Investigation of Herbicide Resistance in Oriental Mustard (*Sisymbrium orientale* L.) in Australia

by

Hue Thi Dang

B.Sc. (Crop Sci.), M.Sc. Agriculture

A thesis by **prior publications** submitted to the University of Adelaide, South Australia **in the fulfilment of the degree of** Doctor of Philosophy

Faculty of Sciences

School of Agriculture, Food and Wine

June 2018

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Hue Thi Dang _____ _____ Date 18/6/2018

TABLE OF CONTENTS

ABSTRACT

Oriental mustard (*Sisymbrium orientale* L.), called Indian hedge mustard in Australia, is an important broadleaf weed of southern Australia. It has become more difficult to control in field crops due to the evolution of herbicide resistance. This study investigated the extent of resistance to four different herbicide modes of action, used to control oriental mustard in Australia. Herbicide resistance status was determined in 75 populations collected in southern Australia from 2010 to 2016 with resistance confirmed to herbicides inhibiting acetolactate synthase, photosystem II, phytoene desaturase (PDS) and auxinic herbicides. Populations resistant to PS-II, PDS-inhibitors and auxinic herbicides and two known susceptible populations (S1 and S2) were used to investigate the level of resistance, its mechanism, inheritance and fitness cost associated with resistance.

Populations P17 and P18 were 311 and 315-fold, respectively, more resistant to atrazine than the susceptible populations as determined by the comparisons of their LD_{50} values. However, there was no resistance detected in these populations to diuron. Sequencing of the chloroplastic *psb*A gene identified a missense mutation of serine 264 to glycine in both herbicide-resistant populations, known to confer high-level of atrazine resistance in other species.

P2 and P13 populations were 81 and 67-fold more resistant to 2,4-D at the LD_{50} level compared to the susceptible populations, respectively. No predicted amino acid modification was detected in sequences of potential target-site genes [Auxin binding protein (ABP), Transport inhibitor response 1 (TIR 1) and Auxin F-box protein 5 (AFB5)]. Further studies showed resistant populations had reduced 2,4-D translocation compared to the susceptible populations. At 72 h after herbicide treatment, 77% of [^{14}C]2,4-D was retained in the treated leaf in the resistant population compared to 32% of [^{14}C]2,4-D retention in the susceptible populations. Studies on inheritance of resistance to PDS-inhibitors confirmed that resistance

to diflufenican in P3 population is inherited as a single dominant gene trait. Likewise, resistance to diflufenican and picolinafen in population P40 is also due to a single dominant gene. Resistance to 2,4-D in populations P2 and P13 is inherited as a single partially dominant gene.

Populations P3 and P40 were 140 and 237-fold more resistant to the PDS inhibitor diflufenican, respectively, than the susceptible populations. Both populations contained a Leu498-Val substitution in the PDS gene. An additional mutation, Glu-425-Asp, was only detected in P40, where cross-resistance to picolinafen was identified. These results suggest that Leu498 mutation alone can confer a high level of resistance to diflufenican; however, the presence of both Leu498 and Glu425 mutations increased the level of resistance to diflufenican and also conferred resistance to picolinafen. Fitness studies conducted under competition with wheat in the absence of herbicides in pots revealed that the mutant PDS genes in populations P3 and P40 did not impose any fitness costs. This means once a resistant trait occurs in the field, it will persist in the absence of herbicides.

PUBLICATIONS ARISING FROM THIS THESIS

Dang, H. T., Malone, J. M., Boutsalis, P., Gill, G. and Preston, C. (2017), Identification of a target-site mutation conferring resistance to triazine herbicides in oriental mustard (*Sisymbrium orientale* L.) from Australia. Weed Biol Manag., 17: 153–160

Dang, H. T., Malone, J. M., Boutsalis, P., Krishnan, M., Gill, G. and Preston, C. (2018), Reduced translocation in 2,4-D-resistant oriental mustard populations (*Sisymbrium orientale* L.) from Australia. Pest. Manag. Sci. doi:10.1002/ps.4845

Dang, H. T., Malone, J. M., Boutsalis, P., Gill, G. and Preston, C. (2018), The Mechanism of Diflufenican Resistance and its Inheritance in Oriental Mustard (*Sisymbrium orientale* L.) from Australia. Pest. Manag. Sci. doi:10.1002/ps.4858.

Dang, H. T., Malone, J. M., Gill, G. and Preston, C. (2018), Cross-resistance to diflufenican and picolinafen and its inheritance in oriental mustard (*Sisymbrium orientale* L.). Pest. Manag. Sci. Submitted.

Dang, H. T., Long, W., Malone, J. M., Preston, C. and Gill, G. (2018), No apparent fitness costs associated with mutations in phytoene desaturase providing resistance to diflufenican and picolinafen in oriental mustard (*Sisymbrium orientale* L.). Pest. Manag. Sci. Submitted.

Dang, H. T., Malone, J. M., Boutsalis, P., Preston, C. and Gill, G. (2016), Investigation of herbicide resistance in Indian hedge mustard (*Sisymbrium orientale* L.). Page 93 *in* Randall R, Lloyd S, Borger C, eds. 20th Australasian Weeds Conference. Perth, Western Australia.

ACKNOWLEDGEMENTS

I would like to express my appreciation to all who have been a part of this adventure. It has been a great privilege to spend four years of my life working and studying at the University of Adelaide, Waite Campus, where the lab of Weed Science became my second home in Australia.

I am especially grateful to my supervisors, Associate Professor Gurjeet Gill, Associate Professor Christopher Preston and Dr Jenna Malone for their enthusiasm and support for my study. I cannot express deeply enough my gratitude for their continuous support, competent coaching, insightful suggestions and advice which helped me enormously to complete my research and degree successfully.

I would also like to acknowledge Dr Peter Boutsalis and Dr Mahima Krishnan for their support and friendship. Peter helped me become familiar with the names of particular herbicides, pot trials and the herbicide resistance testing system, much of which I had little prior knowledge. Mahima introduced me to translocation techniques, which again was not my background.

I would also like to thank all members of the Weed Science group: Dr Samuel Kleemann, Benjamin Fleet, Ruwan Lenorage, Dr Geetha Velappan and Malinee Thongmee, who all helped me greatly with technical advice and support in conducting experiments. I could not have completed my experiments on time without your help. Many thanks to lab friends: Dr Fleur Dolman, Alicia Merriam, David Brunton, Dr Rupinder Saini, Dr The Ngo, Dr Lovreet Shergill for sharing with me your knowledge and experiences in weed science. I would also like to thank Dr Tung Anh Pham, Hue Ngo, Alice Tran, Dr Chi Mai Do, Dr Tony Dang, Phuong Nguyen, Dr Thang Lai and Hieu Trung Bui who have been a great source of

support and encouragement that helped me to cope with the difficulties experienced during my stay in Adelaide.

I wish to express my deep appreciation to the Vietnamese Government, The University of Adelaide for providing the privilege of a PhD scholarship and to the Grains Research Development Corporation (GRDC) for their generous financial support. I also would like to acknowledge the continuous academic and technical support of the University of Adelaide and its staff. Without these, this research would not have been possible.

I wish to thank the Department of Crop Production (DCP), the Ministry of Agriculture and Rural Development (MARD) of Vietnam and the Ministry of Education and Training (MoET) of Vietnam for giving me this precious opportunity to study at The University of Adelaide. I also would like to thank Associate Professor Le Quoc Doanh, Dr Nguyen Tri Ngoc, Mr Tran Xuan Dinh, Dr Pham Dong Quang, Mr Ngo Van Viet, Mr Pham Van Thanh and staff of the DCP, MARD for their continuous support and encouragement that made my study in Australia possible. Many thanks to Ms Minh Quach and Ms Tam Thanh Nguyen from the Vietnam International Education Development (VIED), MoET for their continuous support during my studying at The University of Adelaide.

I am indebted to my parents, brother and sisters for their understanding, support and encouragement throughout my life and for this study in Australia. I am especially grateful to my beloved husband for his incredible personal sacrifice, patience and support. I wish to thank my loving children, Thùy Dung and Tiến Dũng who always asked “Mum, when will you come home from the lab?”. You are all a great source of inspiration and all helped make it possible for me to complete this thesis.

