. This was done in order to establish if there are any differences between these two biotypes in the ability of paraquat to be sorbed onto cell walls.

4.2. **Materials and Methods**

Seedlings of resistant and susceptible biotypes of *H. glaucum* were grown in a growth room as described previously (see Chapter 3).

4.2.1 *Isolation of cell wall fraction*

The isolation procedure was carried out at 4°C according to a method modified from Wojtaszck and Bolwell (1995). The cell wall fraction was mechanically isolated from leaves of resistant and susceptible biotypes. Freshly collected seedlings were frozen in liquid N_2 before homogenizing 3 times in 3 ml of homogenized buffer (HB), consisting of 0.4 M sucrose, 50 mM Hepes/NaOH (pH 6.8), 2 mM EDTA, and 5 mM β-mercaptoethanol, per gram fresh weight with a polytron for 30 s at maximum speed. The homogenate was filtered through Miracloth, centrifuged at 10,000 xg for 10 min and the cell-wall-enriched pellet then washed for 10 min with HB with end-over-end mixing. After centrifuging at 15,000 xg for 15 min, the pellet was resuspended in HB containing 0.1% (v/v) Nondiet P-40 and incubated for 45 min with constant stirring to dissolve lipids. Cell walls were recovered by centrifugation at 15,000 xg for 10 min and washed two times, 10 min each, with deionized water containing 2 mM EDTA and 5 mM β-mercaptoethanol centrifuging each time at 10,000 xg. In some experiments purified cell walls were extracted twice with 1 M NaCl in HB for 60 min, centrifuging after each step at 15,000 xg for 10 min, to extract cell wall associated proteins.

4.2.2 *Uptake experiments*

Paraquat binding to different components of resistant and susceptible plant cells was estimated by three different methods. Firstly, paraquat uptake was

determined using an isolated cell wall fraction still containing the lipids. For this experiment, 10 μM of ^{14}C-paraquat was added to the cell wall suspension and incubated for 2 h at room temperature. Uptake of herbicide was stopped by centrifuging the suspension at 15,000 xg and the resulting supernatant was collected. The pellet was washed for 10 min with HB with end-over-end mixing and centrifuging at 15,000 xg for 15 min. The pellet was resuspended in HB containing 0.1% (v/v) Nondiet P-40 and incubated for 45 min with constant stirring prior to two washes, 10 min each, with deionized water containing 2 mM EDTA and 5 mM β-mercaptoethanol centrifuging each time at 10,000 xg. The supernatants were collected after each centrifugation. ^{14}C present in each sample was determined using a Beckman liquid scintillation spectrometer (Beckman Model LS 5000). The final pellet was oxidised using a Biological Oxidiser (R. J. Harvey Instrument Corp. Model OX600) to determine ^{14}C paraquat still bound to the cell walls.

Paraquat binding capacity of cell walls was measured using an isolated cell wall fraction. This was incubated as described above, but with a range of paraquat concentrations from 0 to 5000 μM. After 2 h at 20°C, the cell wall fractions centrifuged and paraquat bound determined using the method described above.

Absorption of paraquat to cell walls was also measured using leaf slices. Freshly harvested leaves of resistant and susceptible plants were cut to 2-3 mm wide segments and immersed for about two hours in solution containing 10 μM ^{14}C-paraquat at room temperature. The uptake solution was collected and the leaves were washed with deionized water and ground by a hand-held glass tissue homogenizer at 4°C for 2-3 min before isolating the cell wall as described above.

Absorption of paraquat to cell walls of intact leaves was determined by applying 1 μl droplets containing 10 μM ^{14}C-paraquat plus surfactant to the surface of

leaves of resistant and susceptible plants. After 2 hours, the leaves were washed and unabsorbed ^{14}C-paraquat collected. The cell wall fraction was prepared from these leaves using the method described above.

4.2.3 Exchange of paraquat from cell walls with other cations

The exchange capacity of bound ^{14}C-paraquat with other cations was measured following addition of different concentrations of calcium (Ca^{2+}), putrescine (Put^{2+}), cold paraquat (PQ^{2+}), benzyl viologen (BV^{2+}) and potassium (K^{+}) to aliquots of isolated cell walls with the lipids removed prepared from leaves of resistant and susceptible biotypes. After a 1 hour incubation, the aliquots were centrifuged and the amount of ^{14}C-paraquat exchanged with the solutions determined. In separate experiments, the effect of different pH and temperature conditions on paraquat desorption was also measured. The aliquots of cell wall fractions were incubated at three different pH (4.8, 6.8 and 7.8) or two temperatures (15 and 30°C).

All experiments were repeated at least three times, unless stated otherwise, with four replicates within each experiment.

4.3. **Results**

4.3.1 *Sorption of paraquat to cell walls*

The sorption of ^{14}C-paraquat to different components of the cell wall in leaf tissue of susceptible and resistance biotypes of *H. glaucum* was measured in three different ways. Fig. 4.1 shows the amount of paraquat bound to the cell wall fraction when paraquat was applied directly to cell wall preparations from

resistant and susceptible biotypes. About 6% of the absorbed paraquat was easily washed from the cell wall preparation. A further 12 to 15% was removed on washing the preparation with detergent and was presumably associated with cell membranes. A third wash removed a further 6% of the absorbed paraquat. Most of the paraquat absorbed was bound tightly to the cell walls. From the total recovered ^{14}C-paraquat, about 75% was bound strongly to the cell walls. Extensive washes were not able to release this bound paraquat from the cell walls. Preliminary experiments indicated that some of this cell wall associated paraquat could be removed following the solubilisation of cell wall associated protein with NaCl; however, most paraquat remained bound to the final cell wall fraction. There were no differences between resistant and susceptible cell walls in the amount of paraquat absorbed.

In another experiment, 2-3 mm wide leaf slices of resistant and susceptible plants were immersed for 2 h in ^{14}C-paraquat. From the total amount of herbicide absorbed by the leaf slices, about 25% was easily washed off in the first two washes of the cell walls, followed by another 15% when membranes were removed by detergent (Fig. 4.2). The rest of the ^{14}C-paraquat, about 60%, remained tightly bound to the cell walls in both resistant and susceptible biotypes and extensive washing could not release it (Fig. 4.2). There were no distinguishable differences between the susceptible and resistant biotypes in the amount of paraquat found in any of the fractions.

Similar results were obtained when paraquat was applied to the leaf surface and the leaf subsequently fractionated into cell wall components (Fig. 4.3). In this case, more paraquat was released by the initial washes and slightly less associated with the final cell wall fraction. Again, no differences between resistant and susceptible biotypes were observed in paraquat associated with either soluble or insoluble parts of cell wall fractions.

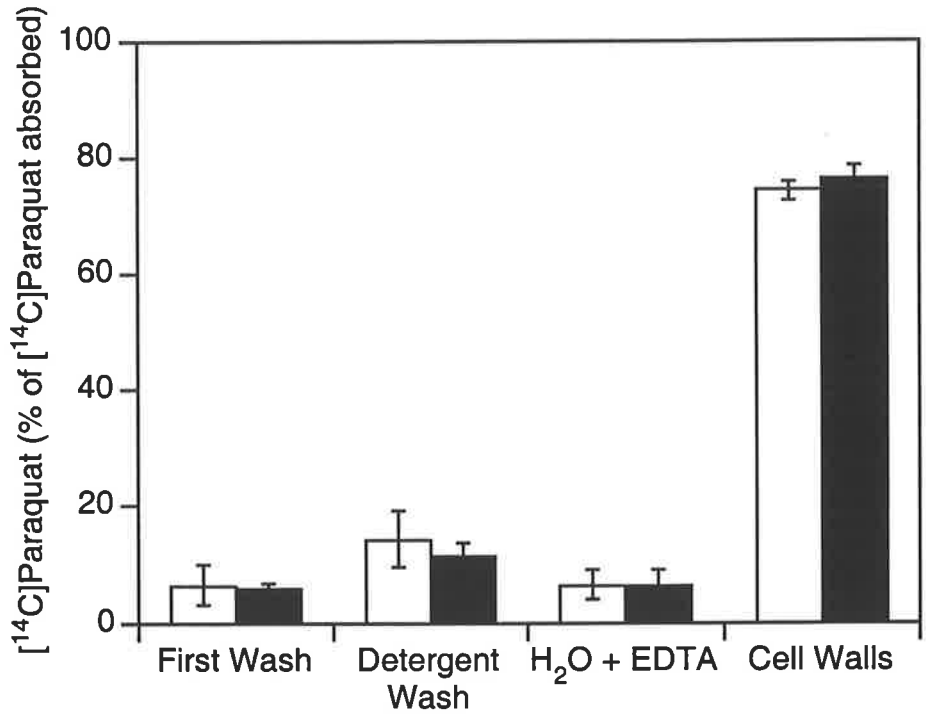


Figure 4.1: Paraquat binding to cell wall fractions of resistant (solid bars) and susceptible (open bars) biotypes of *H. glaucum*. Cell wall fractions were prepared from leaves of resistant and susceptible plants by mechanical isolation. The preparations were incubated in the presence of ^{14}C-paraquat for 2 h before washing extensively. Data points represent means of 3 experiments with 4 replicates ±SE.

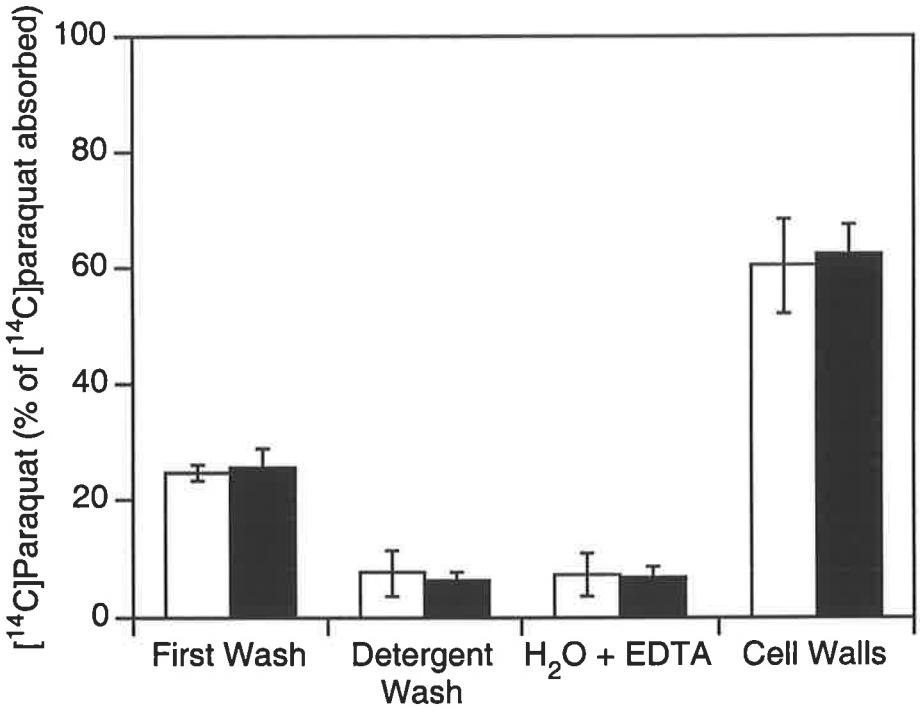


Figure 4.2: Paraquat binding to cell wall fractions prepared from 2-3 mm wide leaf slices of resistant (solid bars) and susceptible (open bars) biotypes of *H. glaucum*. Leaf slices were incubated in 10 μM ^{14}C-paraquat for 2 h at room temperature before preparing the cell wall fractions. Data points represent means of 3 experiments with 4 replicates ±SE.

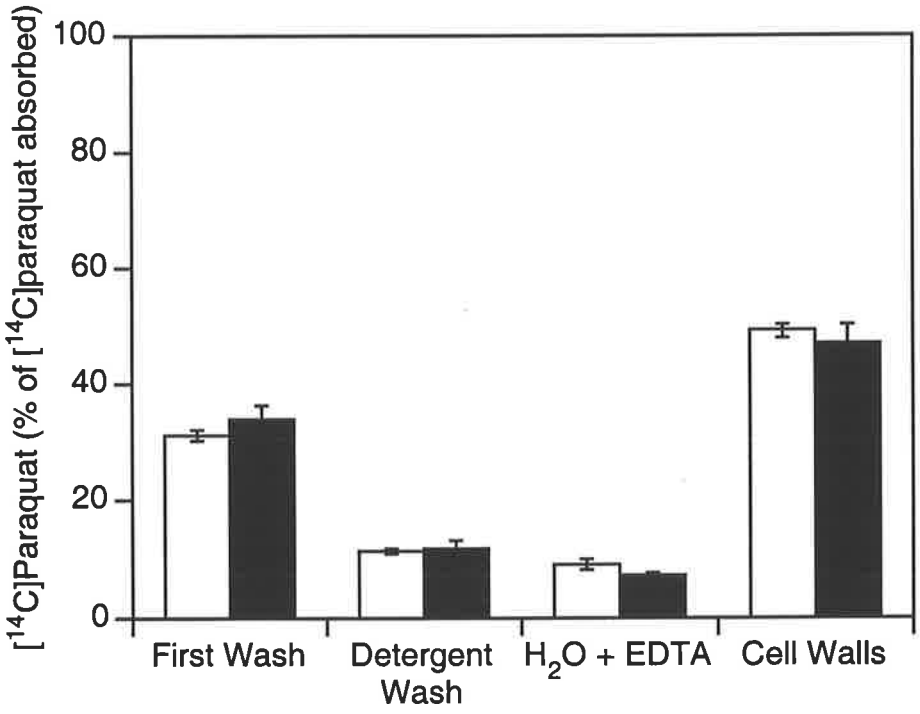


Figure 4.3: Paraquat binding to cell wall fractions prepared from leaves of resistant (solid bars) and susceptible (open bars) biotypes of *H. glaucum*. Droplets of ^{14}C-paraquat were placed on the surface of leaves and incubated for 2 h at room temperature prior to fractionation. Data points represent means of 4 replicates ±SE.

4.3.2 Binding capacity

The capacity of cell walls to bind paraquat was determined following incubation of cell wall preparations with different concentrations of ^{14}C-paraquat. ^{14}C-Paraquat that remained bound to cell walls increased with increasing concentrations of paraquat in the incubation medium and did not saturate at the highest concentration used (Fig. 4.4). At all paraquat concentrations, there were no differences in binding of paraquat to cell walls isolated from resistant and susceptible biotypes.

