



**EVOLUTION AND SPREAD OF PARAQUAT  
RESISTANT BARLEY GRASSES**  
*(Hordeum glaucum Steud. and H. leporinum Link)*

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## ABSTRACT

The bipyridilium herbicide paraquat, a non-selective post emergence herbicide, inhibits Photosystem I (PSI) by diverting electrons from ferredoxin and subsequently producing superoxide. This herbicide acts rapidly on plants and leaves no residual effects on soil. Paraquat has been employed for weed control in crops and pastures for more than three decades in South Australia and is still extensively used. Since 1990, there have been three paraquat-resistant populations of *Hordeum glaucum* documented from lucerne and no-till wheat. Resistance usually occurs following persistent use of paraquat for more than 12 years with at least one application each year. Recent reports suggest that the incidence of paraquat resistance in *Hordeum* spp. is increasing from lucerne (*Medicago sativa* L.) fields. For this reason, this project was designed to investigate new resistant populations of *Hordeum* spp; to assess the role of gene flow in spreading resistance; to determine the fitness of resistant individuals in accelerating resistance evolution; and to identify genetic relationships between resistant and susceptible populations using DNA markers.

A random survey of *Hordeum* spp. populations was conducted in 1999 through the mid-north and lower north of South Australia. There were 50 *Hordeum* populations collected and tested for paraquat resistance. Of these, one population of *H. glaucum* collected from a lucerne field, 1.4 km from Jamestown, was found to be highly resistant to paraquat. A second survey in 2001 targeted lucerne fields and adjacent fields between Jamestown and

Spalding and between Waterloo and Kapunda. From 23 populations collected, five additional paraquat-resistant populations of *H. glaucum* and one paraquat-resistant population of *H. leporinum* were identified with high levels of resistance.

The mechanism of paraquat resistance in *Hordeum* spp. is not fully elucidated, but it is not related to modification of the binding site at PS I, enhanced paraquat metabolism or reduced paraquat uptake. Previous studies have proposed a decrease of paraquat translocation within resistant individuals as the mechanism of resistance. This mechanism is affected by temperature, with the level of resistance decreasing when temperature is increased. Therefore, it is possible to compare resistant mechanisms among paraquat-resistant populations of *Hordeum* spp. using a temperature test. In the present study, the new paraquat-resistant populations demonstrated a similar response to temperature, indicating they may have a similar mechanism of resistance, to previously known populations.

The frequency of gene flow through pollen movement was assessed between paraquat-resistant and -susceptible populations of *H. leporinum* through pot experiments for two consecutive years. Gene flow through pollen movement did occur at a very low frequency, about 0.1 %. As the anthers of *H. glaucum* are never exposed, gene flow could not be expected between paraquat-resistant and susceptible *H. glaucum*. With this low or non-existent frequency of pollen flow, it is concluded that, in *Hordeum* spp., gene flow through seed dispersal plays a more vital role in resistance spread than pollen movement.

The fitness of homozygous and heterozygous resistant, as well as homozygous susceptible, individuals was compared using plants from a single cross in the absence of herbicide application. There was no significant difference in growth and productivity among these three genotypes. The number of tillers increased at a similar rate over time. Likewise, plant dry weight, number of seed per plant and the weight of 100 seeds were not significantly different. Germinability tests found that the seed of paraquat-resistant and -susceptible individuals germinated evenly. This means that when conditions are suitable, resistant individuals will grow as well as susceptible individuals.

Using randomly amplified polymorphic DNA (RAPD) markers; the genetic relationship among paraquat-resistant and -susceptible populations of *Hordeum* spp. was examined. The results showed that individual paraquat-resistant populations of *H. glaucum* and *H. leporinum* had no polymorphic genotypes. In contrast, paraquat-susceptible populations contained polymorphic genotypes. The findings also showed that some paraquat-resistant populations within each species had identical genetic backgrounds. These were *H. glaucum* populations SHG8 and SHG9, and *H. leporinum* populations THL1 and THL3. This indicated the likelihood of gene flow between the fields these populations were collected. However, other paraquat-resistant populations demonstrated genetic dissimilarity, such as that between SHG7 and SHG8 or between THL1 and THL2. This suggests that independent evolutionary events remain important for resistant evolution in fields. These findings suggested management strategies should

incorporate methods to prevent the evolution of paraquat resistance and, at the same time, minimise seed dispersal of resistant populations between fields.

## DECLARATION

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

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Imam Hidayat

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# **Chapter 1: Literature Review**

## 1.1 Introduction

The introduction of the first selective broadleaf herbicides 2,4-D in 1945 followed by MCPA in 1946 became a starting point for widespread chemical use in weed control (Cobb and Kirkwood, 2000). Since then, herbicides have played a significant role in weed management systems for more than five decades resulting in a great increase of crop production worldwide (Powles *et al.*, 1997). Herbicides have been so successful because they provide solutions to a wide range of weed control problems (Preston, 2000), such as decrease weeding costs, controlling weeds in crop areas where other control methods are not able to work, efficiently reducing the frequency of tillage, providing control of perennial weeds, and enabling farmers to employ soil conservation systems (Ross and Lembi, 1985). The use of herbicides has increased greatly with the introduction of minimum tillage systems as an element of sustainable agriculture systems (Aiken, 1998).

As a consequence of the great reliance on herbicides and their intensive use in farming systems, a strong selection pressure for the evolution of herbicide resistance is present (Mortimer, 1997). Herbicide resistance refers to the heritable ability of weeds to grow and produce seeds after being exposed to a recommended rate of herbicide that is previously sufficient to control normal populations of that species (Heap and LeBaron, 2001; Valverde *et al.*, 2000). The occurrence of herbicide resistance in weeds was predicted more than a decade before it happened (Harper, 1956), as resistance had by then already occurred in insects and micro-organism to chemical compounds used for their control. The first reported incidence of herbicide resistance



was to triazine herbicides in a population of *Senecio vulgaris* (Ryan, 1970). Since then, the number of weed species with populations resistant to herbicides has steadily increased (Figure 1.1) at rate of more than nine new incidences per year (Heap, 1997). Once herbicide resistance appears, the population can no longer be controlled by that herbicide. Hence, the incidence of resistance threatens the usefulness of herbicides and vastly complicates weed management practices (Peterson, 1999; Powles *et al.*, 1997).

Herbicides also have been the main element of control for *Hordeum leporinum* and *Hordeum glaucum*, important grass weeds, in both pasture and crop fields in southern Australia for long period of time. Therefore, it is not surprising that after more than two decades of use of the non-selective herbicides paraquat and diquat to control *Hordeum* spp. in lucerne (*Medicago sativa*), the first incidence of resistance in *H. glaucum* was reported in Australia (Warner and Mackie, 1983). This was the second case of weed resistance in Australia after *Lolium rigidum* resistance to diclofop-methyl (Heap and Knight, 1982). Paraquat resistance was also later identified in *H. leporinum* (Tucker and Powles, 1991). In addition to resistance to paraquat and diquat, *H. leporinum* and *H. glaucum* populations have also evolved resistance to fluazifop-p-butyl and sethoxydim (Matthews *et al.*, 2000b). Resistance to paraquat is becoming widespread in populations of *Hordeum* spp. throughout the mid-north of South Australia. A recent survey found that a number of populations collected from cropping fields contained individuals with resistance.

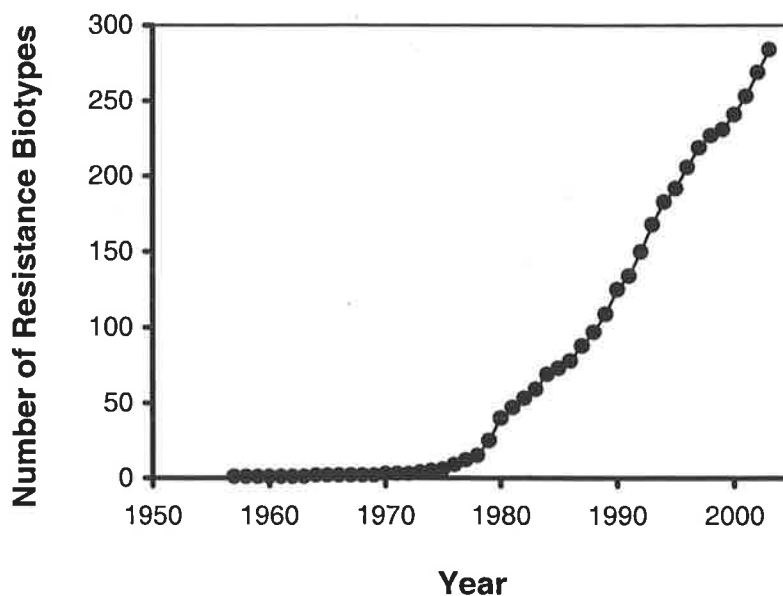


Figure 1.1 Increase in number of resistance biotypes worldwide (adapted from Heap, 2003).

This review will discuss the evolution of herbicide resistance and potential mechanisms of resistance. In addition, the behaviour of paraquat will be reviewed as a herbicidal option commonly used for controlling *Hordeum* spp. DNA marker techniques will also be discussed as a tool to elucidate genetic variation between and within populations of resistant and susceptible weed populations. Lastly, *Hordeum* spp. will be reviewed from biological,

agronomic and resistance aspects, as they are the study species for this project.

## 1.2 Evolution of Herbicide Resistance

Following the first report of resistance in a population of common groundsel (*Senecio vulgaris*) to the triazine herbicides, simazine and atrazine, after ten years successful control with these herbicides (Ryan, 1970), the number of documented herbicide resistance in weeds has significantly increased to 170 species (101 dicots and 69 monocots) and over 270,000 fields in 59 countries (Heap, 2003). The largest number of cases of weed resistance has occurred to the ALS inhibiting herbicides (29%) followed by triazines (23%), ACCase inhibitors (12%), synthetic auxins (8%) and bipyridiliums (8%) respectively (Table 1).

Up to 1999, resistance to triazine herbicides was the most widespread accounting for 27% of all incidences. However, recently there has been a large increase in the evolution of resistance to ALS inhibitors worldwide. The reasons for the rapid increase in resistance to ALS inhibitors are due to the persistence use of these herbicides over large areas, the herbicides providing high efficacy, the lack of herbicide rotation with other modes of action, and the lengthy effect of herbicide residues (Tranel and Wright, 2002).

Table 1.1 Reports of weed resistance to some herbicide groups worldwide (Heap, 2003).

Herbicide Group	Mode of Action	Example	Total
ALS inhibitors	Inhibition of acetolactate synthase	Chlorsulfuron	81
Triazines	Inhibition of photosystem II	Atrazine	65
ACCase inhibitors	Inhibition of acetyl CoA carboxylase	Diclofop-methyl	33
Synthetic auxins	Synthetic auxins (like IAA)	2,4-D	23
Bipyridiliums	Electron diversion at Photosystem I	Paraquat	22
Ureas and amides	Inhibition of photosystem II	Chlorotoluron	20
Dinitroanilines and others	Microtubule assembly inhibition	Trifluralin	10
Thiocarbamates and others	Inhibition of lipid synthesis (not ACCase inhibition)	Triallate	8
Glycines	Inhibition of EPSP synthase	Glyphosate	6
Triazoles, ureas, isoxazolidiones	Inhibition of carotenoid biosynthesis (bleaching)	Amitrole	4
Protoporphyrinogen oxidase inhibitors	Inhibition of Protoporphyrinogen oxidase	Oxyfluorfen	2
Carotenoid biosynthesis inhibitors	Inhibition of carotenoid biosynthesis at the phytoene desaturase (PDS)	Flurtamone	2
Chloroacetamides and others	Inhibition of cell division (inhibition of very long chain fatty acids)	Butachlor	2
Arylamino propionic acids	Unknown	Flamprop-methyl	2
Nitriles and others	Inhibition of photosynthesis at photosystem II	Bromoxyl	1
Mitosis inhibitors	Inhibition of mitosis (mitrotubule polymerazation inhibitor)	Propham	1
Pyrazoliums	Unknown	Difenzoquat	1
Organoarsenicals	Unknown	MSMA	1
Total number of herbicide-resistant biotypes			284

In Australia, the first occurrence of resistance was reported in 1982 (Heap and Knight, 1982), in a biotype of *Lolium rigidum* resistant to diclofop-methyl. One year later *H. glaucum* was reported resistant to the bipyridylum herbicide paraquat (Warner and Mackie, 1983). There are now 27 weed species with resistance to one or more herbicides from 10 different modes of action across Australia (Table 1.2).

Although some other species have also been documented as being resistant to several herbicides, resistance in *L. rigidum* is extremely threatening to the use of chemical control methods because this species shows multiple- and cross-resistance to nine herbicidal classes (Burnet *et al.*, 1994; Powles and Howat, 1990) with five modes of action (Preston *et al.*, 1996a). The incidence of multiple-resistance has occurred because of the ability of this species to evolve multiple mechanisms of resistance (Preston *et al.*, 1996b). It is not surprising that this species has also successfully evolved resistance to glyphosate despite the long held assumption that glyphosate would be immune to resistance development (Lorraine Colwill *et al.*, 2002; Powles *et al.*, 1998). Without proper management in the field, the evolution of resistance might lead to no herbicidal choices in the future for *L. rigidum* (Powles *et al.*, 1997).

Table 2. Weed species documented resistance to herbicides in Australia (Heap, 2003).

Species	States	Herbicide Mode of Action
<i>Arctotheca calendula</i>	Vic	Bipyridiliums
<i>Avena fatua</i>	NSW,SA,WA	ACCCase inhibitors
<i>Avena sterilis</i>	NSW,SA	ACCCase inhibitors
<i>Brassica tournefortii</i>	WA,SA	ALS inhibitors
<i>Bromus diandrus</i>	Vic	ACCCase inhibitors
<i>Cyperus difformis</i>	NSW	ALS inhibitors
<i>Damasonium minus</i>	NSW	ALS inhibitors
<i>Digitaria sanguinalis</i>	SA,WA	ACCCase inhibitors
	SA	ALS inhibitors
<i>Echium plantagineum</i>	SA,WA	ALS inhibitors
<i>Fallopia convolvulus</i>	Qld	ALS inhibitors
<i>Fumaria densiflora</i>	NSW,SA	Dinitroanilines/others
<i>Hordeum glaucum</i>	SA,Vic	Bipyridiliums
	SA	ACCCase inhibitors
<i>Hordeum leporinum</i>	Vic,Tas,SA	Bipyridiliums
	SA	ACCCase inhibitors
<i>Lactuca serriola</i>	SA	ALS inhibitors
<i>Lolium rigidum</i>	NSW, SA,Vic,WA	Multiple Resistance: ACCCase inhibitors ALS inhibitors Dinitroanilines/others Triazoles Mitosis inhibitors Thiocarbamates /others Chloroacetamides/others Triazines Glycines
	SA	Triazines Mitosis inhibitors Thiocarbamates /others Chloroacetamides/others Triazines Glycines
	WA,SA,Vic NSW,Vic,SA,WA	Thiocarbamates/others
<i>Nassella trichotoma</i>	Vic	ACCCase inhibitors
<i>Phalaris paradoxa</i>	NSW	ALS inhibitors
<i>Raphanus raphanistrum</i>	SA,WA	ALS inhibitors
	WA	Triazines
	WA	Multiple Resistance: ALS inhibitors Carotenoid biosynthesis inhibitors
<i>Rapistrum rugosum</i>	Qld	ALS inhibitors
<i>Sagittaria montevidensis</i>	NSW	ALS inhibitors
<i>Sinapis arvensis</i>	NSW	ALS inhibitors
<i>Sisymbrium orientale</i>	NSW,Qld,WA,SA	ALS inhibitors
<i>Sisymbrium thellungii</i>	Qld	ALS inhibitors
<i>Sonchus oleraceus</i>	Qld	ALS inhibitors
<i>Urochloa panicoides</i>	Qld	Triazines
<i>Urtica urens</i>	Vic	Triazines
<i>Vulpia bromoides</i>	Vic	Bipyridiliums

NSW = New South Wales; Vic = Victoria; SA = South Australia; Qld = Queensland; WA = Western Australia, Tas = Tasmania

### **1.3 Factors Influencing Resistance Evolution**

The rate of resistance evolution in weeds is influenced by several factors. These can be summarised as follows: the initial frequency of genes conferring resistance in populations, the level of dominance of the resistant allele, gene flow through cross pollination or seed migration, the strength of the selection pressure by herbicides, the comparative fitness of resistant individuals and the longevity of the weed seed banks (Gressel and Segel, 1982a; Maxwell and Mortimer, 1994; Powles, 1993). These factors all interact, influencing the rate of evolution of herbicide resistance. Therefore, variations in these factors result in differences in the rate of resistance evolution amongst weed species and between herbicides (Powles *et al.*, 1997).

#### **1.3.1 Initial Frequency of Resistance Genes**

The evolution of resistance in a weed population must be preceded by the presence of a gene, or complex of genes, that confers the resistant trait in the population (Christoffers, 1999; Jasieniuk *et al.*, 1996). These genes can be introduced into the population in several ways; including mutation, pollen flow, or migration of seeds or propagules (Maxwell and Mortimer, 1994). Following herbicide application, the frequency of resistant individuals in the population increases and this population will be considered resistant when

about 10-20% of individuals present survive herbicide application (Moss, 2002).

The initial gene frequency for resistance will also influence the rate of resistance evolution given similar selection pressure. It was initially assumed that a resistant gene, reflected as resistant individuals in a population, exists at about  $10^{-5}$  to  $10^{-6}$  for mono-dominant gene or between  $10^{-9}$  to  $10^{-11}$  for mono-recessive genes (Gressel and Segel, 1982a). However, recent investigations have shown that the frequency may be much higher. In a population of *Chenopodium album* the number of individuals resistant to triazine herbicides was found to be about  $3 \times 10^{-3}$  (Darmency and Gasquez, 1990). The most recent finding was that a frequency of chlorsulfuron resistant individuals in populations of *Lolium rigidum* was about  $1 \times 10^{-4}$  (Preston and Powles, 2002a). This higher frequency of resistant individuals could explain why the evolution of resistance occurs more rapidly to some herbicides than others.

### **1.3.2 Mode of Inheritance of Resistance**

Resistance genes in weed species can be transmitted to their progeny through either the nuclear or the organelle genome (Gressel, 1986). All cases of herbicide resistance examined so far are inherited as nuclear genes, with the exception for resistance to triazine herbicides, which is cytoplasmically inherited (Jasieniuk *et al.*, 1996). In addition, resistance traits are mostly controlled by semi dominant or dominant genes (Darmency,



1994; Tardif *et al.*, 1996). Resistance traits controlled by dominant genes will be expressed in both the homozygous and heterozygous states, so both will react positively to selection pressure provided by herbicides (Christoffers, 1999). Dominant genes are also a critical component for an outcrossing weed species, because pollen dispersal can expand the distribution of the resistant population. This has been observed in *Xanthium strumarium* (Ohmes and Kendig, 1999).

Regarding the number of genes, a single major gene has been proven to be responsible for all major cases of herbicide resistance (Jasieniuk *et al.*, 1996). There are two main reasons for this: firstly, most herbicides are targeting specific sites and inhibiting single enzymes in major metabolic pathways, so mutation directly occurs in the genes that encode these enzymes. Secondly, herbicides typically provide an effective control of 90 to 95 %, so only individuals with genes that provide a large effect can survive. In practice, these tend to be single genes. In examples of multiple resistance, the accumulation of several genes, each providing resistance to a separate herbicide, occurs (Preston, 2003). There are also occasional examples where the accumulation of genes encoding different resistance mechanism can occur (Preston and Powles, 1998; Preston and Powles, 2002b; Tardif and Powles, 1993).

### 1.3.3 Selection Pressure

In addition to genetic variation for resistance, there must be persistent and powerful selection pressure to eliminate susceptible individuals from the population (Christoffers, 1999; Maxwell and Mortimer, 1994). Selection pressure refers to the ratio of survival of resistance to susceptible individuals during a growing season after herbicide application (Gressel, 1991; Moss, 2002). Selection pressure can be estimated by effective kill, *i.e.*, the percentage of seed lost, both in resistant or susceptible biotypes, due to herbicide application (Gressel and Segel, 1982b; Jasieniuk *et al.*, 1996). The stronger the selection pressure, the faster the evolution of resistance (Gressel, 1991). Herbicides provide enormous selection pressure on a weed population (Jasieniuk *et al.*, 1996; Mortimer, 1997), because they are generally designed to give more than 90 % control on a susceptible population when applied at the recommended rate (Gressel and Segel, 1982b; Howat, 1987). A 90 % kill rate will cause a ten-fold enrichment of resistance in populations in each year (Howat, 1987).

If herbicides are applied at a lower rate, the evolution of resistance may be slowed because fewer susceptible individuals are killed. However, low rates alone do not mean less selection pressure as they may, in the right environment, also result in high mortality rates. Farmers using herbicides aim to achieve high mortality because poor weed control is not desirable. Therefore, in reality the rate of herbicide used *per se* makes little difference

to the rapidity with which resistance evolves (Preston and Roush, 1998) because efficacy varies with spatial and temporal conditions.

### **1.3.4 Gene Flow**

Gene flow is defined as the movement of genes by gametes, diaspores, or individuals from one site in space to another (Golenberg, 1986). This process can occur through pollen dispersal or seed migration (Golenberg, 1986; Levin and Kerster, 1969; Maxwell and Mortimer, 1994; Schaal, 1980). Of these two means, gene flow via pollen dispersal holds a more important role in plant populations (Campbell and Waser, 1989; Rasmussen and Brodsgard, 1992). However, if mating systems are considered, there is a difference between selfing and outcrossing species. In selfing species, gene flow is mostly through seed migration, while, in outcrossing species, gene flow mediated by pollen dispersal plays a more important role in spreading genes (Darmency, 1996). Factors influencing pollen movement are wind direction and speed, insect pollinators, spacing, plant density, and environmental stress (Golenberg, 1986; Harper, 1977; Levin and Kerster, 1969).

Gene flow plays an important role in building genetic diversity within and between spatially separated populations (Campbell and Waser, 1989; Harper, 1977; Slatkin, 1985). The contribution of gene flow from neighbouring populations can maintain or increase the frequency of an allele responsible for certain traits in a population (Futuyma, 1979). The net

outcome of gene flow is the eventual acquiring of genetic similarity between the populations where it occurs (Fritstrom and Clegg, 1987; Schaal, 1980). The amount of gene flow can be estimated through rare alleles in populations where the progeny have established (Golenberg, 1986; Slatkin, 1985)

Herbicide resistance can evolve in a field from a pre-existing mutation and it can equally be transferred to the field from elsewhere. As well as spreading resistance between populations, gene flow plays a significant role in expanding resistant weed populations (Maxwell and Mortimer, 1994). Gene flow, mediated by pollen dispersal between individuals of the same species, has been documented from herbicide resistant to susceptible populations of *Avena fatua* at a rate of 0.05 % to 0.16 % (Murray *et al.*, 2002), *Setaria faberi* at a rate of 0.24 to 0.73 % (Volenberg and Stoltenberg, 2002), and *Kochia scoparia* at a rate of about 13.1% (Stallings *et al.*, 1995). Surprisingly, gene flow from individuals of closely related but compatible species has also been reported from three different studies for transgenic *Brassica napus* carrying herbicide resistance genes to weed relatives; *Raphanus raphanistrum*, with very low frequency in field experiments (Darmency *et al.*, 1998; Rieger *et al.*, 2001; Warwick *et al.*, 2003).

Weed seed dispersal through farm equipment, contaminated crop seeds, animals and manure also contributes to the spread of resistant populations (Buhler *et al.*, 1997). Tucker and Powles (1988) found that resistant individuals of *H. glaucum* were likely spread by means of seed migration between lucerne fields in an area around Willaura, Victoria through stock,

hay and machinery movement. In addition to these, crop seed has also been suggested as a means of seed spread in *Avena fatua* biotypes resistant to acetyl-Coenzyme A carboxylase (ACCase)-inhibiting herbicides (Andrews *et al.*, 1998). It has also been suggested that seed migration, mediated by hay, was a possible mechanism whereby the target site based resistance to sethoxydim was added to a metabolism based fluazifop-p-butyl resistant *Hordeum leporinum* population (Matthews *et al.*, 2000b).

The fact that gene flow contributes to resistant weed evolution means that management systems for resistant weeds must include methods of restricting gene flow (Christoffers, 1999). The prevention of seed introduction on farming areas and regulation of the level of weed seed infestation are two important actions in resistance weed management strategies (Dekker, 1999).

### **1.3.5 Fitness of Resistant Individuals**

Fitness is related to the capacity of an individual to contribute to the gene pool of the next generation. This is affected by viability, fecundity, and fertility of individual weeds in the population (Roughgarden, 1979). To measure the individual fitness, growth parameters, total biomass and seed production are normally used (Holt and Thill, 1994; Maxwell and Mortimer, 1994).

Weed management practices, such as herbicide application, always causes mortality and survival of individuals in populations. However, survivors often suffer from a decrease in fitness in the absence of the herbicide application (Mortimer, 1997). Therefore, relative fitness of resistance individuals in populations is a factor that may accelerate the rate of resistance evolution.

The effect of fitness on the rate of evolution can be calculated using mutation-selection equilibrium formula (Jasieniuk *et al.*, 1996) as follows:

$$q_e = \mu/hs$$

The frequency of resistance alleles at equilibrium ( $q_e$ ) is a function of mutation rate ( $\mu$ ), selection coefficient ( $s$ ) and the level of dominance ( $h$ ).  $\mu$  is estimated at about  $10^{-6} - 10^{-10}$ , and  $h$  is 1 for dominant, and 0.5 for co-dominant alleles. Selection coefficient ( $s$ ) is determined by the fitness of the homozygous susceptible as 1 and the fitness of resistance as  $1-s$ . With few exceptions, herbicide resistance is mostly controlled by single dominant genes, and therefore, the level of fitness of resistance in most cases will determine the frequency of resistant alleles in population (Preston and Mallory-Smith, 2001). If a recessive gene governs resistance, the formula will be as follows:

$$q_e = (\mu/s)^{0.5}$$

Individuals carrying herbicide resistance genes have been assumed to have a lower fitness compared to normal individuals.