ABBREVIATIONS

a.e	acid equivalent
a.i	active ingredient
ABP	auxin binding protein
AFB	auxin F-box protein
AFLP	amplified fragment length polymorphism
AGRF	Australian genome research facility
ALS	acetolactate synthase
ANOVA	analysis of variance
CAPS	cleaved amplified polymorphic sequence
DAP	day after treatment
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
GR_{50}	herbicide dose required for 50% growth reduction
HAT	hour after treatment
LD_{50}	herbicide dose required for control 50% of the plants
LSD	least significant difference
NCBI	national center for biotechnology information
PCR	polymerase chain reaction
PDS	ehytoene desaturase
*psb*A	photosystem II
RFLP	restriction fragment length polymorphism
RI	resistance index relative to sensitive biotype
RR	homozygous resistant
RS	heterozygous resistant

SEM	standard error of mean
SS	susceptible
TIR	transport inhibitor response
MCPA	2-Methyl-4-Chlorophenoxyacetic Acid

CHAPTER 1: LITERATURE REVIEW

1.1 Oriental mustard

Oriental mustard (*Sisymbrium orientale* L.), commonly called Indian hedge mustard in Australia, is also named "eastern rocket" or hedge mustard, oriental mustard, mustard or wide mustard (McGillion and Storrie, 2006). Oriental mustard is native to Europe, Asia and North Africa, however, it can be found throughout temperate areas of the World as an introduced species.

1.1.1 Biology and ecology of the oriental mustard

Oriental mustard is a diploid species (2n=14) belonging to the Brassicaceae family (or cruciferous family). The plants are bisexual and very highly self-compatible (Boutsalis, 1996). When it is young, oriental mustard plants produce rosette leaves with four pairs of broadly triangular lobes. The basal leaves are divided into deep lobes or toothed leaflets (Fig 1). The longest leaves can be more than 10 cm long. The upper leaves of oriental mustard are alternate and spear-shaped (Richardson et al., 2011). At maturity, plants can reach a height of up to 1 m. The flowers are pale yellow and form clusters on the top of the stems (Fig 1). The pods are 2-celled, cylindrical and slender that open when ripe (Fig 1). Each pod of oriental mustard contains a large number of small, yellow brown seeds. It is estimated that a healthy plant can produce up to 10,000 seeds (McGillion and Storrie, 2006).

In southern Australia, oriental mustard is often present in winter crops such as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), oats (*Avena sativa*), canola (*Brassica napus* L.) and chickpea (*Cicer arietinum*) (Chauhan et al., 2006), where it competes for resources to cause crop yield loss. As the growing season of oriental mustard is associated with the winter

crops, seeds usually germinate at the start of the winter cropping season. Flowers are produced during spring and seeds set in late spring before the plants die off in early summer. In some cases, plant cans survive into their second season in moist areas (McGillion and Storrie, 2006; Wilding et al., 1986).

(a) A young oriental mustard plant

(b) Flowers and young pods of oriental mustard

(c) Ripe pods of oriental mustard

(d) Seeds of oriental mustard

Fig 1: The young seedling (a), flowers and young pods (b), ripe pods (c) and seeds (d) of oriental mustard.

Oriental mustard seeds have a relatively short seed-bank persistence and a very short dormancy period that enables seed to germinate any time when soil moisture is adequate, and exposure to light is known to stimulate germination (Boutsalis and Powles, 1998). As a consequence of the light requirement for germination, oriental mustard germination is inhibited by seed burial in soil at the depth greater than 10 mm (Chauhan et al., 2006). As the species can adapt to different soil types and climatic conditions, it can be found in most arable land across Australia, invades pastures, rangelands, open woodlands, roadsides and disturbed sites, and even in waste areas or grazed woodlands, but it is especially prevalent in cereal crops (Richardson et al., 2011) (Figure 2).

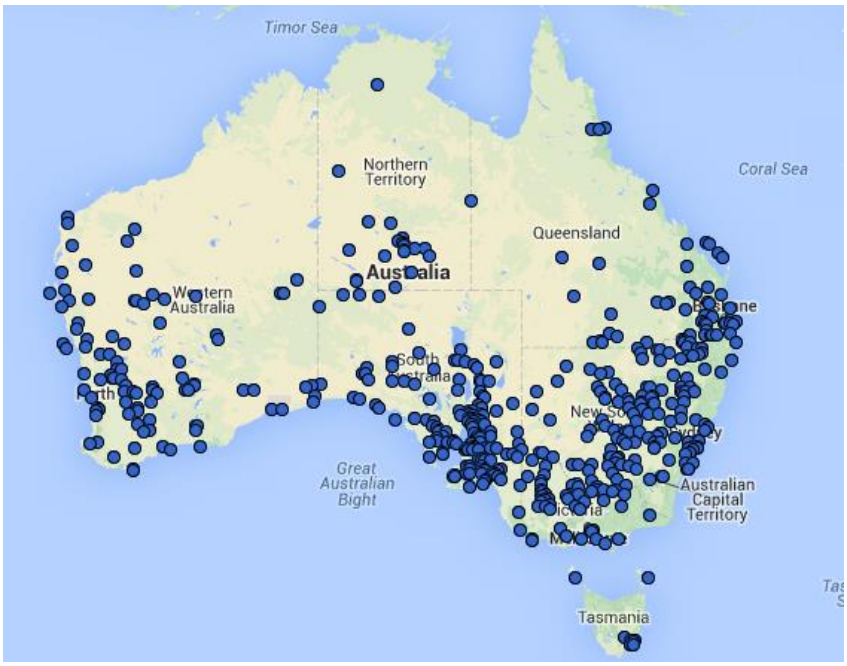


Figure 2: Distribution of oriental mustard in Australia

(Australia's Virtual Herbarium 2014)

1.1.2 Evolution of herbicide resistance in oriental mustard

The first case of herbicide resistance in oriental mustard in Australia occurred in the 1990s, when some populations were found resistant to ALS-inhibitors in wheat and barley crops in

South Australia (Boutsalis and Powles, 1995). Since then, many herbicide resistant biotypes have been found in other states including Queensland, New South Wales, Western Australia and Victoria. In most cases, populations are resistant to acetolactate synthase (ALS) inhibitors (Table 1). Some oriental mustard populations have evolved resistant to photosystem II (PSII) inhibitors (Heap, 2017), phytoene desaturase (PDS) inhibitors (Dayan et al., 2014) and multiple-resistance to ALS and synthetic auxin inhibitors (Preston et al., 2013).

Table 1: Herbicide resistant oriental mustard in Australia (Heap, 2017)

First year	Situation	Active ingredients	Site of action	Location
1990	Spring baley, wheat	chlorsulfuron, imazethapyr, metosulam, metsulfuron-methyl, and triasulfuron	ALS inhibitors (B/2)	South Australia
1993	Wheat	chlorsulfuron	ALS inhibitors (B/2)	Queensland
1994	Cereals, spring barley, and wheat	chlorsulfuron, and metosulam	ALS inhibitors (B/2)	Western Australia and New South Wales
2005	Cereals	2,4-D, imazethapyr, MCPA, metosulam, and metsulfuron-methyl	Multiple Resistance to ALS inhibitors and Synthetic Auxins (O/4)	South Australia
2010	Spring barley, and wheat	chlorsulfuron, and metosulam	ALS inhibitors (B/2)	Victoria
2011	Peas	diflufenican	Carotenoid biosynthesis inhibitors (F1/12)	Victoria
2011	Canola	atrazine	Photosystem II inhibitors (C1/5)	Victoria

1.2 Mechanisms of herbicide resistance

Many mechanisms can confer herbicide resistance in weed populations (Cobb and Reade, 2010). To date, the known mechanisms of herbicide resistance include target site modification, enhanced detoxification, compartmentalization or sequestration and reduced absorption or translocation. However, they can be grouped into target-site-resistance (TSR) and non-target-site resistance (NTSR) mechanisms.