When this data were analysed as a Scatchard plot, a plot of paraquat bound/ paraquat free in solution versus paraquat bound, it appears that there are two different types of binding sites in the cell walls (Fig. 4.5). Most of the paraquat absorbed bound to a low affinity-binding site. This low affinity site was clearly distinguishable from a high affinity site in cell walls from both susceptible and resistant plants. The kinetic constants of binding calculated show that two sites with about 80-fold difference in affinity for paraquat were determined (Table 4.1). The high affinity site had a binding capacity of about 30 nmoles paraquat g^{-1} fw whereas the low affinity site had a binding capacity of 1.2 to 1.4 μmoles paraquat g^{-1} fw. The cell walls from the resistant biotype had about a 10% increased binding capacity for paraquat at high concentrations compared to the susceptible, but this is not considered important for resistance.

4.3.3 Effect of temperature on paraquat binding to cell walls

The mechanism of resistance to paraquat in *H. glaucum* has been shown to be strongly temperature dependent (Purba *et al.*, 1995, Chapter 2). Resistant plants are much less paraquat resistant at higher temperatures than at lower temperatures. The possibility of enhanced binding of paraquat to cell walls in the

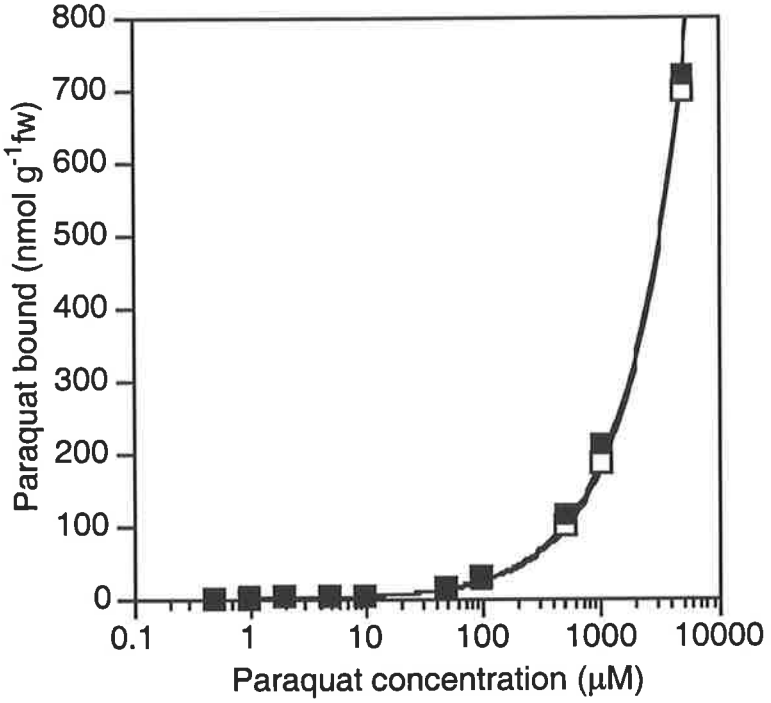


Figure 4.4: Concentration-dependence of binding of ^{14}C-paraquat to isolated cell walls of resistant (■) and susceptible (□) leaves of *H. glaucum* prepared following mechanical isolation. Data points represent means of 2 experiments with 3 replicates ±SE.

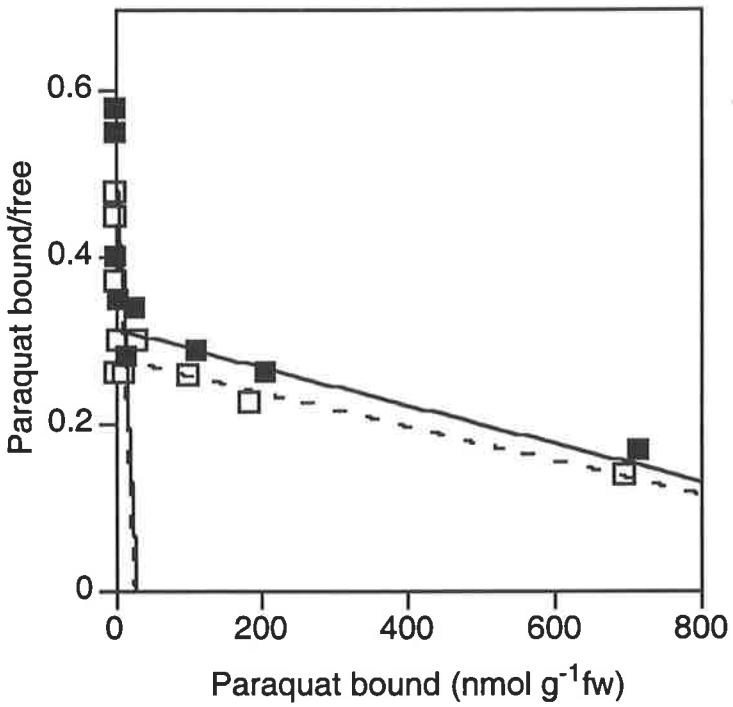


Figure 4.5: Scatchard plot of ^{14}C-paraquat binding to isolated cell walls of resistant (■) and susceptible (□) biotypes. Data points represent means of 2 experiments with 3 replicates ±SE.

Table 4.1: The kinetic constants of paraquat binding to cell walls of resistant and susceptible biotypes of *H. glaucum.*

Biotypes	Low affinity site		High affinity site	
	K_f (M^{-1})	A_{max} (nmoles paraquat g^{-1} fw)	K_f (M^{-1})	A_{max} (nmoles paraquat g^{-1} fw)
Susceptible	2.2×10^{-4}	1260	1.82×10^{-2}	26
Resistant	2.3×10^{-4}	1380	1.85×10^{-2}	29

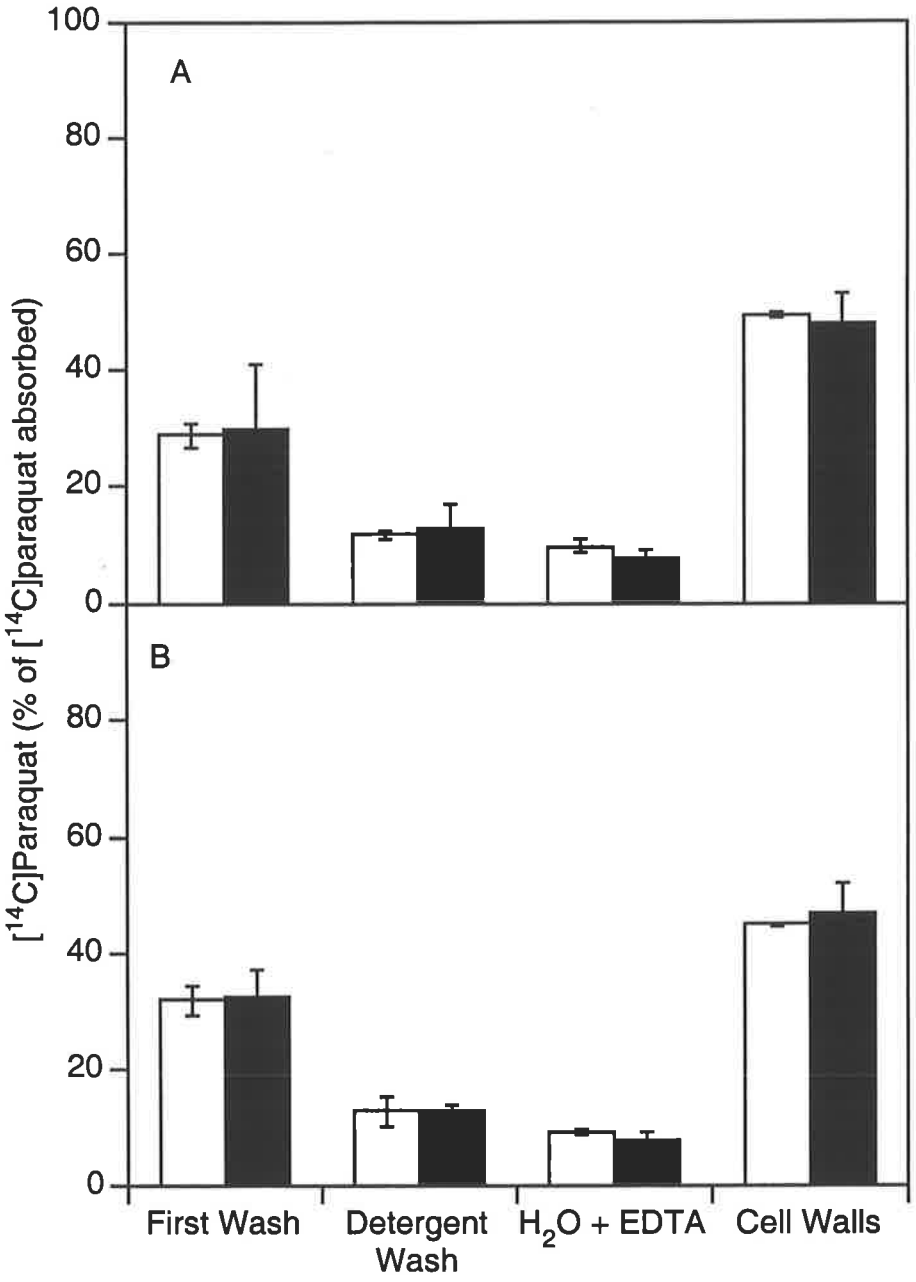


Figure 4.6: Paraquat associated with various fractions of cell walls of susceptible (A) and resistant (B) biotypes of *H. glaucum* following a 2 h incubation of isolated cell walls at 15°C (open bars) or 30°C (solid bars). Data points represent means of 2 experiments with 3 replicates ±SE.

resistant biotype of *H. glaucum* at lower temperature was examined. Binding of ^{14}C-paraquat to both soluble and insoluble parts of the cell wall fraction was measured and shown in Fig. 4.6. The amount of paraquat associated with all fractions of cell walls was the same at 30°C and 15°C. From this experiment, it appears that the mechanism of resistance to paraquat in *H. glaucum* is not related to increased binding of paraquat to cell walls in the resistant biotype.

4.3.4 *Effect of pH on binding of paraquat to isolated cell walls*

The effect of pH on binding of paraquat to cell walls of resistant and susceptible biotypes was determined. Cell wall fractions prepared from resistant and susceptible leaves were incubated at pH 4.8, 6.8, or 7.8 for 2 hours. Paraquat was found to bind strongly to the isolated cell walls irrespective of pH and there were no differences between resistant and susceptible biotypes (Fig. 4.7).

4.3.5 *Exchange of bound paraquat with other cations*

The ability of other cations to exchange with the tightly bound paraquat on cell walls was determined using calcium (Ca^{2+}), putrescine (Put^{2+}), cold paraquat (PQ^{2+}), benzyl viologen (BV^{2+}) and potassium (K^{+}). Aliquots of cell wall fractions prepared from leaves of resistant and susceptible biotypes loaded with ^{14}C-paraquat were incubated in the presence of different cation concentrations. The cell wall fraction was then centrifuged and the amount of ^{14}C-paraquat in the cell walls and ^{14}C-paraquat exchanged with the solution were measured. The results were compared with controls where the cell walls were incubated in the same solution for the same length of time but without additional cations. With increasing concentration of cations in solution, the amount of paraquat bound to the cell walls decreased in both resistant and susceptible biotypes (Fig. 4.8). In

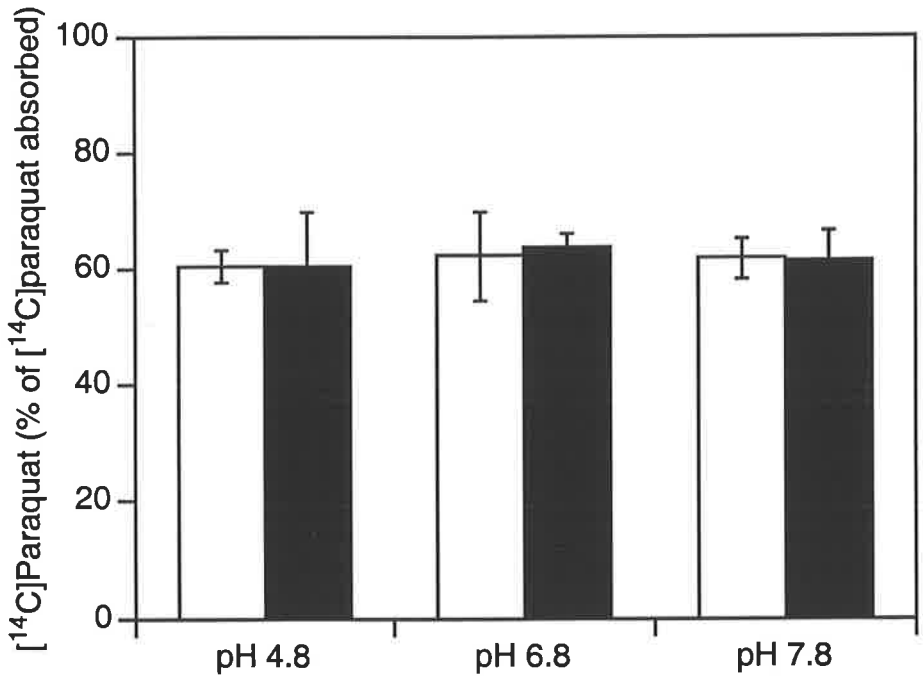


Figure 4.7: The effect of pH on paraquat bound to cell walls isolated from resistant (closed bars) and susceptible (open bars) biotypes of *H. glaucum*. The cell wall preparations were incubated at pH 4,8; 6.8; or 7.8. Data points represent means of 4 replicates ±SE.