There is considerable evidence that the mutation endowing triazine resistance in *Senecio vulgaris*, *Brassicca rapa* and *Amaranthus hybridus* reduces fitness compared to the wild biotype (Jordan, 1999; Plowman *et al.*, 1999; Radosevich and Holt, 1982), because the mutation causes a decrease in photosynthetic efficiency (Moss, 2002). As a result, the resistant plants are less competitive. However, not all cases of weed resistance are associated with a fitness penalty. Resistance to triazines in *Chenopodium album* has been shown to have equal fitness compared to susceptible individuals as resistance frequency increased from 50 % to about 67 – 77 % over two consecutive years of observation (Plowman *et al.*, 1999). Other studies found that individuals resistant to diclofop methyl in *L. rigidum* (Matthews and Powles, 1992) and to fluazifop-p-butyl in *Digitaria sanguinalis* (Wiederholt and Stoltenberg, 1996) have similar fitness to susceptible individuals. This could explain why these two incidences of resistance appeared rapidly (Tardif and Powles, 1993). In brief, without a significant fitness penalty, resistance tends to evolve faster in the population (Jasieniuk *et al.*, 1996).

Previous fitness studies tended to use herbicide-resistant and -susceptible populations from different origins. It is possible that two such populations

developed from different culture and control techniques. Therefore, these studies have been questioned because observed differences in fitness could correspond to other factors rather than the resistance mutation alone (Cousens *et al.*, 1997; Tranel and Wright, 2002). As an alternative, the use of near isogenic populations in fitness studies has been suggested to reduce imprecision (Holt and Thill, 1994).

### 1.3.6 Seed Bank Characteristics

The extent and turnover of the seed bank can also influence the rate of resistance evolution. For a weed population, the native seed bank is the main source of propagules to replace a population rather than seeds recently introduced into an area (Radosevich and Holt, 1984; Streibig *et al.*, 1992). The size of seed bank and longevity of seed within the seed bank significantly influence the population dynamics of weed species (Holt and Thill, 1994; Qi *et al.*, 1996). When a single herbicide is applied, susceptible plants in the population will be killed before producing seeds. This situation will give more opportunity for resistant plants to deposit their seeds. However, the seed bank of susceptible plants will, if large enough, act as a buffer to delay the onset of resistance. The longer the seed life of susceptible biotypes the greater its buffering effects in the seed bank, resulting in a decreased rate of resistance evolution (Gressel and Segel, 1982a). Weed species with a short dormant period in the seed bank will react more rapidly to the selection pressure associated with herbicide application (Powles *et al.*, 1997). This is why resistance occurs more rapidly



in plants with a short-lived seed bank, such as *L. rigidum*, than in plants where the seed bank is longer lived, such as *Raphanus raphanistrum*. This can also mean that resistance will persist for longer in populations where significant dormancy occurs.

## 1.4 Mechanisms of Herbicide Resistance

To be effective in controlling target weeds, herbicides must be absorbed, translocated to the site of action, and not rapidly degraded so they can inhibit physiological process that eventually lead to the death of weeds (Devine *et al.*, 1993; Preston, 2000; Zimdahl, 1993). As resistance has evolved in weeds, laboratory experiments have been conducted to elucidate the basic mechanisms of resistance in weeds towards herbicides. There are four major mechanisms proposed to explain the phenomenon of weed resistance, namely: decreasing the sensitivity of enzyme target sites to herbicidal action, increasing the capability of metabolically detoxifying herbicides, reducing absorption or translocation of herbicides, and sequestering herbicides away from the active sites (Powles *et al.*, 1990; Rubin, 1991). A single difference in one or more steps can lead to herbicide resistance in weed species (Cole, 1994). In some cases the differences may be small, but are sufficient to discriminate susceptible and resistant biotypes (Devine *et al.*, 1993).

In terms of the number of resistant cases, the most common mechanisms found are the alteration of target sites and more rapid detoxification (Devine

and Preston, 2000). Some examples of weed species developing resistance due to the target site alteration are *Alopecurus myosuroides*, *Avena fatua*, *A. sterilis*, *Galium spurium*, *Lolium rigidum* and *Setaria viridis*, (Christopher *et al.*, 1994; Hall *et al.*, 1998; Maneechote *et al.*, 1997; Marles *et al.*, 1993; Moss *et al.*, 2003; Shukla *et al.*, 1997; Tardif *et al.*, 1996), while enhanced herbicide metabolism confers resistance in *Alopecurus myosuroides*, *Digitaria sanguinalis* and *Lolium rigidum* (Burnet *et al.*, 1993; Cocker *et al.*, 1999; Hidayat and Preston, 1997; Prado *et al.*, 1997; Preston and Powles, 1997; Reade and Cobb, 1999). Often, although not always, resistance related to a change in target site confers a greater degree of resistance (Tardif and Powles, 1993), while resistance due to enhanced metabolism causes a broader spectrum of resistance (Powles *et al.*, 1997).

In certain cases, one population may have more than one mechanism of resistance. This was found in *L. rigidum*, which has target site resistance to acetyl coenzyme-A carboxylase and acetolactate synthase inhibiting herbicides, and an increased capability to detoxify the herbicides chlorotoluron, simazine, chlorsulfuron, tralkoxydim and diclofop-methyl (Preston *et al.*, 1996b). Another example is *Hordeum leporinum*, where resistance to sethoxydim is due to decreased sensitivity of the target enzyme and resistance to fluazifop due to increased herbicide detoxification (Matthews *et al.*, 2000b).

Reduced herbicide translocation has been suggested to confer herbicide resistance in a few weed species, such as paraquat resistance in *H. glaucum*, *H. leporinum* (Preston *et al.*, 1992; Purba *et al.*, 1995), *Erigeron*

*philadelphicus* and *E. canadensis* (Tanaka *et al.*, 1986). Another example of sequestration as a mechanism of resistance can be seen in *L. rigidum* resistance to glyphosate. Resistant individuals have the capacity to sequester glyphosate in the leaf tips, while in susceptible individuals glyphosate accumulated in the roots (Lorraine Colwill *et al.*, 2002).

Multiple-resistance refers to situations where certain weed species possess two or more mechanisms of resistance. The following weed species have been documented (Heap and LeBaron, 2001) to have multiple resistant mechanisms: *Lolium rigidum* with eight mechanisms of resistance in 16 countries, *Echinochloa crus-galli* with six mechanisms of resistance in 15 countries, *Avena fatua* with six mechanisms of resistance in 16 countries, *Poa annua* with six mechanisms of resistance in 15 countries, *Alopecurus myosuroides* with five mechanisms of resistance in 8 countries and *Eleusine indica* with 5 mechanisms of resistance in 5 countries.

Management practices to prevent or delay multiple-resistance in weeds should be carefully designed, because resistance can evolve to a number of herbicides at the same time (Powles *et al.*, 1997). When this happens, growers will face limited herbicidal options for weed control. Incorporation of other control techniques along with chemical controls into a package of weed management systems will assist in combating multiple resistance (Preston *et al.*, 1996a).

## 1.5 Paraquat

### 1.5.1 Properties

Paraquat and diquat are classified as bipyridinium herbicides. The herbicidal properties of these compounds were discovered in 1955. Amongst the properties that led to their wide use are: that they have herbicidal effects at low concentration; they rapidly cause bleaching when in contact with green tissue of plants; and they do not have residual effects in the soil because they are adsorbed to clay minerals (Summers, 1980). Paraquat, (1,1'-dimethyl-4,4'-bipyridylium ion, Figure 1.2), is a contact and non selective herbicide. Before its herbicidal properties was found, paraquat, known as methyl viologen, was used as redox indicator (Cobb, 1992; Dodge, 1971). Paraquat is converted to a radical cation by the transfer of one electron. This can be reversed by the presence of oxygen. This redox system has a potential ( $E_0$ ) of  $-0.45$  V (Summers, 1980).

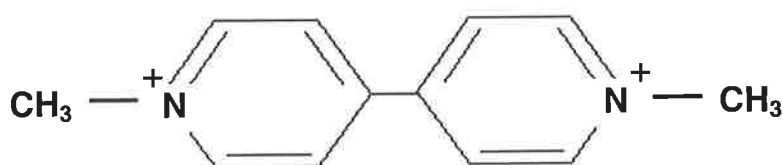


Figure 1.2 Chemical structure of paraquat ion

### 1.5.2 Mode of Action of Paraquat

Paraquat is rapidly absorbed by leaf cells and quickly penetrates to the chloroplast (Dodge, 1971). For paraquat to be active in plants, oxygen and light are required (Bowyer and Cammilleri, 1987). The symptoms of paraquat action can be observed within a few hours of application, forming dark green areas as a result of membrane disruption followed by cell leakage. After two days, necrotic symptoms appear because of plant tissue desiccation leading to plant death (Dodge, 1971; Hess, 2000).

Paraquat action takes place in the chloroplast, disturbing Photosystem I (Preston, 1994) by diverting electrons from the Fe-S centre, a complex iron-sulfur protein (Fujii *et al.*, 1990; Golbeck and Cornelius, 1986) to form the paraquat radical,  $PQ^{\cdot+}$  (Figure 1.3) (Farrington *et al.*, 1973). The paraquat radical is not itself highly toxic, but is unstable and is rapidly oxidised by oxygen, to re-form paraquat (Hess, 2000; Preston, 1994). This reaction with oxygen ( $O_2$ ) produces the superoxide radical,  $O_2^{\cdot-}$  (Bowyer and Cammilleri, 1987).  $O_2^{\cdot-}$  is a toxic agent that can damage the chloroplast envelope and tonoplast (Farrington *et al.*, 1973).

Within chloroplasts, the superoxide radical is normally detoxified by superoxide dismutase (SOD) to make hydrogen peroxide ( $H_2O_2$ ) and molecular oxygen (Figure 1.3). The  $H_2O_2$  then reacts with ferrous salts in the Fenton reaction to produce the hydroxy radical ( $OH^{\cdot}$ ). Hydroxy radicals can also be produced directly from the interaction of hydrogen peroxide and  $PQ^{\cdot+}$  (Cobb, 1992; Fuerst and Norman, 1991; Hess, 2000). It is  $OH^{\cdot}$  that is

the main toxic agent produced by paraquat action (Babbs *et al.*, 1989; Dodge, 1991; Farrington *et al.*, 1973; Hess, 2000; Kunert and Dodge, 1989).

### 1.5.3 Paraquat Use

Paraquat is used in various weed control systems because of its rapid and effective action against weeds. Paraquat is also rapidly adsorbed by soil particles so it has no residual effects (Calderbank and Slade, 1969). This herbicide provides excellent control, particularly of grass weed species. Paraquat is employed in orchards, plantation crops and forests to replace mechanical cultivation. It can also be used to control weeds between rows of arable crops and before sowing or planting crops (Dodge, 1971; Summers, 1980). In pasture, paraquat is applied at low rate for spray topping, after anthesis, but before seed maturity, to reduce seed production (Powles and Bowran, 2000). In rice, paraquat is used to control annual and broadleaf weeds prior to sowing or pre-crop emergence, whereas, in lucerne, this herbicide is applied in autumn or early winter to control winter growing weeds while the lucerne is dormant. In pulse crops, paraquat is applied at the end of the season to reduce seed set of *L. rigidum*. Paraquat is also used in non-agricultural application such as around sheds, and beside roadways and paths (Parsons, 1995).

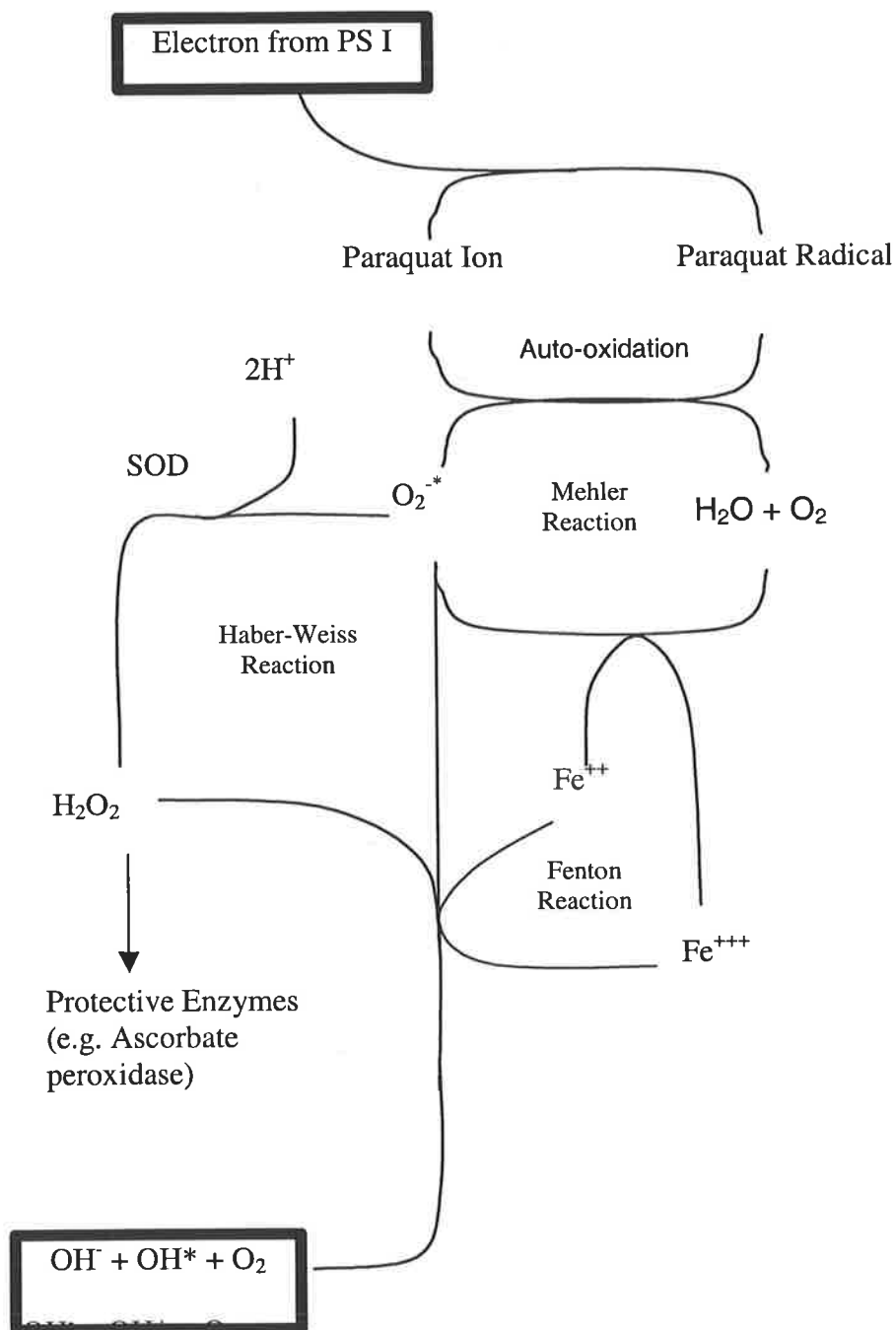


Figure 1.3 Reaction of paraquat ion after receiving electron from Photosystem I and the production of toxic oxygen radicals such as the hydroxyl radical (Hess 2000).

### 1.5.4 Resistance to Paraquat

The first incidence of paraquat resistance was found in *Conyza bonariensis* in Egypt in the early 1980s (Fuerst *et al.*, 1985.). To date, there are 22 weed species documented with resistance to paraquat in 13 countries, Table 1.3 (Heap, 2003). Most paraquat resistance has resulted from repeated applications of paraquat over 10 to 24 years (Alizadeh *et al.*, 1998; Powles, 1986; Powles *et al.*, 1989; Purba, 1993a; Smisek *et al.*, 1998).

In Australia, there are now four weed species with resistance to paraquat, namely: *H. glaucum* (Alizadeh *et al.*, 1998; Powles, 1986), *H. leporinum* (Purba *et al.*, 1995; Tucker and Powles, 1991), *Arctotheca calendula* (Powles *et al.*, 1989), and *Vulpia bromoides* (Purba *et al.*, 1993b). The paraquat-resistant populations were found in perennial and annual cropping systems, such as lucerne and no till-wheat.



Table 1.3 Paraquat resistance in weed species worldwide (Heap, 2003)

Species	Common name	Country/Year
<i>Amaranthus lividus</i>	Livid Amaranth	Malaysia (1990)
<i>Arctotheca calendula</i>	Capeweed	Australia (1986)
<i>Bidens pilosa</i>	Hairy Beggarticks	Kenya (1991)
<i>Conyza bonariensis</i>	Hairy Fleabane	Egypt (1989), Japan (1989), South Africa (2003)
<i>Conyza canadensis</i>	Horseweed	Japan (1980), Canada (1993), USA (1994), Belgium (1998)
<i>Conyza sumatrensis</i>	Sumatran Fleabane	Japan (1980), Taiwan (1980), Malaysia (1990), Sri Lanka (1998).
<i>Crassocephalum crepidioides</i>	Redflower Ragleaf	Malaysia (1990)
<i>Cuphea carthagenensis</i>	Tarweed Cuphea	Fiji (1984)
<i>Eleusine indica</i>	Goosegrass	Malaysia (1990) USA (1996)
<i>Epilobium adenocaulon</i>	American willowherb	Belgium (1982), United Kingdom (1989)
<i>Erigeron philadelphicus</i>	Philadelphia Fleabane	Japan (1989)
<i>Hordeum glaucum</i>	Wall Barley	Australia (1984)
<i>Hordeum leporinum</i>	Barley Grass	Australia (1988)
<i>Ischaemum rugosum</i>	Saramollagrass	Malaysia (1989)
<i>Lipidium virginicum</i>	Virginia Pepperweed	Canada (1993)
<i>Lolium rigidum</i>	Rigid Ryegrass	South Africa (2002)
<i>Monochoria korsakowii</i>	Mizuaoi (Japan) Moolokzam (Korea)	Japan (1994)
<i>Poa annua</i>	Annual Bluegrass	United Kingdom (1981) Belgium (1993)
<i>Solanum americanum</i>	American Black Nightshade	USA (1985)
<i>Solanum nigrum</i>	Black Nightshade	Malaysia (1990)
<i>Vulpia bromoides</i>	Silvergrass	Australia (1990)
<i>Youngia japonica</i>	Asiatic Hawksbeard	Japan (1980)

### 1.5.5 Mechanism of Resistance to Paraquat

As mentioned previously, there are four potential mechanisms of herbicide resistance in weeds. So far there are no reports of any weed species with resistance to paraquat due to differences in paraquat absorption, modification of the paraquat target site, or increases in paraquat detoxification (Preston, 1994). Several studies using [<sup>14</sup>C] paraquat to monitor herbicide movement have found that translocation of paraquat decreased in resistance biotypes compared to susceptible biotypes. This type of resistance mechanism has been observed in *C. bonariensis* (Fuerst et al., 1985.), *E. philadelphicus*, *E. canadensis* (Tanaka et al., 1986), *H. glaucum* (Bishop et al., 1987), and *H. leporinum* (Purba et al., 1995). As a result of reduced translocation, the inhibition of photosynthesis by paraquat is also delayed in resistant populations of *H. glaucum* and *H. leporinum* compared to susceptible populations (Preston et al., 1992).

Another potential mechanism of resistance to paraquat is sequestration. In this mechanism, paraquat is possibly trapped into the cell wall or the vacuole so it is no longer able to reach Photosystem I. Sequestration has been proposed as mechanism of paraquat resistance in *C. bonariensis* (Fuerst et al., 1985.; Norman et al., 1993) and *H. glaucum* (Bishop et al., 1987; Lasat et al., 1997; Powles and Cornic, 1987).

An increase in the activity of superoxide dismutase was also proposed as a possible mechanism of paraquat-resistant in a population of *C. bonariensis* (Shaaltiel and Gressel, 1986). There was a 60 % increase of superoxide

dismutase activity in the chloroplast of resistant individuals compared to susceptible individuals. The amounts of ascorbate peroxidase and glutathione reductase that were also responsible for detoxifying oxygen radical in chloroplast increased up to 150% and 190% respectively in resistant individuals. However, in a further study on this species Amsellem *et al.* (1993) reported that at certain stages of growth the level of increase in antioxidant activity was not consistently observed in resistant individuals, although whole plants still showed 20-fold resistance to paraquat. In this case, increased amounts of these enzymes might be partly responsible for conferring resistance, but another mechanism must also play significant role in facilitating resistance (Amsellem *et al.*, 1993).

In a recent study (Soar *et al.*, 2003) reported, using intact plants treated in the dark, no difference in paraquat translocation between resistant and susceptible populations of *A. calendula*. However, differential translocation of paraquat occurred when plants treated with paraquat were exposed to light. Following the onset of light, paraquat induced massive damage to cells in susceptible plants. This damage then became a means of paraquat translocation due to the damaged region having a water potential of 0 compared to a negative water potential in the rest of the plant. This resulted in paraquat moving extensively from the site of paraquat application to other parts of the plant. This process has been described by Smith and Sagar (1966) as reverse xylem flow. In resistant plants, there was much less cell damage and paraquat movement was much more limited. Therefore, it is possible that there is another mechanism, rather than just reduced

translocation, is responsible for resistance in this species because resistant biotypes are able to avoid the initial severe damage that precipitates paraquat movement.

Other researchers have suggested that physiological mechanisms within the chloroplast are responsible for conferring resistance in *Solanum americanum*, rather than sequestration. It was suggested that a decrease in electron supply from Photosystem I to the paraquat ion occurred in the resistant biotype resulting in the production of fewer hydroxyl radicals (Chase *et al.*, 1998a; Chase *et al.*, 1998b; Chase *et al.*, 1998c).

Another unique characteristic of paraquat resistance in some species is that the level of resistance is significantly reduced by an increase in temperature. In *Hordeum* spp. this is related to increased paraquat translocation (Purba *et al.*, 1995). In contrast, the level of sensitivity to paraquat slightly decreased in susceptible biotype following the increase of temperature (Alizadeh, 1998; Lasat *et al.*, 1996; Purba *et al.*, 1995).

## **1.6. Molecular Markers**

### **1.6.1 DNA**

As the substance of genes, deoxyribonucleic acid (DNA) is a long unbranched polymer composed of four main nucleotides containing a

deoxyribose residue, a phosphate group, and the bases adenine (A), thymine (T), cytosine (C), and guanine (G). The nucleotides are joined together forming polynucleotides by covalent phosphodiester bonds that link the 5' carbon of one deoxyribose group to the 3' carbon of the other to form a long chain. Two complementary chains are held together by hydrogen bonds at bases paired exclusively between A and T, G and C forming a double helix structure (Alberts *et al.*, 1994; Watson, 1977).

The discovery of the double helix structure of (DNA) was a significance breakthrough in molecular biology because it provided an understanding of the mechanism of heredity. Genetic information is carried in a linear sequence of nucleotides with the bases A, T, C, and G functioning as written codes for biological information (Alberts *et al.*, 1994). The genomes of most plants are estimated to contain about  $10^8$  to  $10^{10}$  nucleotides (Paterson *et al.*, 1991).

Individual organisms have variations in the DNA sequences in their genomes. This variation can be detected at the level of individual genotypes using genetic markers (Sunnucks, 2000). The genetic variation can be the result of a difference in a single nucleotide in a gene, a piece of repetitive DNA or through DNA arrangements (Jones *et al.*, 1997).

### 1.6.2 DNA Marker Systems

Genetic analysis of plant populations can be conducted more rapidly and in greater detail with modern genetic analysis techniques such as DNA markers. These techniques can reveal the relationship between individuals or populations at the genetic level (Paterson *et al.*, 1991; Sunnucks, 2000). Unlike morphological markers that are affected by environmental factors, genetic markers are more convenient because they express precise information about the variation in the DNA sequence (Jones *et al.*, 1997). Moreover, DNA markers can be inherited by progeny with the potential for multiple states for each trait (Sunnucks, 2000), so the presence of the trait can be conveniently monitored over time and space. Therefore, the use of DNA markers can efficiently provide genetic information for fingerprinting genotype differentiation, phylogenetic analysis, forensic investigation, paternity tests, and identification of genes responsible for certain diseases (Kumar, 1999; Paterson *et al.*, 1991).

In weeds, molecular studies can be useful in the examination of genetic diversity, population genetics, and reproductive biology (Marshall, 2001). Genetic diversity between and within weed species can result in morphological variation that provides a key for weed identification and management in fields. Therefore, knowledge of genetic polymorphism can be useful to improve strategies of weed management systems (Dekker, 1999), because differential responses of individuals towards weed

management tactics are controlled from the genetic level (Ransom *et al.*, 1998).

From a herbicide resistance perspective, weed populations that have high genetic variation tend to evolve resistance more rapidly to herbicides (Clark and Yamaguchi, 2002). This occurs because it is more likely for a resistant individual to be present and selected by herbicide application (Tranel and Wright, 2002). This suggests the possibility that molecular-based techniques might enable the prediction of the onset of resistance to herbicides (Marshall, 2001).

#### **1.6.2.1 RFLP**

Restriction fragment length polymorphism (RFLP) was the first widely used DNA marker technique to detect polymorphism in the genomes of two or more individuals. This technique uses a restriction endonuclease to digest genomic DNA and to produce differences in fragment lengths of cut DNA as a result of variations in DNA sequence. The polymorphisms detected by RFLP are related to changes in base pairs in the recognition sites of restriction enzymes or to insertions or deletions in the regions between cutting sites (Whitkus *et al.*, 1994). A small piece of DNA labelled with a radioactive or a fluorescent tag from the same sample of DNA can act as probe to recognise one or more homologs of DNA fragments using Southern hybridisation (Jones *et al.*, 1997).