1.2.1 Target-site Resistance Mechanism

Target-site resistance mechanism is the result of a modification of the herbicide-binding site which occurs when there is an alteration at the target-site (Preston et al., 2001). Generally, herbicides target specific proteins where they act to disrupt bio-chemical processes and exert negative effects on plant growth or metabolism leading to plant death (Cobb and Reade, 2010). However, the interaction between herbicide and target site can be weakened if there is a modification in the primary structure (i.e. mutation) or increased amount of the enzyme (Cobb and Reade, 2010; Preston and Mallory-Smith, 2001). Therefore, due to the modification of the target site, herbicides may reach the site of action, but fail to cause any lethal action to the plant at the recommended doses (Powles and Yu, 2010). This enables resistant plants to survive the herbicide treatment.

Resistance due to TSR has been confirmed in a number of weed species with different herbicide modes of action. These include resistance to inhibitors of photosystem II (PSII), acetolactate synthase (ALS), Acetyl CoA Carboxylase (ACCase) and 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*). Resistance to PSII inhibitors due to Ser264Gly mutation in *psb*A gene has been reported in many weed species such as *Poa annua* (Svyantek et al., 2016), *Sonchus oleraceus* (Fraga and Tasende, 2003) and *Vulpia bromoides* (Ashworth et al., 2016) as well as resistance due to Val219Ile mutation identified in *P. annua*

(Mengistu et al., 2000) and *Cyperus difformis* (Pedroso et al., 2016). A double mutation, Thr97Ile Pro101Ser in the *EPSPS* gene was confirmed to confer resistance to glyphosate in *Escherichia coli* (Funke et al., 2009) or Thr102Ile and Pro106Ser mutations in the *EPSPS* gene of glyphosate resistant *Eleusine indica* (Chen et al., 2015). Multiple mutations in the ALS gene have been confirmed as the main cause of resistance to ALS-inhibiting herbicides including Pro197Ser, Ala205Phe, Asp376Glu, Trp574Leu, Ser 653Asn/Thr, Phe, or Ile and Ala122Tyr (Yu et al., 2008). For examples, six mutations, Pro-197-Ala, Pro-197-Arg, Pro-197-Gln, Pro-197-Leu, Pro-197-Ser and Trp-574-Leu were identified as the main cause of resistance to ALS-inhibitors in *Lolium rigidum* (Yu et al., 2008); or the mutation Ala205Phe confers resistance to ALS-inhibitors in *Poa annua* (Brosnan et al., 2016).

1.2.2 Non-target Site Resistance Mechanisms

Non–target-site resistance (NTSR) mechanisms in weeds describes all mechanisms other than changes at the target-site that enables a plant to survive herbicide treatment. Mechanisms of NTSR include (1) reduced herbicide uptake, where absorption of the herbicide into the mesophyll is prevented by the leaf cuticle or other structural barriers (Kohler et al., 2004); (2) rapid metabolic detoxification or enhanced metabolism where the plant has the ability to degrade the herbicide to non-toxic forms before it can affect the plant (Preston, 2004); and (3) reduced translocation, where the herbicide remains at its site of application instead of reaching its site of action at sufficient concentration to cause death. In some cases, the development of hairy epidermis and waxy cuticles may also limit the availability of herbicide at the target site (Ferreira and Reddy, 2000).

Resistance due to NTSR mechanisms have been reported in several field-evolved resistance cases worldwide. Resistance caused by decreased herbicide absorption were reported in 2,4-D-resistant *Glechoma hederacea* (Kohler et al., 2004) and imazamox-resistant

Bromus tectorum (Pester et al., 2017). Resistance conferred by enhanced herbicide metabolism has been reported in *Galeopsis tetrahit* (Weinberg et al., 2006) and *Papaver rhoeas* (Torra et al., 2017). Reduction of herbicide translocation is a common NTSR mechanism in weeds. Examples include reduced translocation of glyphosate in *Conyza canadensis* (Koger and Reddy, 2005) or impaired translocation of 2,4-D in *Raphanus raphanistrum* (Goggin et al., 2016).

1.3 Factors contributing to the evolution of herbicide resistance in weeds

There are a number of factors contributing to the evolution of herbicide resistance in weeds. These include the variations in genes, the intensity of selection, genetic inheritance of resistance, gene flow within and between populations, and fitness of both susceptible and resistant biotypes under herbicide or non-herbicide treatment (Diggle et al., 2003; Jasieniuk et al., 1996). As all these factors influence the rate of evolution of herbicide resistance, weed species and herbicides differ in the rate of evolution of resistance (Powles et al., 1997). However, here two major factors, inheritance and fitness costs of resistance will be the focus.

1.3.1 Genetic inheritance of resistance

Inheritance is defined as a process of passing genetic traits from a parent to the next generations (Rao 2000). In weed species, resistance genes can be transmitted to their progeny via the nuclear or organelle genomes (Gressel, 1986). The transmission of nuclear inheritance is performed by both pollen and ovules, while cytoplasmic inheritance transmission typically happens only through the ovules. However, most cases of herbicide resistance studied are inherited as nuclear genes except for resistance to triazines herbicides, which has maternal inheritance (Jasieniuk et al., 1996). Inheritance of target-site resistance to triazine herbicides is cytoplasmic in most species as the chloroplast genome contains the target gene (*psb*A gene),

the D1 protein of photosystem II), therefore resistance to triazines is passed with the chloroplasts from the maternal parent (Hirschberg et al., 1984; Hirschberg and McIntosh, 1983). In addition, resistant traits in weeds are mainly the result of semi dominant or dominant genes (Darmency, 1994; Tardif et al., 1996), suggesting that resistant traits will be expressed in both homozygous and heterozygous states (Christoffers, 1999). Preston and Malone (2015) demonstrated that resistance to synthetic auxins in oriental mustard (i.e. 2,4-D, MCPA) was due to a single dominant gene. Resistance to other herbicide groups in oriental mustard is not fully understood.

1.3.2 Fitness of resistant biotypes

Fitness is defined as the measure of survival and reproduction of viable offspring. Mutations conferring resistance in a plant are expected to produce a fitness cost in individuals in stress free conditions (Coustau and Chevillon, 2000). The fitness penalties in plants can be expressed as the reduction in plant growth, the decrease of photosynthetic activity and the ability to compete for resources, and reproduction ability (Vila-Aiub et al., 2009). If there are no differences in the fitness of two biotypes (resistant and susceptible plants), without herbicide application, their relative frequency will not be affected during the absence of herbicide use. However, if the resistant populations suffer a fitness penalty, their relative frequency will decrease in the absence of herbicide selection pressure (Gill et al., 1996). This also means that resistant weed biotypes do not grow and reproduce as well as the susceptible biotypes in the absence of herbicides.

As a high fitness cost can affect the destiny of resistance alleles, this phenomenon is considered an advantage for management of resistant biotypes in any weed species (Paris et al., 2008). The higher the fitness cost, the sooner the replacement of resistance with susceptibility will occur, as there should be a decrease in the frequency of resistant traits.

Therefore, the prediction of the evolutionary dynamics of herbicide resistance in any weed genotype should be based on the knowledge of fitness consequences in the presence and absence of herbicide resistance alleles (Neve et al., 2003). However, fitness is a dynamic entity and can be changed over time with the selection under different environments for more fit individuals (Maxwell & Mortimer 1994), and without a significant fitness penalty, the evolution of resistance tends to occur faster in any weed population (Jasieniuk et al., 1996). At this stage, there is very little information available on the presence of fitness penalty in herbicide resistant oriental mustard populations.

1.4 Major herbicides used to control oriental mustard in Australia

A number of herbicide modes of action have been used to control oriental mustard in Australia such as PSII-inhibitors, PDS-inhibitors, synthetic auxins, ALS-inhibitors and glyphosate. However, this project has focused mainly on resistance to PSII-inhibitors, PDS-inhibitors, and synthetic auxins.

1.4.1 Inhibitors of photosynthesis at photosystem II (PSII inhibitors)

Photosystem II (PSII) inhibiting herbicides (i.e. atrazine) kill weeds by blocking electron transport at PSII leading to excessive oxygen radical production. These herbicides were first introduced in the late 1950s (Müller, 2008) and since then, have been registered and used for weed control in more than 100 countries all over the world (LeBaron et al., 2008). In Australia, triazine herbicides have been widely used in cropping systems as PRE and POST emergence herbicides to control broadleaf weeds in field crops, especially in triazine-tolerant canola (Ashworth et al., 2016). However, both atrazine and diuron herbicides are also regularly used in a range of non-cropping areas including roadside, garden paths and railway lines, etc. (Giacomazzi and Cochet, 2004).