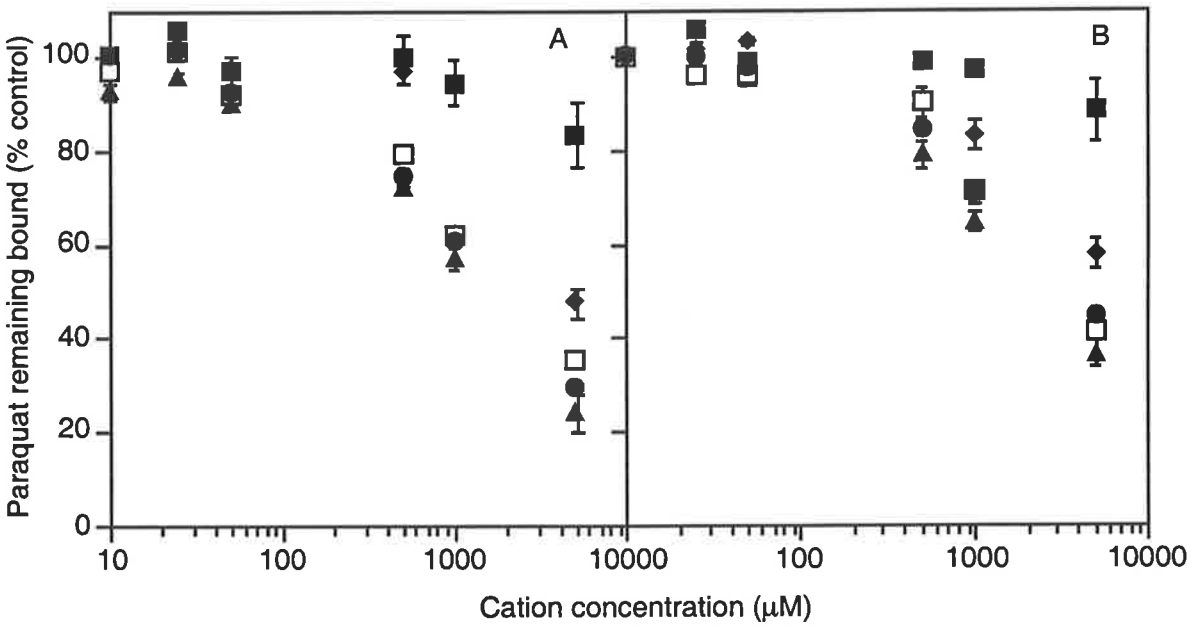


Figure 4.8: Competition between bound ^{14}C-paraquat and the cations unlabelled paraquat (▲), benzyl viologen (□), putrescine (●), calcium (◆), potassium (■) in isolated cell walls of susceptible (A) and resistant (B) biotypes of *H. glaucum*. Data points represent means of 2 experiments with 4 replicates ±SE.

cell walls isolated from both resistant and susceptible biotypes, paraquat, benzyl viologen, and putrescine were equally effective at displacing bound paraquat. Calcium was slightly less effective at displacing bound paraquat, and K^+ was ineffective at displacing bound paraquat. The addition of 500 μM divalent cation removed about 20% of the bound paraquat and addition of 5 mM divalent cations removed 70% of the bound paraquat. The bound paraquat was slightly more easily removed from cell walls of the susceptible biotype than the resistant biotype. However, it is clear that paraquat exchanges slowly from tight binding sites within the cell walls of both biotypes.

4.4. **Discussion**

Binding of paraquat to the cell walls of the resistant biotype of *H. glaucum* has been suggested to be a possible mechanism of resistance to paraquat. If there was such increased binding in the resistant biotype this would lead to sequestration of herbicide outside of the cell and, therefore, there would be less paraquat available to enter to its active site in the chloroplasts. Bishop *et al.* (1987) suggested that enhanced paraquat binding in a resistant biotype of *H. glaucum* might be the reason for exclusion of paraquat from the active site. In contrast, studies on an isolated cell wall fraction from *C. bonariensis* indicated no differences in ^{14}C-paraquat binding between resistant and susceptible biotypes (Fuerst *et al.*, 1985). Similar studies with cell wall preparations from *Arctotheca calendula* also established no differences (Preston et al., 1994b). Paraquat is a highly polar di-cation and can easily bind to cell walls which have large numbers of negatively charged binding sites due to the presence of de-esterfied galacturonans (Baydoun and Brett, 1988). Paraquat binding to the cell wall can vary with plant species. Twice as much paraquat was bound to the cell wall of *R. glutinosa* leaves than to the cell wall of corn or soybean leaves (Chun *et al.*, 1997a). However, these authors also reported isolation of an extract from cell

walls able to bind paraquat (Chun *et al.*, 1997b), and this material may contribute extensively to binding of paraquat. In the experiments reported here, paraquat was found to bind strongly to cell wall preparations from leaves of resistant and susceptible biotypes (Figs. 4.1, 4.2, 4.3). When paraquat was applied to the surface of leaves, the amount of paraquat bound to the cell walls was about 45% of the herbicide absorbed by leaves (Fig. 4.3). This increased considerably when leaf tissue was mechanically disrupted and the cell contents removed before addition of ^{14}C-paraquat (Fig. 4.1). This increased binding may be due to the fact that the amount of free space or the number of binding sites available to the herbicide was increased following destruction of the plasma membrane.

Paraquat binding to the cell wall is a major obstacle for translocation of this herbicide to its site of action. This is an important factor that has to be considered in any efflux experiment. Hart *et al.* (1992b) suggested that cell wall-bound paraquat, in maize roots, desorbed considerably faster than herbicide compartmentalized within the cytoplasm or vacuole and used this as a means to estimate the amount of paraquat within the apoplast. However, from the experiments reported in this Chapter (Figs. 4.1, and 4.8), it is clear that most of the applied paraquat is bound tightly to the cell walls and is not easily exchangeable with solution without the presence of strong competition from other cations.

Paraquat was also bound to membrane fractions and cell wall associated proteins (Figs. 4.1-4.3). However, there was no significant difference between resistant and susceptible biotypes in binding to membrane and the cell wall associated proteins. Therefore, the possibility of paraquat being preferentially bound to membrane negative charge sites and/or cell wall associated proteins was ruled out. There were no large differences in binding of paraquat to either

cell walls or any soluble constituent of the cell walls that may be responsible for increased binding in the resistant biotype.

The mechanism of resistance to paraquat in *H. glaucum* has been shown to be temperature dependent (Purba et al, 1995; Chapter 2). This temperature sensitivity provides a good tool to examine the possible relationship between cell wall binding and mechanism of resistance. Temperature had no effect on binding capacity of cell walls of either the resistant or susceptible biotypes. The effect of temperature on binding was also studied by Lasat *et al.* (1996) when they estimated the amount of paraquat bound to the cell walls of *H. glaucum* by measuring paraquat released to the external solution under warm and cool conditions. They were not able to find any differences between paraquat binding to root cell walls of resistant and susceptible biotypes at either temperature.

In concentration-dependent binding experiments, cell wall fractions prepared from leaves of resistant and susceptible biotypes were incubated in concentrations up to 5 mM ^{14}C-paraquat. The amount of paraquat binding to the soluble and insoluble parts was then measured. The kinetics of ^{14}C-paraquat binding were similar in both biotypes (Fig. 4.4). The binding in both resistant and susceptible biotypes was increased with increasing concentrations of herbicide and was not saturated up to 5 mM. This demonstrates a strong capacity of cell walls to bind paraquat. Paraquat binding to the cell walls of maize roots has been demonstrated to be linear (Hart *et al.*, 1992b). These authors showed that concentration-dependent kinetics for paraquat influx were non-saturating up to 1 mM with a linear component supposedly representing interaction of paraquat with the cell wall. The kinetics of paraquat binding to cell walls were also reported to be the same in roots of resistant and susceptible biotypes of *H. glaucum* (Lasat *et al.*, 1996).

A plot of paraquat bound against paraquat bound/free demonstrated two different types of binding sites with different affinities for paraquat in both biotypes. Much of the considerable binding of paraquat to cell walls can be attributed to the low affinity-binding site. Such paraquat is also more likely to be more easily exchanged off the cell wall by other cations.

Paraquat bound to the cell walls can exchange with the other cations in the solution. However, most paraquat bound was not exchangeable at lower concentrations of cations (Fig. 4.8). The original concentration of ^{14}C-paraquat loaded into the cell walls was about 10 μM. About 20% of the bound paraquat remained strongly bound to the cell walls even when the concentration of other divalent cations reached 5 mM. This may be due to some ^{14}C-paraquat being irreversibly trapped and bound to the cell walls and not able to exchange with other cations in the solution. Cold paraquat, putrescine and benzyl viologen were demonstrated to be good competitors for bound paraquat while calcium was less effective, and K^+ was generally ineffective at removing bound paraquat. Generally, there were few differences between resistant and susceptible biotypes in the ability of cations to exchange with bound paraquat. However, the susceptible biotype showed slightly better exchange capacity at higher cation concentrations than the resistant biotype.

This study of paraquat binding to cell walls *in vivo* and *in vitro* has demonstrated that considerable quantities of applied paraquat become tightly bound to cell walls. This paraquat is clearly unavailable for action within the cell. This large binding of paraquat to cell walls will clearly mask the ability to determine uptake of paraquat into cells and distribution within cell organelles if the cell wall is still present. The studies of Hart *et al.* (1992b) and Lasat *et al.* (1996) which have attempted to determine the distribution of paraquat within cell organelles by efflux experiments attributed the slowest eluting phase to the vacuole. However, given

the large and tight binding of paraquat to cell walls, this slow effluxing phase must be, at least in part, the result of paraquat binding to the cell wall. The outcome of the present experiments has been to establish that increased binding of paraquat to cell walls is not the mechanism of resistance in *H. glaucum*.

Chapter 5

Paraquat uptake into protoplasts of resistant and susceptible biotypes of *H. glaucum*

5.1. **Introduction**

In Chapter 4, it was demonstrated that enhanced paraquat binding to cell walls in the resistant biotype of *H. glaucum* is not the reason for exclusion of paraquat from the active site. Because the cell wall has large numbers of negatively charged binding sites to which paraquat strongly binds, protoplasts appear to be a convenient and more homogeneous system to study paraquat transport. The protoplast is a naked plant cell, surrounded by the plasma membrane, which lies within the cell wall and can be isolated by removing the cell wall (Vasil, 1976). Isolated protoplasts are a useful tool in the laboratory to study the absorption of a variety of chemicals by cells. One of the advantages of using protoplasts in absorption studies as indicated above is the lack of cell wall material. Quantitative measurement of solute can be achieved without the complications of the free space or apoplastic volume. Plant protoplasts have been used in studies of auxin metabolism (Delbarre *et al.*, 1994), triazine herbicide movement (Darmstadt *et al.*, 1984; McCloskey and Bayer, 1990), and paraquat transportation (Hart *et al.*, 1993; Norman *et al.*, 1993; Powles and Cornic, 1987).

Paraquat exits as a divalent cation in solution, and therefore, direct diffusion of paraquat across the plasmalemma lipid bilayer is unlikely. Studies of animal tissue and some plants suggest that paraquat transports actively across cell membranes and its transport across the membrane is facilitated by a carrier that normally functions in transport of polyamines (Smith and Wyatt, 1981; Hart *et al.*, 1992a). Active transport requires energy from metabolic processes to move a molecule across a membrane against its electrochemical potential (Nobel, 1991).

The presence of paraquat at the active site (PSI) can be detected by measuring photosynthetic activity. Therefore, photosynthetic parameters have been used to examine the effects of paraquat in leaves of resistant and susceptible biotypes of

H. glaucum and *H. leporinum* (Bishop *et al.*, 1987; Preston *et al.*, 1992b), *A. calendula* (Preston *et al.*, 1994), *Conyza bonariensis* (Fuerst *et al.*, 1985; Norman *et al.*, 1993), and *Solanum americanum* (Chase *et al.*, 1998a; 1998b).

In *H. glaucum*, the mechanism of resistance to paraquat was suggested to be sequestration of paraquat away from its site of action by enhanced apoplastic binding which results in reduced transport of paraquat into the cytoplasm (Bishop *et al.*, 1987). However, this mechanism was not observed in studies examining isolated cell walls (Chapter 4). Another possibility for conferring resistance would be a change at one of the plant cell membranes which results in inhibiting net movement of paraquat across the membrane, thereby sequestering it from its site of action. Preston *et al.* (1992b) reported that translocation of paraquat from older leaves to younger leaves was reduced by more than 50% in a resistant biotype of *H. glaucum*, compared to a susceptible biotype. In this study, paraquat that was translocated in the resistant biotype did not reach its site of action in chloroplasts as no reduction in photosynthetic activity was observed in the resistant biotype compared to a 62% reduction of photosynthesis in a susceptible biotype. Preston *et al.* (1992b) proposed that a barrier at the plasmalemma may be the mechanism of resistance. However, Powles and Cornic (1987) showed that the effects of paraquat on intact chloroplast and protoplasts isolated from leaves of *H. glaucum* did not differ between resistant and susceptible biotypes. In addition, isolated protoplasts of resistant and susceptible *C. bonariensis* have also shown no differences in response to paraquat (Norman *et al.*, 1993).

The aim of the experiments in this chapter was to study the movement of paraquat across the plasmalemma of protoplasts prepared from leaves of resistant and susceptible biotypes of *H. glaucum*.

5.2. **Materials and Methods**

5.2.1 *Isolation of protoplasts*

Protoplasts were prepared according to a method modified from Kaiser *et al.* (1982). Seedlings of the resistant and susceptible biotypes of *H. glaucum* were grown for about 2 weeks in a growth chamber with a 12h 15°C light/12 h 10°C dark cycle as described in Chapter 3. Protoplasts were prepared from freshly harvested leaves. The leaves were manually sliced into approximately 1-mm segments with a new razor blade under solution A, which contained 0.5 M sorbitol, 1 mm $CaCl_2$, 0.1% BSA, 0.05% PVP, 5 mM MES adjusted to pH 5.5 with KOH. The leaf segments were floated on the surface of solution A plus 0.7 % Cellulase "Onozuka" R-10 (Yakult, Tokyo, Japan) and 0.05% Macerozyme R-10 (Yakult, Tokyo, Japan) adjusted to pH 5.5 with KOH. The leaf segments were digested for 3 hours without agitation at room temperature under lights. At the end of the digestion period, the enzyme solution was removed and discarded. The leaf segments were washed twice with solution B containing 0.5 M sorbitol, 1 mM $CaCl_2$, 5 mM MES, pH 6.0 (KOH) and filtered through a nylon filter to remove undigested materials. The crude protoplast preparation was transferred into 15-ml centrifuge tubes and centrifuged at 100 xg for 5 min. The supernatant was discarded and pellets were resuspended by gentle shaking in a small amount of solution B. Protoplasts were kept on ice in subsequent procedures of purification and storage. The protoplasts were purified by floating through a stepped gradient of Percoll (Pharmacia). Five ml of 30% Percoll solution was added to each tube and thoroughly mixed. Three ml of 20% Percoll solution was then layered onto this, followed by 1 ml solution B without Percoll. After centrifugation at 250 xg for 10 min, the purified protoplasts were collected from a band between the 20% Percoll layer and upper layer with an enlarged-bore disposable pipette.