In the past, RFLP has been used extensively in identification of genes responsible for important characters, such as resistance to the fungus

*Erysiphe graminis* in *Hordeum vulgare* (Hinze *et al.*, 1991) or construction of molecular maps of *H. vulgare* and other cereal genomes (Langridge *et al.*, 1995; Sherman *et al.*, 1995). In weeds, this technique was recently used to detect hybrids between *Brassica napus* and *Raphanus raphanistrum* (Rieger *et al.*, 2001). The effectiveness of RFLPs have been limited because this technique is expensive and time consuming, requiring a library of cloned DNA sequences from the species of interest or related species and demanding a large amount of genomic DNA (Whitkus *et al.*, 1994).

#### **1.6.2.2 PCR Based Markers**

The polymerase chain reaction (PCR) is an *in vitro* technique for amplifying specific DNA or RNA sequences by the extension of primers on complementary DNA strands (Bej *et al.*, 1991; Taylor, 1991). DNA is extracted from the organism being studied and is mixed with two oligonucleotide primers, deoxyribose nucleotides (dNTPs), and a high temperature stable DNA polymerase, usually TAC or similar. DNA is denatured at high temperature and then, on lowering the temperature, the oligonucleotide primers anneal to DNA. Following annealing, the reaction is set at the optimal temperature for the polymerase to function. Extension of the complementary DNA strand occurs and the reaction is heated again to denature the DNA strands. This cycle is repeated 25 – 40 times resulting in an exponential increase in the concentration of the DNA segment of interest (Bej *et al.*, 1991; Brown, 2000; Buffery, 1993; Hoelzel and Green, 1998; Taylor, 1991).



Some PCR based marker techniques currently available to be employed for various genetic analyses are listed in Table 1.5. In addition to advantages and disadvantages of each technique, other factors should be considered in choosing the technique to be used, such as budget and time. Here, the principles and usefulness of three common techniques, RAPD, AFLP and microsatellites, are discussed.

Table 1.5. DNA marker techniques employed in genetic analysis (adapted from Sunnucks, 2000).

Techniques	Single locus	Co-dominant	Loci available	Connectibility among studies	Transfer to new taxa	Variability
Mitochondria/ Chloroplast Sequence	Yes	Yes	Single	Direct	Yes	Low-High
RAPD	No	No	Many	Limited	Yes	High
AFLP	No	No	Many	Limited	Yes	High
Microsatellites	Yes	Yes	Many	Indirect	Some	High
rDNA	No	No	Few	Limited	Yes	Moderate-High

### 1.6.2.3. Randomly amplified polymorphic DNAs (RAPD)

RAPDs were introduced in 1990 and have been a popular tool in DNA marker analysis (Williams *et al.*, 1990). This technique uses the ability of a

single PCR primer consisting of about 10 nucleotides that are GC-rich to bind to two homologous regions of DNA. The sequence between the primer binding sites, of between 200 – 2000 kb in length, is amplified. Following separation by electrophoresis, the PCR products can be readily visualised by staining with ethidium bromide (Jones *et al.*, 1997). The main advantages of using RAPDs are that no prior knowledge of DNA sequences is needed and no radioactive probes are used. Also RAPDs are cheaper and simpler to use than some other techniques (Abad *et al.*, 1998; Jones *et al.*, 1997; Moodie *et al.*, 1997; Nissen *et al.*, 1995; Woolley *et al.*, 2000).

The stability and consistency of the RAPD technique has been proven in studying the DNA profiles of rye (*Secale cereale* L.) from 10 individuals (Iqbal and Rayburn, 1994). Similar success was also experienced by Wooly *et al.* (2000) in classifying 50 accessions of almond cultivars collected from Australia, California, Europe and the Middle East. RAPD markers have also been chosen in several studies to examine: genetic relationship amongst some cultivars of *H. vulgare* (Noli *et al.*, 1997; Tinker *et al.*, 1993), the results of backcross experiments in *H. vulgare* (Komatsuda *et al.*, 1997), the difference between in *H. vulgare* and *H. spontaneum* (Ordon *et al.*, 1997), and the phylogenetic relationships among 39 wild *Hordeum* species, subspecies, and *H. vulgare* (Marillia and Scoles, 1996).

In weed populations, RAPDs have been successfully employed to study genetic diversity in *Sinapsis arvensis* (Moodie *et al.* 1997), *Euphorbia esula* (Rowe *et al.* 1997), *Cyperus esculentus* (Abad *et al.* 1998), *Apocynum cannabinum* (Ransom *et al.* 1998), *Chromolaena odorata* (Scott *et al.* 1998),

and *Echinochloa* spp. (Lopez-Martinez *et al.* 1999). DNA markers, based on RAPD techniques, have been used to study the centre of origin of introduced weeds, to consider whether a population has developed from one or multiple introductions, and to understand the relationship between target weeds and potential biological agents in weed biological control systems (Nissen *et al.* 1995).

RAPDs are also widely used to study genetic relationships of weed species from genus *Hordeum*. The result of these studies showed a high level of polymorphisms between and within species which were relatively consistent to those based on morphological assessments (Gonzalez and Ferrer, 1993; Marillia and Scoles, 1996) and confirmed, in particular, the close relationship between *H. murinum* and *H. leporinum* (De Bustos *et al.*, 1998), *H. marinum* and *H. scalinum*, the unique nature of *H. bulbosum* (DeBustos *et al.*, 1999), and high genetic diversity between and within populations of *H. spontaneum* (Dawson *et al.*, 1993).

#### **1.6.2.4 AFLP and Microsatellites**

AFLP (Amplified Fragment Length Polymorphism) and microsatellites are alternative techniques for studying genetic variation in crops and weeds. AFLP was introduced in 1995 and consists of three major steps: restriction of DNA and ligation of oligonucleotide adapters, specific amplification of sets of restriction fragments, and gel analysis of amplified fragment (Vos *et al.*, 1995). This technique is now widely employed because it is quick, robust, no prior knowledge of DNA sequence is required, and it is able to identify

multiple loci and genetic polymorphisms in a single PCR reaction (Aggarwal *et al.*, 1999; Ajmone Marsan *et al.*, 1998; Maughan *et al.*, 1996). The selectiveness of AFLPs is due to the use of selected restricted fragment amplification to generate AFLPs from whole digested genomic DNA (Lu *et al.*, 1996; Vos *et al.*, 1995).

Studies on genetic diversity and similarity within and between populations of *Oryza* spp. (Aggarwal *et al.*, 1999; Zhu *et al.*, 1998) *Helianthus annuus* (Hongtrakul *et al.*, 1997) and *Cynodon* spp (Zhang *et al.* 1999) have been successfully conducted with AFLP markers. In weeds, AFLP detected little variation within populations, but a great variation between populations of *Setaria italica* and *S. viridis* (Thierry d' Ennequin *et al.*, 2000). AFLP has also been used to detect genetic relationship among resistant populations of wild oat (*Avena fatua*) collected from different fields (Andrews *et al.*, 1998).

Microsatellites or Simple Sequence Repeats (SSRs) is a PCR based marker technique that detects repetitive DNA sequences in plant genomes. As the plant genome contains a large number of repetitive sequences of less than 6 bp, tandemly repeated and randomly dispersed throughout chromosome, microsatellites can reveal high polymorphism and detect variation within a population (Jones *et al.*, 1997; Kresovich *et al.*, 1995; Morchen *et al.*, 1996). However this technique is expensive, needs a long time to develop and requires specific primers (Jones *et al.*, 1997).

Using the microsatellite technique, genetic diversity of *Anisantha sterilis* within and between farms was studied (Green *et al.*, 2001). A high level of

polymorphism between individuals within a field, from different fields and between different farms was found. Microsatellites have also been successfully used to determine genetic relationships and the possibility of gene flow between cultivated, wild and weedy forms *Beta vulgaris*. The level of polymorphism was low in cultivated beets, higher in wild biotypes and moderate in weed beets (Desplanque *et al.*, 1999; Morchen *et al.*, 1996; Viard *et al.*, 2002).

## **1.7 Weedy *Hordeum* spp.**

### **1.7.1 Biology**

The *Hordeum* spp. are described as having flat leaf blades, a dense spike with single flowered spikelets arranged in threes. The central spikelet is bisexual with two glumes resembling awns placed side by side in front of the spikelet (Black, 1978). The awns on the central spikelet are shorter than those on the outside and spikelets have pale brown or brown anthers. When the spikelets are mature they fall as triads making them very useful dispersal tools (Auld and Medd, 1987; Cocks *et al.*, 1976).

Three species of the *Hordeum murinum* complex are present in Australia. These are *Hordeum murinum*, only recorded from near Hobart, Tasmania, *H. leporinum* and *H. glaucum*. The later two are widespread across southern Australia and are the most troublesome weeds from the *Hordeum* genus

(Cocks *et al.*, 1976). Although *H. leporinum* and *H. glaucum* have relatively similar features, *H. leporinum* can be distinguished due to the exertion of anthers while *H. glaucum* has included anthers (Cocks *et al.*, 1976). Cytogenetic studies (Rajhathy and Morrison, 1962) showed that *H. leporinum* and *H. glaucum* have a different chromosome numbers. *H. leporinum* is tetraploid with 28 chromosomes, whereas *H. glaucum* is diploid with 14 chromosomes. It is believed that *H. glaucum* is an ancestor of *H. leporinum* and *H. murinum*.

Rainfall is a determining factor of weedy *Hordeum* spp. distribution in southern Australia. *H. leporinum* prefers to grow in wetter areas where the rainfall is more than 425 mm, whereas *H. glaucum* is found in the semiarid areas with the rainfall less than 425 mm (Cocks *et al.*, 1976).

### 1.7.2 Agronomic Aspects

The barley grasses (*Hordeum* spp) are annual winter growing weeds native to Europe and Asia (Dashorst and Jessop, 1998). They are thought to have entered Australia following first settlement and their presence can be found almost everywhere in southern Australia's pastoral zones (Smith, 1968a). With the exception of *Hordeum vulgare*, cultivated barley, other members of *Hordeum* spp. are not economically important because their grain size is small (Smith, 1972).

*Hordeum* spp. are important weeds in pasture (Smith, 1972) as well as in crop areas, particularly in minimum tillage systems (Heap and Stephenson, 1986; Poole *et al.*, 1986). *Hordeum* spp. have been categorised as one of the most difficult weeds to control in Australia (Jones *et al.*, 2000). This species is also an alternative host for cereal diseases (Code, 1986). *Hordeum* spp. are more competitive in wheat crops compared to ryegrass (*Lolium rigidum*) and silver grass (*Vulpia myuros*) (Poole and Gill, 1986).

In southern Australia *H. leporinum* can be a major component of pastures. This weed grows well in fertile and lighter textured soils (Poole *et al.*, 1986). Under high soil nitrogen, *H. leporinum* grows vigorously and often invades legume-based pastures (Smith, 1968b). In some pastures, *H. leporinum* amounts to 50 to 90 % of total dry matter production and produces about 25,000 seeds per m<sup>2</sup> (Smith, 1968c). Barley grasses produce seeds with long and barbed awns. These awns can aggravate the mouths, eyes, and noses of cattle or sheep as well as contaminate wool and meat (Smith, 1968a).

*Hordeum* spp. seeds have an after-ripening period of up to 130-140 days after which they germinate if conditions are suitable. This seed character may explain why *Hordeum* spp. have successfully adapted to southern Australia cropping zones with hot-dry summers (Popay, 1981). In general, *Hordeum* species germinate in May and produce seeds in October (Poole, 1986).

Several herbicides have been registered for the control of *Hordeum* in pastures and crops (Table 1.6). Among these, paraquat and diquat have been intensively used over a long period of time, particularly in lucerne crops and pastures.



Table 1.6. Some herbicides introduced for barley grass control in Australia (APVMA, 2003).

Chemical Name	Example of Product Name	Crops
Amitrole	Amitrole	Vineyards, orchards, potatoes
Atrazine	Nu-trazine	Ryegrass seed crops, Triazine-tolerant canola
Bromacil	Hyvar	Asparagus, citrus, pineapple
Chlorsulfuron	Glean	Wheat and triticale
Clethodim	Select	Lupins, faba bean, soybeans, mungbeans, chickpeas
Cyanazine	Bladex	Field peas, Chickpeas, faba beans, lentils
Diuron	Diuron	Lucerne, cotton, sugar cane, asparagus
Ethofumesate	Tramat	Oilseed poppy, beet crops, onions, ryegrass pasture
Fluazifop-p-butyl	Fusilade	Lupins, canola, faba beans, field peas, chick peas
Fluometuron	Convoy	Cotton
Glufosinate ammonium	Basta	Vineyards, citrus orchards, fruit plantations
Glyphosate	Roundup	Peanuts, cotton, sugarcane, vine crops, soybeans, onions
Haloxifop-methyl	Verdict	Canola, chickpeas, faba beans, lucerne, lupins, medic and clover pasture
Imazamox	Raptor	Field peas, soybean, peanuts
Imazapic+Imazapyr	OnDuty	Canola (Clearfield)
Imazapic + Imazapyr + MCPA	MIDAS	Wheat (Clearfield)

Table 1.6 Continued from previous page

Chemical Name	Example of Product Name	Crops
Imazethapyr	Spinnaker	Faba beans, field peas, soybeans, peanuts, Lucerne, subterranean clover
Metribuzin	Sencor	Barley and faba beans
Norflurazon	Solicam	Citrus, grapes, pome fruits, stone fruits, nuts
Oxyfluorfen	Goal	Cereals, cotton, onions, brassica vegetables, tobacco
Phenmedipham	Betanal	Beet crops
Paraquat	Gramoxone	Lucerne, orchards, vineyards
Propaquizafop	Correct	Lucerne, canola, faba beans, pasture, lupins, chickpeas, lentils
Propyzamide	Kerb	Lucerne, clover, medics, lettuce
Quizalofop-p-ethyl	Targa	Canola, cabbage, carrot, cauliflowers, clover and subclover pastures, faba beans, onions
Sethoxydim	Sertin	Canola, chickpeas, faba bean, lupins, field peas, lentils
Simazine	Simazine	Lucerne, lupins, asparagus, faba beans, Triazine-tolerant canola
Sulfosulfuron	Monza	Wheat
Tepaloxymid	Aramo	Canola, chickpeas, faba bean, lupins, field peas
Terbacil	Sinbar	Lucerne, pasture, apples, peaches
Trifluralin	Triflur	Cereals, legumes, cotton, orchards, vineyards

### 1.7.3 Resistance to Herbicides in *Hordeum* spp.

The first incidence of herbicide resistance in the genus *Hordeum* was found in a population of *H. glaucum* resistant to paraquat at Willaura near Ararat, Victoria (Warner and Mackie, 1983). Paraquat failed to kill this population following 10-12 years of continual application. The resistant population was 250 times as resistant as susceptible plants (Powles, 1986). In this case, the factors responsible for resistance evolving included: no crop rotation for 10 years; continuous application of paraquat/diquat every year; no integrated weed management; and no cultivation for at least 10 years (Tucker and Powles, 1988a). Paraquat resistance was also found in *H. leporinum* from a field of lucerne near Elmhurst, Victoria. In this case, there was a 24-year history of paraquat and diquat application (Tucker and Powles, 1991).

The first incidence of paraquat resistance in a *H. glaucum* population in lucerne fields in South Australia was reported by Purba (1993). Following this, two populations of *H. glaucum* from zero tillage were also found to be resistant to paraquat after 10-15 years of use (Alizadeh *et al.*, 1998). A survey of lucerne fields in Victoria in 1989 discovered a number of *Hordeum* spp. populations with resistance to paraquat (Tucker and Powles, 1988b). A survey, conducted in 1999 in South Australia, established that further populations of *Hordeum* spp. resistant to paraquat were present in both lucerne and cereal fields (Preston unpublished).

In addition to resistance to paraquat and diquat, *H. leporinum* and *H. glaucum* populations have also been reported with resistance to fluazifop-p-

butyl and sethoxydim (Matthews et al., 2000b). To date, six populations of *H. leporinum* and one population of *H. glaucum* with resistance to aryloxyphenoxypropanoate herbicides have been discovered (Preston unpublished).

The incidence of resistance to herbicides in *Hordeum* spp. may continue to increase in Southern Australia, because the control of this weed is highly dependent on chemical use. Therefore, studies on factors contributing to resistance evolution in this weed species are urgent to prevent or delay further evolution.

## **1.8 Conclusion and Objectives of this Project**

Herbicide resistance in weeds has become a major issue in weed management because it limits the long-term use of herbicides and complicates weed management practices. In Australia the number of weed species documented with herbicide resistance is increasing as a result of continued intensive herbicide use. Herbicide use is a driving factor for weed resistance, but the rate of evolution is influenced by initial frequency of resistance genes, the fitness of resistant individuals and the soil seed bank. Pollen dispersal and seed migration can play important roles in spreading resistant genes. Movement of resistance via pollen and seeds can also create new sources for resistance evolution.

*Hordeum* spp. are important winter weeds in both pasture and crop fields in Australia. Some populations of this weed species have successfully evolved resistance to the herbicides paraquat, fluazifop-p-butyl and quizalofop-p-ethyl, resulting in a limitation of herbicidal choices for future control.

A recent survey suggests paraquat-resistant barley grass is more widespread in South Australia than previously thought, particularly in lucerne fields. The advent of glyphosate resistance in *L. rigidum* (Lorraine Colwill *et al.*, 2002; Powles *et al.*, 1998; Pratley *et al.*, 1999) in Australia means that paraquat is being promoted as an alternative knockdown herbicide to glyphosate. Consequently, paraquat will be used in a more intensive manner in the future increasing the risk of evolution of resistance to this herbicide.

This study will seek to determine whether paraquat-resistant *Hordeum* spp. are being moved between lucerne fields and crop fields in South Australia. The study will undertake surveys for resistant populations in fields and will then seek to determine the similarity among resistance populations. RAPD markers will be employed to detect genetic variation within and between resistant and susceptible populations. The role of gene flow mediated by pollen movement will also be determined, as well as, the relative fitness of resistant homozygote, heterozygotes, and susceptible homozygote individuals.

**Chapter Two:**  
**Survey and Testing for Paraquat-resistant Populations**  
**of *Hordeum glaucum* and *H. leporinum* from Crops**  
**and Pastures**  
**in South Australia**

## 2.1 Introduction

The herbicide paraquat has been used for a long time in South Australia to control weed species in crops and pastures. In lucerne fields, this herbicide is employed to control winter growing weeds such as *Hordeum* spp. while lucerne is dormant (Preston, 1994). In cereal crops, paraquat is used for weed control prior to sowing the crops, whereas in pasture paraquat is applied for spray-topping in spring to reduce seed production (Powles and Bowran, 2000). Paraquat does not have any residual soil effects and its long-term use is safe for both crops and pasture (Summers, 1980). However, the intensive application of paraquat for several years provides continuous selection pressure on populations that enables the evolution of herbicide resistance (Alizadeh et al., 1998; Powles et al., 1989; Purba, 1993a; Tucker and Powles, 1991).

The first incidence of paraquat resistance *H. glaucum* in South Australia occurred in a lucerne field at Spalding in 1990. Further experiments showed that this population was highly resistant to paraquat (Purba 1993). Later, three more paraquat-resistant populations of *H. glaucum* from separate fields were investigated. Two resistant populations were reported from no-till wheat at Avon (Alizadeh et al., 1998) and a population from a lucerne field at Mount Bryan (Alizadeh 1998).

Recently, there have been increasing reports of failure of paraquat to control *Hordeum* spp. in lucerne fields in South Australia. These failures can be an indication of the incidence of paraquat resistance. As the incidence of

paraquat resistance increases, the need for early detection of herbicide resistance becomes crucial for management. Resistant individuals need to be stopped from enriching seed banks (Matthews, 1994) and spreading between fields. Collecting mature seeds, followed by testing of herbicide efficacy on seedling of suspected resistant populations, forms the initial steps in identifying resistance.

In both *H. glaucum* and *H. leporinum*, paraquat resistance has been shown to be the result of reduced basipetal movement of paraquat from leaves (Purba et al., 1995). This mechanism is temperature-dependent and correlated with a dramatic reduction of resistance under warm conditions (Alizadeh et al., 1998; Purba et al., 1995). Therefore, a simple way to determine whether populations have a similar mechanism of resistance is to investigate whether higher temperatures reduce the level of resistance. This chapter describes the results of surveys to evaluate the extent of resistance in two prominent lucerne-growing areas of South Australia and the testing undertaken to determine the resistance status of populations collected.

## **2.2 Materials and Methods**

### **2.2.1 Surveys**

Two surveys were conducted, one in 1999 and one in 2001, to collect sample populations in South Australia. The area included in the surveys is



shown in Figure 2.1. In 1999, populations were collected from cereal crops, pastures and lucerne fields. In 2001, the survey was targeted to lucerne fields and adjacent fields. The surveys were conducted in November 1999 and 2001, when *H. glaucum* and *H. leporinum* had set seeds. From each population the seeds were harvested from as many individual plants as possible. The seeds were air dried for one week and then stored in cold room at 4°C. The plants were tested for resistance in the following winter.

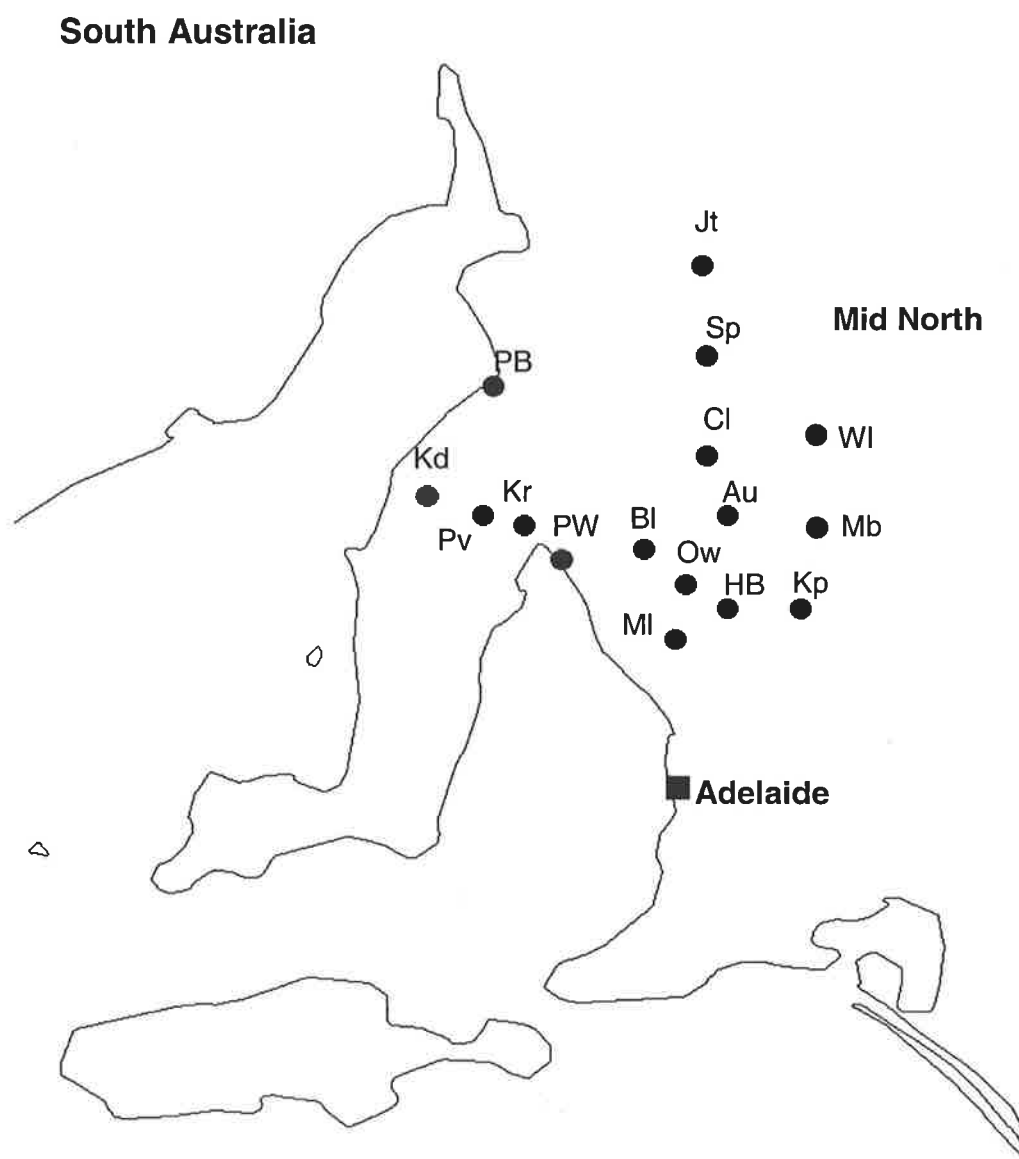


Figure 2.1 Survey locations of *Hordeum* sp in South Australia. The sample populations were collected between towns; Port Broughton (PB), Kadina (Kd), Paskerville Pv), Kulpara (Kr), Port Wakefield (PW), Balaklava (Bl), Owen (Ow), Mallala (MI), Hamley Bridge (HB), Kapunda (Kp), Marrabel (Mb), Waterloo (WI), Auburn (Au), Clare (Cl), Spalding (Sp) and Jamestown (Jt)

## 2.2.2 Plant Materials

The seeds collected from surveys were germinated on 0.6 % agar in 1 L plastic containers for 7 days in an incubator with a 12 h/12 h (20°C/20°C) light ( $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and dark regime. Individual seedlings were transplanted at the one-leaf stage into 17 cm pots containing potting mix. When plants had two to five leaves they were sprayed with a commercial formulation of paraquat (Gramoxone 250 g L<sup>-1</sup> paraquat, Crop Care Australia) at 200 g a.i. ha<sup>-1</sup>. All survivors were considered to be resistant and allowed to set seed. The seeds harvested from these individuals were bulked within sites and used for dose response experiments and DNA analysis. As a comparison, known paraquat-resistant populations of *H. glaucum* (SHG4 from no till wheat in South Australia) and *H. leporinum* (THL1 from a lucerne field in Tasmania) were used in dose response experiments. Susceptible controls consisted of populations of *H. glaucum* (VHG2, the fourth generation grown from seeds collected in 1986 from a pasture field at Elmhurst, Victoria, with no history of paraquat application) and *H. leporinum* (THL4, a population from a pasture at Ouse – Tasmania). In addition, a second susceptible population of *H. leporinum* was harvested from unsprayed plants of a population where all plants were controlled by 200 g ha<sup>-1</sup> paraquat when tested. In the following years, the seeds were germinated as described above and transplanted into pots containing potting soil for dose response experiments. There were ten plants per pot and three to five replicates for each rate of herbicide.