Atrazine and diuron kill weeds by inhibiting photosynthesis at photosystem II. Atrazine (2-chloro-4-ethylamine-6-[isopropylamino]-s-triazine) is a triazine herbicide which was invented and developed by Geigy chemical company in Switzerland in 1958 (Moreland, 1967; Müller, 2008). When applied, these herbicides inhibit photosynthesis in all organisms with oxygen-evolving photosystems (Moreland, 1967). They block the electron transport chain by displacing plastoquinone from its binding site on the D1 protein subunit of photosystem II (PS II) (Erickson et al., 1984; Trebst, 2008). This leads to the excess production of singlet oxygen which subsequently results in the destruction of lipids and chlorophyll (Preston et al., 2001), leading to death of sensitive plants (Lossli, 1994).

The intensive use of PSII inhibitors in agriculture has resulted in the evolution of herbicide resistance in a number of weed species over the world. To date, resistance to triazine and urea herbicides has been confirmed globally in 74 and 28 weed species, respectively (Heap 2017). In contrast with this global situation, triazine resistance has only been confirmed in a few weed species in Australia including *Urochloa panicoides* (Adkins *et al*. 1997), *Lolium rigidum* (Burnet *et al*. 1994), *R. raphanistrum* (Hashem *et al*. 2001) and *V. bromoides* (Ashworth *et al*. 2016). A substitution of a single amino acid (Ser-264-Gly) in the *psb*A gene was confirmed as the main factor conferring high-levels of resistance in many atrazine resistant biotypes (Goloubinoff *et al*. 1984; Hirschberg *et al*. 1984; Powles & Yu 2010). Although, resistance to triazines has been well documented in several weed species, understanding the mechanism(s) of resistance to triazines in oriental mustard is still a knowledge gap in Australia.

1.4.2 2,4-Dichlorophenoxyacetic acid (2,4-D)

The synthetic auxin herbicides were developed in the 1940's with three major classes including phenoxyacetic acids [(i.e., 2,4-D and MCPA (2-methyl-4-chlorophenoxyacetic

acid)], benzoic acids (i.e., dicamba, chloramben), and pyridine acids (i.e., picloram, fluroxypyr) marketed (Kelley and Riechers, 2007). Among these, 2,4-D has been the most widely used herbicide worldwide for the control of dicot weeds in crop and non-crop situations during the last seven decades (Chinalia et al., 2007; Mithila et al., 2011; Peterson, 1967).

The use of 2,4-D herbicide for weed control in cereals, especially maize and wheat created a revolution in agricultural production all over the world (Holt et al., 1993; Mithila et al., 2011). 2,4-D interfere with numerous plant pathways and causes symptomatic metabolic abnormalities or disruption of growth processes in susceptible plants leading to plant epinasty, followed by growth inhibition and death (Chinalia et al., 2007; Grossmann, 2010). Three auxin receptors have been identified, which include the auxin-binding protein 1 (ABP1) (Shi and Yang, 2011; Thomas et al., 2003), auxin-signalling F-box (TIR1/AFB) receptor protein homologs (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Ruegger et al., 1998), and the S-phase kinase-associated protein 2 (SKP2) (Jurado et al., 2010).

Even though the first case of resistance to 2,4-D was reported in *Daucus carota* in 1957 (Hilton, 1957), resistance to 2,4-D is still at a low level and the mechanisms of resistance in several cases remains unknown (Christoffoleti et al., 2015). Many putative TSR and NTSR mechanisms have been suspected to confer resistance to this herbicide group (Mithila et al., 2011). However, the evolution of resistance to auxinic herbicides in weeds is considered to be more likely associated with NTSR due to its complex mode of action (Goggin et al., 2016). To date, resistance to 2,4-D due to decreased herbicide absorption has been identified in *Glechoma hederacea* (Kohler et al., 2004) or increased metabolism in *Bromus inermis*, *Phleum pratense*, *Dactylis glomerata* (Hagin et al., 1970), and *Galeopsis tetrahit* (Weinberg et al., 2006). Resistance conferred by reduced translocation was confirmed in *R. raphanistrum* (Goggin et al., 2016), *Galeopsis tetrahit* (Weinberg et al., 2006), *Papaver rhoeas* (Rey-Caballero et al., 2016) and *Lactuca serriola* (Riar et al., 2011). Meanwhile, molecular studies

in *Arabidopsis thaliana* (Gleason et al., 2011; Walsh et al., 2006) and *Brassica kaber* (Zheng and Hall, 2001) found that resistance to auxinic herbicides were conferred by mutations in the TIR1 gene and its homologs (SGT and AFB genes) in Arabidopsis (Walsh et al., 2006) or ABP gene in *B. kaber* (Zheng and Hall, 2001). In Australia, resistance to 2,4-D in oriental mustard was identified in 2005 (Preston et al., 2013). However, the mechanism of resistance to 2,4-D in oriental mustard is still incompletely understood.

1.4.3 Inhibitors of carotenoid biosynthesis

Diflufenican and picolinafen are pyridinecarboxamide herbicides belonging to Group 15 [according to the Weed Science Society of America (WSSA) designation], which inhibit carotenoid biosynthesis in the plant. Diflufenican was developed in 1979 and has been used as a PRE- and early POST-emergent herbicide for the selective control of certain broadleaf weeds, especially Brassicaceae family, in winter cereals since the mid-1980s (Ashton et al., 1994; Haynes and Kirkwood, 1992; Rouchaud et al., 1991). Picolinafen is a selective, post-emergence herbicide, which has been used to control annual broadleaf weeds in winter crops in Australia since 2001. Carotenoids are essential pigments of the photosynthetic apparatus that play important roles in plants, especially in photosynthesis (Armel et al., 2007; Hashimoto et al., 2016). They not only participate in light harvesting, but also protect the chloroplasts during photosynthesis from the harmful effects of singlet oxygen formed (Boger, 1996). The enzyme phytoene desaturase (PDS), which initiates the desaturation sequence starting from phytoene in carotenoid biosynthesis, has been the target of many bleaching herbicides such as diflufenican and picolinafen (Misawa et al., 1994; Ohki et al., 2003). These herbicides inhibit the formation of carotenoids (Bartley et al., 1991; Hashimoto et al., 2016; Sandmann et al., 1991) to cause a lack of carotenoids, destruction of chloroplast membranes and degradation

of chlorophyll. This results in pronounced bleaching symptoms and necrosis of the tissues of susceptible plants, leading to plant death (Armel et al., 2007; Boger and Sandmann, 1998).

Even though the PDS-inhibiting herbicides have been used for many years to control weeds in agriculture and aquaculture, only a few cases of field-evolved resistance have been recorded worldwide (Dayan et al., 2014; Heap, 2017). These include *Hydrilla verticillata* (Michel et al., 2004), *R. raphanistrum* (Walsh et al., 2004), *P. annua*, *Apera spica-venti* and oriental mustard (Dayan et al., 2014; Heap, 2017). To date, the mechanism(s) of resistance as well as inheritance of resistance to PDS inhibitors in oriental mustard have not been investigated.