The number and viability of protoplasts was determined by using a hemocytometer and fluorescein diacetete (FDA). The FDA mainly accumulates in the cytoplasm of intact protoplasts. The purified protoplast preparations were examined with a light microscope and were found to be free of cellular debris and microorganisms. When stained with FDA, more than 90% of the protoplasts brightly fluoresced a yellow green colour indicating that the plasmalemma of the protoplasts was intact.

5.2.2 *O_2 evolution measurements*

O_2 evolution of protoplasts of resistant and susceptible biotypes was measured using a Clark-type O_2 electrode. O_2 evolution of protoplasts at 25°C was measured by adding 200 µl of protoplast suspension to 1 ml of assay medium which contained 0.4 M Sorbitol, 1 mM $CaCl_2$, 15 mM Tricine-KOH (pH 7.5) plus 10 mM $NaHCO_3$ in the oxygen electrode. To determine the effect of paraquat on O_2 evolution by protoplasts, protoplasts were incubated in the dark for 10 min with paraquat. Where other cations were used, they were added with the paraquat. The rate of O_2 evolution was determined after illuminating for 5 min at 1000 µE m^{-2} s^{-1}. Chlorophyll concentration was determined as described by Arnon (1949).

5.2.3 *Uptake of ^{14}C-paraquat into protoplasts*

Uptake of ^{14}C-paraquat into the protoplasts was measured in 400-µl centrifuge tubes using a silicone oil centrifugation technique. The experiment was started by adding 50 µl of protoplasts to 400 µl microtubes already containing, from bottom to top: 10 µl of Dow 550 fluid (silicon oil)(Dow Corning), 100 µl of 4:1 (v/v) AR200 (Fluka): Dow 510 fluid, and 150 µl of uptake solution containing the appropriate concentrations of ^{14}C-paraquat and 2 mM other cations if desired.

Unless otherwise stated, the uptake period was 10 s. Uptake was stopped by centrifuging the tubes at 3500 xg for 40 s in a Microfuge. This pelleted the protoplasts through the upper silicone oil layer. The microtubes were snapfrozen in liquid nitrogen and the tips, containing protoplasts pellets, were cut off the frozen tubes and placed in scintillation vials. Benzoyl peroxide/toluene was added to the vials and the vials sonicated to disrupt the pellet. The benzoyl peroxide acted to bleach the protoplasts to reduce quenching effects during LSS. Scintillant (Ultima Gold-XR) was added to the vials and radioactivity associated with the pellet determined by LSS.

5.3. **Results**

5.3.1 *Paraquat inhibition of O_2 evolution*

The presence of paraquat in the chloroplast in the light results in production of a paraquat radical that reacts with O_2 producing superoxide, which reduces net O_2 evolution (Preston *et al.*, 1992b). Measurement of net O_2 evolution was used to estimate the presence of paraquat in the chloroplasts of protoplasts prepared from leaves of resistant and susceptible biotypes of *H. glaucum*. O_2 evolution in protoplasts of resistant and susceptible biotypes was measured in concentrations of 2 to10 mM paraquat (Fig. 5.1). Increasing the concentration of paraquat inhibited O_2 evolution of intact protoplasts; however, very high paraquat concentrations were required to fully inhibit O_2 evolution. O_2 evolution was inhibited by 50% with 6 mM paraquat. In contrast, the same level of inhibition was obtained in isolated chloroplasts of this biotype with about 7 μM paraquat (Powles and Cornic, 1987). This demonstrates that paraquat is much less effective when applied to protoplasts compared with chloroplasts. Despite this,

there were no appreciable differences between protoplasts of resistant and susceptible biotypes in inhibition of O_2 evolution by paraquat (Fig. 5.1).

In the above experiment, O_2 evolution was measured in the presence of 1 mM $CaCl_2$. Calcium is required for stability of membranes, but has been shown to inhibit the uptake of paraquat into protoplasts of maize roots (Hart *et al.*, 1993). Therefore, the effect of a single dose of paraquat (500 μM) in the presence of different concentrations of Ca^{2+} was measured. Ca^{2+} had a protective effect on inhibition of O_2 evolution by paraquat (Fig. 5.2). Increasing concentrations of Ca^{2+} increased O_2 evolution of protoplasts in the presence of paraquat for both resistant and susceptible biotypes. At 0.05 mM Ca^{2+}, 500 μM paraquat inhibited O_2 evolution of protoplasts by about 70%. Increasing the concentration of Ca^{2+} to 5 mM reduced this inhibition to about 35%. There were no differences between protoplasts isolated from resistant and susceptible plants in the ability of Ca^{2+} to protect against paraquat damage.

5.3.2 *Time-course of paraquat accumulation in protoplasts*

When protoplasts from resistant and susceptible biotypes were incubated with 100 μM of ^{14}C-paraquat for varying times, paraquat accumulation in protoplasts increased in a time-dependent manner and was saturated after 2 min (Fig. 5.3). Paraquat uptake was very rapid with about half the maximum absorption occurring within 10 s. The time course of paraquat absorption by protoplasts isolated from resistant and susceptible plants was similar.

5.3.3 *Paraquat uptake into protoplasts*

Paraquat absorption by mesophyll protoplasts prepared from resistant and susceptible leaves of *H. glaucum* is shown in Fig. 5.4. Protoplasts were

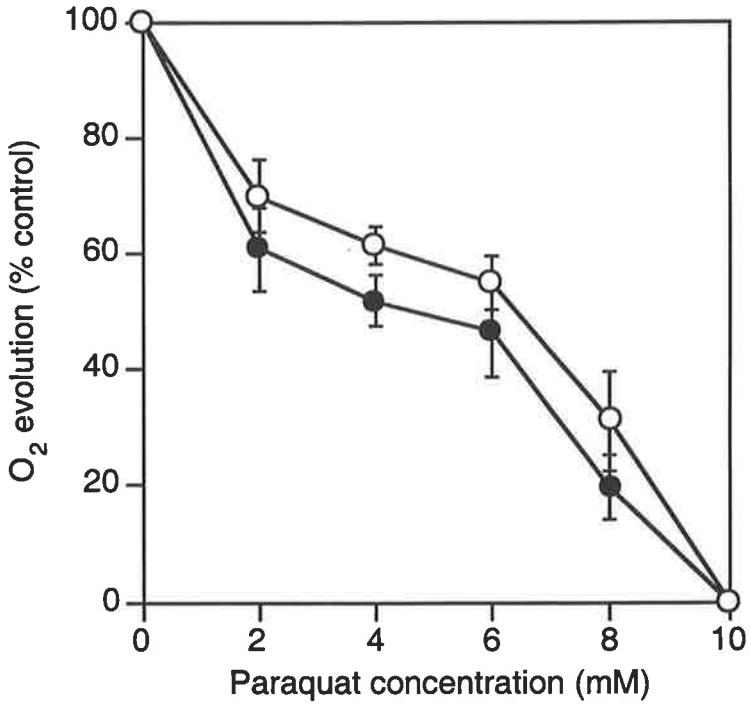


Figure 5.1: Inhibition of O_2 evolution by paraquat in protoplasts prepared from resistant (●) and susceptible (O) biotypes of *H. glaucum*. Each point represents the mean of three experiments ±SE.

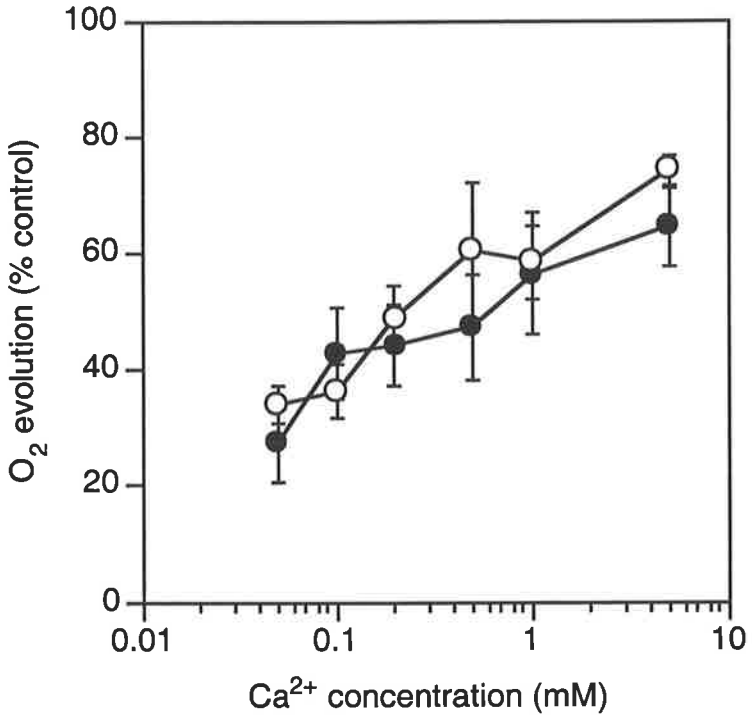


Figure 5.2: Effect of varying concentrations of $CaCl_2$ on inhibition of O_2 evolution by paraquat in protoplasts of resistant (●) and susceptible (O) biotypes of *H. glaucum*. Data points represent the means of two experiments ±SE.

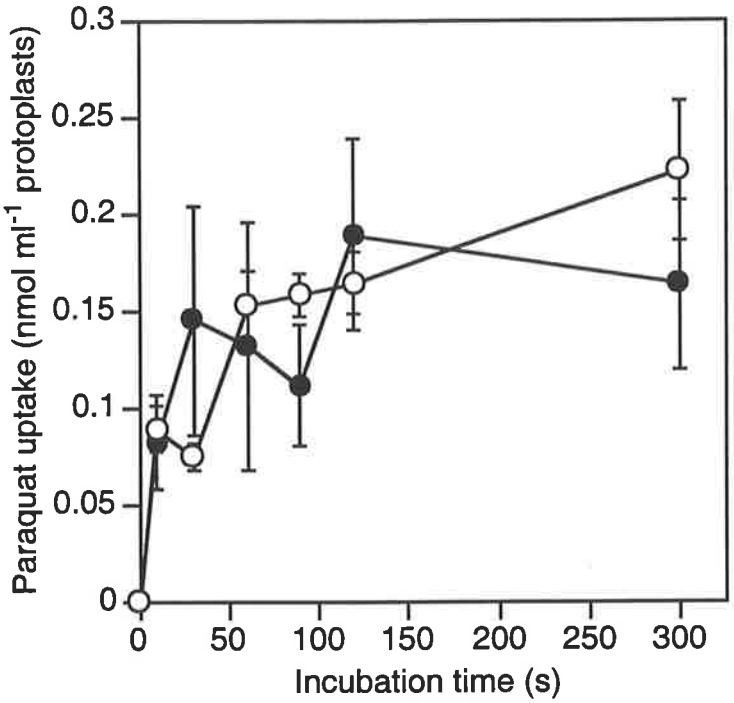


Figure 5.3: Time-course of ^{14}C-paraquat uptake in protoplasts of resistant (●) and susceptible (O) biotypes of *H. glaucum*. Protoplasts were incubated with 100 μM ^{14}C-paraquat and uptake was measured at different time intervals. Each point represents the mean of three experiments ±SE.

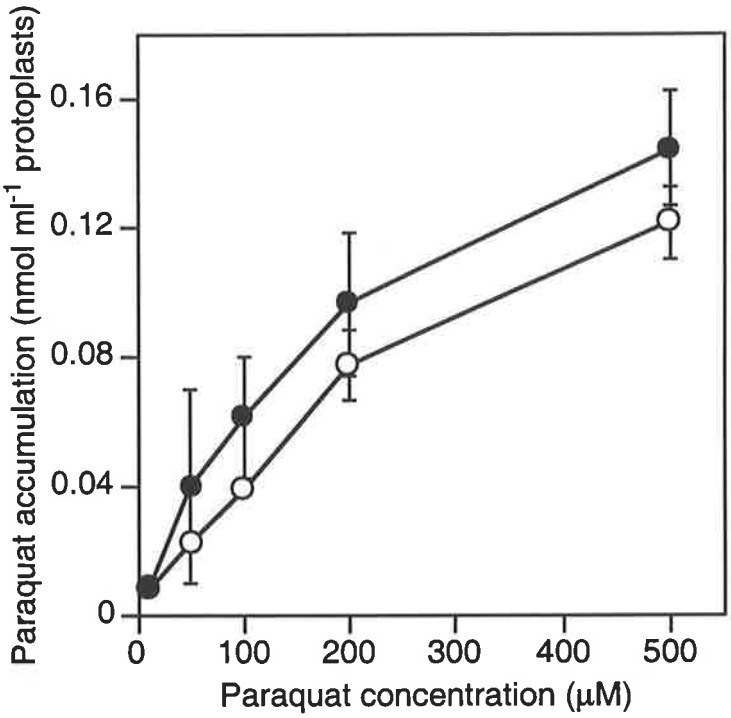


Figure 5.4: Concentration-dependent uptake of paraquat into protoplasts of resistant (●) and susceptible (O) biotypes of *H. glaucum*. The protoplasts were incubated in varying concentrations of ^{14}C-paraquat for 10 s before centrifugation through silicon oil. Each point represents the mean of two experiments, each containing duplicate samples, ± SE.

incubated in varying concentrations of ^{14}C-paraquat and were separated from the solution using the silicon oil separation method. Accumulation of ^{14}C-paraquat into the protoplasts increased with increasing concentrations of paraquat. However, there were no obvious differences in accumulation of paraquat into protoplasts of resistant compared to susceptible biotypes other than a general tendency for more paraquat to accumulate in protoplasts from resistant plants.

Further experiments were also performed incubating protoplasts with Ca^{2+} and putrescine to determine the effect of these cations on uptake of paraquat into the protoplasts. Divalent cations have been shown to compete with paraquat and non-competitively inhibit its uptake into plant cells (Hart *et al.*, 1992b; 1993). Both Ca^{2+} and putrescine inhibited uptake of ^{14}C-paraquat into the protoplasts; however, putrescine was a more effective inhibitor (Fig. 5.5). Addition of Ca^{2+} and putrescine together was no more effective at inhibiting paraquat uptake than the addition of putrescine alone. The addition of cations inhibited paraquat uptake in protoplasts isolated from both resistant and susceptible biotypes.