### 2.2.3 Herbicide Application

Experiments to evaluate the response of susceptible and resistant populations to paraquat were carried out during winter at the Waite Campus. Plants at the three to five-leaf stage were sprayed with paraquat at rates ranging from 0 to 1600 g ha<sup>-1</sup> (plus 0.2 % non ionic surfactant) in the late afternoon and were kept indoors overnight. The plants were returned outdoors the following morning. The herbicide was applied using a custom-built moving belt (1 m s<sup>-1</sup>), twin nozzle laboratory sprayer delivering a total volume of 103 L ha<sup>-1</sup> at 250 kPa pressure. The boom was positioned 40 cm above the foliage. Plant mortality and survival were determined at 21 days after herbicide application. Plants were considered alive if they had produced new green leaf material.

### 2.2.4 Effects of temperature on paraquat resistance

Dose response experiments were also conducted during summer to observe the effect of high temperature on the degree of resistance. The susceptible and resistant populations of *H. leporinum* and *H. glaucum* were germinated and transplanted to pots as described above. The plants were kept in a glasshouse for two days to allow seedlings to recover from transplanting. After this, the plants were grown outdoors with daily watering. At the three to five-leaf stages, the plants were treated with paraquat in a dose response

and spraying conditions as described above. Plants were scored as alive or dead 14 days after paraquat application.

## 2.3 Results

### 2.3.1 Surveys

From the first survey in 1999, 44 sample populations were collected and, of these, seven samples did not germinate. Of the remaining populations, seven contained individuals that survived application of paraquat (Table 2.1). Further testing showed that only one population of *H. glaucum*, from 1.4 km between Jamestown and Spalding South Australia, was resistant. One possible explanation for this was that the first test was conducted in early spring and some susceptible individuals were able to survive at a rate of 200 g ha<sup>-1</sup> paraquat as temperature increased.

Table 2.1. Data from survey and testing of *Hordeum* spp. in 1999

Population	Location	Crop	Status
99001	3.3 km Pt Wakefield - Balaklava	Wheat	Susceptible
99002	10.4 km Balaklava - Salter Springs	Barley	Susceptible
99003	6.2 km Rhynie - Auburn	Wheat	Susceptible
99006	19.4 km Balaklava - Salter Springs	Wheat	Susceptible
99007	1.8 km Balaklava - Auburn	Wheat	Susceptible
99008	16.6 km Mallala - Owen	Wheat	Susceptible
99009	23.0 km Balaklava - Salter Springs	Wheat	Susceptible
99010	2.3 km Owen - Hamley Bridge	Wheat	Susceptible
99012	8.5 km Pt Wakefield - Balaklava	Barley	Susceptible
99013	2.3 km Owen - Hamley Bridge	Wheat	Susceptible
99014	13.0 km Hamley Bridge - Tarlee	Barley	Susceptible
99015	7.0 km Tarlee - Marrabel	Canola	Susceptible
99016	18.6 km Tarlee - Marrabel	Wheat	Susceptible
99019	15.6 km Tarlee - Marrabel	Wheat	Susceptible
99020	12.0 km Owen - Hamley Bridge	Wheat	Susceptible
99021	20.3 km Mallala - Owen	Barley	Susceptible
99022	12.3 km Mallala - Owen	Wheat	Susceptible
99023	9.5 km Owen - Hamley Bridge	Wheat	Susceptible
99024	9.0 km Hamley Bridge - Tarlee	Pasture	Susceptible
99025	13.0 km Hamley Bridge - Tarlee	Lucerne	Susceptible
99026	10.6 km Tarlee - Marrabel	Wheat	Susceptible
99027	16.6 km Mallala - Owen	Wheat	Susceptible
99028	1.4 km Jamestown - Spalding	Lucerne	Resistant (SHG7)
99029	22.5 km Jamestown - Spalding	Wheat	Susceptible
99030	17.9 km Jamestown - Spalding	Wheat	Susceptible
99031	8.5 km Kulpara - Paskerville	Pasture	Susceptible
99032	1.7 km Paskerville - Kadina	Wheat	Susceptible
99033	8.8 km Alford - Pt Broughton	Barley	Susceptible
99034	17.8 km Alford - Wokuna	Wheat	Susceptible
99035	13.2 km Wallaroo - Alford	Wheat	Susceptible
99036	10.0 km Spalding - Clare	Lucerne	Susceptible
99037	2.5 km Jamestown - Caltowie	Lucerne	Susceptible
99038	26.5 km Jamestown - Spalding	Lucerne	Susceptible
99039	5.5 km Paskerville - Kadina	Wheat	Susceptible
99040	2.9 km Alford - Pt Broughton	Lentils	Susceptible
99043	11.6 km Paskerville - Kadina	Wheat	Susceptible
99044	8.8 km Alford - Pt Broughton	Wheat	Susceptible

The second survey was conducted in 2001 to search for additional resistant populations between Jamestown, Spalding and Clare and to explore other areas between Waterloo and Kapunda. This later survey also sampled *Hordeum* spp. from fields adjacent to lucerne fields. The aim was to determine whether resistant populations were evolving locally or were being imported from elsewhere. Of 23 populations sampled, one sample did not germinate. There were five populations that contained resistant individuals of *H. glaucum* (Table 2.2). In addition, one sample of *H. leporinum* from a wheat field contained paraquat-resistant individuals. This is the first report of paraquat resistance in *H. leporinum* from South Australia.

Table 2.2 Data from survey and testing of *Hodeum* spp. in 2001

Population	Location	Crop	Status
01001	1.4 km Jamestown - Spalding	Lucerne	Susceptible
01002	1.4 km Jamestown - Spalding	Pasture	Susceptible
01003	1.4 km Jamestown - Spalding	Lucerne	Resistant (SHG7)
01004	9.8 km Jamestown - Spalding	Lucerne	Resistant (SHG8)
01005	9.8 km Jamestown - Spalding	Wheat	Susceptible
01006	16.8 km Jamestown - Spalding	Lucerne	Resistant (SHG9)
01007	16.8 km Jamestown - Spalding	Wheat	Susceptible
01009	21.4 km Jamestown - Spalding	Wheat	Susceptible
01010	4.0 km Spalding - Clare	Pasture	Resistant (SHG10)
01011	4.0 km Spalding - Clare	Pasture	Susceptible
01012	5.0 km Spalding - Clare	Oat	Susceptible
01013	5.0 km Spalding - Clare	Pasture	Susceptible
01014	5.0 km Spalding - Clare	Pasture	Resistant (SHG11)
01015	5.0 km Waterloo - Marrabel	Luc/Oats	Resistant (SHG12)
01016	5.1 km Waterloo - Marrabel	Wheat	Susceptible
01017	0.2 km Marrabel - Waterloo	Luc/Oats	Susceptible
01018	1.6 km Marrabel - Kapunda	Lucerne	Susceptible
01019	1.6 km Marrabel - Kapunda	Pasture	Susceptible
01020	1.6 km Marrabel - Kapunda	Wheat	Resistant (SHL2)
01021	7.3 km Marrabel - Kapunda	Lucerne	Susceptible (SHL1)
01022	7.3 km Marrabel - Kapunda	Wheat	Susceptible
01023	7.3 km Marrabel - Kapunda	Wheat	Susceptible



### 2.3.2 Dose response experiments

The known susceptible population of *H. glaucum* (VHG2) was controlled with 200 g a.i ha<sup>-1</sup> paraquat. The known resistant population of *H. glaucum* (SHG4) was highly resistant to paraquat (Figure 2.2 and 2.3). When an additional six populations of *H. glaucum* (designated SHG7, SHG8, SHG9, SHG10, SHG11 and SHG12) were tested for resistance, all proved to be resistant with dose responses similar to SHG4.

SHL1, determined as a susceptible population because all individuals were controlled by 200 g a.i. ha<sup>-1</sup> paraquat in initial testing, proved to be highly susceptible to paraquat in dose response experiments (Figure 2.4 and 2.5). The known resistant population, THL1, proved highly resistant to paraquat in this experiment, with little mortality even at 1600 g a.i ha<sup>-1</sup>. The suspected resistant population SHL2 also proved highly resistant to paraquat, with a similar dose response to THL1.



Figure 2.2 Response to paraquat of a known susceptible *H. glaucum* population (VHG2), known resistant populations (SHG4 and SHG5), and six additional populations suspected to be resistant, three weeks after paraquat application.

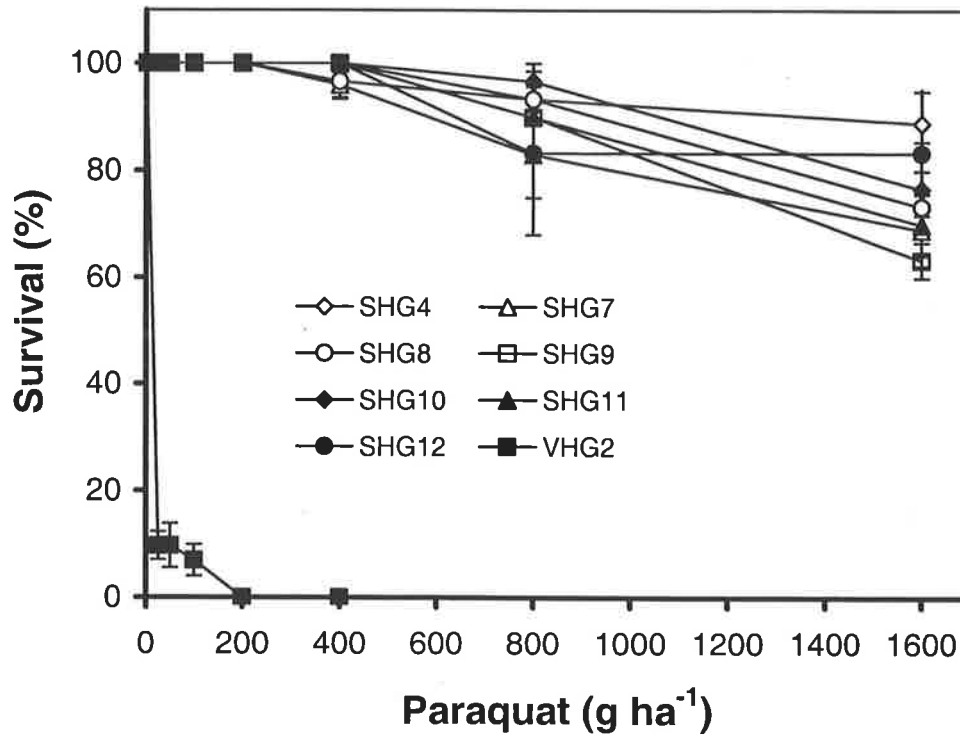


Figure 2.3 Response of a known susceptible *H. glaucum* population (VHG2), a known resistant population (SHG4), and six additional populations suspected to be resistant, to paraquat. Populations were grown outdoors in winter. Each point is the mean of 3 replicates  $\pm$  SE.



Figure 2.4 Response to paraquat of susceptible (SHL1) and resistant (SHL2) populations of *H. leporinum* three weeks after paraquat application.

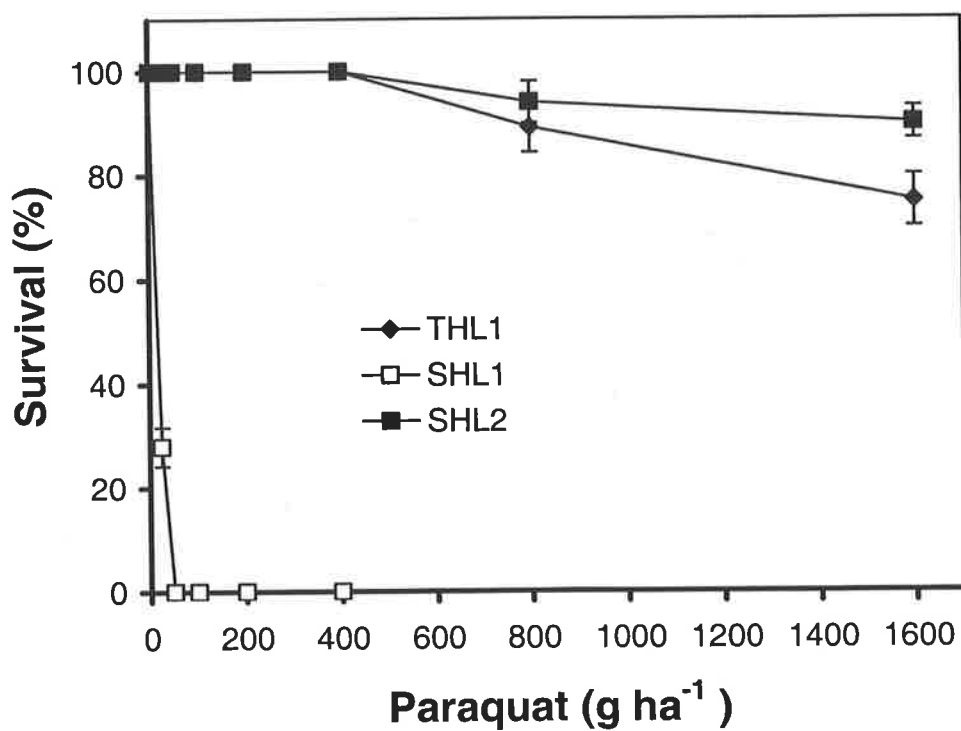


Figure 2.5 Response of a known susceptible *H. leporinum* population (SHL1), a known resistant population (THL1), and a suspected resistant population (SHL2), to paraquat. Populations were grown outdoors during winter. Each point is the mean of 5 replicates  $\pm$  SE.

### 2.3.3 Response to different temperatures

Previous work has established that paraquat efficacy on *H. glaucum* and *H. leporinum* varies with temperature (Alizadeh, 1998; Purba *et al.*, 1995). Control of susceptible *Hordeum* spp. plants is slightly more difficult in summer. Surprisingly, paraquat-resistant populations became considerably easier to control in summer than winter (Alizadeh, 1998; Purba *et al.*, 1995). This improved control of resistant plants under warm temperatures correlated with much greater translocation of paraquat under these conditions (Purba *et al.*, 1995). Slightly greater translocation of paraquat occurred in susceptible plants.

In order to quickly determine whether the mechanisms of paraquat resistance were likely to be similar between resistant populations, dose response experiments were conducted in summer as well as in winter. The SHG4 and SHG7 populations of *H. glaucum* were chosen to represent resistant populations of *H. glaucum*. In winter these populations were highly resistant to paraquat with only about 15 and 30 % mortality at a rate 1600 g a.i. ha<sup>-1</sup> (Figure 2.4.A). However, in summer the level of resistance sharply decreased with more than 60 % mortality at a rate 400 g ha<sup>-1</sup> of paraquat. In contrast, survival of the susceptible population, VHG2, increased slightly in summer. Similarly, the resistant populations of *H. leporinum* THL1 and SHL2 were highly resistant to paraquat in winter, but much less resistant in summer (Figure 2.5). The susceptible population, SHL1, had slightly higher survival in summer compared to winter. The reasons for this are unknown,

but have been observed in other paraquat-resistant populations of *H. leporinum* and *H. glaucum* (Purba *et al.* 1995).

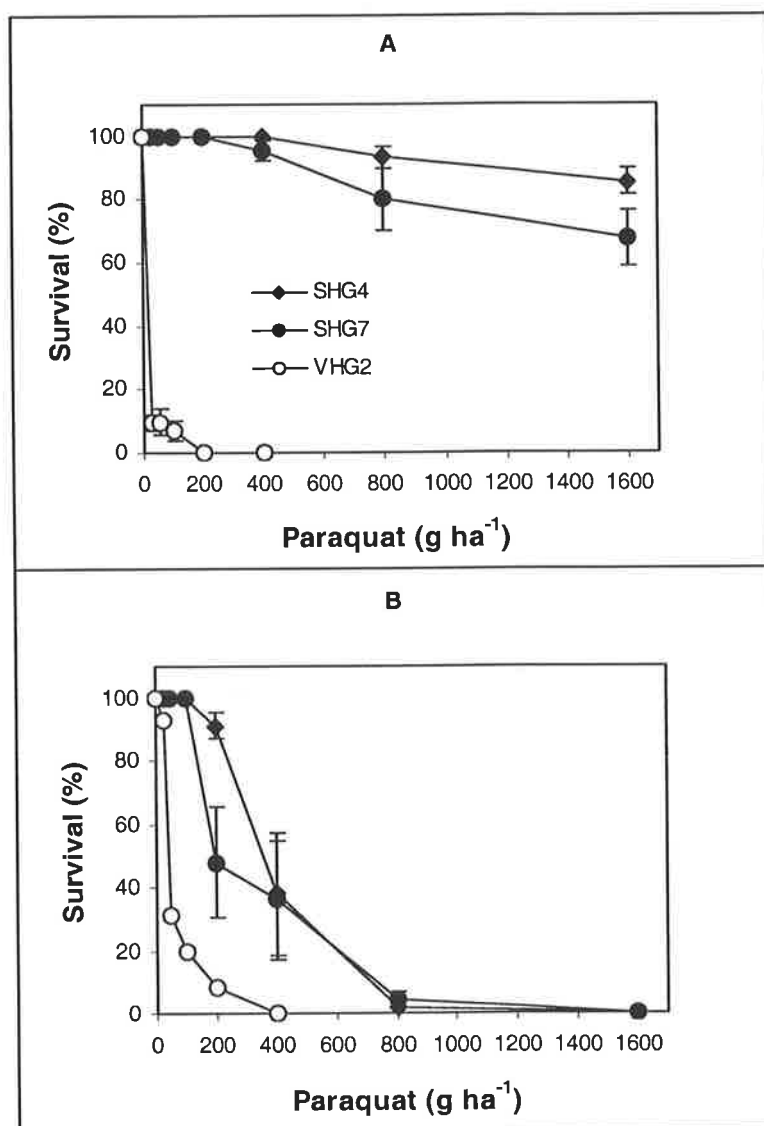


Figure 2.6 Response of resistant (SHG4 and SHG7) and susceptible (VHG2) populations of *H. glaucum* to paraquat in winter (A) and summer (B). Each point is the mean of 5 replicates  $\pm$  SE.

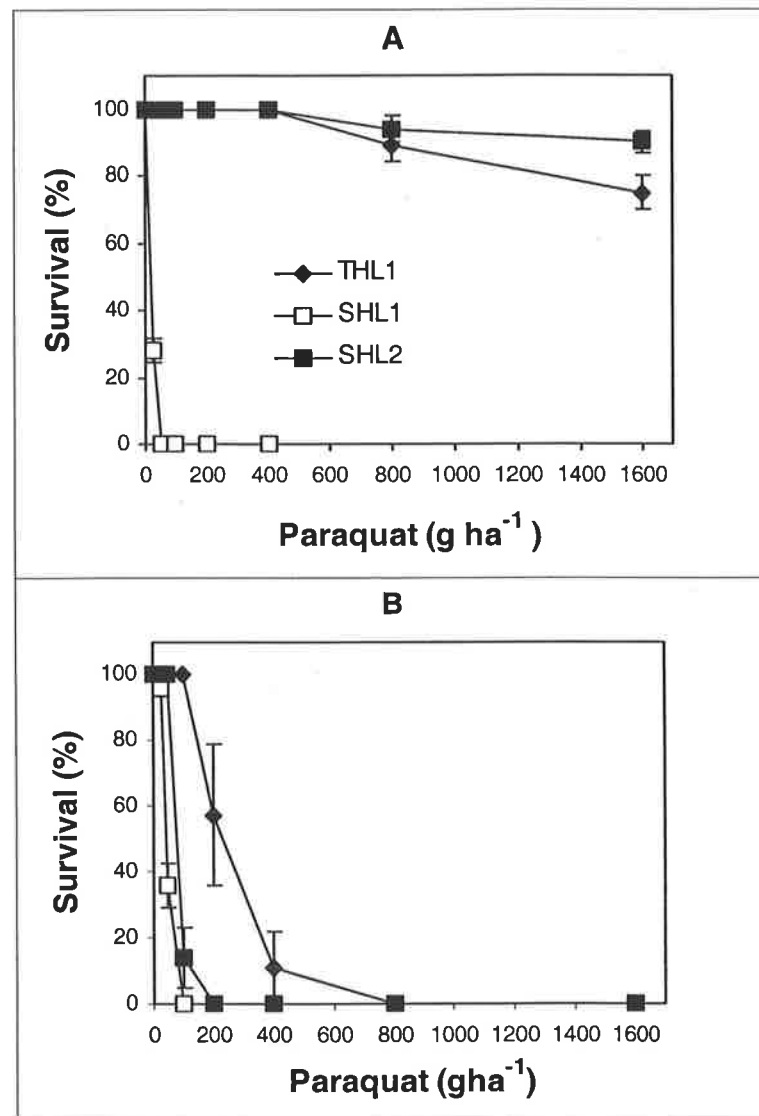


Figure 2.7 Response of resistant (THL1 and SHL2) and susceptible (SHL1) populations of *H. leporinum* to paraquat in winter (A) and summer (B). Each point is the mean of 5 replicates  $\pm$  SE.



## 2.4 Discussion

The random survey identified only a single paraquat-resistant population of *H. glaucum*. This survey was initially undertaken with the intent of determining the distribution of paraquat-resistant *Hordeum* spp. in this area. The single population found was in a lucerne field. As a result of the intensive use of paraquat for winter cleaning of lucerne crops in Australia, herbicide resistant populations evolve quite readily in this system (Powles, 1986; Purba, 1993a; Tucker and Powles, 1991). In contrast, paraquat resistance occurs much more rarely in annual grain cropping systems, however, it can occur (Alizadeh *et al.*, 1998). The survey undertaken here identified that paraquat-resistant *Hordeum* spp. was not widespread in cropping regions of the mid-north and lower north of South Australia.

The second survey targeted lucerne fields in particular and, in addition, sampled adjacent fields. The aim of this survey was to determine whether paraquat-resistant *Hordeum* spp. was being spread from lucerne fields to adjacent fields. Of the nine populations surveyed, from Jamestown to Spalding, three contained paraquat-resistant *H. glaucum*. Paraquat-resistant populations of *H. glaucum* were also discovered in two pastures, between Spalding and Clare. The two pastures were adjacent to each other and adjacent to a site where paraquat resistance was discovered in 1990 (Purba, 1993a). One paraquat-resistant population of *H. leporinum* was found in a wheat field, 1.6 km Marabell - Kapunda. It is possible that the wheat field had previously been a lucerne field and the resistance had evolved while the field had lucerne in it, but the history of the field is not known.

Dose response experiments were conducted on all the paraquat-resistant populations discovered in the survey. All resistant populations of *H. glaucum* were highly resistant to paraquat and had very similar dose response. Likewise, one resistant population of *H. leporinum* showed a high degree of resistance to paraquat. Most paraquat-resistant populations of *Hordeum* spp. previously discovered are also highly resistant to this herbicide (Alizadeh et al., 1998; Powles, 1986; Purba, 1993a; Tucker and Powles, 1991). However, lower levels of resistance have been discovered in a *H. leporinum* population (Purba, 1993a) and in resistant populations of other weed species (Chase et al., 1998; Ismail et al., 2001; Powles et al., 1989; Purba, 1993b; Smisek et al., 1998). As all paraquat-resistant populations discovered in this survey are equally highly resistant, it is tempting to assume they all contain the same mechanism of resistance and, possibly, also the same resistance allele.

Previous work has shown that paraquat resistance in both *H. glaucum* and *H. leporinum* is temperature sensitive. Resistant plants lose much of their resistance in warm temperatures (Alizadeh, 1998; Purba, 1993a; Purba et al., 1995). This effect appears to be related to the mechanism of resistance (Purba et al., 1995). Pot experiments here confirmed that paraquat resistance in *Hordeum* spp. is highly temperature dependent (Figure 2.4 and 2.5). The fact that all the resistant populations found in the surveys had similar responses to paraquat and, where tested, showed temperature dependence of resistance suggests a similar mechanism of resistance. This

mechanism probably involves changes in paraquat translocation patterns (Bishop *et al.*, 1987; Preston *et al.*, 1992; Purba *et al.*, 1995).

If a single mechanism is responsible for resistance to paraquat in *Hordeum* spp. it could be speculated that this resistance phenomenon is controlled by the same allele. As some resistant populations such as SHG4, SHG7 and SHG12 were collected from separate locations, independent evolutionary events resulting from intensive and lengthy use of paraquat may be the major cause. However, some resistant populations of *H. glaucum* originated from fields in the same vicinity, such as SHG10 and SHG11. This suggests that gene flow may play an important role in spreading resistance (Christoffers, 1999). Gene flow through pollen movement can be eliminated as a means of spreading resistance in *H. glaucum* because the anthers are never exposed (Black, 1978; Cocks *et al.*, 1976). However, gene flow through seed migration is possible because of stock, hay and machinery movement between fields (Matthews *et al.*, 2000a; Tucker and Powles, 1988c), as well as buying and selling of crop seed between growers (Andrews *et al.*, 1998). Unlike *H. glaucum*, *H. leporinum* has exerted anthers and, therefore, there is an opportunity for pollen exchange between paraquat-resistant and susceptible individuals. That two documented resistant populations of *H. leporinum* from adjacent fields occurred in lucerne fields in Tasmania (Purba, 1993a) has raised speculation that gene flow may contribute to spreading resistance.