1.5 Research objectives

The use of herbicides has been the most effective solution for weed control in Australia. However, the effectiveness of this option has been reduced by the rapid increase in herbicide resistance in many weed biotypes in Australia. The increase in resistance to several herbicides in oriental mustard populations has made it a serious broadleaf weed in crop production systems in southern Australia. Resistance to ALS inhibitors in oriental mustard has been well documented since the 1990s. Evolution of resistance to other herbicide groups such as *psb*A inhibitors, PDS-inhibitors and synthetic auxin herbicides identified more recently has made it more challenging to control this weed species. Despite some previous research, mechanisms of resistance (i.e. mutation of genes), inheritance, and fitness of resistant oriental mustard individuals to different herbicide groups have not been fully investigated. Therefore, the objectives of this study were:

- To screen oriental mustard populations to determine their resistance status to different herbicide groups including ALS inhibitors, *psb*A inhibitors, PDS-inhibitors, glyphosate and synthetic auxinic herbicides;

- To conduct dose response investigations to quantify the level of resistance (LD_{50} and GR_{50}) in different oriental mustard populations to different herbicide groups;
- To undertake laboratory investigations to determine the mechanisms of herbicide resistance including TSR and NTSR mechanisms;
- To undertake crosses between resistant and susceptible parents and screen the progeny to determine the mode of inheritance of resistance to PDS-inhibitors and 2,4-D in oriental mustard; and
- To undertake competition experiments to determine the fitness cost of PDS-inhibitor resistant genotypes of oriental mustard.

REFERENCES

Armel GR, Rardon PL, McComrick MC, Ferry NM (2007) Differential response of several carotenoid biosynthesis inhibitors in mixtures with atrazine. Weed Technology 21:947-953

Ashton IA, Abulnaja KO, Pallett KE, Cole DJ, Harwood JL (1994) The mechanism of inhibition of fatty acid synthase by the herbicide diflufenican. Phytochemistry 35:587-590

Ashworth MB, Han H, Knell G, Powles SB (2016) Identification of triazine-resistant vulpia bromoides. Weed Technology 30:456-463

Bartley GE, Viitanen PV, Pecker I, Chamovitz D, Hirschberg J, Scolnik PA (1991) Molecular cloning and expression in photosynthetic bacteria of a soybean cDNA coding for phytoene desaturase, an enzyme of the carotenoid biosynthesis pathway. Proceedings of the National Academy of Sciences 88:6532-6536

Boger P (1996) Mode of action of herbicides affecting carotenogenesis. Journal of Pesticide Science 21:473-478

Boger P, Sandmann G (1998) Carotenoid biosynthesis inhibitor herbicides-mode of action and resistance mechanisms. Pesticide Outlook (United Kingdom)

Boutsalis, Powles (1998) Seedbank characteristics of herbicide-resistant and susceptible *Sisymbrium orientale*. Weed Research 38:389-395

Boutsalis P (1996) Resistance to acetolactate synthase-inhibiting herbicides in Sonchus oleraceus, *Sisymbrium orientale* and *Brassica tournefortii*. PhD desertation.

Boutsalis P, Powles SB (1995) Resistance of dicot weeds to acetolactate synthase (ALS)-inhibiting herbicides in Australia. Weed Research 35:149-155

Brosnan JT, Vargas JJ, Breeden GK, Grier L, Aponte RA, Tresch S, Laforest M (2016) A new amino acid substitution (Ala-205-Phe) in acetolactate synthase (ALS) confers broad spectrum resistance to ALS-inhibiting herbicides. Planta 243:149-159

Chauhan BS, Gill G, Preston C (2006) Seedling recruitment pattern and depth of recruitment of 10 weed species in minimum tillage and no-till seeding systems. Weed Science 54:658-668

Chen J, Huang H, Zhang C, Wei S, Huang Z, Chen J, Wang X (2015) Mutations and amplification of EPSPS gene confer resistance to glyphosate in goosegrass (*Eleusine indica*). Planta 242:859-868

Chinalia FA, Regali-Seleghin MH, Correa EM (2007) 2, 4-D toxicity: cause, effect and control. Terr Aquat Environ Toxico. 1:24-33

Christoffers MJ (1999) Genetic aspects of herbicide-resistant weed management. Weed Technology 13: 647-652

Christoffoleti PJ, Figueiredo MRAd, Peres LEP, Nissen S, Gaines T (2015) Auxinic herbicides, mechanisms of action, and weed resistance: A look into recent plant science advances. Scientia Agricola 72:356-362

Cobb AH, Reade JPH (2010) Herbicide Resistance. Herbicides and Plant Physiology: Wiley-Blackwell. Pp, 216-237

Coustau C, Chevillon C (2000) Resistance to xenobiotics and parasites: can we count the cost? Trends Ecol Evol. 15:378-383

Darmency H (1994) Genetics of herbicide resistance in weeds and crops. Herbicide resistance in plants: Biol Biochem: 263-298

Dayan FE, Owens DK, Tranel PJ, Preston C, Duke SO (2014) Evolution of resistance to phytoene desaturase and protoporphyrinogen oxidase inhibitors--state of knowledge. Pest Manag Sci 70:1358-66

Dharmasiri N, Dharmasiri S, Estelle M (2005) The F-box protein TIR1 is an auxin receptor. Nature 435:441-445

Diggle A, Neve P, Smith F (2003) Herbicides used in combination can reduce the probability of herbicide resistance in finite weed populations. Weed Research 43:371-382

Erickson JM, Rahire M, Bennoun P, Delepelaire P, Diner B, Rochaix J-D (1984) Herbicide resistance in *Chlamydomonas reinhardtii* results from a mutation in the chloroplast gene for the 32-kilodalton protein of photosystem II. Proc Natl Acad Sci 81:3617-3621

Ferreira JF, Reddy KN (2000) Absorption and translocation of glyphosate in *Erythroxylum coca* and *E. novogranatense.* Weed Science 48:193-199.

Fraga MI, Tasende MG (2003) Mechanisms of resistance to simazine in *Sonchus oleraceus*. Weed Research 43:333-340

Funke T, Yang Y, Han H, Healy-Fried M, Olesen S, Becker A, Schönbrunn E (2009) Structural basis of glyphosate resistance resulting from the double mutation Thr97→ Ile and Pro101→ Ser in 5-enolpyruvylshikimate-3-phosphate synthase from Escherichia coli. Journal of Biological Chemistry 284:9854-9860

Giacomazzi S, Cochet N (2004) Environmental impact of diuron transformation: a review. Chemosphere 56:1021-1032

Gill GS, Cousens RD, Allan MR (1996) Germination, growth, and development of herbicide resistant and susceptible populations of rigid ryegrass (*Lolium rigidum*). Weed science:252-256

Gleason C, Foley RC, Singh KB (2011) Mutant analysis in Arabidopsis provides insight into the molecular mode of action of the auxinic herbicide dicamba. PLoS One 6:e17245

Goggin DE, Cawthray GR, Powles SB (2016) 2,4-D resistance in wild radish: reduced herbicide translocation via inhibition of cellular transport. J Exp Bot. 67:3223-3235

Gressel J (1986) Model and genetics of herbicide resistace in plants. Pesticide resistance: strategies and tactics for management. Washington DC: National Academy Press. Pages 54-73

Grossmann K (2010) Auxin herbicides: current status of mechanism and mode of action. Pest Manag Sci 66:113-120

Hagin RD, Linscott DL, Dawson JE (1970) 2, 4-D metabolism in resistant grasses. J. Agric Food Chem. 18:848-850

Hashimoto H, Uragami C, Cogdell RJ (2016) Carotenoids and Photosynthesis. *in* Stange C, ed. Carotenoids in nature: biosynthesis, regulation and function. Cham: Springer International Publishing. Pages 111-139

Haynes C, Kirkwood RC (1992) Studies on the mode of action of diflufenican in selected crop and weed species: Basis of selectivity of pre-and early post-emergence applications. Pest Manag Sci 35:161-165

Heap I (2017) The International Survey of Herbicide Resistant Weeds. http://weedscience.org (acessed 20 Feb 2017).