5.4. **Discussion**

Uptake of paraquat has been examined extensively in leaves of resistant and susceptible plants of *H. glaucum* (Bishop *et al.*, 1987; Preston *et al.*, 1992b). However, the presence of the cell wall that binds considerable quantities of paraquat (Chapter 4) could interfere with such studies. Therefore, protoplasts were re-examined as a model system to study paraquat absorption by cells.

Paraquat is a photosystem I electron acceptor that causes rapid membrane damage by accepting electrons and transferring these electron to oxygen (Summer, 1980). Therefore, measurement of photosynthetic activity is a useful

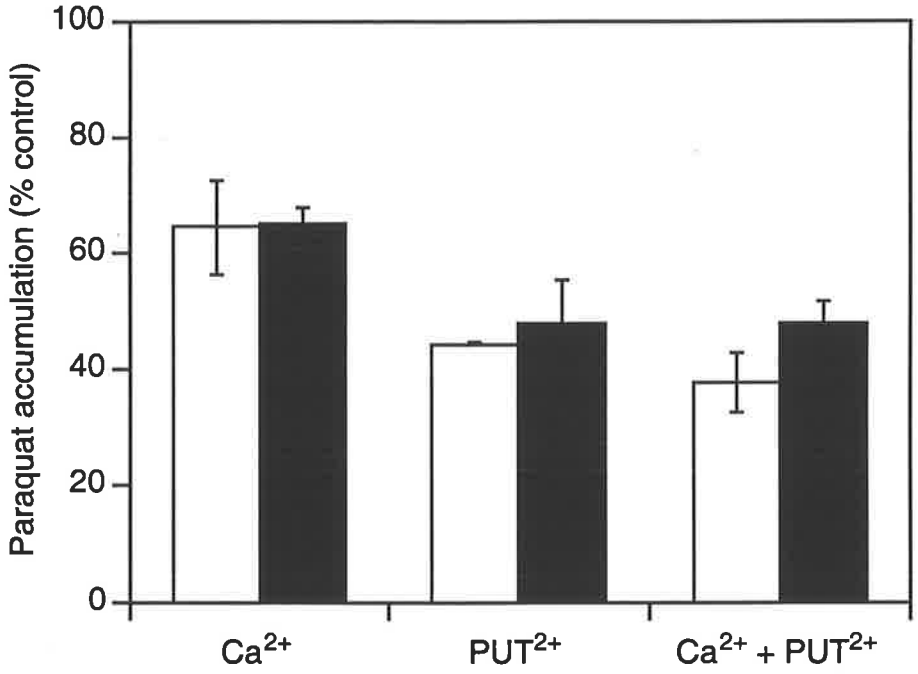


Figure 5.5: Effect of 2mM putrescine and 2 mM $CaCl_2$ on paraquat (100μM) accumulation into protoplasts of resistant (solid bars) and susceptible (open bars) biotypes of *H. glaucum*. Data are means ± SE of three replicates.

method of estimating paraquat appearance at its active site in the chloroplasts (Preston *et al.*, 1992b; Preston, 1994; Chase *et al.*, 1998a). In concentration-dependent experiments, increasing concentrations of paraquat inhibited O_2 evolution by protoplasts from resistant and susceptible plants (Fig. 5.1). However, a paraquat concentration of 10 mM was needed to completely inhibit O_2 evolution while complete inhibition of photosynthesis in chloroplasts was obtained by concentrations of paraquat as low as 15 μM (Powles and Cornic, 1987). The results obtained here confirm those of Powles and Cornic (1987) in that a high concentration of paraquat is needed to inhibit O_2 evolution by isolated protoplasts. Also, complete inhibition of photosynthesis in intact leaves of resistant biotypes was obtained when the concentration of paraquat inside the leaf was about 0.8 mM (Bishop *et al.*, 1987). It seems that protoplasts of *H. glaucum* are generally much more resistant to paraquat than are chloroplasts or leaf slices. The same conclusion was also reached by Norman *et al.* (1993) for *Conyza bonariensis.* These authors reported that paraquat-induced chlorosis (an indicator of paraquat sensitivity) was similar between biotypes for chloroplasts and protoplasts of *C. bonariensis.* However, leaf sections of the resistant plants exhibited an 81-fold level of resistance similar to the 100-fold level observed in whole plants. Chl_{50} values for protoplasts from resistant and susceptible biotypes were 72 μM and 71 μM compared with 24.3 μM and 0.3 μM for leaf slices, respectively (Norman *et al.*, 1993).

Paraquat is a highly polar divalent cation and its fast penetration into cells is most likely through active transport across membranes. There have been reports suggesting that paraquat may be carried across membranes by transport proteins that normally carry other cations such as polyamines (Byers *et al.*, 1987; Hart *et al.*, 1992a). Therefore, the low sensitivity of protoplasts to paraquat compared with chloroplasts and the whole leaf, and its general resistance to this herbicide may be attributed to protoplasts lacking the cell wall. A structurally

intact cell wall may be required for a carrier located in the plasmalemma to effectively transport paraquat from the apoplast into the cytoplasm (Norman *et al.*, 1993). The low sensitivity of protoplasts may also be due to low permeability of the plasmalemma to paraquat compared with higher permeability of the chloroplast envelope. Ca^{2+} may also be having an impact on binding sites on the plasmalemma membrane. For example, in carrot protoplasts, it has been suggested that Ca^{2+} may compete with spermidine for the same anionic sites involved in transporting spermidine into the protoplasts (Pistocchi *et al.*, 1988).

Ca^{2+} is essential for membrane stability and has been reported to inhibit uptake of paraquat into root cells of maize (Hart *et al.*, 1993) as well as influencing the transport processes of polyamines (Antognoni *et al.*, 1995; Pistocchi and Bagni, 1990). Increasing concentrations of Ca^{2+} resulted in a reduction in the impact of paraquat on O_2 evolution in protoplasts of both resistant and susceptible plants (Fig. 5.2). It has been speculated that Ca^{2+} concentrations within the cell are highly regulated, therefore, its inhibitory effect may be the result of its known effects on ionic channels, ATPases or other enzymes in membranes (Antognoni *et al.*, 1993). Ca^{2+} and putrescine also had an inhibitory effect on accumulation of paraquat into the protoplasts. Accumulation of ^{14}C-paraquat into protoplasts of both resistant and susceptible plants was reduced considerably when they were incubated in the presence of putrescine or mixtures of putrescine and Ca^{2+} (Fig. 5.5). This inhibition of paraquat uptake indicates that putrescine and Ca^{2+} can interfere with paraquat movement across the plasmalemma. Ca^{2+} alone had a lesser effect on inhibition of paraquat uptake. Ca^{2+} had no measurable impact on increasing the impact of putrescine on uptake as was reported in carrot protoplasts (Antognoni *et al.*, 1993). The same interfering effect of putrescine and Ca^{2+} on paraquat uptake has been also reported in maize protoplasts and intact maize roots (Hart *et al.*, 1992b; 1993).

Uptake of paraquat by protoplasts appeared to follow saturation-type kinetics (Fig. 5.4), suggesting that the transport process was carrier mediated. Carrier-mediated transport of paraquat has been shown for cells of some plants and animal tissue (Hart *et al.,* 1992a; Smith and Wyatt, 1981). A large amount of ^{14}C-paraquat accumulated during the first 20 s of incubation (Fig. 5.3). This indicates that the initial rate of paraquat uptake into protoplasts is rapid. Hart *et al.* (1993) reported that most of the paraquat accumulated into protoplasts of maize suspension cultures occurred during the first 10 s of incubation. This fast uptake of paraquat across the plasmalemma may be due to the initial electrochemical potential gradient for paraquat transport across the plasmalemma which is substantial (Hart *et al.*, 1993).

The proposed sequestration mechanism of resistance is not demonstrated to be the result of any change in plasmalemma membranes in resistant biotypes as there were no or little differences in response to paraquat between protoplasts from the two biotypes. However, given the general tolerance of protoplasts to paraquat, particularly in the presence of Ca^{2+}, questions must be raised about the general suitability of protoplasts for these studies. Other methods may be required to clarify the role of the plasmalemma in paraquat resistance.

Lasat *et al.* (1997) have proposed an alternative hypothesis that resistance in *H. glaucum* is due to sequestration of paraquat in the vacuole. The numerous attempts to isolate intact vacuoles from *H. glaucum* leaves were unsuccessful. Therefore, this hypothesis could not be tested explicitly.

Chapter 6

General discussion

Paraquat has been widely used in agriculture for more than 30 years for weed control prior to crop sowing and to control weeds in perennial cropping systems. As a result of the intensive use of paraquat, resistance has appeared in populations of 27 species of weeds. All previously-reported cases of paraquat resistance have been observed in perennial cropping operations where weeds have evolved resistance following intense selection pressure from up to several applications of paraquat per year (reviewed by Preston, 1994). In Australia, all previously reported paraquat resistant populations have been found in lucerne crops in southern Australia following a long history of once annual paraquat use.

In 1994 failure of paraquat to control *Hordeum glaucum* from two fields devoted to continuous cereal cropping was reported. These fields had a long history of use of paraquat and diquat to control weeds in a zero tillage system. Both populations of *H. glaucum* proved highly resistant to paraquat (Chapter 2). This is the first report, world-wide, of paraquat resistance in a weed species from the most common usage of paraquat, zero- and minimum-tillage annual cropping. The occurrence of paraquat resistant biotypes in this zero-tillage system is a direct consequence of farmers being dependent strongly on such herbicides for weed management in these systems. The populations of *H. glaucum* examined here demonstrate that paraquat resistance is not restricted to perennial cropping systems, but can occur anywhere paraquat is used intensively. The paraquat resistant biotypes are controlled by herbicides with different modes of action such as fluazifop-p-butyl, or glyphosate (Chapter 2).

The mechanism of resistance to paraquat in *H. glaucum* is unknown but is correlated with reduced translocation of paraquat (Preston *et al.*, 1992b). It has previously been established that paraquat resistance in *H. glaucum* and *H. leporinum* demonstrates a dependence on temperature (Purba *et al.*, 1995). The resistant populations are much less tolerant of paraquat at higher temperatures

compared to low temperatures. The reduction in resistance at high temperatures was demonstrated to be the result of increased translocation of paraquat (Purba *et al.*, 1995). This temperature dependence of paraquat resistance appears restricted to grass species as no temperature dependence was observed in resistant populations of *Arctotheca calendula* (Purba and Preston, unpublished results) or *Conyza bonariensis* (Preston, unpublished results). This temperature dependence of paraquat resistance provides a useful test of similarity of mechanisms of resistance. The two biotypes of *H. glaucum* from zero-till cropping demonstrated temperature dependence of paraquat resistance as previously known paraquat resistant biotypes from lucerne crops did (Chapter 2). In essence, the paraquat resistance mechanism is relatively ineffective at high temperatures in all resistant biotypes of *H. glaucum*.

Previous studies have demonstrated that paraquat resistance in *H. glaucum* is not the result of altered activity of the herbicide at its target site, photosystem I (Powles and Cornic, 1987). Decreased cuticular penetration of paraquat and reduced import of paraquat into the chloroplast were also eliminated as possible mechanisms of resistance in *H. glaucum* (Bishop *et al.*, 1987; Powles and Cornic, 1987). Enhanced activities of superoxide dismutase or other oxygen radical detoxifying enzymes have been proposed as a mechanism of resistance to paraquat in other species (Shaaltiel and Gressel, 1986), but have been eliminated as a potential resistance mechanism in *H. glaucum* (Powles and Cornic, 1987). Previous work has also established that paraquat does not appear at the active site within the chloroplast as readily in the resistant population of *H. glaucum* (Preston*et al.*, 1992b) despite similar absorption of paraquat through the leaf cuticle (Bishop *et al.*, 1987). Therefore, it seems that the main reason for resistance to paraquat lies in a reduction in the movement of paraquat from intercellular spaces in the leaf to the chloroplast. Paraquat may be prevented from entering to the cell by either enhanced apoplastic binding to cell wall

material, decreased uptake into cells, and/or sequestration in the vacuole. These possible mechanisms of resistance were examined in this thesis.

Research with animal and plant tissue has established that paraquat may enter animal and plants cells on a transporter that normally transports polyamines such as putrescine (Smith and Wyatt, 1981; Hart *et al.*, 1992a). The work of Hart *et al.* (1992a) has established that polyamines and paraquat are competitive inhibitors with a high polyamine concentration restricting the transport of paraquat, and vice versa. From this research, it is possible to hypothesise that, in plants, paraquat may be transported across cell membranes by a polyamine transporter. A possible resistance mechanism could be a change in the polyamine transporter that reduces the efficiency of the paraquat transporter, thereby restricting paraquat entry into the cell. Equally, if the plant had significantly higher constitutive or induced levels of polyamines in the apoplast, this would restrict paraquat entry into the cell. It was not possible to study the polyamine transporter as it has not yet been characterised. However, it was possible to establish if there are higher polyamine levels in leaves of paraquat resistant plants. It is apparent from Chapter 3 that constitutive putrescine contents are generally higher in resistant biotypes compared to susceptible biotypes of *H. glaucum* and *H. leporinum* although there was considerable variation amongst biotypes (Chapter 3). Higher constitutive putrescine content has also been reported in leaves of some paraquat-resistant biotypes of *Conyza canadensis* and *Conyza bonariensis* and suggested to play a role in paraquat resistance in these species (Szigeti *et al.*, 1996; Ye *et al.*, 1997). The application of paraquat to both resistant and susceptible plants of *H. glaucum* and *H. leporinum* resulted in an increase in putrescine content of leaf tissue (Chapter 3). Paraquat induced putrescine contents to higher levels in resistant compared to susceptible plants.

While increased putrescine content in the apoplast would tend to decrease paraquat uptake in resistant biotypes of *H. glaucum* and *H. leporinum*. This does not appear to be a primary paraquat resistance mechanism in *Hordeum* spp. There was a poor correlation between constitutive leaf putrescine content and the degree of resistance to paraquat measured by GR_{50} (Chapter 3). In addition, induction of putrescine was also observed following application of oxyfluorfen, a herbicide of different mode of action that also imposes oxygen radical stress and to which the paraquat resistant biotypes are not resistant (Chapter 3).