Given that there is potential for resistance genes to move and contaminate new fields, it is necessary to understand the relative importance of

independent evolution and gene flow to the spread of herbicide resistance. Understanding the factors that facilitate the spread of resistance would be useful in aiding management.

## **Chapter 3**

### **Assessment of Gene Flow Mediated by Pollen Exchange between Paraquat-resistant and Susceptible *Hordeum leporinum* Link.**

### 3.1 Introduction

The development of resistance to herbicides in weed populations is an evolutionary process (Christoffers, 1999) involving several factors including gene flow (Lenormand et al., 1998; Maxwell and Mortimer, 1994). During the last few years gene flow through pollen flow has been a great concern because of the widespread appearance of herbicide resistance weeds and the introduction of transgenic crops carrying herbicide resistance genes. Several studies have established that gene flow occurs between herbicide resistant to susceptible individuals of weed species (Murray et al., 2002; Stallings et al., 1995; Volenberg and Stoltenberg, 2002) and from transgenic crops to wild relatives or volunteer weeds (Darmency et al., 1998; Hall et al., 2000; Marshall et al., 2001; Messeguer et al., 2001; Rieger et al., 2001; Warwick et al., 2003).

*H. leporinum* has evolved resistance to paraquat at several sites in Australia since 1990 (Purba et al., 1995; Tucker and Powles, 1991). Inheritance experiments proved that resistance to paraquat in *H. leporinum* was conferred by a single, partially dominant, nuclear gene (Purba et al., 1993). This gene can be a useful marker in detecting pollen dissemination between resistant and susceptible plants (Messeguer et al., 2001).

*H. leporinum* is predominantly self pollinating (von Bothmer et al., 1991), however, the anthers are exerted from the floret at maturity (Black, 1978; Cocks et al., 1976). This provides a possible mechanism for exchange of

pollen between resistant and susceptible individuals within this species. Such an exchange of pollen would allow outcrossing to occur.

There is no information so far on whether gene flow through pollen exchange has played, or could play, an important role in spreading paraquat resistance in *H. leporinum*. Therefore, in the present study, the extent of gene flow mediated by pollen movement between paraquat-resistant and susceptible individuals of *H. leporinum* was determined. This information could lead to better understanding of the mechanisms of resistance spread in paraquat resistance *H. leporinum* and contribute to practical management of resistance spread.

## **3.2 Materials and Methods**

### **3.2.1 Plant Materials**

Seeds of the resistant population of *H. leporinum*, identified as THL1, were originally collected from a lucerne field at Ouse, Tasmania with a history of annual applications of paraquat for 12 years. Seeds of a susceptible population, THL4, were collected from a nearby pasture with no history of paraquat application (Purba, 1993a). The seeds of THL1 and THL4 that were used in these experiments were the fourth generation of self-pollinated individuals maintained at Waite Campus. The seeds were germinated on

0.6 % agar in 1 L plastic containers for 7 days in seed incubator with a 12 h/12 h (20 °C/20°C) light ( $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and dark regime.

### 3.2.2 Dose Response Experiments

To examine the response of the current seed stocks of THL1 and THL4 to paraquat and to choose the appropriate rates for the remaining experiments, dose response experiments were conducted during winter. There were two dose response experiments with three replicates. When seedlings had one leaf, they were transplanted to pots containing potting mix with 10 plants per pot, as one replicate. At the three to five-leaf stage, plants were sprayed with a commercial formulation of paraquat (Gramoxone,  $250 \text{ g L}^{-1}$  paraquat, Crop Care Australia) at rates ranging from 0 to  $1600 \text{ g a.i. ha}^{-1}$  (plus 0.2 % non ionic surfactant) as described in Chapter 2.

### 3.2.2 Pot Experiments for Gene Flow

The experiments were conducted in pots during 2001 and 2002 at the Waite Campus. At the one-leaf stage, a single individual each of THL1 and THL4 were planted in pots 30 cm in diameter containing potting soil. The distance between plants was about 12 cm. There were 12 pots per year placed at 5 m intervals. Any wild *H. leporinum* growing around the experiment site were



controlled to prevent pollen contamination of experimental plants. Plants were hand watered as required during spring and summer.

To confirm that the susceptible parents of THL4 were truly susceptible, a single spike from each THL4 individual was bagged before anthesis with glassine bags, 5 cm x 18 cm. After anthesis, each plant was bagged separately with porous plastic bags to prevent seeds scattering. Plant phenology was monitored including the number of tillers. Plants were harvested in December (2001) and January (2003) and seeds from each plant were stored separately. Seeds from the bagged spikes were also harvested and stored in separate envelopes. The seeds were air dried for a week after which they were stored in cold room  $\pm 4^{\circ}\text{C}$ . Seed number per plant was counted. Data were analysed using least significant different (LSD) at the 5 % level.

### **3.2.3 Germinability Test**

These tests aimed to evaluate the quality of seeds that were harvested from plants grown in pots. About 100 seeds from each individual plant were tested for germinability. The seeds were germinated as described above. The percentage of seed germination was determined after 10 days.

### 3.2.4 Determination of Gene Flow Frequencies

Gene flow frequencies of seeds harvested from THL1 and THL4 were tested during winter 2002 and 2003. More than 4000 and 6000 seeds from THL1 and THL4 respectively were tested in 2002 and more than 3000 seeds from each of THL1 and THL4 were tested in 2003. The seeds were germinated as described above and seedlings were transplanted at one-leaf stage into 28 cm x 39 cm trays containing potting soil with 63 individuals per tray. The seedlings were grown outdoors.

At the three to five-leaf stage, plants were sprayed with a commercial formulation of paraquat at a rate of 75 g a.i. ha<sup>-1</sup> for THL4 to select for resistant F<sub>1</sub> progeny and 400 g a.i. ha<sup>-1</sup> for THL1 to select for susceptible F<sub>1</sub> progeny. In addition to the tested plants, one pot of THL4, from non-experimental seeds, containing 10 individual was added as controls when THL1 plants were sprayed. Plants from bagged spikes of THL4 individuals were also treated with 75 g ha<sup>-1</sup> paraquat in a similar manner. The herbicide was applied as describe above in Chapter 2. Plants were sprayed in the afternoon and kept indoors overnight. They were returned outdoors in the following morning. The survival and mortality of plants were determined 21 days after herbicide application. Plants were considered alive if they produced new leaves. The survivors of THL4 individuals from the 2001 experiment were transplanted to new pots (30 diameter) and allowed to produce seeds.

### 3.2.5 Segregation Test

The seeds harvested from F<sub>1</sub> survivors of THL4 individuals from the 2001 experiment were germinated and grown as described above. At the three-leaf stage, they were sprayed with 50 g ha<sup>-1</sup> paraquat. Plants were scored for survival and mortality two weeks after herbicide application. Data were analysed by X<sup>2</sup> analysis for a 3:1 survival:mortality segregation ratio.

## 3.3 Results

### 3.3.1 Response to Paraquat

Dose response experiments proved that THL1 was highly resistant to paraquat and displayed no mortality at a rate of 400 g ha<sup>-1</sup> paraquat (Figure 3.1). In contrast, THL4 was very susceptible to paraquat application and all plants were controlled by 50 g ha<sup>-1</sup> of paraquat. This result indicates that 75 g ha<sup>-1</sup> paraquat would be expected to kill all susceptible individuals and 400 g ha<sup>-1</sup> paraquat would kill no resistant individuals. Therefore, these rates would be appropriate for determining heterozygotes (Purba et al., 1993).

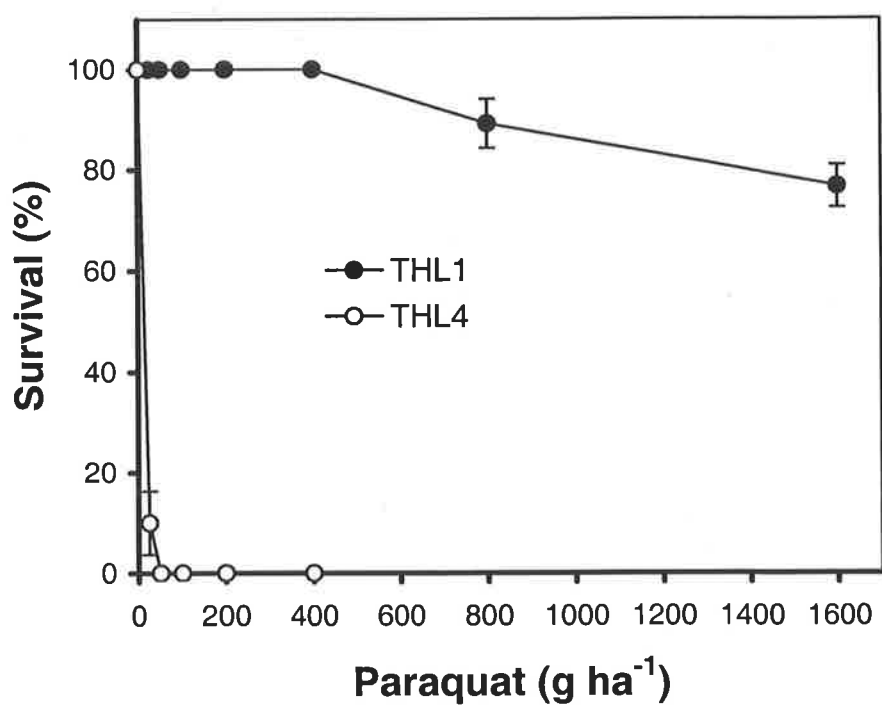


Figure 3.1 Response of resistant (THL1) and susceptible (THL4) populations of *H. leporinum* to paraquat in winter. Each point is the mean  $\pm$  SE of two experiments each with three replicates.

### 3.3.2 Plant Growth and Gene Flow Assessment

All THL1 and THL4 individuals grew normally in pots (Figure 3.2). Transplanting, flowering and harvest dates for the experiment in each of 2001 and 2002 are given in Table 3.1. The advantage of using populations from the same geographical region is that they have a relatively similar biological rhythms, such as flowering time. Flowering for both populations started in October in both 2001 and 2002 and finished in two to three weeks. At flowering, the inflorescences of two plants mixed maximising the opportunities for gene flow between two plants (Figure 3.2). At maturity, the pale yellow anthers were exerted from the florets. All of these properties maximised the likelihood of pollen exchange between flowers taking place.

The early transplanting in 2001 resulted in a longer period of growth and development compared to the later transplanting in 2002. However, seed production of plants that were grown in 2002 was higher than plants grown in 2001 (Table 3.2). This may be due to better watering and nutrition in 2002.



Figure 3.2 THL1 and THL4 individuals growing together in a pot at flowering.

Table 3.1 Transplanting, flowering and harvest time for the pot experiments in 2001 and 2003. DAT = Days after transplanting.

Date	Activity	DAT
31 May 2001	Transplanting	0
10 October 2001	Start of flowering	132
10 December 2001	Harvest	193
19 July 2002	Transplanting	0
18 October-2002	Start of flowering	91
02 January 2003	Harvest	167

Tiller number was only assessed in 2002. THL1 produced less tillers compared to THL4 (Table 3.2). This is because THL1 stopped tillering earlier than THL4. Therefore, THL1 individuals also finished flowering earlier and had earlier maturity than THL4 individuals. THL1 individuals produced less productive tillers (data not shown) and produced fewer seed per tiller compared to THL4 individuals. Therefore, total seed production was much lower for THL1 compared to THL4. THL1 produced only 68% of the amount of seed produced by THL4 in 2001 and 69% of the amount in 2002. However, both biotypes produced good seed quality as more than 95% of the seeds set germinated (Table 3.2).

Table 3.2 Number of tillers, number of seeds and germinability of seeds from resistant (THL1) and susceptible (THL4) biotypes of *H. leporinum* grown in pots in 2001 and 2002.

Year	Biotype	Tiller number per plant	Seed number per plant	Seed germination (%)
2001	THL1	-	820.67 a	95.50 a
	THL4	-	1,186.50 b	97.08 a
	LSD (5%)	-	156.70	6.04
2002	THL1	66.58 a	962.67 a	97.42 a
	THL4	120.92 b	1,422.67 b	98.25 a
	LSD (5%)	12.31	188.06	1.10

Values in each column in each year followed by the same letter are not significantly different ( $P = 0.05$ ).

Gene flow, in this study, was assessed as the rate of pollination between the resistant biotype (THL1) and susceptible biotype (THL4). Testing for gene flow was conducted during winter, as this is the normal growing time for *Hordeum* spp. in southern Australia. Gene flow was examined in both directions; from THL1 to THL4 and vice versa.

Of the 9,848 seeds harvested from THL1 in 2001, 4,720 seedlings were grown in 2002 and sprayed with 400 g ha<sup>-1</sup> paraquat (Table 3.3). Of these plants, only five plants or 0.12% died. In 2003, of the 11,552 seeds collected from THL1 individuals in 2002, 3,126 seedlings were grown and treated with 400 g ha<sup>-1</sup> paraquat. Of these plants, only two or 0.06% were killed.



THL4 individuals produced a total of 14,238 and 17,072 seeds in 2001 and 2002 respectively. In 2002, 6,642 seedlings were grown and sprayed with 75 g ha<sup>-1</sup> paraquat, of these nine plants or 0.13% survived. In 2003, 3,176 seeds were grown and sprayed with 75 g ha<sup>-1</sup> paraquat. Five plants or 0.15% survived at this rate of herbicide.

Table 3.3 Number of seed tested and frequency of gene flow between susceptible and resistant *H. leporinum* and vice versa.

Year	Biotype	Number of plants tested	Survival	Mortality	Gene flow frequency (%)
2001	THL1	4720	4715	5	0.12 ± 0.07
	THL4	6642	9	6633	0.13 ± 0.04
2002	THL1	3126	3124	2	0.06 ± 0.03
	THL4	3176	5	3171	0.15 ± 0.06

### 3.3.4 Segregation Test

To examine whether the survivors of paraquat application at a rate of 75 g ha<sup>-1</sup> from the susceptible (THL4) biotype in 2002 were the result of pollination from THL1, a segregation test was conducted on the F<sub>2</sub> progeny. If pollen from resistant plants were responsible for the observed survivors at 75 g ha<sup>-1</sup> paraquat, their genotypes would be heterozygous. Therefore, it

could be expected that F<sub>2</sub> progeny would segregate in a 3:1 survival:mortality ratio. Of nine F<sub>1</sub> individuals, one set no seed but eight F<sub>1</sub> individuals produced seeds. When the progeny were tested, seven samples segregated with both survivors and mortality at the rate of 50 g ha<sup>-1</sup> (Table 3.4). However, F<sub>2</sub> progeny of plant no. 3 were all killed by 50 g ha<sup>-1</sup> paraquat. This indicates that plant no. 3 was not a hybrid of THL1 and THL4. Therefore, only seven F<sub>1</sub> were confirmed to segregate. Segregation of six of these seven fitted the expected 3:1 survival:mortality ratio for a single gene with dominance at this rate (Purba *et al* 1993). Surprisingly, plant no. 7 did not segregate in this ratio. Re-spraying survivors of this experiment with 400 g ha<sup>-1</sup> paraquat (data not shown) demonstrated that more of the progeny were homozygous resistant than would have been expected. Lack of time precluded further investigation of the progeny of this plant.

There were 166 seeds (in 2001) and 218 seeds (in 2002) harvested from spikes bagged prior to flowering (control). When they were germinated and seedlings were tested, they were all killed by 75 g ha<sup>-1</sup> paraquat. This indicates that each THL4 parent used in the gene flow experiment was truly susceptible.

Table 3.4 Segregation of F<sub>2</sub> progeny after paraquat application at a rate of 50 g ha<sup>-1</sup>.

Plant No.	Number of progeny tested	Survival	Mortality	X <sup>2</sup> (3:1)	p
1.	63	46	17	0.132	0.716
2.	63	48	15	0.048	0.827
3.	19	0	19	57.000	< 0.001
4.	81	66	15	1.815	0.178
5.	42	33	9	0.286	0.593
6.	77	55	22	0.524	0.469
7.	63	10	53	117.466	< 0.001
8.	42	36	6	2.571	0.109

## 2.4 Discussion

It is often difficult to determine the amount of gene flow between plants, particularly when levels of gene flow are small. It is relatively easy to determine that plant species are fully outcrossing simply by isolating a single plant and determining whether it can set seed. However, it is often more difficult to determine accurately the amount of selfing in species. For example, *H. leporinum* is normally considered to be selfing species (von Bothmer et al., 1991). However, as the anthers of *H. leporinum* are exerted from the floret at anthesis, the possibility of gene flow between plants exists.

Herbicide resistance has proven to be an ideal marker for gene flow (Messeguer *et al.* 2001). Herbicide resistance is usually a single gene trait and normally dominant. Resistance is also very easily to test for and allows a large number of individuals to be tested easily for rare events (Rieger et al., 2002).

The design of the experiment here maximised the possibility of gene transfer by growing the plants together in the same pot. The amount of gene flow observed was very small, so it is likely that gene flow will be negligible at any distance. It is most likely that pollen movement between *H. leporinum* plants occurred when wind physically brought the heads together. Likewise, (Rognli et al., 2000) found that growing *Festuca pratensis* plants together greatly increased the rate of gene flow compared to plants standing alone.

This study demonstrated the usefulness of herbicide resistance alleles as a marker in determining the frequency of gene flow through pollen movement,

even in a self-pollinating species. The results of this experiment are consistent with the expectation that pollen flow could happen between paraquat-resistant and susceptible biotypes of *H. leporinum*. The rate of gene flow from THL1 to THL4 at 0.11 to 0.15% and from THL4 to THL1 at 0.6 to 0.12% was higher compared to previous studies on *Avena fatua* (Murray et al., 2002), but was lower than *Setaria faberi* (Volenberg and Stoltenberg, 2002), both also normally selfing species.

The frequency of gene flow in selfing species was predicted to be close to the rate of mutation (Maxwell and Mortimer, 1994; Volenberg and Stoltenberg, 2002) which is estimated at between  $10^{-5}$  to  $10^{-6}$  for a mono-dominant gene or between  $10^{-9}$  to  $10^{-11}$  for a mono-recessive gene (Gressel and Segel, 1982a; Jasieniuk et al., 1996). However, the rate of gene flow reported here was higher so resistance evolution could occur slightly faster under constant herbicide application than might otherwise be the case.

The segregation test in this study confirmed the hybrid status of most  $F_1$  individuals. Of the eight individuals tested, the progeny of six segregated as expected and are clearly the result of outcrossing. The progeny of one individual were all susceptible (Table 3.4). This plant probably escaped herbicide application during screening. The progeny of the final plant segregated in an unusual manner. The reason for this is not known. Therefore, at least 75 % of the survivors detected proved to carry the paraquat resistance gene. As the heterozygotes produced on THL1 by THL4 pollen were killed, it was impossible to test their progeny.

Gene flow between THL1 and THL4 occurred in both directions. The frequency of gene flow from THL4 to THL1 was similar to that from THL1 to THL4. This is despite THL1 producing only 68% of the seed of THL4. The larger production of tillers, and presumably pollen, by THL4 did not appear to favour increased gene flow.

To a certain extent, gene flow between plants is able to contribute to the evolution of multiple herbicide resistance in weed populations (Preston et al., 1996). Pollen mediated gene flow could allow two resistant alleles to come together in the same plant. *H. leporinum* has also developed resistance to acetyl-coenzyme A carboxylase-inhibiting herbicides (Matthews et al., 2000a) and if these appeared in the same field as paraquat-resistant plants, gene flow could allow multiple resistance to occur. However, gene flow between THL1 and THL4 was low that such an event might only happen when plants were growing together. In consequence, it is unlikely to expect that pollen-mediated gene flow would contribute significantly to the evolution of multiple-resistance in this species unless the plants were growing together.

It is unlikely that *H. leporinum* pollen moves far between plants. While pollen movement might play a part in local gene movement to accelerate resistance evolution (Rieseberg and Burke, 2001), it is unlikely to be important as a factor in movement between fields. Therefore, this study confirms a previous investigation that the gene flow through seed migration could be the major cause of distribution of *H. leporinum* resistance between fields (Tucker and Powles 1988).

The contribution of gene flow in accelerating resistance evolution to paraquat in *H. leporinum* could be a more significant factor if the hybrid progeny possess an equal or greater fitness than homozygous parents. In some other examples, the hybrid progenies display lower fitness (Arnold et al., 1999). Therefore, evaluation of fitness amongst three genotypes (homozygous resistance, heterozygous, and homozygous susceptible) in paraquat resistance *H. leporinum* is relatively important in determining the importance of gene flow.

**Chapter 4**  
**Comparison of Fitness among Homozygous and  
Heterozygous Paraquat-Resistant and Susceptible  
populations of *Hordeum leporinum***



## 4.1 Introduction

Herbicide resistant individuals in weed species exist in populations as a result of the constant selection pressure applied by herbicides. Fitness plays an important role in the establishment of resistant populations. The fitness impacts of the resistance gene will determine whether resistant individuals will persist in unsprayed populations. Herbicide resistant individuals will have higher fitness compared to susceptible individuals under herbicide application (Holt and Thill, 1994; Maxwell and Mortimer, 1994); however, in the absence of herbicide, the fitness of a resistant individual can be equal to or less than the susceptible individuals (Christoffers, 1999; Maxwell and Mortimer, 1994). If a fitness penalty on resistant individuals is not significant in unsprayed populations, the frequency of resistant alleles will be greater and resistant populations will evolve faster in fields once selection is applied (Jasieniuk *et al.*, 1996; Tranel and Wright, 2002). In addition, the presence of susceptible individuals will not seriously delay the onset of resistance through competition when the herbicide is not used.

The fitness of heterozygous resistant individuals is also an important factor for the rapid evolution of herbicide resistance as selection initially occurs on the heterozygotes. If performance of heterozygous individuals is lower than that of susceptible individuals, evolution will be slowed. Typically, fitness comparisons have been made between a single resistant population and a single susceptible population. There are a lot of studies concerning the fitness of hybrid individuals between crops and closely related weeds compared to their parents (Campbell and Waser, 2001; Snow *et al.*, 2001;

Spencer and Snow, 2001) or comparative fitness of herbicide resistant and susceptible individuals (Hartzler and Battles, 2001; Jordan, 1999; Parks *et al.*, 1996; Plowman *et al.*, 1999; Purba *et al.*, 1996; Radosevich and Holt, 1982). However, despite the importance of heterozygotes in the selection for resistance there are few studies on the fitness of heterozygous compared to homozygous individuals related to herbicide resistance genes in weed species.

As previously discussed, resistance to paraquat in *H. leporinum* is controlled by a single semi dominant nuclear gene. The F<sub>2</sub> progeny between susceptible (SS) and resistant (RR) individuals segregate at ratio of 1:2:1 (RR:RS:SS) when sprayed with appropriate rates of paraquat (Purba *et al.*, 1993a). The presence of heterozygous individuals (RS) provides an opportunity to design experiments to compare the fitness of these three genotypes simultaneously. Therefore, the purpose of this study was to examine the extent to which the resistance gene can affect growth and seed production of paraquat-resistant homozygous and heterozygous individuals. The results will provide information about the potential contribution of heterozygous individuals to the evolution of herbicide resistance populations.

## 4.2 Materials and Methods

### 4.2.1 Plant Materials

Ten F<sub>3</sub> seeds from a cross between resistant and susceptible biotypes of *H. leporinum* were grown during winter in an experimental site that would be used for the fitness experiment. The plants were allowed to set seed. The seeds harvested from these plants were grown and then sprayed with paraquat at rate of 200 g ha<sup>-1</sup>. In general, individuals homozygous for paraquat resistance in *H. leporinum* survive paraquat application at this rate with a little damage. In contrast, individuals homozygous for paraquat susceptibility in this species are killed by paraquat at this rate. Individuals heterozygous for paraquat resistance survive, but experience severe damage and the plants require a longer time to recover. Of ten families tested, one (Family no. 9) was found to segregate for homozygous resistance and susceptible, as well as heterozygous resistant individuals (Table 4.1). The rest of the seeds of Family no. 9 were used in the experiment, described in this section.

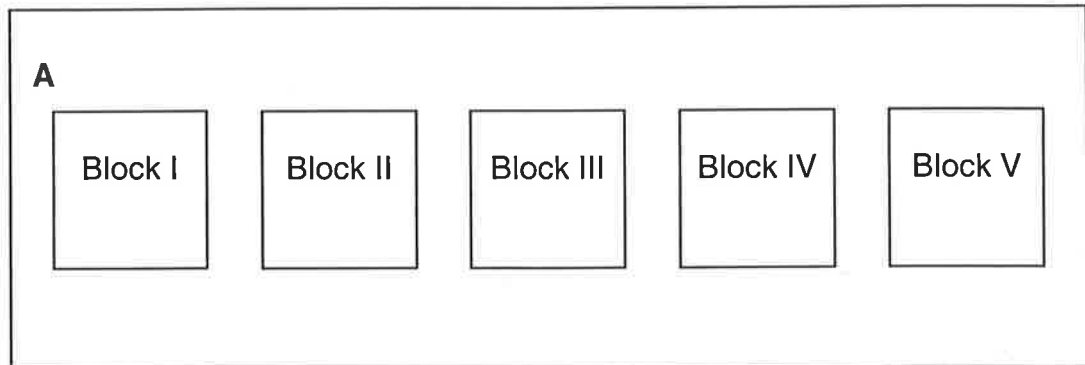
## 4.2.2 Methods

### 4.2.2.1 Experimental Design

This experiment was conducted at an experimental site at the Waite Campus in 2002. The site was a 9 m x 9 m block filled to a depth of 1 m with Mt Compass loam. The experiment consisted of five blocks and each block contained 25 test plants surrounded by two rows of buffer plants (Figure 4.1). Permanent grid meshes, 10 cm x 10 cm, were laid over the soil of each block prior to transplanting to determine plant position and to maintain a constant distance between individuals.