Hilton H (1957) Herbicide tolerant strains of weeds. Hawaiian sugar planters association annual report: Hawaiian Sugar Planters Association. Pages 69-72

Hirschberg J, Bleecker A, Kyle DJ, McIntosh L, Arntzen CJ (1984) The molecular basis of triazine-herbicide resistance in higher-plant chloroplasts. Zeitschrift für Naturforschung C 39:412-420

Hirschberg J, McIntosh L (1983) Molecular basis of herbicide resistance in *Amaranthus hybridus*. Science 222:1346-1349

Holt JS, Powles SB, Holtum JA (1993) Mechanisms and agronomic aspects of herbicide resistance. Annual Review of Plant Biology 44:203-229

Jasieniuk M, Brule-Babel AL, Morrison IN (1996) The evolution and genetics of herbicide resistance in weeds. Weed science (USA)

Jurado S, Abraham Z, Manzano C, López-Torrejón G, Pacios LF, Del Pozo JC (2010) The Arabidopsis cell cycle F-box protein SKP2A binds to auxin. The Plant Cell 22:3891-3904

Kelley KB, Riechers DE (2007) Recent developments in auxin biology and new opportunities for auxinic herbicide research. J. Agric. Food Chem 89:1-11

Kepinski S, Leyser O (2005) The Arabidopsis F-box protein TIR1 is an auxin receptor. Nature 435:446

Koger CH, Reddy KN (2005) Role of absorption and translocation in the mechanism of glyphosate resistance in horseweed (*Conyza canadensis*). Weed Science 53:84-89

Kohler EA, Throssell CS, Reicher ZJ (2004) 2, 4-D rate response, absorption, and translocation of two ground ivy (*Glechoma hederacea*) populations. Weed Technol 18:917-923

LeBaron HM, McFarland JE, Burnside OC (2008) Chapter 1 - The Triazine Herbicides: a milestone in the development of weed control technology. Pages 1-12 *in* LeBaron HM, McFarland JE, Burnside OC, eds. The Triazine Herbicides. San Diego: Elsevier

Lossli R (1994) Chapter 8 Triazines. Toxicology 91:59-62

Maxwell BD, Mortimer AM (1994) Selection for herbicide resistance. Pages 1-26 *in* Powles SB, Holtum JAM, eds. Herbicide Resistance in Plants: Biology and Biochemistry. Boca Raton, FL: Lewis Publishers

McGillion T, Storrie A (2006) Integrated weed management in Australian cropping systems– a training resource for farm advisors. CRC for Australian Weed Management: Adelaide

Mengistu LW, Mueller-Warrant GW, Liston A, Barker RE (2000) psbA mutation (valine(219) to isoleucine) in Poa annua resistant to metribuzin and diuron. Pest Manag Sci 56:209-217

Michel A, Arias RS, Scheffler BE, Duke SO, Netherland M, Dayan FE (2004) Somatic mutation-mediated evolution of herbicide resistance in the nonindigenous invasive plant hydrilla (*Hydrilla verticillata*). Molecular Ecology 13:3229-3237

Misawa N, Masamoto K, Hori T, Ohtani T, Böger P, Sandmann G (1994) Expression of an Erwinia phytoene desaturase gene not only confers multiple resistance to herbicides interfering with carotenoid biosynthesis but also alters xanthophyll metabolism in transgenic plants. The Plant Journal 6:481-489

Mithila J, Hall JC, Johnson WG, Kelley KB, Riechers DE (2011) Evolution of resistance to auxinic herbicides: historical perspectives, mechanisms of resistance, and implications for broadleaf weed management in agronomic crops. Weed science 59:445-457

Moreland DE (1967) Mechanisms of action of herbicides. Annu. Rev. Plant Biol 18:365-386

Müller G (2008) Chapter 2 - History of the Discovery and Development of Triazine Herbicides. Pages 13-29 *in* LeBaron HM, McFarland JE, Burnside OC, eds. The Triazine Herbicides. San Diego: Elsevier

Neve P, Diggle A, Smith F, Powles S (2003) Simulating evolution of glyphosate resistance in Lolium rigidum I: population biology of a rare resistance trait. Weed Research 43:404-417

Ohki S, Miller-Sulger R, Wakabayashi K, Pfleiderer W, Boger P (2003) Phytoene desaturase inhibition by O-(2-phenoxy)ethyl-N-aralkylcarbamates. J Agric Food Chem 51:3049-55

Paris M, Roux F, Berard A, Reboud X (2008) The effects of the genetic background on herbicide resistance fitness cost and its associated dominance in *Arabidopsis thaliana*. Heredity 101:499-506

Pedroso RM, Al-Khatib K, Alarcon-Reverte R, Fischer AJ (2016) A *psb*A mutation (Val(219) to Ile) causes resistance to propanil and increased susceptibility to bentazon in *Cyperus difformis*. Pest Manag Sci 72:1673-1680

Pester TA, Nissen SJ, Westra P (2017) Absorption, translocation, and metabolism of imazamox in jointed goatgrass and feral rye. Weed Sci 49:607-612

Peterson GE (1967) The discovery and development of 2, 4-D. Agricultural History 41:243-254

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

Powles SB, Yu Q (2010) Evolution in action: plants resistant to herbicides. Annual review of plant biology 61:317-347

Preston C (2004) Herbicide resistance in weeds endowed by enhanced detoxification: complications for management. Weed Sci 52:448-453

Preston C, Dolman FC, Boutsalis P (2013) Multiple resistance to acetohydroxyacid synthase–inhibiting and auxinic herbicides in a population of oriental mustard (*Sisymbrium orientale*). Weed Sci 61:185-192

Preston C, Mallory-Smith CA (2001) Biochemical mechanisms, inheritance, and molecular genetics of herbicide resistance in weeds. Herbicide Resistance and World Grains. CRC Press, Boca Raton, Florida 13: 23-60

Preston C, Malone JM (2015) Inheritance of resistance to 2,4-D and chlorsulfuron in a multiple-resistant population of Sisymbrium orientale. Pest Manag Sci 71:1523-8

Rao VS (2014) Transgenic herbicide resistance in plants: Crc Press

Rey-Caballero J, Menendez J, Gine-Bordonaba J, Salas M, Alcantara R, Torra J (2016) Unravelling the resistance mechanisms to 2,4-D (2,4-dichlorophenoxyacetic acid) in corn poppy (*Papaver rhoeas*). Pest Biochem Physiol 133:67-72

Riar DS, Burke IC, Yenish JP, Bell J, Gill K (2011) Inheritance and physiological basis for 2,4-d resistance in prickly lettuce (*Lactuca serriola* L.). J. Agric. Food Chem 59:9417-9423

Richardson FJ, Richardson RG, Shepherd RCH (2011) Weeds of the south-east.

Rouchaud J, Gustin F, Van Himme M, Bulcke R, Benoit F, Maddens K (1991) Metabolism of the herbicide diflufenican in the soil of field wheat crops. J. Agric. Food Chem. 39:968-976

Ruegger M, Dewey E, Gray WM, Hobbie L, Turner J, Estelle M (1998) The TIR1 protein of Arabidopsis functions in auxin response and is related to human SKP2 and yeast Grr1p. Genes & Development 12:198-207

Sandmann G, Schmidt A, Linden H, Boger P (1991) Phytoene desaturase, the essential target for bleaching herbicides. Weed Sci 39:474-479

Shi J-H, Yang Z-B (2011) Is ABP1 an auxin receptor yet? Molecular plant 4:635-640

Svyantek AW, Aldahir P, Chen S, Flessner ML, McCullough PE, Sidhu SS, McElroy JS (2016) Target and nontarget resistance mechanisms induce annual bluegrass (poa annua) resistance to atrazine, amicarbazone, and diuron. Weed Technol 30:773-782

Tardif F, Preston C, Holtum J, Powles S (1996) Resistance to acetyl-coenzyme A carboxylase-inhibiting herbicides endowed by a single major gene encoding a resistant target site in a biotype of *Lolium rigidum*. Funct. Plant Biol. 23:15-23

Thomas C, Meyer D, Wolff M, Himber C, Alioua M, Steinmetz A (2003) Molecular characterization and spatial expression of the sunflower ABP1 gene. Plant Molecular Biology 52:1025-1036

Torra J, Rojano-Delgado AM, Rey-Caballero J, Royo-Esnal A, Salas ML, De Prado R (2017) Enhanced 2,4-D metabolism in two resistant *Papaver rhoeas* populations from Spain. Front Plant Sci 8:1584

Trebst A (2008) Chapter 8 - The Mode of Action of Triazine Herbicides in Plants. Pages 101-II *in* LeBaron HM, McFarland JE, Burnside OC, eds. The Triazine Herbicides. San Diego: Elsevier

Vila-Aiub MM, Neve P, Powles SB (2009) Fitness costs associated with evolved herbicide resistance alleles in plants. New Phytologist 184:751-767