The higher putrescine content in resistant biotypes may be due to a change in the carrier responsible for influx of putrescine into the cell or vacuole, or efflux from the cell or vacuole. A change in the carrier might lead to increased accumulation of both putrescine and paraquat either in the vacuole or outside the cell. Other factors are also likely to influence this higher putrescine content in resistant biotypes. These include a change in the activity of polyamine biosynthetic enzymes, which usually increase in response to different stress (Flores and Galston, 1982a; Young and Galston, 1983). For example, it was recently reported that a greater activity of polyamine biosynthetic enzymes resulted in production of more putrescine in a paraquat resistant biotype of *C. bonariensis* (Ye *et al.*, 1997). Another possibility is that the mutation providing resistance to paraquat imposes some other stress on the resistant plants, which respond by increasing putrescine contents.

In Chapter 4, the possibility of enhanced binding of paraquat to cell wall components was examined as a possible reason for exclusion of paraquat from the cell. Increased binding in the resistant biotype would lead to sequestration of herbicide outside of the cell and, therefore, there would be less paraquat available to enter to the active site in the chloroplasts. The study of paraquat binding to cell walls *in vivo* and *in vitro* has demonstrated that considerable

quantities of applied paraquat become tightly bound to cell walls. Paraquat binding increased considerably following removal of cell contents by mechanical isolation. This increased binding may occur because the amount of free space or the number of binding sites available to paraquat was increased following destruction of the plasma membrane. Paraquat was also found to bind to the membrane fractions and cell wall associated proteins (Chapter 4). However, there were no significant differences between resistant and susceptible biotypes in binding to membrane, cell wall, or the cell wall associated proteins. The results obtained in Chapter 4 are in agreement with other reports that differential paraquat binding to the cell wall does not account for resistance in other species (Norman *et al.*, 1993; Preston *et al.*, 1994; Lasat *et al.*, 1996).

Paraquat, due to its negative charge, binds strongly to the cell wall and is not easily exchangeable without the presence of strong competition from other cations. High concentrations of other divalent cations were required to release bound paraquat. Paraquat, benzyl viologen, and putrescine were the most effective competing cations. However, not all of the paraquat bound can be exchanged with the solution. About 20% of the bound paraquat remained strongly bound to the cell walls even when the concentration of other divalent cations was high (Chapter 4). This may be the result of paraquat being irreversibly trapped and bound to the cell walls and not able to exchange with other cations in the solution. The kinetics of ^{14}C-paraquat binding were similar in resistant and susceptible biotypes (Chapter 4) and displayed two different types of binding sites.

Paraquat binding to the cell wall is a major obstacle for translocation of this herbicide to its site of action. A considerable quantity of paraquat applied to plants will not reach the active site in chloroplast because of binding to cell walls. This has to be considered in any uptake or efflux experiment because the cell

wall binding will interfere with the ability to determine uptake of paraquat into cells and distribution within cell organelles. In an attempt to determine the distribution of paraquat within cell organelles by efflux experiments, Hart *et al.* (1992) and Lasat *et al.* (1997) attributed the slowest eluting phase to the vacuole. However, considering the large and tight binding of paraquat to cell walls, this slow effluxing phase must be, at least in part, due to binding of paraquat to the cell wall.

The presence of the cell wall, which binds considerable quantities of paraquat (Chapter 4), could interfere with paraquat uptake. Therefore, protoplasts were used as a model system to study paraquat absorption by cells. Protoplasts appear to be a convenient and more homogeneous system than intact leaves for such studies. Intact tissue constitutes a complex system where the cuticle and cell cohesion hamper the study of solute transport (March and Tremolieres, 1985).

Measurement of photosynthetic activity was used as a useful method for estimating paraquat appearance at its active site in the chloroplasts of protoplasts. As described in Chapter 1, the presence of paraquat in the chloroplast in the light results in production of a paraquat radical that reacts with O_2 producing superoxide. This reduces net O_2 evolution. Protoplasts of *H. glaucum* are generally much more resistant to paraquat than were chloroplast or leaf slices (Chapter 5; Powles *et al.*, 1987; Preston *et al.*, 1992b). In *Conyza bonariensis*, Norman *et al.* (1993) observed the same effect when they showed that a high concentration of paraquat was needed to bleach chlorophyll of protoplasts compared to leaf slices. One possible reason for this general tolerance of protoplasts is that calcium is a non-competitive inhibitor of paraquat uptake (Preston *et al.*, 1992a; Hart *et al.*, 1993). When the calcium concentration was reduced, paraquat was considerably more effective at inhibiting O_2 evolution

by protoplasts (Chapter 5). At low concentrations of Ca^{2+}, protoplasts of both susceptible and resistant *H. glaucum* were equally sensitive to paraquat. Ca^{2+} is needed to maintain membrane integrity and some of the increased sensitivity at low concentrations of Ca^{2+} may be due to deterioration of membranes.

Experiments with ^{14}C-paraquat demonstrated that paraquat absorption by protoplasts was initially rapid. No differences were observed in absorption of paraquat between protoplasts of susceptible and resistant *H. glaucum* (Chapter 5). As no differences were observed in ^{14}C-paraquat absorption by protoplasts or in paraquat inhibition of O_2 evolution between protoplasts of resistant and susceptible *H. glaucum*, it could be argued that resistance is not the result of a change in a plasmalemma membrane transporter for paraquat. However, given the general tolerance of protoplasts to paraquat, particularly in the presence of Ca^{2+}, other methods such as isolation of plasmalemma vesicle may be required to clarify the role of the plasmalemma in paraquat resistance.

Following the present study, there remain two possible mechanisms that could account for paraquat resistance in *H. glaucum*. These are exclusion of paraquat from the cell as proposed by Preston *et al.* (1992a; 1992b) or sequestration of paraquat in the vacuole as proposed by Lasat *et al.* (1997).

The plasmalemma exclusion hypothesis was proposed based on reduced inhibition of photosynthetic activity by paraquat in leaves and leaf slices (Preston *et al.*, 1992a; 1992b). This hypothesis is supported by reduced basipetal translocation of paraquat (Preston *et al.*, 1992b; Purba *et al.*, 1995) and similar acropetal translocation (Purba *et al.*, 1995). This type of hypothesis is also supported by the generally recessive genetics of resistance in *H. glaucum* and *H. leporinum* (Islam and Powles, 1988; Purba *et al.*, 1993b). Protoplast uptake experiments (Chapter 5) do not support this hypothesis; however, given the

general high tolerance of protoplasts to paraquat, these experiments need to be interpreted with care.

The vacuolar sequestration hypothesis is based on measurement of efflux of paraquat from intact roots of susceptible and resistant *H. glaucum* (Lasat *et al.*, 1997). These studies demonstrated three phases of efflux of paraquat, which were attributed to efflux from the apoplast, cytoplasm and vacuole. The large tight binding of paraquat to cell walls (Chapter 4) means that these attributions may contain a phase that comes from the cell wall. The vacuolar sequestration hypothesis is supported by reduced basipetal translocation of paraquat (Preston *et al.*, 1992b; Purba *et al.*, 1995); however, it would be expected that acropetal translocation might also be reduced, which does not happen (Purba *et al.*, 1995). The protoplast uptake experiments (Chapter 5) do not support vacuolar sequestration as absorption should be greater in the resistant protoplasts. However, the questions raised about protoplasts above also apply here. The initial inhibition of photosynthesis followed by a rapid recovery following application of paraquat to leaves of *Conyza bonariensis* (Shaaltiel and Gressel, 1987) and *Conyza canadensis* (Lehoczki *et al.*, 1992) would support a vacuolar sequestration hypothesis. However, this phenomena is not seen with either resistant *H. glaucum* or *H. leporinum* (Preston *et al.*, 1992b). Mornet *et al.* (1997) demonstrated that paraquat could be absorbed by intact vacuoles of barley, however, high external concentrations of paraquat (4 mM) were required to obtain significant uptake. Lastly, vacuolar sequestration, if mediated by a pump, would be expected to be the result of a partially dominant gene.

In conclusion, the mechanism of resistance to paraquat in *H. glaucum* appears to be sequestration of paraquat away from the site of action. Increased apoplast binding does not seem to be involved in the process, although paraquat binds strongly to the cell wall. A change in a paraquat transporter is most likely the

mechanism of resistance. As yet it is unclear whether sequestration occurs in the apoplast or the vacuole. Experiments with plasmalemma and tonoplast vesicles isolated from resistant and susceptible biotypes will further help to locate the mechanism of resistance.

References

Alizadeh, H. (1993). Mechanism of resistance to paraquat germination *Hordeum glaucum* seedlings in the dark. *MSc thesis*. The University of Adelaide, Adelaide.

Anthony, R. G., Waldin, T. R., Ray, J. A., Bright, S. and Hussey, P. J. (1998). Herbicide resistance caused by spontaneous mutation of the cytoskeletal protein tubulin. *Nature*. **393**: 260-263.

Antognoni, F., Pistocchi, R., and Bagni, N. (1993). Uptake competition between polyamines and analogues in carrot protoplasts. *Plant Physiology and Biochemistry*. **31**: 693-698.

Antognoni, F., Pistocchi, R., casali, P., and Bagni, N. (1995). Does calcium regulate polyamine uptake in carrot protoplasts? *Plant Physiology and Biochemistry*. **33**: 701-708.

Appleby, A. P. and Brenchley, R. G. (1968). Influence of paraquat on seed germination. *Weed Science*. **16**: 484-485.

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

Babbs, F. C., Pham, J. A. and Coolbaugh, R. C. (1989). Lethal hydroxyl radical production in paraquat treated plants. *Plant Physiology*. **90**: 267-270.

Bagni N, and Pistocchi R (1985) Uptake and translocation of polyamines in higher plants. In *Recent Progress in Polyamine Research*. (Edited by L. Selmeci, and M. E. Brosnan, N. Seiler), pp. 545-550. New Yorh. Raven.

Baker, D. A. and Hall, J. L. (1988). Introduction and general principles. In *Solute Transport in Plant Cells and tissues.* (Edited by D. A. Baker and J. L. Hall), pp. 1-27. Longhman Scientific and Technical. New York.

Baldwin, B. S. (1963). Translocation of diquat in plants. *Nature.* **198**: 872-873.

Baydoun, E. A. H. and Brett, C. T. (1988). Properties and possible physiological significance of cell wall calcium binding in etiolated pea epicotyls. *Journal of Experimental Botany*. **39**: 199-208.

Beversdorf, W. D., Hume, D. J. and Vanderloo, D. (1988). Agronomic performance of triazine-resistance and susceptible reciprocal spring Canola hybrids. *Crop Science*. **28**: 932-934.

Bhargara, S. (1993). Paraquat tolerance in a photomixotrophic culture of *Chenopodium rubum*. *Plant Cell Reports*. **12:** 230-232.

Bishop, A., Britton, R. S., Dellow, J. J. and Incerti, M. (1996). Herbicide resistance extension strategy for south-eastern Australia. In *Eleventh Australian Weeds Conference Proceedings*. (Edited by R. C. H. Shepherd), pp. 132-134, Weed Science Society of Victoria, Melbourne.

Bishop, T., Powles, S. B. and Cornic, G. (1987). Mechanism of paraquat resistance in *Hordeum glaucum*. II. Paraquat uptake and translocation. *Australian Journal of Plant Physiology*. **14**: 539-547.

Bowyer, J. R., Hallahen, B. J., Camilleri, P. and Howard (1989). Mode of action studies on nitrodiphenyl ether herbicides. II. The role of photosynthetic electron transport in *Scenedesmus obliquus*. *Plant Physiology*. **89**: 674-680.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of micro quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. **72**: 248-254.

Brian, R. C. (1967). The uptake and adsorption of diquat and paraquat by tomato, sugar beet, and cocksfoot. *Annals of Applied Biology*. **60**: 77-85.

Brian, R. C. and Headford, D. W. R. (1968). The effect of environment on the activity of bipyridylium herbicides. *Proceedings 9th British Weed Conference*. pp. 108-114.

Brian, R. C. and Ward, J. (1967). The influence of environment on potato haulm killed by diquat and its residue in the tubers. *Weed Research*. **7**: 117-130.

Briggs, G. G., Rigitano, R. L. O. and Bromilow, R. H. (1987). Physico-chemical factors affecting uptake by roots and translocation to shoots of weak acids in barley. *Pesticide Science*. **19**: 101-112.

Briskin, D. P. (1994). Membrane and transport systems in plants: An overview. *Weed Science*. **42**:255-262.

Bromilow, R. H., Chamberlain, K. and Evans, A. A. (1990). Physiochemical aspects of phloem translocation of herbicides. *Weed Science*. **38**: 305-314.

Bus, J. S., Aust, S. D., and Gibson, J. E. (1974). Superoxide and singlet oxygen-catalysed lipid peroxidation as a possible mechanism for paraquat (methyl viologen) toxicity. *Biochemical and Biophysical Research Communications*. **58**: 749-752.

Byers, T. L., Kameji, R., Rannels, D. E., and Pegg, A. E. (1987). Multiple pathways for uptake of paraquat, methylglyoxal bis(guanylhydrazone), and polyamines. *American Journal of Physiology*. **252**: C663-C669.

Cakmak, I., and Marschner, H. (1992). Magnesium deficiency enhances resistance to paraquat toxicity in bean leaves. *Plant, Cell and Environment*. **15**: 955-960.

Calderbank, A. (1968). The bipyridylium herbicides. *Advances in Pest Control Research*. **8**: 127-235.

Calderbank, A., and Slade, P. (1976). Diquat and paraquat. In: *Herbicides: Chemistry, Degradation, and Mode of Action*. (Edited by P. C. Kearney and D. D. Kaufman) Vol 2. Marcel Dekker, New York. pp 501-540.

Carroll, E. W., Schwarz, O. J., and Hickock, L. G. (1988). Biochemical studies of paraquat-tolerant mutants of the fern *Ceratopteris richardii*. *Plant Physiology*. **87**: 651-654.

Chase, C., Bewick, T. A., and Shilling, D. G. (1998a). Differential photosynthetic electron transport and oxidative stress in paraquat-resistant and sensitive biotypes of *Solanum americanum*. *Pesticide Biochemistry and physiology*. **60**: 83-90.

Chase, C., Bewick, T. A., and Shilling, D. G. (1998b). Characterization of paraquat resistance in *Solanum americanum* Mill. II. Evidence for a chloroplasts mechanism. *Pesticide Biochemistry and Physiology*. **60**: 23-30.

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., Liljegren, D. R., and Holtum, J. A. M. (1992). Resistance to acetolactate synthase-inhibiting herbicides in annual ryegrass (*Lolium rigidum*) involves at least two mechanisms. *Plant Physiology.* **100**: 1909-1913.