Table 4.1 Response of F<sub>4</sub> progeny of *H. leporinum* to paraquat application at a rate of 200 g ha<sup>-1</sup>

Family	No. of plants tested	Survived	Severe damage	Mortality
1	75	0	0	75
2	113	0	0	113
3	100	0	0	100
4	100	0	0	100
5	100	0	0	100
6	63	0	0	63
7	70	0	0	70
8	63	63	0	0
9	53	8	22	23
10	63	0	0	63



**B**

*	*	*	*	*	*	*	*	*
*	*	*	*	*	*	*	*	*
*	*	5	10	15	20	25	*	*
*	*	4	9	14	19	24	*	*
*	*	3	8	13	18	23	*	*
*	*	2	7	12	17	22	*	*
*	*	1	6	11	16	21	*	*
*	*	*	*	*	*	*	*	*
*	*	*	*	*	*	*	*	*

Figure 4.1 The position of blocks 1-5 (A) and individuals plants 1-25 in a block (B) in field of fitness experiment. \* = buffer plants

#### **4.2.2.2 Planting**

Seedlings were transplanted into each block in the mid winter (24 June 2002), the growing season for *Hordeum* spp. Seedlings of even size were planted at the centres of each grid. Within one week after planting, the few seedlings that had died were replaced by similar aged plants. During the experiment, all blocks were kept free from non-experimental plants by regular hand weeding. Plants were hand watered as required during spring and summer. The number of tillers per plant was counted at 3, 6, 9, 12 and 15 weeks after transplanting. The number of reproductive tillers was counted 18 weeks after transplanting. At the end of flowering, each plant was bagged using porous plastic bags to prevent seeds scattering. The plastic bags were kept on until plants harvested and total dry weight was obtained.

#### **4.2.2.3 Plant Harvest**

Plants were harvested when the heads had turned golden-yellow indicating seed maturity. Prior to harvest, the buffer plants were cut and removed. All plant samples were harvested individually by excising at ground level and were kept in the porous plastic bags. The plant materials were air dried for two weeks (summer time) after which total dry weight of each plant was measured and the number of seed per plant counted. The weight of 100 seeds was measured after the genotype of each plant was determined.

#### 4.2.2.4 Germinability Test

To examine the germinability of seeds harvested from test plants, seeds were germinated on 0.6 % of agar in 1 L plastic containers as described in Chapter 3. The percentage of seed germinating after 10 days was determined. The seeds were considered germinated when their radicle appeared outside the seed coat.

#### 4.2.2.5 Genotype Determination

This test was aimed to determine the genotype of each plant used in the experiment. About 20 seeds of each plant were germinated and at the one-leaf stage 10 seedlings were transplanted into 17 cm pots containing potting mix. When the plants had three leaves, they were sprayed with paraquat at 200 g a.i. ha<sup>-1</sup>. The status of each plant was determined 21 days after herbicide treatment. The parent plants were considered homozygous resistant (RR) when all progeny survived at this rate with a little leaf bleaching. Parent plants were considered homozygous susceptible (SS) when all progeny were killed by 200 g ha<sup>-1</sup> paraquat. When progeny plants showed a mix of survival, survival with severe bleaching and death, the parents were determined as heterozygous (RS).

#### **4.2.2.6 Statistical Analysis**

The data on number of reproductive tillers, dry weight, number of seeds, the weight of 100 seeds, and germinability were subject to analysis of variance in Genstat using a strategy that allowed for unequal replication.

### **4.3 Results**

#### **4.3.1 Plant Genotypes**

The results of the genotype test confirmed that the population had three genotypes, namely: homozygous resistance (RR), homozygous susceptible (SS), and heterozygote resistance (RS). Each block of this experiment contained all three genotypes (Table 4.2). The total numbers of individuals of each genotype were 22 RR (18%), 56 RS (46%), and 43 SS (36%). This ratio of genotypes did not fit the typical ratio 1:2:1 for inheritance of resistance controlled by a single semi partially dominant gene. Homozygous susceptible individuals were over represented in the experiment. The reasons for this are unknown.



Table 4.2 Genotype of each individual from each block (RR = THL1; RS = hybrid of THL1 and THL4; SS = THL4; and D = Dead).

No.	Block I	Block II	Block III	Block IV	Block V
1	RS	RR	RS	RS	RR
2	RR	SS	SS	SS	SS
3	SS	SS	RR	RS	RS
4	RS	SS	RR	SS	SS
5	SS	RS	RS	RS	RS
6	RR	RS	SS	SS	D
7	RR	RS	SS	D	RS
8	RR	SS	SS	SS	RS
9	RS	RR	RS	RS	SS
10	RR	RS	RS	RR	RS
11	RS	RS	SS	SS	RS
12	RS	D	RR	SS	RS
13	SS	RS	SS	D	SS
14	RS	RS	RS	RS	SS
15	RS	RS	RS	SS	SS
16	SS	RR	SS	RR	SS
17	SS	RR	RS	RR	RS
18	RS	SS	RS	SS	RR
19	RS	RR	RR	SS	SS
20	RS	RS	RS	RS	SS
21	RS	SS	RS	RS	RS
22	RS	SS	RS	SS	RR
23	RS	SS	RS	SS	RR
24	RS	RS	RS	RS	RR
25	RS	RS	SS	SS	SS
Total SS	5	8	8	12	10
Total RS	15	11	13	8	9
Total RR	5	5	4	3	5

### 4.3.2 Plant Growth

In general most plants grew well. Although seedlings were transferred directly from seed incubator into the soil, they required only a short period to establish. The second leaf was mostly initiated within one week after transplanting. After the primary shoot had three mature leaves, about three weeks from transplanting, the first tiller emerged (Figure 4.1). The number of tillers sharply increased from about 3-4 tillers at week 6 to about 23-25 tillers per plant at week 12. This six-week period was the main active vegetative stage for this species. Rust appeared on some plants between 15-16 weeks after planting and was controlled with tebuconazole at a rate of 63 g ha<sup>-1</sup>. After the maximum number of tillers was achieved, the number of tillers decreased slightly. This decrease in tiller number could be related to the failure of young tillers to compete with old tillers for space and nutrition.

There was no significant difference in number of tillers between RR, RS and SS over time. This means under these field conditions the three genotypes had similar growth rates. More than 50% of plants of each block started flowering in the first week of October 2002, 14 weeks after transplanting. Four plants were later found dead because of competition effects and were not considered in the analysis, as they could not be genotyped. Plants were harvested in mid December 2002, 26 weeks after transplanting.

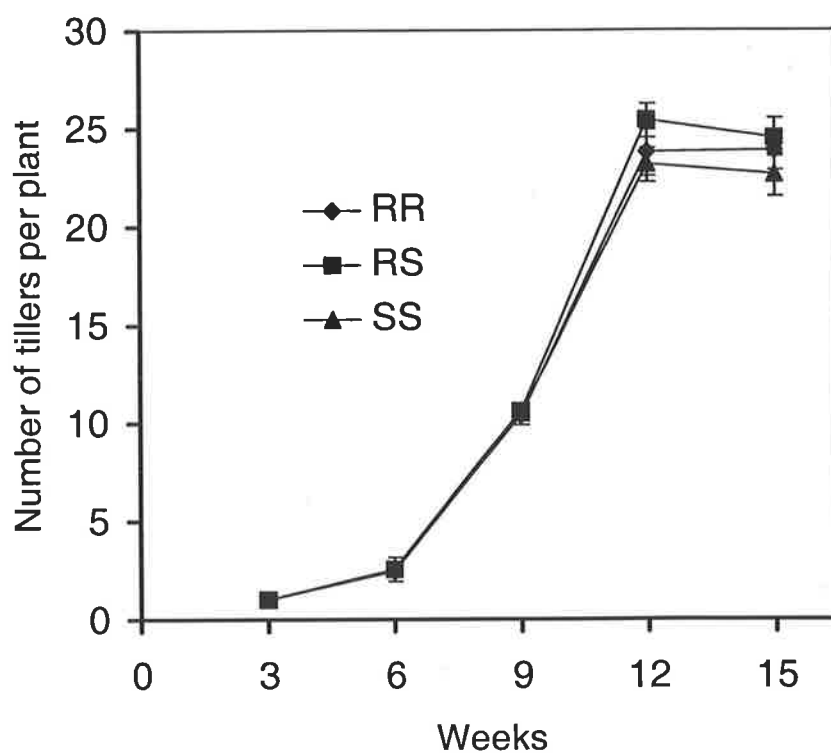


Figure 4.2 Growth, as measured by number of tillers per plant, of plants homozygous resistant (RR), susceptible (SS) and heterozygous resistant (RS) to paraquat over time.

### 4.3.3 Productivity and seed quality

Productivity of RR, RS, and SS plants was individually calculated as the number of reproductive tillers, total above ground dry matter at harvest, and number of seeds (Table 4.3). Seed quality was estimated by measuring the weight of 100 seeds and testing their germinability (Table 4.4).

Table 4.3 The average of number of productive tillers, dry weight above ground, and number of seed per plant for paraquat homozygous resistance (RR), homozygous susceptible (SS) and heterozygous resistance to of *H. leporinum* when grown under competitive conditions.

Genotype	Number of reproductive tillers	Dry weight at harvest (g plant <sup>-1</sup> )	Number of seed per plant
RR	11.86	10.00	291.36
RS	14.02	12.00	343.48
SS	12.70	10.11	289.79
LSD (P=5%)	ns	ns	ns

The number of reproductive tillers per plant did not vary significantly between RR, RS and SS genotypes (Table 4.3). The dry weight of individual plants for RR, RS and SS genotypes was also not significantly different. Total seed production was also similar, at about 300 seeds per plant. As a whole, these results indicated that the RR, RS and SS individuals had similar fitness in this experiment.

Table 4.4 The average of weight of 100 seeds and germinability of seeds for paraquat homozygous resistance (RR), homozygous susceptible (SS) and heterozygous resistance (RS) *H. leporinum* when grown under competitive conditions.

Genotypes	Weight of 100 seeds (g)	Germinability (%)
RR	0.72	97.05
RS	0.71	97.46
SS	0.74	97.67
LSD (5%)	ns	ns

In this study, seed quality was measured as weight of 100 seeds and germinability. The weight of 100 seeds per plant was not significantly different between RR, RS, SS individuals. The average weight of 100 seeds was about 0.71 to 0.74 g for all individuals. Seeds from all plants, regardless of genotype, had high viability with more than 97% germinating. As the seed germinability and weight of 100 seeds were similar, this indicates that in a field situation, if moisture availability and temperature were suitable, the seeds of RR, RS, and SS are likely to germinate simultaneously.

## 4.4 Discussion

Herbicide resistance fitness studies in the past have been criticised because these studies tended to use herbicide resistant and susceptible populations from different sites that might have different history of culture or control methods. Hence, any differential fitness observed in these studies might have reflected a broader spectrum of genetic polymorphism or environmental effects rather than have been caused solely by the resistant mutation (Cousens *et al.*, 1997; Jasieniuk *et al.*, 1996; Tranel and Wright, 2002). It has been suggested that using near isogenic populations in fitness studies could give a more accurate interpretation of fitness in relation to the mutation conferring herbicide resistance (Holt and Thill, 1994). However, to establish these kind of studies, near isogenic plant materials must be derived from several backcross experiments over a long period of time (Eberlein *et al.*, 1999).

In this study, an alternative approach was taken with the fitness of three different genotypes measured simultaneously. The population used here was the F<sub>4</sub> progeny from an initial cross of resistance to susceptible individuals (Purba *et al.*, 1993a). Individuals had been selfed three times to create the F<sub>4</sub> generation. The problem with this experiment was the status of each individual was unknown prior to the experiment and their status was only later determined from their progeny. This is unlike fitness comparisons of resistant and susceptible populations where their status is known prior to the experiment and, therefore, it is possible to arrange the position of individuals following a set of experimental design. Because genotype was

unknown at the start of this experiment, all individuals were distributed randomly. Secondly, the number of individuals of each genotype was not the same so the replication is unequal. Consequently, to establish ANOVA, a specific statistical analysis was required (Gomez and Gomez, 1984).

Competition is an ecological process and can vary depending on the availability of resources. Competition for space and resources starts when the availability of these items are inadequate to supply plant requirements (Berkowitz, 1988). Therefore, it has been suggested that the measurement of fitness parameters should represent plant situations over the full life cycle (Maxwell and Mortimer, 1994). In this experiment, several parameters were observed ranging from tillering stage to germinability of the seed produced. All parameters observed in this experiments were not significantly different between genotypes. Heterozygous resistant individuals were as fit as homozygous resistant and homozygous susceptible individuals. Therefore, this result is in agreement with an assumption that hybrid individuals do not always have lower fitness compared to their parents (Arnold *et al.*, 1999).

A previous fitness study (Purba *et al.*, 1996) on this species also found that a similar fitness was evident between paraquat-resistant and susceptible populations when they were grown in fields. However, in this previous fitness study, in the absence of competition the susceptible populations had a higher tiller number, plant dry weight, and seed production. This was also observed in this study (Chapter 3), suggesting that competition may be changing the seed output differently between genotypes. Perhaps susceptible individuals produce more seeds in circumstances of lower intra-

specific competition. Another possible reason is the time of transplanting in this experiment (24 June 2002) was one month later compared to the experiment in Chapter 3 (31 May 2001). The late transplanting could shorten the vegetative growth period through limiting soil moisture or increasing photoperiod. The rapid change from vegetative to reproductive stage can decrease seed production overall masking differences in seed production (Swarbrick and Mercado, 1987). In this case, it reduces the opportunity for susceptible individuals to maximise seed production.

An alternative explanation for the failure to measure a significant fitness penalty in this experiment could relate to when the fitness penalty is expressed. The genotyping of the plants used in the experiment showed a much lower than expected number of homozygous resistant individuals. The strategy of selecting equal sized individuals for planting out may have inadvertently discriminated against individuals carry the resistant allele.

Germinability was similar for three genotypes. This simultaneous germination might provide a similar opportunity for each biotype to exploit space and resources for their growth when the herbicide is not used. Also, the similarity of germinability and the number of seeds per plant amongst three genotypes suggested that the seed bank dynamic among genotypes is stable.

It is commonly assumed that herbicide resistant populations have lower fitness than susceptible populations (Maxwell and Mortimer, 1994; Moss, 2002). The reports of fitness studies on triazine resistance in *Senecio*



*vulgaris* (Radosevich and Holt, 1982), *Brassicca rapa* (Plowman *et al.*, 1999) and *Amaranthus hybridus* (Jordan, 1999) acted to confirm this assumption. Plowman *et al.* (1999), for example, found a remarkable decrease in frequency of resistance in *Brassicca rapa* from 50 % to 13 % over two years because of competition with susceptible counterparts. Therefore, it is possible to exploit the lack of fitness to delay the onset of resistance in populations. However, not all resistant populations suffer from fitness penalties. Populations of *Chenopodium album* resistant to atrazine (Plowman *et al.*, 1999), *Lolium rigidum* resistant to diclofop methyl (Matthews and Powles, 1992) and *Digitaria sanguinalis* resistant to fluazifop-p-butyl (Wiederholt and Stoltenberg, 1996) have similar fitness compared to susceptible populations. In these cases, there would be no buffering effect from susceptible populations to suppress the onset of resistance. As a consequence, the evolution of herbicide resistance could be expected to be faster in fields for these species (Jasieniuk *et al.*, 1996).

Paraquat resistance in *H. leporinum* has resulted from repeated applications of this herbicide for 12 to 24 years (Purba, 1993a; Tucker and Powles, 1991). It is not yet clear why this species required more than 12 years to evolve resistance to paraquat in lucerne fields if reduced fitness was not evident in resistant populations. It is possible that herbicide and crop factors played important roles in delaying the onset of resistance in the fields. An alternative experiment using isogenic plants and conducted in lucerne fields should be considered to answer this remaining question.

**Chapter 5**  
**The Use of DNA Markers to Determine  
Genetic Relationships between Paraquat  
Resistant and Susceptible Populations of  
*Hordeum leporinum* and *H. glaucum***

## 5.1 Introduction

The ability of weed populations to respond to environmental changes caused by management practices is dependent on selectable traits being present in populations (Jasieniuk and Maxwell, 2001). Populations with low genetic diversity are less likely to be able to positively respond to changing environments. In relation to herbicide use, genetic information about weed populations can be used to understand how herbicide resistance evolves and spreads in populations (Weller *et al.*, 2001). It is commonly assumed that weed populations with high genetic variation tend to evolve resistance more rapidly to herbicides (Clark and Yamaguchi, 2002), because it is more likely resistant individuals will be present in these populations and herbicide application will select for these genotypes (Tranel and Wright, 2002). Therefore, knowledge of genetic information about presence and absence of herbicide resistance is vital to establish effective weed management practices.

DNA markers have been important tools to study genetic variability of weed populations (Jasieniuk and Maxwell, 2001). Some of these techniques can distinguish genetic differences carried by two or more individuals within and between populations (Sunnucks, 2000; Whitkus *et al.*, 1994). Using DNA markers the genetic variation of weed populations can be determined and, potentially, the onset of weed resistance to herbicides is more likely to be predicted (Marshall, 2001)

Among DNA marker techniques, RAPD markers have been widely used for genetic analysis of populations in weeds (Arnholdt Schmitt, 2000; Barcaccia *et al.*, 1997; Lopez Martinez *et al.*, 1999; Pester *et al.*, 2003; Ransom *et al.*, 1998; Rutledge *et al.*, 2000). The reason for this is that the RAPD technique is simple, quick, cheap, and no prior knowledge of DNA sequences is required (DeBustos *et al.*, 1998; Muralidharan and Wakeland, 1993). RAPD markers can also be used with small quantities of genomic DNA, require no DNA digestion and labelling of probe, give a large number of markers throughout the genome and provide a high level of polymorphism (de Vienne *et al.*, 2003; Pester *et al.*, 2003; Whitkus *et al.*, 1994).

*Hordeum glaucum* and *H. leporinum* are two important weedy grasses that have documented resistance to the herbicide paraquat since 1983 and 1991 respectively (Tucker and Powles, 1991; Warner and Mackie, 1983). To date there are about eleven and five cases of paraquat resistance in *H. glaucum* and *H. leporinum* respectively reported in Australia (C. Preston, pers. comm.). Paraquat is still the herbicide of choice to control *Hordeum* spp. in lucerne and is being used increasingly in crop fields, so the occurrence of resistance will continue and this will threaten the usefulness of this herbicide.

Genetic studies will provide a better understanding of resistance evolution and distribution of paraquat-resistant *Hordeum* spp. in fields. The use of DNA markers in this study was intended to detect genetic differences and similarities within and between populations of paraquat susceptible and resistant populations of *H. glaucum* and *H. leporinum*. This will confirm the relative importance of local mutation(s) and gene flow in the evolution of

resistant populations. The benefits of this study are that they can assist the construction of a proper management strategy to prevent or delay the incidence of local resistance and to reduce the spread of resistance in both weed species (Brown, 1996).

## **5.2 Materials and Methods**

### **5.2.1 Plant Materials**

#### **5.2.1.1 *Hordeum glaucum***

Five resistant populations and seven susceptible populations of *H. glaucum* were used in this study (Table 5.1). Seeds of SHG4 and SHG5 were harvested from plants that survived application of 200 g ha<sup>-1</sup> paraquat. The original seeds of SHG4 and SHG5 were collected from Avon (1994) and Mt Bryan (1996), South Australia, after the failure of paraquat to control plants following 14 years of application. Seeds of SHG7, SHG8, and SHG9 were collected from lucerne fields along the road between Jamestown and Spalding, South Australia. Likewise, seed of susceptible populations were collected from fields adjacent to these resistant populations (Table 5.1). About 20 seeds of each population were germinated on 0.6 % agar in 1 L plastic containers, placed for 7 days in seed incubator with a 12 h/12 h (20°C/20°C) light (30  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and dark regime. At the one-leaf stage

seedlings were transplanted to pots containing soil and allowed to grow to produce sufficient leaf material for DNA analysis.

Table 5.1 List of paraquat-resistant and susceptible populations of *H. glaucum* used in this study, their origin and codes.

Codes	Location	Crops	Status
01001	1.4 km Jamestown - Spalding	Lucerne	Susceptible
01002	1.4 km Jamestown - Spalding	Pasture	Susceptible
01003	1.4 km Jamestown - Spalding	Lucerne	Susceptible <sup>1)</sup>
SHG8	9.8 km Jamestown - Spalding	Lucerne	Resistant
01005	9.8 km Jamestown - Spalding	Wheat	Susceptible
SHG9	16.8 km Jamestown - Spalding	Lucerne	Resistant
01007	16.8 km Jamestown - Spalding	Wheat	Susceptible
99030	17.9 km Jamestown - Spalding	Wheat	Susceptible
99038	26.5 km Jamestown - Spalding	Lucerne	Susceptible
SHG4	Avon	Wheat <sup>2)</sup>	Resistant
SHG5	Mt Bryan	Lucerne	Resistant
SHG7	1.4 km Jamestown - Spalding	Lucerne	Resistant

<sup>1)</sup> Susceptible individuals from the same field as SHG7

<sup>2)</sup> No till wheat

### **5.2.1.2 *Hordeum leporinum***

Three paraquat-resistant *H. leporinum* populations, THL1, THL2 and THL3, and one susceptible population, THL4, all from near Ouse in Tasmania were used in this investigation. The resistant populations of *H. leporinum* were from lucerne fields with histories of annual applications of paraquat for 12 to 24 years. Seeds of a susceptible population, THL4, were collected from a nearby pasture with no history of paraquat application (Purba, 1993a). The seeds that were used in these experiments were about the fourth generation of self-pollinated individuals maintained at Waite Campus. About 20 seeds of each population were germinated and grown as previously described.

### **5.2.2 DNA extraction**

Fresh young leaves were harvested separately from eight plants per population on the day of DNA extraction. About 0.5 g of leaf material was excised from each individual using clean scissors, wrapped in aluminium foil and then frozen directly in liquid nitrogen.

The leaf tissue was ground to a fine powder in liquid nitrogen with a mortar and pestle. Genomic DNA was extracted from the leaf tissue using a CTAB (cetyltrimethyl-ammonium bromide) method (Doyle and Doyle, 1990; Rogers and Bendich, 1985). The powder was transferred using a spatula into a 10 ml centrifuge tube containing 5 ml CTAB isolation buffer [2% (w/v) CTAB, 1.4 M NaCl, 0.2% (v/v) mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl

(pH 8.0)] that had been preheated at 60°C in water bath. The tube was gently inverted several times and incubated at 60°C in water bath for 30 minutes. Approximately 5 ml of chloroform:isoamyl alcohol (24:1) was added to the tube to extract the DNA and the mixture and was centrifuged at 12,000 x g for 15 minutes at 4°C. The upper aqueous phase was removed and transferred to a new tube. About 2.5 volumes of cold ethanol (99.7%) were added and this mixture was gently inverted several times to precipitate DNA. At this stage, DNA could be observed as flocculent and was pelleted by centrifugation at 12,000 x g for 10 minutes. The ethanol was poured off and the pellet was air dried in a 37°C incubator or on the bench. The pellet was then washed with 70% ethanol for at least 20 minutes and centrifuged at 12,000 x g for 10 minutes. The supernatant was removed and the pellet was air dried for at least 60 minutes. After this, the DNA pellet was resuspended with 200 µl of TE buffer pH 8.0 (10mM Tris-HCl, 1mM EDTA) and stored overnight at 4°C.

To remove RNA, the DNA pellet was dissolved in 2 ml TE buffer and ribonuclease [10 mg ml<sup>-1</sup> RNAase A, 71 U/mg<sup>-1</sup> (SIGMA)] was added at 1:100 dilution. This mixture was incubated at 37°C in water bath for 30 minutes. The mixture was extracted with 3 ml phenol:chloroform:isoamyl-alcohol (25:24:1), followed by centrifugation at 12,000 x g for 15 minutes at 4°C. The upper layer was collected and 3 ml chloroform:isoamyl alcohol was added. After gently inverting, the mixture was again centrifuged for 15 minutes. The upper aqueous phase was transferred to a new tube and was precipitated with 6 ml cold alcohol (98%). The DNA was pelleted by



centrifugation at 12,000 x g for minutes. The ethanol (98%) was carefully poured off and the DNA pellet was washed with 4 ml ethanol (75%), followed by centrifugation at 12,000 x g for 15 minutes. The ethanol was removed and the DNA pellet was air dried for at least 60 minutes. The DNA was then resuspended with 400  $\mu$ l TE buffer and was stored at 4°C for a short-term use or at -20°C (or -80°C) for a long-term storage.

The amount of DNA in samples was determined approximately by comparing with a  $\lambda$ HindIII marker on 1% (w/v) agarose gels (Gibco BRL) prepared with 5  $\mu$ l of ethidium bromide (10 mg ml<sup>-1</sup>) for every 100 ml of 0.5 x TBE buffer (1xTBE: 90 mM Trizma base, 90 mM boric acid, 2 mM EDTA, pH 8.0).  $\lambda$ HindIII marker (1  $\mu$ l) and DNA samples (4  $\mu$ l) were mixed with 2  $\mu$ l volume of gel-loading buffer [0.25% bromophenol, 0.25% xylene cyanol FF, 15% Ficoll (Type 400; Pharmacia) made up to 10 ml in water] and then placed on well in the gel. The gels were submerged in 0.5xTBE and were run by electrophoresis at approximately 100 volts for 60 minutes. The DNA bands were visualised by illumination with ultraviolet light and photographed for comparison. Based on this visual comparison, the DNA was diluted to an approximate concentration of 20-40 ng  $\mu$ l<sup>-1</sup> for PCR amplification.