Walsh MJ, Powles SB, Beard BR, Parkin BT, Porter SA (2004) Multiple-herbicide resistance across four modes of action in wild radish (*Raphanus raphanistrum*). Weed Sci 52:8-13

Walsh TA, Neal R, Merlo AO, Honma M, Hicks GR, Wolff K, Matsumura W, Davies JP (2006) Mutations in an auxin receptor homolog AFB5 and in SGT1b confer resistance to synthetic picolinate auxins and not to 2,4-dichlorophenoxyacetic acid or indole-3-acetic acid in Arabidopsis. Plant Physiol 142:542-52

Weinberg T, Stephenson GR, McLean MD, Hall JC (2006) MCPA (4-chloro-2-ethylphenoxyacetate) resistance in hemp-nettle (*Galeopsis tetrahit* L.). J. Agric. Food Chem. 54:9126-9134

Wilding JL, Barnett AG, Amor RL (1986) Crop weeds: Inkata Press

Yu Q, Han H, Powles SB (2008) Mutations of the ALS gene endowing resistance to ALS-inhibiting herbicides in *Lolium rigidum* populations. Pest Manag Sci 64:1229-1236

Zheng H-g, Hall JC (2001) Understanding auxinic herbicide resistance in wild mustard: physiological, biochemical, and molecular genetic approaches. Weed Sci 49:276-281

CHAPTER 2

IDENTIFICATION OF A TARGET-SITE MUTATION CONFERRING RESISTANCE TO TRIAZINE HERBICIDES IN ORIENTAL MUSTARD (*SISYMBRIUM ORIENTALE* L.) FROM AUSTRALIA

Hue Thi Dang, Jenna M Malone, Peter Boutsalis, Gurjeet Gill and Christopher Preston

School of Agriculture, Food and Wine, University of Adelaide

PMB1, Glen Osmond, South Australia 5064, Australia

Weed Biology and Management **17** (4): 153–160 (2017)

(with permission from the Weed Biology and Management and John Wiley and Sons)

JOHN WILEY AND SONS LICENSE
TERMS AND CONDITIONS

Feb 25, 2018

This Agreement between Mrs. Hue Dang ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	4286950096352
License date	Feb 13, 2018
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	Weed Biology and Management
Licensed Content Title	Identification of a target-site mutation conferring resistance to triazine herbicides in oriental mustard (*Sisymbrium orientale* L.) from Australia
Licensed Content Author	Hue T. Dang,Jenna M. Malone,Peter Boutsalis,Gurjeet Gill,Christopher Preston
Licensed Content Date	Oct 17, 2017
Licensed Content Pages	8
Type of Use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Electronic
Portion	Full article
Will you be translating?	No
Title of your thesis / dissertation	Investigation of Herbicide Resistance in Oriental Mustard (Sisymbrium orientale L.) in Australia
Expected completion date	Mar 2018
Expected size (number of pages)	160
Requestor Location	Mrs. Hue Dang School of Agriculture, Food and Wine PMB 1, Glen Osmond Adelaide, South Australia 5064 Australia Attn: Mrs. Hue Dang
Publisher Tax ID	EU826007151
Total	**0.00 AUD**

Statement of Authorship

Title of Paper	Identification of a Target-Site Mutation Conferring Resistance to Triazine Herbicides in Oriental Mustard (*Sisymbrium orientale* L.) from Australia
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Dang HT, Malone JM, Boutsalis P, Gill G and Preston C, Identification of a target-site mutation conferring resistance to triazine herbicides in oriental mustard (*Sisymbrium orientale* L.) from Australia. Weed Biology and Management **17** (4): 153–160 (2017)

Principal Author

Name of Principal Author (Candidate)	Hue Thi Dang		
Contribution to the Paper	Planned the study, conducted all experiments, analyzed and interpreted data and wrote manuscript.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	23 - 02 - 2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Peter Boutsalis		
Contribution to the Paper	Supervised the whole plant studies and helped with data interpretation		
Signature		Date	27 Feb 2018

Name of Co-Author	Jenna Moira Malone		
Contribution to the Paper	Supervised the molecular work, data interpretation and edited manuscript		
Signature		Date	26/2/18

Name of Co-Author	Christopher Preston		
Contribution to the Paper	Supervised development of research, data interpretation and analysis, and edited manuscript		
Signature		Date	23/02/2018

Name of Co-Author	Gurjeet Gill		
Contribution to the Paper	Supervised development of research, data interpretation and analysis, and edited manuscript		
Signature		Date	23. 2. 2018

Weed Biology and Management
Weed Biology and Management **17**, 153–160 (2017)

RESEARCH PAPER

Identification of a target-site mutation conferring resistance to triazine herbicides in oriental mustard (*Sisymbrium orientale* L.) from Australia

HUE T. DANG*, JENNA M. MALONE, PETER BOUTSALIS, GURJEET GILL and CHRISTOPHER PRESTON
[1]*School of Agriculture, Food and Wine, The University of Adelaide, Glen Osmond, SA, Australia*

In southern Australia, oriental mustard (*Sisymbrium orientale*) has been controlled successfully by triazine herbicides for several decades. The screening of 40 populations that were collected from the southern grain belt of Australia during 2010 and 2013 for resistance to six different herbicides (glyphosate, diflufenican, imazamox, chlorsulfuron, atrazine and 2,4-dichlorophenoxyacetic acid) identified two oriental mustard populations as highly resistant to atrazine. Compared to the known oriental mustard-susceptible populations (S1 and S2), these two resistant populations (P17 and P18) from near Horsham, Victoria, Australia, were 311- and 315-fold resistant to atrazine, as determined by a comparison of the LD_{50} values. However, there was no resistance to diuron detected in these populations. Sequencing of the chloroplast *psb*A gene identified a missense mutation of serine 264 to glycine in both herbicide-resistant oriental mustard populations, which is known to confer high-level atrazine resistance in other species.

Keywords: oriental mustard, *psb*A, *Sisymbrium orientale*, target-site mutation, triazines.

The use of herbicides in weed control is critical in field crop production in many countries, including Australia. However, the intensive use of herbicides with the same mode of action has been a major contributor to the evolution of herbicide resistance over the last 50 years. To date, there are 480 resistant biotypes of 252 weed species (147 dicots and 105 monocots) in 68 countries (Heap 2017). Resistance has been confirmed to 22 out of the 26 known herbicide sites of action and to 161 different herbicides.

Oriental mustard (*Sisymbrium orientale* L.) is a troublesome broad-leaved weed of field crops in southern Australia. It is a diploid species (2n = 14) that belongs to the *Brassicaceae* family. The plants are bisexual and self-compatible (Boutsalis 1996). In southern Australia, oriental mustard is often present in winter crops, such as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), oat (*Avena sativa*) and canola (*Brassica napus* L.) and chickpea (*Cicer arietinum*) (Chauhan *et al.* 2006a), where it competes with crops for resources and causes difficulties in crop harvest. The seeds of oriental mustard usually germinate at the start of the winter cropping season. The flowers are produced during spring and seeds are set in late spring before the plants die off in early summer (Wilding *et al.* 1986; McGillion & Storrie 2006). Oriental mustard seeds have a relatively short seedbank persistence and a very short dormancy that enables seed to germinate at any time when the soil moisture is adequate and exposure to light is known to stimulate germination (Boutsalis & Powles 1998; Chauhan *et al.* 2006b). However, the germination of oriental mustard seeds is inhibited by burial in soil at a depth of >10 mm (Chauhan *et al.* 2006b).