Christopher, J. T., Preston, C., and Powles, S. B. (1994). Malathion antagonizes metabolism-based chlorsulfuron resistance in *Lolium rigidum. Pesticide Biochemistry and Physiology* **49**: 172-182.

Chun, J. C., Ma, S. Y., Kim, S. E., and Lee, H. J. (1997a). Physiological response of *Rehmannia glutinosa* to paraquat and its tolerance mechanisms. *Pesticide Biochemistry and Physiology.* **59**: 51-63.

Chun, J. C., Kim, S. E., and Ma, S. Y. (1997b). Inactivation of paraquat by an aqueous extract of *Rehmannia glutinosa. Pesticide Science.* **50**: 5-10.

Coupland, D. (1991). The role of compartmentation of herbicides and their metabolites in resistance mechanisms. In *Herbicide Resistance in Weeds and Crops.* (Edited by J. C. Caseley, G. W. Cussans, and R. K. Atkin). pp. 263-278. Butterworth-Heinemann. Oxford.

Darmstadt, G. L., Balke, N. E., and Price, T. P. (1984). Triazine absorption by excised corn root tissue and isolated protoplasts. *Pesticide Biochemistry and Physiology.* **21**: 10-21.

Delbarre, A., Muller, P., Imhoff, V., Morgat, J. L., and Barbierbrygoo, H. (1994). Uptake, accumulation and metabolism of auxins in tobacco leaf protoplasts. *Planta.* **195**: 159-167.

Delmer, D. P., Read, S. M. and Cooper, G. (1987). Identification of a receptor protein in cotton fibers for the herbicide 2,6-dichlorobenzonitrile. *Plant Physiology.* **84**: 415-420.

DiTomaso, J. M., Rost, T. L., and Ashton, F. M. (1988). Herbicide-induced diamine accumulation in pea roots. The effect of napropamide on polyamine levels. *Plant and Cell Physiology*. **29**: 1367-1372.

Dodge, A. D. (1971). The mode of action of the bipyridylium herbicides, paraquat and diquat. *Endeavour*. **30**: 130-135.

Edlich, W., and Lyr, H. L. (1987). Mechanism of action of dicarboximide fungicides. in *Modern Selective Fungicides*. (Edited by H. Lyr), pp. 107-118. Fisher Verlag, Jena.

Evans, P. T., and Malmberg, R. L. (1989). Do polyamines have roles in plant development. *Annual Review of Plant Physiology and Plant Molecular Biology*. **40**: 235-269.

Faulkner, J. S. (1975). A paraquat tolerant line in *Lolium perenne*. In *Symposium on Status, Biology and Control of Grass Weeds in Europe*. pp. 349-359. European Weed Research Society. Paris.

Faulkner, J. S., and Harvey, B. M. R. (1981). Paraquat tolerance *Lolium perenne* L.: effects of paraquat on germinating seedlings. *Weed Research*. **21**: 29-36.

Federico, R., and Angelini, R. (1986). Occurrence of diamine oxidase in the apopolast of pea epicotyls. *Planta*. **167**: 300-302.

Flores, H. E., and Galston, A. W. (1982a). Polyamine and plant stress: activation of putrescine biosynthesis by osmotic shock. *Science*. **217**: 1259-1261.

Flores H. E., and Galston A. W. (1982b). Analysis of polyamines in higher plants by High Performance Liquid Chromatography. *Plant Physiology*. **69**: 701-706.

Fuerst, E. P., and Norman, M. A. (1991). Interactions of herbicides with photosynthetic electron transport. *Weed Science*. **39**: 439-458.

Fuerst, E. P., and Vaughn, K. C. (1990). Mechanisms of paraquat resistance. *Weed Technology*. **4**: 150-156.

Fuerst, E. P., Nakatani, H. Y., Dodge, A. D., Penner, D., and Arntzen, C. J. (1985). Paraquat resistance in *Conyza*. *Plant Physiology*. **77**: 984-989.

Fuji, T., Yokoyama, E., Inoue, K., and Sakuri, H. (1990). The site of electron donation of photosystem I to methyl viologen. *Biochemica et Biophysica Acta*. **1015**: 41-48.

Funderburk, H. H., and Bozarth, G. A. (1967). Review of the metabolism and decomposition of diquat and paraquat. *Journal of Agriculture and Food Chemistry*. **15**: 563-567.

Funderburk, H. H., and Lawrence, J. M. (1964). Mode of action and metabolism of diquat and paraquat. *Weeds*. **12**: 259-264.

Golbeck, J. H. (1992). Structure and Function of Photosystem I. *Annual Review of Plant Physiology and Plant Molecular Biology*. **43**: 293-324.

Golbeck, J. H., and Bryant, D. A. (1991). Photosystem I. *Current Topisc in Bioenergetics*. **16**: 83-177.

Gronwald, J. W., Anderson, R. N. and Yee, C. (1989). Atrazine resistance in velvet leaf (*Abutilon theophrasti*) due to enhanced atrazine detoxification. *Pesticide Biochemistry and Physiology*. **34**: 149-163.

Hall, L. M., and Devine, M, D. (1990). Cross-resistance of a chlorsulfuron-resistant biotype of *Sellaria media* to a triazolopyrimidine herbicide. *Plant Physiology*. **93**: 962-966.

Hamblin, A. (1987). The effect of tillage on soil physical conditions. In: *Tillage: New Directions in Australian Agriculture*. (Edited by P. S. Cornish and J. E. Pratley). pp. 128-170. Inkata Press, Melbourne, Australia.

Harper, J. L. (1956). The evolution of weeds in relation to the resistance to herbicides. *Proceeding of 3rd British Weed Control Conference*. **1**: 179-788.

Harper, D. B., and Harvey, B. M. R. (1978). Mechanism of paraquat tolerance in perennial ryegrass. II. Role of superoxide dismutase, catalase and peroxidase. *Plant, Cell and Environment*. **1**: 211-215.

Harris, N., and Dodge, A. D. (1972). The effect of paraquat on flax cotyledon leaves: physiological and biochemical changes. *Planta*. **104**: 210-219.

Hart, J. J., DiTomaso, J. M., Linscott, D. L., and Kochian, L. V. (1992a). Transport interactions between paraquat and polyamines in roots of intact maize seedlings. *Plant Physiology*. **99**: 1400-1409.

Hart, J. J., Ditomaso, J. M., and Kochian, L. V. (1993). Characterization of paraquat transport in protoplasts from maize (*Zea mays* L.) suspension cells. *Plant Physiology*. **103**: 963-969.

Hart, J. J., Ditomaso, J. M., Linscott, D. L., and Kochian, L. V. (1992b). Characterization of the transport and cellular compartmentation of paraquat in roots of intact maize seedlings. *Pesticide Biochemistry and Physiology*. **43**: 212-222.

Harvey, B. M. R., and Harper, D. B. (1982). Tolerance to bipyridylium herbicides. in *Herbicide Resistance in Plants*. (Edited by H. M. LeBaron and J. Gressel), pp 215-233. John Wiley and Sons, New York.

Harvey, B. M. R., Muldoon, J., and Harper, D. B. (1978). Mechanism of paraquat tolerance in perennial ryegrass. I: Uptake, metabolism and translocation of paraquat. *Plant, Cell and Environment*. **1**: 203-209.

Hassan, H. M., and Fridovic, I. (1977). Regulation of the synthesis of superoxide dismutase in *E. coli.* Induction by methyl-viologen. *Biological Chemistry*. **252**: 7667-7672.

Heap, I. M. (1997). The occurrence of herbicide-resistant weeds worldwide. *Pesticide Science*. **51**: 235-234.

Heap, J., and Knight, R. (1982). A population of ryegrass tolerant to the herbicide diclofop-methyl. J*ournal of Australian Institute of Agricultural Science*. **48**: 156-157.

Hess, F. D. (1985). Herbicide absorption and translocation and their relationship to plant tolerances and susceptibility. In *Weed Physiology. Vol II. Herbicide*

Physiology. (Edited by S. O. Duke), pp. 191-214. CRC Press. Boca Raton. Florida.

Holt, J. S., Powles, S. B., and Holtum, J. A. M. (1993). Mechanisms and agronomic aspects of herbicide resistance. *Annual Review of Plant Physiology and Plant Molecular Biology*. **44**: 203-229.

Holtum, J. A. M., Matthews, J. M., Hausler, R. E., Liljegren, D. R. and 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.

Hughes, K. H. (1979). Isolation of a herbicide-resistant line of soybean cells. In *Plant cell and tissue culture: principle and applications*. (Edited by W. R. Sharp, P. O. Larson, E. F. Paddock, and V. Ragharan), pp. 874. Ohio State University Press, Columbia, Ohio.

Hughes, K. W., Negretto, D., Daub, M. E., and Meeusen, R. L. (1984). Free-radical stress response in paraquat-sensitive and resistant tobacco plants. *Environment and Experimental Botany*. **24**: 151-157.

Imai, Y., and Kuwatsuka, S. (1989). Characteristics of paraquat-degrading microbes. *Journal of Pesticide Science*. **14**: 475-480.

Islam, A. K. M. R., and Powles, S. B. (1988). Inheritance of resistance to paraquat in barley grass *Hordeum glaucum* Steud. *Weed Research*. **28**: 393-397.

Itoh, K., and Matsunaka, S. (1990). Parapatric differentiation of paraquat resistant biotypes in some Compositae species. In *Biological approaches and evolutionary trends in plants*. (Edited by S. Kawano), pp. 33-49. Academic Press, London.

Jansen, M. A. K., Malan, C., Shaaltiel, Y., and Gressel, J. (1990). Mode of evolved photooxidant resistance to herbicides and xenobiotics. *Zeitschrift fur Naturforschung*. **45c**: 463-469.

Kaiser, G., Martinoia, E., and Wiemken, A. (1982). Rapid appearance of photosynthetic products in the vacuoles isolated from Barley mesophyll protoplasts by a new fast method. *Zeitschrift fur Pflanzenphysiologie.* **107**: 103-113.

Kapusta, G., and Krausz, R. F. (1993). Weed control and yield are equal in conventional, reduced-, and no-tillage soybean (*Glycine max*) after 11 years. *Weed Technology.* **7**: 443-451.

Kasai, F., and Bayer, D. E. (1991). Relationship between intracellular pH and absorption by root protoplasts under the influence of metabolic inhibitors and antiauxins. *Journal of Pesticide Science.* **16**: 171.

Kitayama, K., and R. K. Togasaki (1992). Characterization of paraquat resistant mutants of *Chlamydomonas reinhardtii.* in Research in Photosynthesis, Volume IV. (Edited by N. Murata), pp. 487-490. Kluwer Academic Publishers, Dordrect, The Netherlands.

Kohen, R. (1985). Paraquat toxicity is enhanced by iron and reduced by desferrioxamine in laboratory mice. *Biochemical Pharmacology.* **34**: 1841-1843.

Korbashi, P., Kohen, R., Katzhendler, J., and Cherion, M. (1986). Iron-mediated paraquat toxicity in *Escherichia coli. Journal of Biological Chemistry.* **261**: 12472-12476.

Kraus, T., and R. A. Fletcher. (1994). Paclobutrazol protects wheat seedlings from heat and paraquat injury - is detoxification of active oxygen involved. *Plant and Cell Physiology.* **35**: 45-52.

Kunert K. J., and Dodge, A. D., Mets, L. E., Thiel, A. E. (1989). Herbicide-induced radical damage and antioxidative systems. In *Target Sites of Herbicide Action.* (Edited by P. Böger, and G. Sandmann), pp 45-65. CRC Press, Boca Raton, Fl.

Langebartels, C., Kerner, K., Leonardi, S., Schaudner, M., Trost, M., Heller, W., and Sandermann, N. (1991). Biochemical plant responses to ozone. 1.

Differential induction of polyamine and ethylene biosynthesis in tobacco. *Plant Physiology.* **95**: 882-889.

Lasat, M. M., Ditomaso, J. M., Hart, J. J., and Kochian, L. V. (1996). Resistance to paraquat in *Hordeum glaucum* is temperature dependent and not associated with enhanced apoplasmic binding. *Weed Research.* **36**: 303-309.

Lasat, M. M., Ditomaso, J. M., Hart, J. J., and Kochian, L. V. (1997). Evidence for vacuolar sequestration of paraquat in roots of a paraquat-resistant *Hordeum glaucum* biotype. *Physiologia Plantarum.* **99**: 255-262.

LeBaron, H. M., and McFarland, J. (1990). Herbicide resistance in weeds and crops: an overview and prognosis. In *Managing Resistance to Agrochemicals: From Fundamental Research to Practical Strategies.* (Edited by M. B. Green, H. M. LeBaron, and W. K. Moberg), pp. 336-352. American Society, Washington, DC.

Lee, T. M., Lur, H. S., and Chu, C. (1995). Abscisic acid and putrescine accumulation in chilling-tolerant rice cultivars. *Crop Science.* **35**: 502-508.

Lehoczki, E., Laskay, G., Gaal, I. and Szigeti, Z. (1992). Mode of action of paraquat in leaves of paraquat-resistant *Conyza canadensis* (L.) Cronq. *Plant Cell and Environment.* **15**: 531.

Lewinsohn, E., and Gressel, J. (1984). Benzyl viologen-mediated counteraction of diquat and paraquat phytotoxities. *Plant Physiology.* **76**: 125-130.

Licochhev, S., and Fridovich, I. (1993). Effect of paraquat on *Escherichia coli*: Sensitivity to small changes in pH of the medium- A cautionary note. *Archives of Biochemistry and Biophysics.* **306**: 518-520.

March, G., and Tremolieres, A. (1985) Protoplast growth and photoregulation. In *The physiological properties of plant protoplasts.* (Edited by P. E. Pile), pp. 258-266. Spring-Verlag, New York, NY.

McCloskey, W. B., and Bayer, D. E. (1990). Atrazine absorption and effects on photosynthesis in wheat leaf protoplasts. *Pesticide Biochemistry and Physiology.* **37**: 227-238.

Minton, K. W., Tabor, H. and Tabor, C. W. (1990). Paraquat toxicity is increased in Escherichia coli defective in the synthesis of polyamines. *Proceedings Of The National Academy Of Sciences Of The United States Of America.* **87**: 2851-2855.

McDonald, R. E., and Kushed, M. M. (1986). Accumulation of putrescine during chilling injury of fruit. *Plant Physiology.* **82**: 324-326.

Mornet, C., Mondory, C., Gaillard, C. and Martinoia, E. (1997). Transport of paraquat and polyamines across the vacuolar membrane of barley mesophyll cells. *Plant Physiology and Biochemistry.* **35**: 589-594.