### 5.2.3 PCR Amplification

A set of 40 random decamer primers (kit R and T, Operon<sup>TM</sup> Technologies Inc.) was screened for this experiment. The amplification reactions were

performed in a 30  $\mu\text{l}$  volume containing 1  $\mu\text{l}$  of diluted DNA, 3  $\mu\text{l}$  of 10x buffer [1x is 10 mM Tris-HCl (pH 8.8 at 25 °C), 50 mM KCl, 0.1% Triton X-100], 0.25  $\mu\text{M}$  primer, 200  $\mu\text{M}$  dNTPs, 2 mM  $\text{MgCl}_2$ , and 1 unit of Taq DNA polymerase (Promega). A negative control without DNA template was included in each amplification reaction. The mixture was gently agitated and was transferred to a Programmable Thermal Controller (PTC-100<sup>TM</sup>, MJ Research, Inc.). The machine was programmed for 30 cycles with the following thermal profile: 15 s for denaturation at 94°C, 15 s for annealing at 36°C, and 30 s for extension at 72°C. At the end of 30 cycles, a final extension was applied at 72°C for 2 minutes. The reactions were then held at 4°C until used.

#### 5.2.4 Agarose gels

To the each of the PCR products 5  $\mu\text{l}$  loading buffer was added. Then 20  $\mu\text{l}$  of each mixture was loaded into the wells of 1.5% agarose gels (Gibco BLR) stained with 5  $\mu\text{l}$  of ethidium bromide (10 mg  $\text{ml}^{-1}$ ) for every 100 ml of 0.5 x TBE buffer. The gels were submerged in 0.5 x TBE buffer. Each gel included a 100-bp ladder (Fermentas) as a molecular weight standard. The PCR products were separated by electrophoresis at approximately 70 volts for 90 minutes. The DNA bands were visualised by illumination with ultraviolet light and photographed.

All PCR products of paraquat susceptible and resistant populations of *H. leporinum* were visualised using agarose gels. PCR products of paraquat susceptible and resistant populations of *H. glaucum* were run in polyacrylamide gels.

### 5.2.5 Polyacrylamide Gels

To increase resolution and visualisation of PCR products polyacrylamide gels (Code No. 17-1198-06, Pharmacia Biotech) were employed. This technique used DNA Silver Staining Kit (Code No. 17-6000-30, Pharmacia Biotech). Sample buffer (2  $\mu$ l), made as described by the manufactures' notes, was added to 5  $\mu$ l of each PCR product and the mixture applied to the gel. The running condition was as follows: 200V, 20mA, 10W for 20 mins, 380V, 30mA, 20W for 50 mins, and 450V, 30mA, 20W for 30 mins. The temperature was set at 10°C. The silver staining procedure followed the instructions of manufacture.

### 5.2.6 Statistical analysis

The polyacrylamide gels were observed using a light box (Medilite), while the picture of agarose gels was directly used for analysis. For the same primer, bands of identical size were considered to be equivalent for the analysis of similarity. The bands were scored for presence (1) and absence (0). This

information was used to calculate the genetic distance between populations using Nei's unbiased (Nei, 1978) formula. The unweighted pair group method with arithmetic average (UPGMA) was used to construct dendograms. The software package Tools for Population Genetic Analysis (Miller, 1997) was used to analyse the data.

## **5.3 Results**

### **5.3.1 DNA Extraction**

Genomic DNA was successfully extracted from leaf tissue of *H. glaucum* and *H. leporinum* using CTAB method. When 4  $\mu$ l of each DNA sample was loaded on agarose gel, the band was about equal to 1  $\mu$ l of DNA  $\lambda$  HindIII (Figure 5.1). The DNA samples were suitable for PCR.

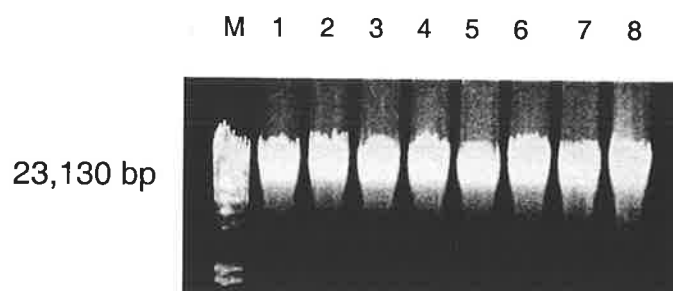


Figure 5.1 Gel electrophoresis of DNA extracted from *H. leporinum* using CTAB method. Lane 1: Marker ( $\lambda$  HindIII). Lane 2-8: DNA extracted from *H. leporinum*.

### 5.3.2 RAPD analysis

From 40 primers screened in this study, three primers (OPT-07, OPT-13 and OPT-15) were able to amplify DNA sequences. Results from a further test using DNA samples from different populations of *Hordeum* spp. showed that primers OPT-13 and OPT-15 produced no polymorphisms. Therefore, only OPT-07 (GGCAGGCTGT) was employed in this study. PCR amplification using OPT-07 primer produced numerous bands of which 13 bands could be easily distinguished on agarose gels. On polyacrylamide gels 20 bands could be easily visualised. The size of amplified fragments detected ranged

from 400 to 2000 bp for agarose gels and 400 to 3000 bp for polyacrylamide gels. The comparison between paraquat-resistant and susceptible populations was carried out using pooled DNA samples of 8 individuals from each population. The RAPD banding patterns were highly reproducible if the reaction conditions were maintained as described.

### **5.3.3 *Hordeum glaucum***

The DNA extracted from paraquat susceptible and resistance *H. glaucum* samples was successfully amplified by RAPD-PCR technique. Using primer OPT-07, polymorphisms could be detected from within a susceptible population (Figure 5.2). Two different genotypes were observed in the susceptible population 01005. However, paraquat-resistant populations showed no polymorphisms (data not shown).

Variation in banding patterns was evident between paraquat susceptible and resistant populations of *H. glaucum* (Figure 5.3). Genetic differences were also found among susceptible populations. These susceptible populations had one or two bands that consistently differentiated them. For example, the susceptible populations 01005 and 99038 had extra bands between 700 bp and 800 bp, although these two populations shared many other bands.

Genetic similarity was apparent between some resistant populations. Two resistant populations, SHG8 collected 9.8 km from Jamestown and SHG9 collected 16.8 km from Jamestown had identical banding patterns. Likewise,

SHG 7 and 01003 populations had a similar RAPD profiles. SHG4 and SHG5 populations had a relatively similar banding pattern, with only one band difference.

Paraquat-resistant population SHG7 had the same banding pattern with individuals from susceptible population 01001 and 01003, but not with population 01002 (Figure 5.4). These populations were from adjacent fields. SHG8 had an identical genetic profile with individuals within the susceptible population 01005 from an adjacent field. However, paraquat-resistant population SHG9 had a different genotype to the susceptible population 01007 from an adjacent field.

The genetic distance between populations (Table 5.2) was determined using Nei's formula (1978). The closest relationship was evident between population 01003 and SHG7, as well as between SHG8 and SHG9, where the value of genetic distance was 0.000. The furthest genetic distance was found between 99038 and three populations (01003, SHG4 and SHG7), with the value reaching 0.9163.

A dendrogram was constructed for paraquat susceptible and resistant populations of *H. glaucum* using the value for genetic distance (Figure 5.5). This dendrogram grouped the resistant populations SHG8 and SHG9 into a single cohesive sub-cluster. Population SHG7 and population 01003 were grouped into another sub cluster. This suggested SHG8 and SHG9 resistant populations might have evolved from a single source. However, as SHG7 was not genetically identical to SHG8 and SHG9, it is more likely the SHG7 has evolved from a separate evolutionary event. SHG4 and SHG5

populations fell into one sub cluster, but were not identical. Another cluster comprised of susceptible populations 01007 and 99030, indicating the similarity between two populations. Unlike other populations, population 99038 had a distinct band profile; therefore it was exclusively separated from the other populations.



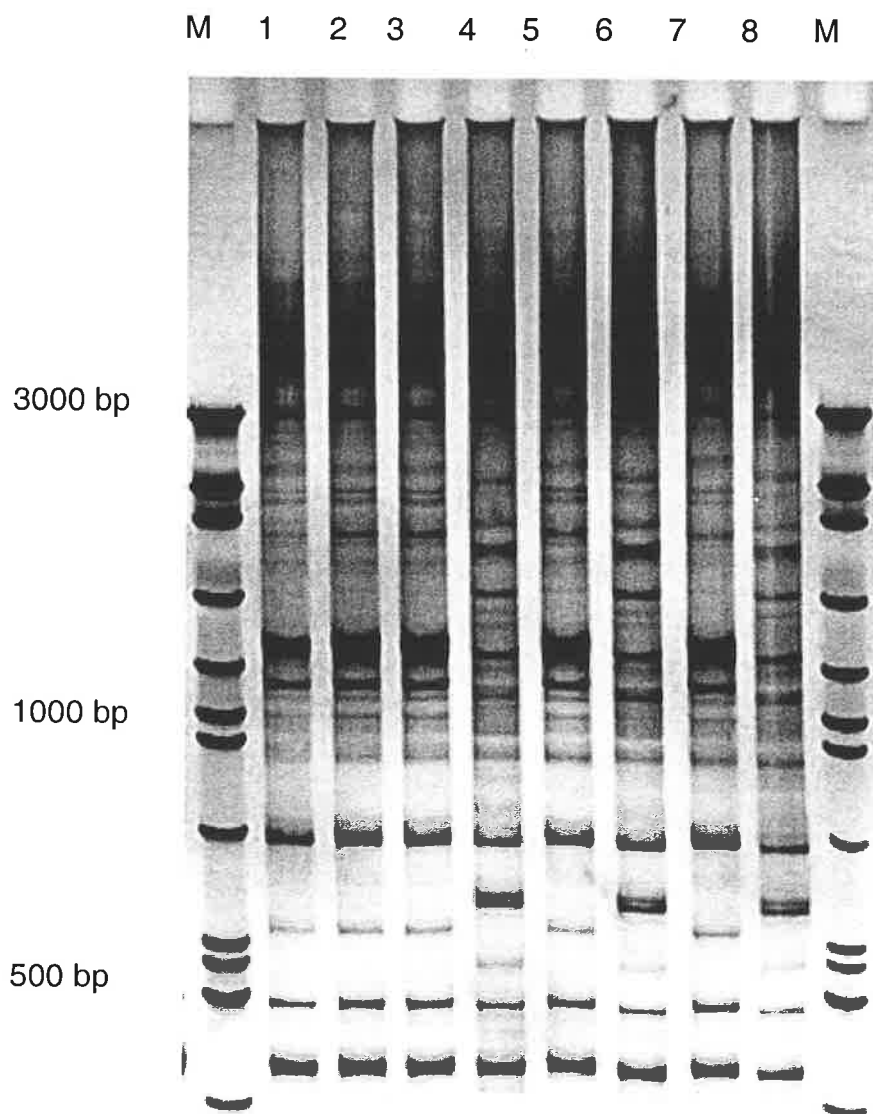


Figure 5.2 RAPD profile showing polymorphism between individuals within a paraquat susceptible population of *H. glaucum* (01005) amplified with primer OPT-07 and visualised in a silver stained Clean-Gel 48S polyacrylamide gel. M = 100 bp marker (Fermentas), Lanes 1-8: individuals 1 to 8.

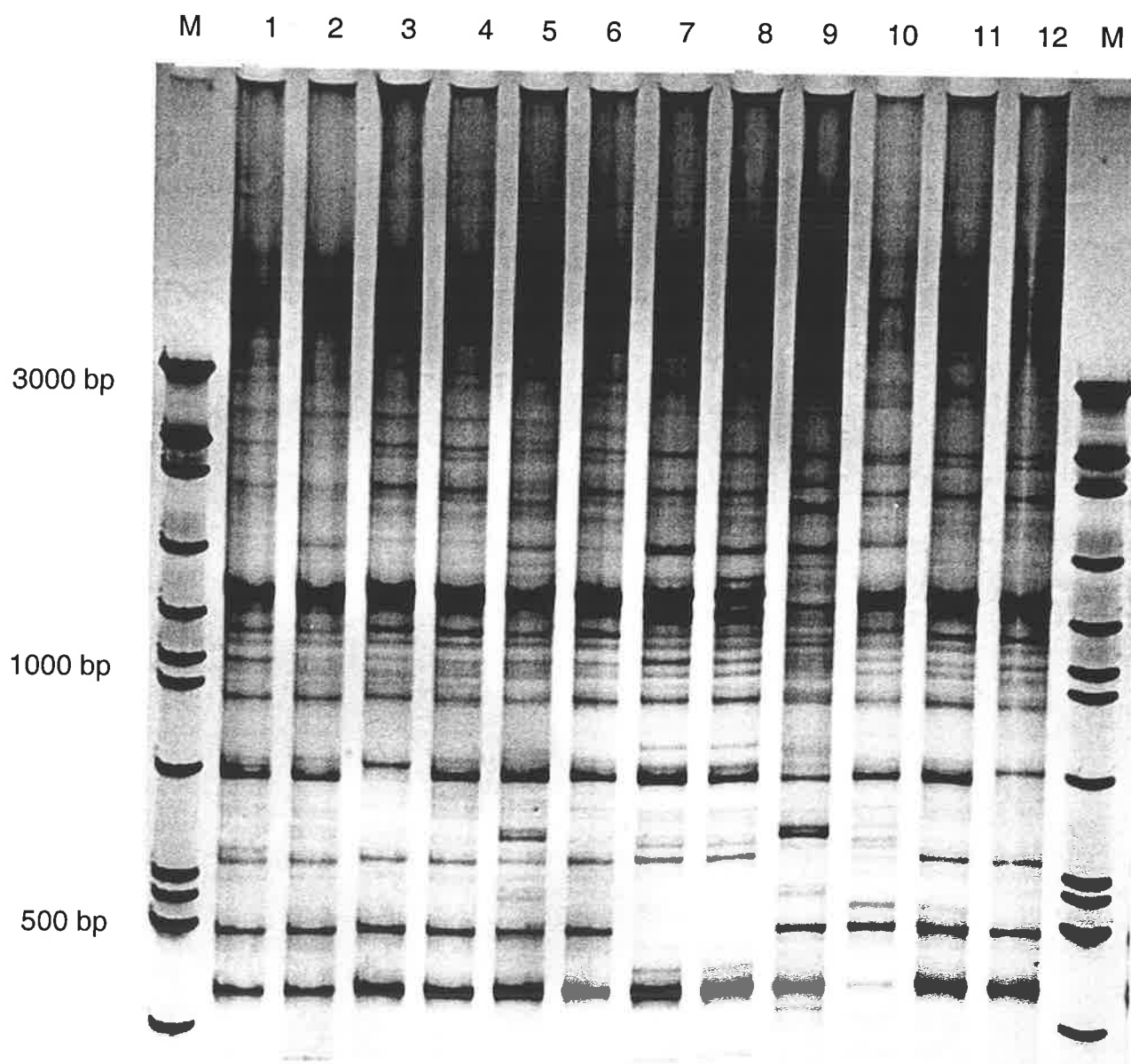


Figure 5.3 RAPD profile of paraquat-resistant and susceptible populations of *H. glaucum* amplified using primer OPT-07 and visualised in a silver stained Clean-Gel 48S polyacrylamide gel. M = 100 bp marker (Fermentas). Lanes 1-12: 1 = Pop.01001 (S), 2 = Pop.01002 (S), 3 = Pop.01003 (R+S), 4 = SHG8 (R), 5 = Pop.01005 (S), 6 = SHG9 (R), 7 = Pop.01007 (S), 8 = Pop.99030 (S), 9 = Pop.99038 (S), 10 = SHG4 (R), 11 = SHG5 (R), and 12 = SHG7 (R)

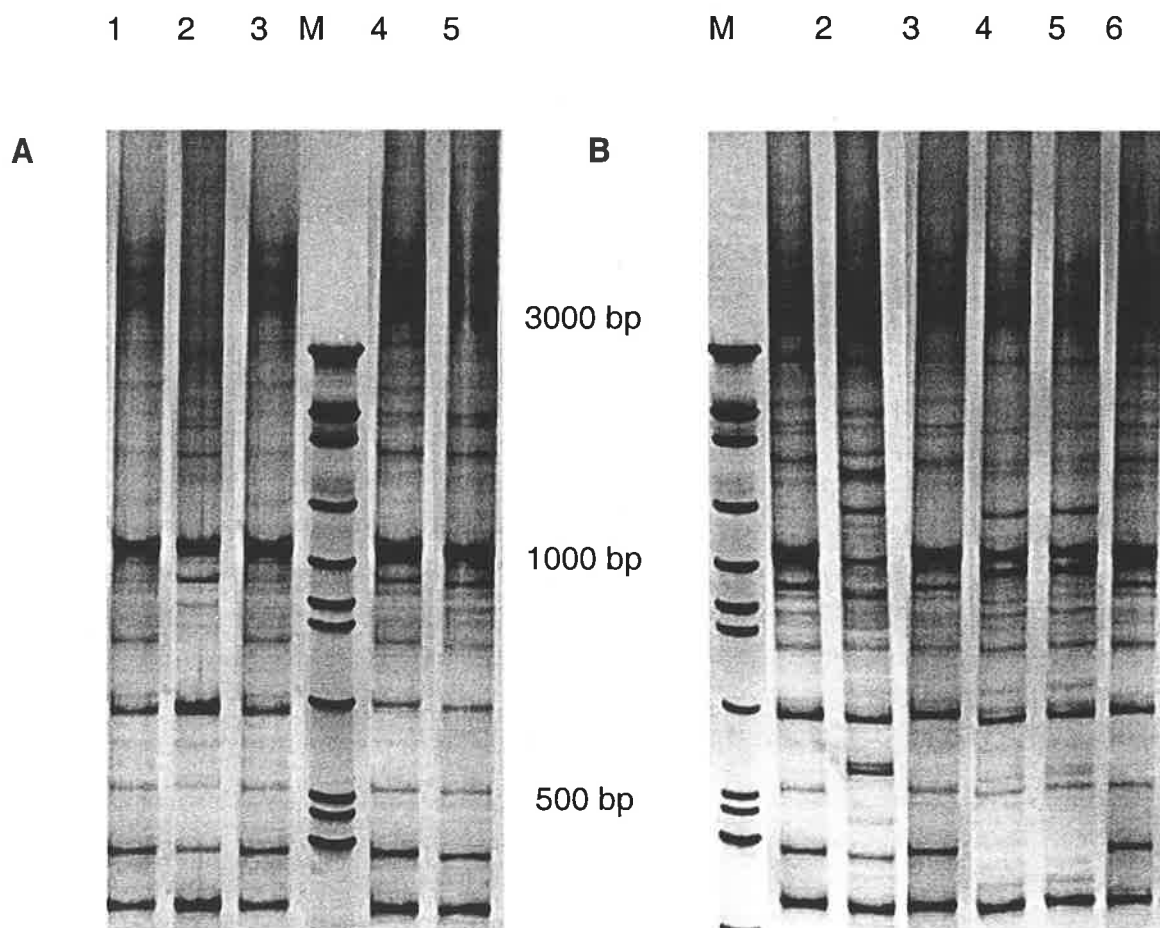


Figure 5.4 RAPD profile showing the genetic relationship between paraquat-resistant (SHG7) and susceptible (01001, 01002 and 01003) populations of *H. glaucum* (A); and the genetic relationship between paraquat-resistant (SHG8 and SHG9) and susceptible (01005 and 01007) populations of *H. glaucum* (B). A). M = 100 bp marker (Fermentas), Lanes 1-5: 1 and 2 = 01001, 3 = 01002, 4 = 01003, and 5 = SHG7. B). M = 100 bp marker (Fermentas), Lanes 1-6: 1 and 2 = 01005, 3 = SHG8, 4 and 5 = 01007, 6 = SHG9.

Table 5.2 Genetic distance between paraquat resistant and susceptible populations of *H. glaucum* calculated using Nei's (1978) formula.

	01001	01002	01003	SHG8	01005	SHG9	01007	99030	99038	SHG4	SHG5	SHG7
01001	0.0000											
01002	0.0513	0.0000										
01003	0.0513	0.1054	0.0000									
SHG8	0.0513	0.1054	0.1054	0.0000								
01005	0.2231	0.1625	0.2877	0.1625	0.0000							
SHG9	0.0513	0.1054	0.1054	0.0000	0.1625	0.0000						
01007	0.3567	0.2877	0.4308	0.2877	0.3567	0.2877	0.0000					
99030	0.4308	0.3567	0.5108	0.3567	0.4308	0.3567	0.0513	0.0000				
99038	0.7985	0.6931	0.9163	0.6931	0.4308	0.6931	0.5978	0.6931	0.0000			
SHG4	0.1625	0.2231	0.2231	0.1054	0.2877	0.1054	0.4308	0.5108	0.9163	0.0000		
SHG5	0.1054	0.1625	0.1625	0.0513	0.2231	0.0513	0.3567	0.4308	0.7985	0.0513	0.0000	
SHG7	0.0513	0.1054	0.0000	0.1054	0.2877	0.1054	0.4308	0.5108	0.9163	0.2231	0.1625	0.0000

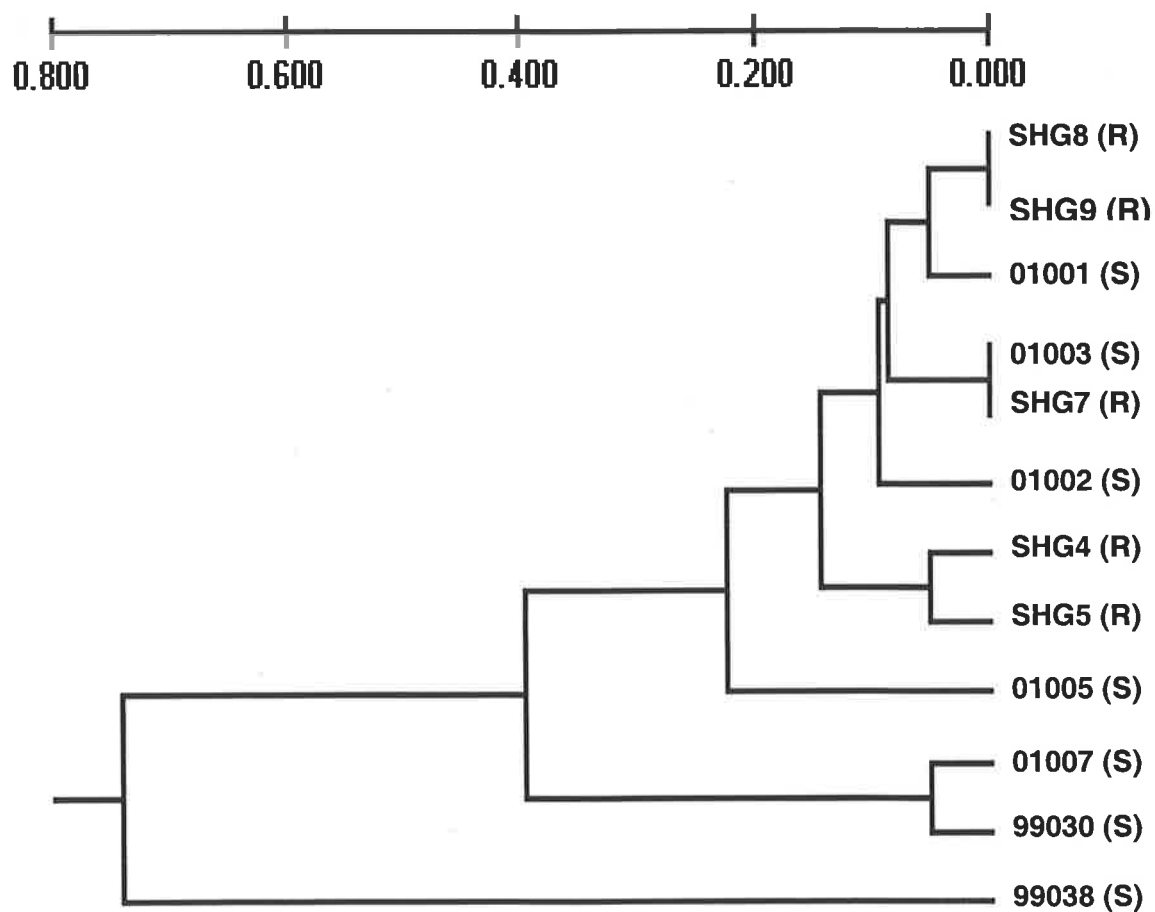


Figure 5.5 Dendrogram illustrating genetic relationships among paraquat-resistant and susceptible populations of *H. glaucum*, generated by the UPGMA cluster analysis.

### 5.3.3 *Hordeum leporinum*

The genomic DNA of paraquat-resistant and susceptible *H. leporinum* individuals was amplified by RAPD-PCR technique using primer OPT-07. The results indicated that there were two genotypes present in the paraquat susceptible population, THL4 (Figure 5.6), with polymorphic bands observed about 550 bp and 900 bp. As for the *H. glaucum* populations, this primer detected no polymorphisms within each paraquat-resistant population (data not shown), although there were differences between the populations.

Using primer OPT-07, genetic polymorphism was detected between paraquat-resistant and susceptible populations (Figure 5.7). THL1 population had an identical banding pattern to THL3, but was different to THL2, which had a unique band between 600 to 700 bp. THL4 population showed an extra band at about 500 to 600 bp, which was not present in any of the resistant populations.

The genetic distance among populations varied between 0.0000 and 0.2624 (Table 5.3). The closest relationship was between THL1 and THL3 populations (0.0000). A similar genetic distance was calculated for THL2 to THL1 and THL2 to THL3 populations. The furthest genetic distance was found between THL 2 and THL4 populations (0.2624).

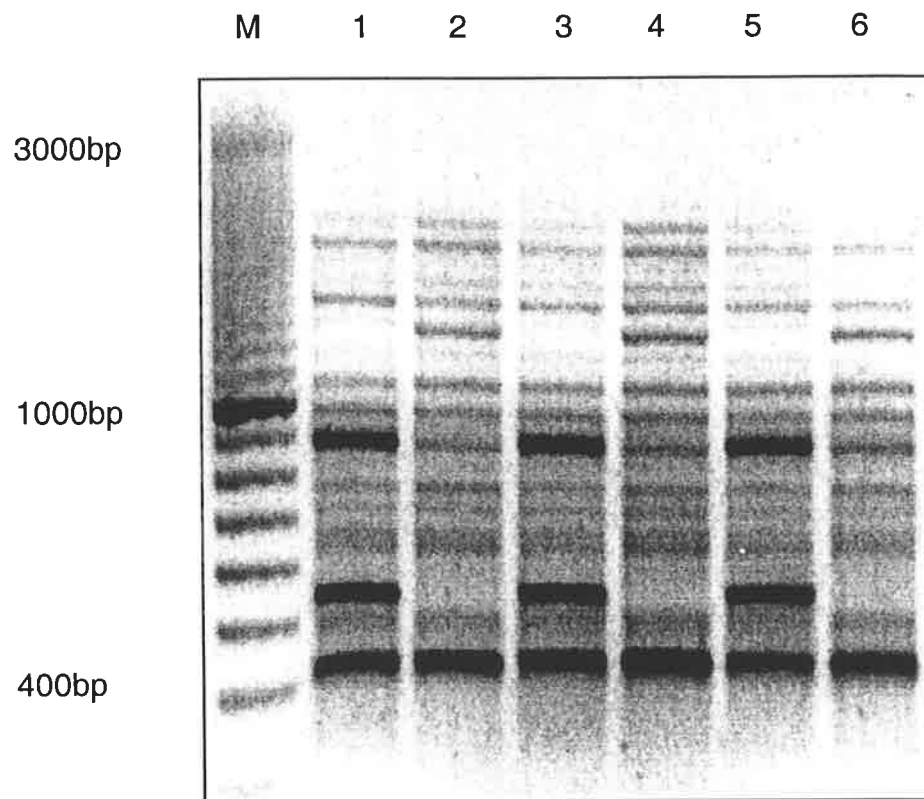


Figure 5.6 RAPD profile showing polymorphism within paraquat susceptible population of *H. leporinum* (THL4) amplified with primer OPT-07 and visualised in agarose gel. M = 100 bp marker (GeneWorks), Lanes 1-6: individuals 1 to 6.

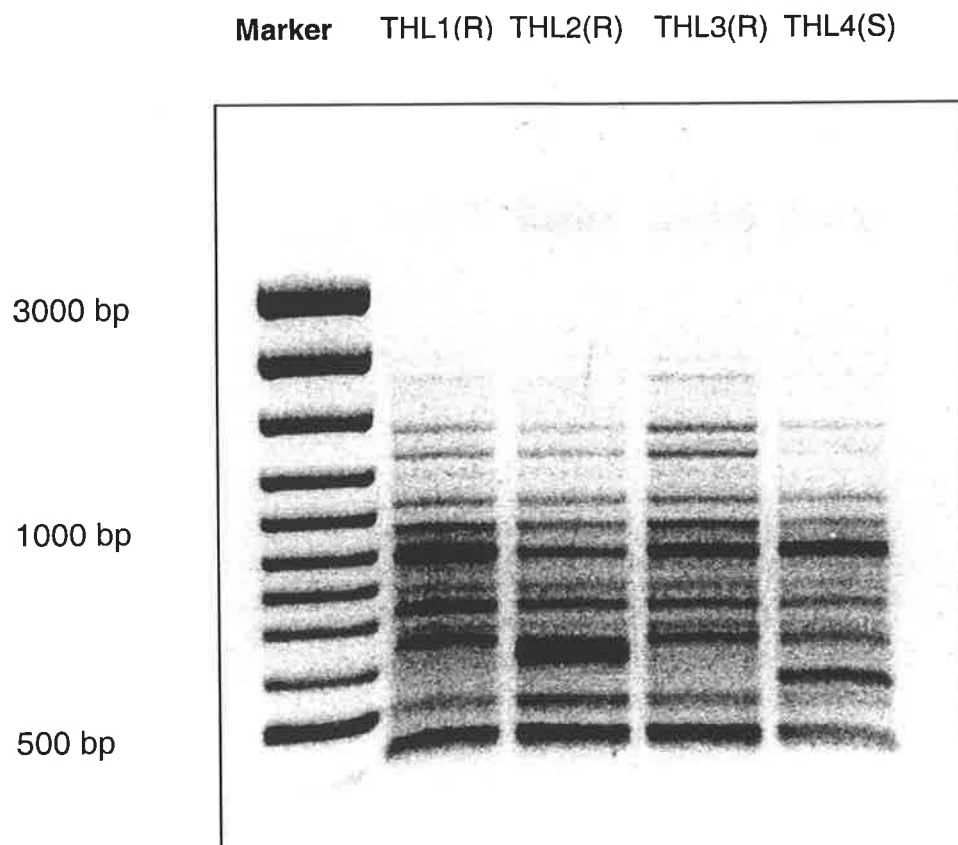


Figure 5.7 RAPD profile showing polymorphism between populations of paraquat-resistant and susceptible *H. leporinum* amplified with primer OPT-07 and visualised in agarose gel. Marker = 100 bp (Fermentas).



Table 5.3 Genetic distance between paraquat-resistant and susceptible populations of *H. leporinum* calculated using Nei's (1978) formula.

Populations	THL1	THL2	THL3	THL4
THL1 (R)	0.0000			
THL2 (R)	0.0800	0.0000		
THL3 (R)	0.0000	0.0800	0.0000	
THL4 (S)	0.1671	0.2624	0.1671	0.0000

The data from genetic distances were used to construct a dendrogram using UPGMA program (Figure 5.8). This dendrogram had two main branches, three populations clustered together, namely: THL1, THL2, and THL3. THL1 and THL3 were identical, while THL4 was separated from all three resistant populations in terms of genetic distance. This indicated that the resistant the resistant populations had all experienced a bottleneck, while THL4, with more than one genotype present, had retained genetic diversity.

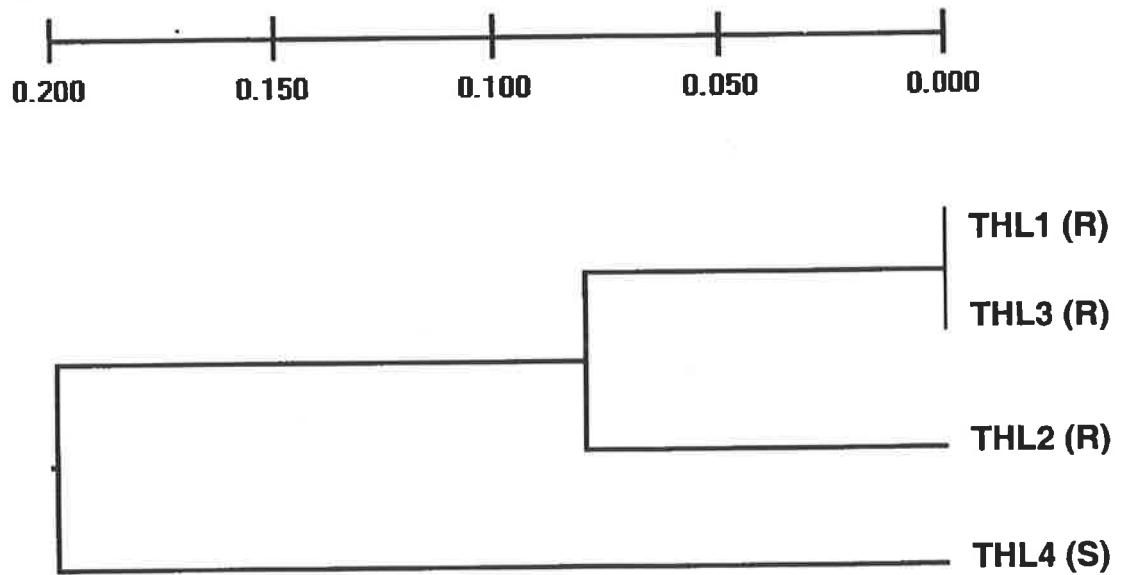


Figure 5.8 Dendrogram illustrating genetic relationship among paraquat-resistant and susceptible populations of *H. leporinum*, generated by the UPGMA cluster analysis.

## 5.4 Discussion

RAPD markers have often been employed to investigate genetic relationships within and between populations of crop and weed species. The polymorphic bands revealed by RAPD markers can be useful information to compare genetic diversity, to determine the origin of introduced plants, and to establish phylogenetic relationships. However, RAPD markers provide no idea of the nature of the amplified sequence nor its function (de Vienne *et al.*, 2003; Gonzalez and Ferrer, 1993; Nissen *et al.*, 1995; Williams *et al.*, 1990). Only in very rare cases would RAPD markers that are linked to resistance genes be found (Kondo *et al.*, 2000). Yet a single primer can generate polymorphic bands within populations making this system versatile and easy to use when establishing differences within and between populations (Parker *et al.*, 1998). Polymorphisms in banding pattern occur among individuals or populations due to alterations of priming sites or the length of fragments. Substitutions, insertions or deletions within the genome that occur over time are responsible for these changes (Muralidharan and Wakeland, 1993; Parker *et al.*, 1998; Williams *et al.*, 1990).

In relation to herbicide resistance, RAPD markers have infrequently been used to observe genetic variations between and within herbicide susceptible and resistant populations. The challenge of using RAPD markers within a single species is to find primers that could generate polymorphisms providing relevant differentiation between populations. In this study, the use of primer OPT-07 was successful in detecting polymorphisms between populations of

both *H. glaucum* and *H. leporinum* species. While RAPD markers have significant advantages in being easy and cheap to use, there are also disadvantages. The main disadvantage is the limited number of bands that are generated. This can lead to errors in assuming genotypes are identical when they are different. RAPD profiles can be improved by separating fragments using polyacrylamide gels, which gives more bands that can be scored. Marker systems such as AFLPs that provide many bands may be preferable under certain circumstances. However, the intent of this study was to establish that different paraquat-resistant populations of *H. glaucum* and *H. leporinum* were genetically distinct. For this purpose, RAPD markers were acceptable.

RAPD markers have previously been used to investigate genetic variation among propanil susceptible and resistant populations of *Echinochloa crus-galli* (Rutledge *et al.*, 2000). RAPD data showed some polymorphism between propanil susceptible and resistant populations. However, among 10 resistant populations investigated, RAPD markers detected no polymorphism for four resistant populations. These resistant populations with the same banding pattern may have originated from the same source and then spread through seed migration.

In the present study, RAPD markers successfully detected genetic variation within paraquat susceptible populations of *H. glaucum* and *H. leporinum*. Using primer OPT-07, there were at least two genotypes detected in RAPD profiles of susceptible populations of both species. These genotypes, although different between species, differed by the presence or absence of

only a few bands with other amplified bands shared among all susceptible individuals.

In contrast, such genetic variation was not found within individual paraquat-resistant populations of *H. glaucum* and *H. leporinum*. Individuals within each paraquat-resistant population shared all amplified bands generated by OPT-07 primer. The persistent application of paraquat may have acted as a bottleneck on these populations so genetic variation has been decreased. Of course, this does not mean that all individuals were identical. Had a greater number of primers been employed polymorphism within resistant populations may have been detected.

Genotype comparison between paraquat susceptible and resistant populations of *H. glaucum* was conducted using pooled DNA samples of eight individuals per population. The RAPD profile on polyacrylamide gels showed polymorphism between populations. A dendrogram constructed from these data shows one large cluster containing all resistant and some susceptible populations. SHG8 and SHG9 resistant populations had identical profiles (genetic distance 0.0000). It is very likely that these populations developed from a single resistance mutation.

SHG7 and 01003 populations also had a similar genetic background (genetic distance 0.000). These populations fell into one cohesive sub cluster. This could be explained because they were sampled from the same site, 1.4 km from Jamestown on the road to Spalding, although collected one year apart (Chapter 2). SHG7 resistant population was generated from survivors of 200

g paraquat ha<sup>-1</sup> application, while population 01003 contained a mix of susceptible and resistant individuals. It is highly likely that the resistant population SHG7 evolved from population 01003.

The presence of the same banding pattern between SHG7 and individuals within population 01003, and between SHG8 and individuals within population 01005 may indicate that these resistant populations had evolved from local susceptible populations. However, SHG9 had a different genotype to susceptible population 01007, so this resistant population has no genetic relationship with the local susceptible population.

SHG4 and SHG5 populations were collected from different locations with different histories of culture and control. They also showed different RAPD profiles, which support the supposition that they evolved from different mutation events conferring resistance. Their locations were more than 100 km from Jamestown and is unlikely they share the same population origin with the paraquat-resistant populations found between Jamestown and Spalding.

The study on paraquat-resistant populations of *H. leporinum* with the primer OPT-07 found paraquat susceptible and resistant populations had different genetic backgrounds. Cluster analysis grouped THL1 and THL3 into one group, indicating that they may have evolved from a single event. This assumption is supported by the fact that THL1 and THL3 populations were collected from different ends of the same lucerne field, despite different management occurring at each end of the field. It is not known which

population evolved resistance first, but resistance is likely to have moved from one end to the other.

The uniqueness of THL2 population was revealed by RAPD profiles in which a distinct band was exclusively present. Previous studies found THL2 displayed moderate resistance to paraquat compared to THL1 and THL3 (Purba, 1993a). This is interesting, because this population has been treated with paraquat for more than two decades, longer than THL1 and THL3. Geographically, the THL2 population was located about 12 km away from THL1 and THL3 and seed or pollen movement between THL1, THL3 and THL2 was unlikely. Therefore, the result of the RAPD analysis presented here, coupled with other investigations, points to THL2 resulting from an independent mutation. THL4 population grouped outside the first grouping of populations THL1, THL2, and THL3, so it could be speculated that none of the resistant populations documented here originated from THL4.

Overall, the RAPD results of this experiment have been useful in understanding the genetic background between paraquat-resistant populations for *H. glaucum* and *H. leporinum* species. Simply, genetic similarity among resistant populations could be associated with seed migration, while polymorphism indicates independent mutations in the evolution of resistance. Effective management strategies could be designed to stop seed dispersal and minimise local selection. DNA marker analysis has found that in non resistant populations, genetic variation could be maintained by crop and herbicide rotations resistance (Moodie *et al.*, 1997).

This means weed management strategies should integrate any practice that have the potential to preserve genetic diversity within populations.



**Chapter 6:**  
**General Discussion and Conclusion**

## 6.1 Discussion

For over half a century herbicides have played a vital role in increasing agricultural production, particularly in developed countries and to lesser extent in developing countries (Heap and LeBaron, 2001; Heap, 1997). With about 240 different herbicides present in the market, herbicides now provide a lot of options for farmers to control weeds (Valverde *et al.*, 2000). However, the presence of weed resistance to herbicides has been a concern for weed control because it has occurred to most herbicides in all cropping systems (Heap and LeBaron, 2001; Powles *et al.*, 1997). The problem of weed resistance is currently worsened by the occurrence of herbicide multiple-resistance in some weed species, such as in *Lolium rigidum*, *Echinochloa crus-galli* and *Avena fatua* (Heap and LeBaron, 2001). These multiple-herbicide resistant populations represent a serious practical problem because herbicide options become limited. Farmers will usually respond to herbicide resistance by using herbicides with new modes of action to provide sufficient weed control (Reade and Cobb, 2002). Nevertheless, if prophylactic strategies to delay or prevent resistance are not consistently adopted by herbicide users, the use of new herbicides will only lead to their failure due to the continuing evolution of herbicide resistance.

The need for continued use of herbicides has encouraged weed scientist to explore the nature of weed resistance. Resistance to herbicides in weeds reflects the phenomenon of evolution in which weed populations have the capability to respond to the detrimental environment created by herbicide

application (Maxwell and Mortimer, 1994). In this regard, the rate of evolution will be determined by the interaction between biological aspects of weeds and selection pressure applied by herbicide use (Clark and Yamaguchi, 2002; Powles *et al.*, 1997; Valverde *et al.*, 2000). Biological factors that influence the rate of evolution are the initial frequency of resistance genes, the rate of resistant gene flow, the degree of dominance of resistance genes and the fitness of resistant individuals. Meanwhile, patterns of herbicide use influence the rate of resistance evolution through frequency of use, persistence, selectivity, and efficacy on weeds (Clark and Yamaguchi, 2002; Maxwell and Mortimer, 1994; Moss, 2002; Valverde *et al.*, 2000).

As a consequence of intensive use of paraquat over a long period of time, paraquat resistance in *Hordeum* spp. is increasing and will probably be more widespread in the future. Paraquat resistance was first reported in 1983 in *H. glaucum* (Warner and Mackie, 1983). To date, there are three other weed species documented with populations resistant to this herbicide, (Powles *et al.*, 1989; Purba, 1993a; Purba *et al.*, 1993b; Tucker and Powles, 1991). On the whole, the appearance of paraquat resistance is a result of repeated annual applications for more than 12 years. The slow evolution of paraquat resistance here could be the result of low initial frequencies of resistant alleles (Purba, 1993a) and a low level of gene flow through pollen dispersal (Chapter 3). More rapid evolution of herbicide resistance has occurred with other herbicides. The nature of paraquat action may contribute to the slow rate of evolution because changes to the target site or detoxification of

paraquat are not possible. This means there are fewer possible mechanisms of resistance able to be selected compared to other herbicides.

Except for one *H. leporinum* population from Tasmania (THL2), all paraquat-resistant populations in Australia have a high degree of resistance to paraquat. THL2 population is distinctive because it appeared following treatment with paraquat for 24 years, but expresses much lower resistance than THL1 and THL3 which have been selected with paraquat for only 12 years (Purba, 1993a). The reason for this lower resistance is unknown.

The first part of this thesis reports an investigation of additional herbicide resistant populations in *Hordeum* spp. A random survey in 1999 across the mid-north of South Australia found resistance to paraquat in only one population of *H. glaucum* (SHG7). This resistant population was obtained from a lucerne field near Jamestown; about 35 km from the location of the first incidence of paraquat-resistant of *H. glaucum* reported in 1990 (Purba, 1993a). A second survey in 2001 targeted this area and found four more paraquat-resistant populations of *H. glaucum*, SHG8, SHG9, SHG10, and SHG11. Another paraquat-resistant population of *H. glaucum* (SHG12) was identified from a lucerne field near Waterloo. Because the distance between Jamestown and Waterloo is more than 200 km, this latter resistant population is probably the result of an independent evolutionary event. Overall, there are seven new paraquat-resistant populations of *Hordeum* spp. documented in this study.

It is important to note that the frequency of paraquat-resistant populations of *Hordeum* spp. in South Australia could be higher than that found in this study for two reasons. Firstly, not all *Hordeum* plants growing in the fields were sampled. There may have been small patches of paraquat-resistant *Hordeum* spp. that were not sampled. Secondly, the use of paraquat in no-till wheat and non-agricultural areas has increased, such that it is possible that the number of resistant populations in this system has also increased. These areas were not surveyed intensively.

The dose response experiments clearly demonstrated that all resistant populations of *H. glaucum* and *H. leporinum* had a high degree of resistance (Figures 2.3 and 2.5), similar to the level of resistance documented from previous studies (Alizadeh *et al.*, 1998; Purba *et al.*, 1995). It could be assumed that these paraquat-resistant populations have occurred as result of repeated application of paraquat for at least 12 years. In addition, it is interesting to highlight that despite the paraquat-resistant populations of *Hordeum* spp. occurring in different fields, a high level of resistance was always attained.

Even though a number of studies have been carried out, the mechanism of resistance to paraquat is so far not fully elucidated. Resistance to paraquat in *Hordeum* spp. is not due to mutation of herbicide binding site (PS I) or an increased capacity to detoxify this herbicide (reviewed by Preston, 1994). Previous research observed a decrease in basipetal paraquat movement within resistant individuals compared to susceptible individuals such that insufficient herbicide reached the site of action (Bishop *et al.*, 1987; Purba *et*

*al.*, 1995). A feature of this mechanism of resistance is sensitivity to temperature such that the level of resistance decreased at increased temperatures (Alizadeh *et al.*, 1998; Purba *et al.*, 1995). The paraquat-resistant populations discovered in the surveys also exhibited this response to temperature (Chapter 2), suggesting they all may have the same mechanism of resistance.

Herbicide resistant populations can occur through separate evolutionary events or by gene flow; through pollen movement or seed migration (Jasieniuk *et al.*, 1996; Maxwell and Mortimer, 1994). Evidence for gene flow between herbicide resistant and susceptible individuals through pollen movement has been established for some weed species (Murray *et al.*, 2002; Stallings *et al.*, 1995; Volenberg and Stoltenberg, 2002). In the present study, *H. leporinum* was chosen for gene flow experiments rather than *H. glaucum* because the anthers are exerted in the former species and not in the later (Chapter 3). The study showed that gene flow did occur in *H. leporinum* at a very low rate, about 0.1%. Hence, the present study supports the previous assumption that the role of gene flow through seed dissemination is probably far greater in spreading resistant genes than pollen movement in these two self-pollinating species. However, it seems that paraquat resistance can spread quite some distance through seeds (Tucker and Powles, 1988b).

The possession of resistance genes is commonly associated with an alteration of fitness of resistant individuals in relation to normal individuals in the absence of herbicide application (Darmency, 1996; Holt and Thill, 1994).

In some weed species the presence of resistance genes is frequently associated with a reduction in fitness (Jordan, 1999; Plowman *et al.*, 1999; Radosevich and Holt, 1982). A study of the fitness of resistant, heterozygote and susceptible individuals under competition was unable to detect any reduction in fitness as a result of possession of paraquat resistance gene (Chapter 4). This is at variance with theory (Jasieniuk *et al.*, 1996) and with other studies of paraquat-resistant weeds (Purba *et al.*, 1996). However, when resistant and susceptible plants were grown with little competition, susceptible plants produced almost twice as much seed as resistant plants (Chapter 3). Given there are conditions where a fitness penalty is not apparent or very low, then resistant individuals may persist in fields. When this occurs, it may be more difficult to use competition to suppress selection for resistance.

Recent developments in DNA-based molecular markers has provided an opportunity to understand the genetic basis and population genetics of herbicide resistance evolution (Brown, 1996). The molecular genetics of paraquat-resistant populations in *Hordeum* spp. was studied using RAPD marker technique (Chapter 5). The results confirmed the fundamental assumption of resistant evolution that repeated paraquat applications on *H. glaucum* and *H. leporinum* have caused a bottleneck in populations by selecting a very few resistant individuals. Consequently, resistant populations are genetically more homogeneous than susceptible populations. Genetic comparison among paraquat-resistant populations found genetic similarity and dissimilarity in both *H. glaucum* and *H. leporinum*

populations. These findings suggested that the existence of paraquat-resistant populations could be the result of both independent evolutionary events and introductions from elsewhere. It was possible to find the same genotype in resistant individuals and susceptible individuals from adjacent fields. This indicated that evolution of resistance was likely to have occurred from the local susceptible population. One clear example of introduction of a resistant genotype from elsewhere was observed which indicated the contribution of gene flow through seed movement in spreading resistance genes.

There have been a few studies only that have attempted to examine the role of independent evolution versus gene flow in herbicide resistance in weeds. Cavan *et al.* (1998) concluded independent evolutionary events were important in resistance in *Alopecurus myosuroides*. Andrews *et al.* (1998) and Stankiewicz *et al.* (2001) argued that seed movement was important in spreading resistance in *Avena fatua* and *Solanum nigrum* respectively. In this study, both processes were shown to occur.

Management strategies to delay paraquat-resistant evolution and to reduce the spread of paraquat-resistant seeds could be proposed as follows: rotate herbicides with different modes of action, use herbicide mixtures that contain no paraquat, spray topping with glyphosate on paraquat-resistant populations to reduce seed production, grazing with sheep or cattle, improve equipment hygiene, rotate cropping systems, use free weed crop seeds, burn hay that contains paraquat-resistant seeds, and employ other control methods to reduce the frequency of herbicide use (Matthews, 1994; Powles



and Bowran, 2000). These contain strategies to reduce weed spread as well as strategies to delay evolution of resistance.

## 6.2 Conclusion

In conclusion, the number of paraquat-resistant populations in *H. galucum* have increased in South Australia, mainly about areas where the first paraquat-resistant population was discovered 1990. The first paraquat-resistant population of *H. leporinum* was also investigated in this study. Like the existing resistant populations, all newly discovered paraquat-resistant populations documented here had high level of resistance with temperature dependence.

The appearance of resistance is not only related to the repeated application of paraquat in one location, but also might result from resistant gene flow from elsewhere. The fact that gene flow through pollen transfer in *Hordeum* spp. was low suggests that gene flow through seed dissemination must be more important for the spread of resistance between fields for these species. The success of paraquat-resistant evolution in fields was also due to equal fitness among homozygous and heterozygous resistance and susceptible populations under some circumstances.

DNA fingerprinting of paraquat-resistant and susceptible populations of *H. glaucum* and *H. leporinum* confirmed the role of both independent evolutionary events and spread of resistance through seed dispersal in development of paraquat-resistant populations. This suggest that

management practices need to be directed not only to prevent or stop the occurrence of resistance in situ by herbicide or crop rotations, but actions must also be taken to limit seed dispersal of resistant individuals between fields.

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