Moss, S. R., and Rubin, B. (1993). Herbicide-resistant weeds: a worldwide perspective. *Journal of Agricultural Science.* **120**: 141-148.

Moyer, J. R., Roman, E. S., Lindwall, C. W., and Blackshow, R. E. (1994). Weed management in conservation tillage systems for wheat production in North and South America. *Crop Protection.* **13:** 243-59.

Nobel, P. S. (1991). Solute. In *Physiochemical and Environmental Plant Physiology.* pp. 109-189. Academic Press, New York.

Norman, M. A., Fuerst, E. P., Smeda, R. J., and Vaughn, K. C. (1993). Evaluation of paraquat resistance mechanisms in *Conyza*. *Pesticide Biochemistry and Physiology.* **46**: 236-249.

Ormrod, D. P., and Beckerson, D. W. (1986). Polyamines as antiozonants for tomato. *HortScience.* **21**: 1070-1071.

Oshima, T. (1983). Novel polyamines. *Methods in Enzymology.* **94**: 401-410.

Pakbaz, H. H., Foda, D., Hasan, I., Berisha, M., and Sami, I. S. (1993). Paraquat-induced lung injury: prevention by vasoactive intestinal peptide and related peptide helodermin. *American Journal of Physiology.* **265**: L369-L373.

Pistocchi, R., and Bagni, N. (1990). Effect of calcium on spermine uptake in carrot cell cultures and protoplasts. *Journal of Plant Physiology.* **136**: 728-733.

Pistocchi, R., Bagni, N., and Creus, J. A. (1987). Polyamine uptake in carrot cell cultures. *Plant Physiology.* **84**: 514-518.

Pistocchi, R., Keller, F., Bagni, N., and Matile, P. (1988). Transport and subcellular localization of polyamines in carrot protoplasts and vacuoles. *Plant Physiology.* **87**: 514-518.

Polos, E., Mikulas, J., Szigeti, Z., Mathovics, B., Do Quy Hai, Parducz, A., and Lehoczki, E. (1988). Paraquat and atrazine co-resistance in *Conyza canadensis*. *Pesticide Biochemistry and Physiology.* **26**: 22-28.

Powles, S. B. (1986). Appearance of a biotype of the weed, *Hordeum glaucum* Steud, resistant to the herbicide paraquat. *Weed Research.* **26**: 167-172.

Powles, S. B., and Cornic, G. (1987). Mechanism of paraquat resistance in *Hordeum glaucum*. 1. Studies with isolated organelles and enzymes. *Australian Journal of Plant Physiology.* **14**: 81-89.

Powles, S. B., and Holtum, J. A. M. (1994). *Herbicide Resistance in Plants: Biology and Biochemistry.* Lewis Publishers, Boca Raton, FL.

Powles, S. B., Tucker, E. S., and Morgan, T. R. (1989). A capeweed (*Arctotheca calendula*) biotype in Australia resistant to bipyridyl herbicides. *Weed Science.* **37**: 60-62.

Powles, S. B., Preston, C., Bryan, I. B., and Jutsum, A. R. (1997). Herbicide resistance: impact and management. *Advances in Agronomy.* **58**: 57-93.

Pratley, J. E. (1995). Long term investigation of the effect of tillage practices on crop production at Wagga Wagga, New South Wales. *Australian Journal of Experimental Agriculture.* **35**: 885-892.

Preston, C. (1994). Mechanisms of resistance to herbicides interacting with photosystem I. In *Herbicide Resistance in Plants: Biology and Biochemistry.* (Edited by S. B. Powles and J. A. M. Holtum), pp 61-82. Lewis Publishers, Boca Raton, FL.

Preston, C., Holtum, J. A. M., and Powles, S. B. (1991). Resistance to the herbicide paraquat and increased tolerance to photoinhibition are not correlated in several weed species. *Plant Physiology*. **96**: 314-318.

Preston, C., Holtum, J. A. M., and Powles, S. B. (1992a). Do polyamines contribute to paraquat resistance in *H. glaucum*?. In *Research in Photosynthesis, Vol III.* (Edited by N. Murata), pp. 571-574. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Preston, C., Holtum, J. A. M., and Powles, S. B. (1992b). On the mechanism of resistance to paraquat in *Hordeum glaucum* and *H. Leporinum*. Delayed inhibition of photosynthetic O_2 evolution after paraquat application. *Plant Physiology*. **100**: 630-636.

Preston, C., Balachandran, S., and Powles, S. B. (1994). Investigations of mechanisms of resistance to bipyridyl herbicides in *Arctotheca calendula* (L.) Levyns. *Plant Cell and Environment*. **17**: 1113-1123.

Price, C. (1979) Movement of xenobiotics in plants-perspectives. In *Advances in Pesticide Science.* (Edited by H. Geissbuhler), pp. 401-409. Pergamon Press, Oxford.

Purba, E. (1993). Factors influencing the development of resistance to the bipyridyl herbicides in Australia. *PhD thesis*. The University of Adelaide, Adelaide.

Purba, E., Preston, C., and Powles, S. B. (1993a). Paraquat resistance in a biotype of *Vulpia bromoides* (L.) S. F. Gray. *Weed Research*. **33**: 409-413.

Purba, E., Preston, C. and Powles, S. B. (1993b). Inheritance of bipyridyl herbicide resistance in arctotheca-calendula and hordeum-leporinum. *Theoretical and Applied Genetics*. **87**: 598-602.

Purba, E., Preston, C., and Powles, S. B. (1995). The mechanism of resistance to paraquat is strongly temperature dependent in resistant *Hordeum leporinum* Link and *H. glaucum* Steud. *Planta*. **196**: 464-468.

Rabinowitch, H. D., Clare, D. A., Crapo, J. D., Fridovich, I. (1982). Positive correlation between superoxide dismotase and resistance to paraquat toxicity in the green alga *Chlorella sorkiniana*. *Archives of Biochemistry and Biophysics*. **225**: 640-648.

Rinaldi, A., Floris, G., and Finazzi-Agro, A. (1982). Purification and properties of diamine oxidase from *Euphorbia* Latex. *European Journal of Biochemistry*. **127**: 417-422.

Rose, M. S., Smith, L. L., and Wyatt, I. (1974). Evidence for energy-dependent accumulation of paraquat into rat lung. *Nature*. **252**: 3134.

Roy, M., and Ghosh, B. (1996). Polyamines, both common and uncommon, under heat stress in rice (*Oryza sativa*) callus. *Physiologia Plantarum*. **98**: 196-200.

Ryan, G. F. (1970). Resistance of common groundsel to simazine and atrazine. *Weed science*. **18**: 614-616.

Saari, L. L., Cotterman, J. C., Primiani, M. M. (1990). Mechanism of sulfonylurea herbicide resistance in the broadleaf weed, *Kochia scoparia*. *Plant Physiology*. **93**: 55-61.

Shaaltiel, Y., and Gressel, J. (1986). Multi enzyme oxygen radical detoxifying systems correlated with paraquat resistance in *Conyza bonariensis*. *Pesticide Biochemistry and Physiology*. **26**: 22-28.

Shaaltiel, Y., and Gressel, J. (1987). Kinetic analysis of resistance to paraquat in *Conyza*. *Plant Physiology*. **85**: 869.

Shaaltiel, Y., Chua, N. H., Gepstein, S., and Gressel, J. (1988). Dominant pleiotropy controls enzymes co-segregating with paraquat resistance in *Conyza bonariensis*. *Theoretical and Applied Genetics*. **75**: 850-856.

Slade, P. (1966a). The fate of paraquat applied to plants. *Weed Research*. **6**: 158-167.

Slade, P. (1966b). The movement of paraquat in plants. *Weed Research*. **6**: 267-273.

Slocum, R. D., Kaur-Sawhney, R., and Galston, A. W. (1984). The physiology and biochemistry of polyamines in plants. *Archives of Biochemistry and Biophysics*. **35**: 283-303.

Smith T. A. (1983) Putrescine and inorganic ions. *In Recent Advances in Phytochemistry*. (Edited by B. N. Timmermann, C. Steelink, F. A. Loewus), pp 7-54. Plenum Press, New York.

Smith, T. A. (1973). Ammonium levels in mineral deficient *Hordeum vulgare* leaves. *Phytochemistry*. **12**: 2093-2100.

Smith, T. A. (1985a) Polyamines in Plants. In *Polyamines in Plants*. (Edited by A. W. Galston, and T. A. Smith), pp 12-21. Martinus Nijhoff, Dordrecht.

Smith, T. A. (1985b). Polyamines. *Annual Reviews of Plant Physiology*. **36**: 117-143.

Smith, T. A., and Barker, J. H. A. (1988). The di- and polyamine oxidases of plants. In *Progress in Polyamine Research*, (Edited by V. Zappia and A. E. Pegg), pp 573-587. Plenum Press, New York.

Smith, L. L., and Wyatt, I. (1981). The accumulation of putrescine into slices of rat lung and brain and its relationship to the accumulation of paraquat. *Biochemical Pharmacology*. **30**: 1053.

Stecko, V. (1974). The effect of diquat and paraquat applied to seeds. *Proceedings 12th British Weed Control Conference*. **1**: 108-114.

Stein, W. D. (1986). Transport and Diffusion Across Cell Membranes. Academic Press, Orland, FL.

Stein, W. D. (1990). Channels, Carriers, and Pumps. Academic Press. San Diego, CA.

Sterling, T. M. (1994). Mechanism of herbicide absorption across plant membranes and accumulation in plant cells. *Weed Science*. **42**: 263-276.

Strange, R. H., and Rogers, R. L. (1971). A microautoradiographic study of ^{14}C-trifluralin absorption. *Weed Science*. **19**: 363.

Summers, L. A. (1980). The Bipyridylium Herbicides. Academic Press, London.

Szigeti, Z., Racz, I., Darko, E., Lasztity, D., and Lehoczki, E. (1996). Are either SOD and catalase or the polyamines involved in the paraquat resistance of *Conyza canadensis*. *Journal of Environmental Science and Health*. **31B**: 599-604.

Tanaka, Y., Chisaka, H., and Saka, H. (1986). Movement of paraquat in resistant and susceptible biotypes of *Erigeron philadelphicus* and *E. canadensis*. *Physiologia Plantarum*. **66**: 605-608.

Tardif, F. J, and Powles, S. B. (1993). Target site-based resistance to herbicides inhibiting acetyl-CoA carboxylase. *Proceedings Brighton Crop Protection Conference-Weeds*. pp. 533-540.

Thomas, B. R., and Pratt, D. (1982). Isolation of paraquat tolerant-mutants from tomato cell cultures. *Theoretical and Applied Genetics*. **63**: 167-176.

Thrower, S. L., Hallam, N. D., and Thrower, L. B. (1965). Movement of diquat, 1,1'-ethylene-2,2'-bipyridylium dibromide in leguminous plants. *Annals of Applied Biology*. **55**: 253-260.

Torrigiani, P., Serafini-Fracassini, D., and Fara, A. (1989). Diamine oxidase activity in different physiological stages of *Helianthus tuberosus* tuber. *Plant Physiology*. **89**: 69-73.

Trebst, A. (1991). The molecular basis of resistance of photosystem II herbicides. In *Herbicide resistance in weeds and crops*. (Edited by J. C. Caseley, G. W. Cussans and R. K. Atkin), pp. 145-164. Butterworth-Heinemann Ltd., Oxford.

Tucker, E. S., and Powles, S. B. (1991). A biotype of hare barley (*Hordeum leporinum*) resistant to paraquat and diquat. *Weed Science*. **39**: 159-162.

Turner, L. B., and Stewart, G. R. (1988). Factors affecting polyamine accumulation in barley (*Hordeum vulgare* L.) leaf section during osmotic stress. *Journal of Experimental Botany*. **39**: 311-316.

Vasi, I. K. (1976). The progress, problem, and prospects of plant protoplast research. *Advances in Agronomy*. **28**: 119-160.

Vaughn, K. C., Vaughan, M. A., and Camilleri, P. (1989). Lack of cross-tolerance of paraquat-resistant hairy fleabane (*Conyza bonariensis*) to other toxic oxygen generators indicates enzymatic protection is not the resistance mechanism. *Weed Science*. **37**: 5-11.

Warwick, S. I. (1991). Herbicide resistance in weedy plants: Physiology and population biology. *Annual Reviews of Ecology and Systematics*. **22**: 95-114.

Watanabe, Y., Honina, T., Ito, K., and Miyrashara, M. (1982). Paraquat resistance in *Erigeron philadelphicus*. *Weed Research (Japan)*. **27**: 49-54.

Wojtaszek, P., and Bolwell, G. P. (1995). Secondary cell-wall-specific glycoprotein(s) from French bean hypocotyls. *Plant Physiology*. **108**: 1001-1012.

Yamasue, Y., Kamiyama, K., Hanioka, Y., and Kusanagi, T. (1992). Paraquat resistance and its inheritance in seed germination of the foliar-resistant biotypes of *Erigeron canadensis* L. and *E. Sumatrensis* Retz. *Pesticide Biochemistry and Physiology*. **44**: 21-27.

Ye, B., Muller, H. H., Zhang, J., and Gressel, J. (1997). Constitutively elevated levels of putrescine and putrescine-generating enzymes correlated with oxidant stress resistance in *Conyza bonariensis* and wheat. *Plant Physiology*. **115**: 1443-1451.

York, W. S., Darvill, A. G., McNeil, M., Stevenson, T. T. and Albersheim, P. (1985). Isolation and characterization of plant cell walls and cell wall components. *Methods in Enzymology*. **118**: 3-40.

Young N. D., Galston, A. W. (1983). Putrescine and acid stress. *Plant Physiology*. **71**: 767-771.

Youngman, R. J., and Dodge, A. D. (1981). On the mechanism of paraquat resistance in *Conyza* sp. In *Photosynthesis VI. Photosynthesis and Environment*. (Edited by G. Akoyunoglou), pp. 537-544. Balaban, Philadelphia.

Zer, H., Peleg I., and Chevion M. (1994). The protective effect of desferrioxamine on paraquat-treated pea (*Pisum sativum*). *Physiologica Plantarum.* **92**: 437-442.

Zhelera, D. I., Alexieva, V. S., and Karanov, E. N. (1993). Influence of atrazine on free and bound polyamine levels in pea leaves. *Journal of Plant Physiology.* **141**: 282-285.