Photosystem II (PSII)-inhibiting herbicides (e.g. atrazine) kill weeds by blocking electron transport at PSII, leading to excessive oxygen radical production. These herbicides were first introduced in the late 1950s (Moreland 1967; Müller 2008) and, since then, have

Communicated by K. Hirase.
**Correspondence to: Hue T. Dang, School of Agriculture, Food and Wine, The University of Adelaide, PMB1, Glen Osmond, South Australia 5064, Australia.*
Email: huethi.dang@adelaide.edu.au

Received 29 July 2017; accepted 3 September 2017

doi:10.1111/wbm.12133

been registered and used for weed control in >100 countries all over the world (LeBaron *et al.* 2008). In Australia, triazine herbicides have been used widely in cropping systems as pre- and postemergence herbicides to control broad-leaved weeds in field crops, especially in triazine-tolerant canola (Ashworth *et al.* 2016). However, both atrazine and diuron herbicides are also regularly used in a range of non-cropping areas, including roadsides, garden paths and railway lines etc. (Giacomazzi & Cochet 2004). When applied, these herbicides inhibit photosynthesis in all organisms with oxygen-evolving photosystems (Moreland 1967). They block the electron transport chain by displacing plastoquinone from its binding site on the D1 protein subunit of PSII (Erickson *et al.* 1984; Trebst 2008). This leads to the excessive production of singlet oxygen that subsequently results in the destruction of lipids and chlorophyll (Devine & Shukla 2000; Preston *et al.* 2001), leading to the death of sensitive plants (Lossli 1994).

The intensive use of PS II-inhibitors in agriculture has resulted in the evolution of herbicide resistance in a number of weed species around the world. To date, resistance to triazine and urea herbicides has been confirmed globally in 74 and 28 weed species, respectively (Heap 2017). In contrast with this global situation, triazine resistance has been confirmed in only a few weed species in Australia, including liverseed grass (*Urochloa panicoides*) (Adkins *et al.* 1997), rigid ryegrass (*Lolium rigidum*) (Burnet *et al.* 1994), wild radish (*Raphanus raphanistrum*) (Hashem *et al.* 2001) and silver grass (*Vulpia bromoides*) (Ashworth *et al.* 2016). A substitution of a single amino acid (Ser-264-Gly) in the *psb*A gene was confirmed as the main factor that confers high levels of resistance in many atrazine resistant biotypes (Goloubinoff *et al.* 1984; Hirschberg *et al.* 1984; Powles & Yu 2010).Various herbicides, including acetolactate synthase (ALS)-inhibitors, phytoene desaturase inhibitors, auxin mimics and PSII-inhibitors, have been used widely to control oriental mustard in field crops in southern Australia. The frequent use of these herbicides has increased resistance to these herbicides' mode of action in oriental mustard populations (Preston *et al.* 2013). After the first case of herbicide resistance in oriental mustard to ALS-inhibitors was reported in South Australia in 1990 (Boutsalis & Powles 1995), the number of herbicide-resistant biotypes has increased gradually and has extended to other states (Heap 2017). Furthermore, resistance to other herbicidal mode of actions, especially multiple resistance to synthetic auxins and ALS-inhibitors, has been confirmed (Preston *et al.* 2013). However, the mechanisms of resistance to different herbicide groups in oriental mustard populations have not been fully investigated.

South Australia and Victoria are two important cereal-growing states of Australia where oriental mustard is common. Weed seed collection surveys were conducted throughout the grain belt of these two states from 2010 to 2013 to study the evolution of resistance to different herbicide groups in oriental mustard. The objective of this study was to screen for atrazine resistance in these surveyed populations, quantify the level of resistance to PSII inhibitors, including atrazine and diuron, and to investigate whether resistance in populations that had been confirmed as atrazine-resistant was associated with known mutations in the *psb*A gene.

MATERIALS AND METHODS

Collection of the oriental mustard populations and screening for atrazine resistance

The seeds of 40 populations of oriental mustard were collected randomly from the grain belt of southern Australia (South Australia and Victoria) during 2010 and 2013. Sampling was conducted just prior to the crop harvest and locations were marked by using a global positioning system, as described by Boutsalis *et al.* (2012). In July 2014, the oriental mustard populations were screened for their response to 1000 g ha^{-1} of atrazine (Gesaprim 900 DF; Syngenta, Sydney, Australia).

Two populations (P17 and P18) that survived atrazine treatment in the screening experiment and two susceptible populations were selected for further studies. S1 is a known standard susceptible population that was collected from an organic field near Roseworthy, South Australia, as described in Preston *et al.* (2013). The S2 population was collected near Port Kenny, Eyre Peninsula, South Australia. These populations (S1 and S2) were confirmed as susceptible to all the tested herbicides, including glyphosate, diflufenican, diuron, imazamox, chlorsulfuron, atrazine and 2,4-dichlorophenoxyacetic acidat the recommended field rates for the postemergent control of oriental mustard in Australia (data not shown).

In order to generate homogeneous populations for the current study, five healthy plants of the P17 and P18 populations that survived the atrazine treatment at 4000 g ha^{-1} were transplanted into large pots (25 cm diameter) that contained standard potting mix, with one plant per pot. In addition, five untreated plants from each susceptible population (S1 and S2) were planted into pots, as described above. As oriental mustard tends to mainly self-pollinate (Boutsalis *et al.* 1999), the pots were transferred outdoors and watered and fertilized as required. The plants were allowed to self-pollinate to produce seeds. The mature seeds from these

plants were collected and stored at 10°C for use in all the experiments.

Seed germination and plant growth

About 200 seeds of each population were sown into a seedling nursery tray that contained standard potting mix (Boutsalis *et al.* 2012). When the seedlings reached the one-to-two true-leaf stage, they were transplanted into small punnet pots (8.5 cm × 9.5 cm × 9.5 cm) (Masrac Plastics, Dry Creek, Australia) that contained standard potting mix, as above, with three replicates of nine seedlings per pot for each herbicide treatment. The pots were maintained outdoors during the normal growing season (May to October) and watered as needed. All the experiments were conducted at the Waite Campus of the University of Adelaide, South Australia (34°58′13.5″S, 138°38′22.7″E). At the three-to-four true-leaf stage, the seedlings were treated with herbicides by using a moving-boom twin nozzle laboratory sprayer (University of Adelaide) that was placed 40 cm above the seedlings, delivering a water volume of 110 L ha^{-1} at a pressure of 250 kPa and at a speed of 1 m s^{-1}. Before the treatment, the number of seedlings per pot was counted. The control plants were not treated with any herbicide.

Dose–response experiments

In the 2015 growing season, the seeds of two triazine-resistant (P17 and P18) and two triazine-susceptible (S1 and S2) populations were germinated in seedling trays. At the one-to-two true-leaf stage, the seedlings were transplanted into small pots (8.5 cm × 9.5 cm × 9.5 cm) that contained standard potting mix, as described above. The experiment was established with three replicates of nine plants per pot for each herbicidal dose treatment. The pots were maintained outdoors during the normal growing season (May to October) and were watered as required.

At the three-to-four true-leaf stage, the seedlings were treated with atrazine (Gesaprim 900 DF) and diuron (Diuron 900 DF; Nufarm Australia, Melbourne, Australia). The resistant populations were treated with atrazine at 0, 62.5, 125, 250, 500, 1000, 2000, 4000, 8000, 16,000, 32,000 and 64,000 g ha^{-1} and diuron at 0, 31.3, 62.5, 125, 250, 500, 1000, 2000, 4000, 8000 and 16,000 g ha^{-1}. Whereas, the susceptible populations (S1 and S2) were treated with atrazine at 0, 62.5, 125, 250, 500, 1000, 2000, 4000, 8000 and 16,000 g ha^{-1} and diuron at 0, 31.3, 62.5, 125, 250, 500, 1000, 2000, 4000 and 8000 g ha^{-1}. In Australia, the recommended field rate for postemergence oriental mustard control in crops for atrazine and diuron are 1000 and 500 g ha^{-1}, respectively. Adjuvant Hasten TM (Victorian Chemical Company, Pty. Ltd., Melbourne, Australia) was added to the atrazine spray solution at 1% (v/v), while no adjuvant was applied for diuron. The plants were maintained outdoors after the herbicide application. The dose–response experiment was repeated in the 2016 growing season.

Identifying target-site mutations

Plant material (~100 mg) from the youngest green leaf tissue of one susceptible (S1) and two triazine-resistant (P17 and P18) populations (five individuals per population) was collected for DNA extraction. For the resistant population, the samples were collected from five survivors of atrazine at 4000 g ha^{-1} at 28 days after treatment (DAT). For the susceptible population, the samples were collected from five plants prior to the treatment with atrazine at the recommended rate to ensure they were susceptible. The obtained samples were snap-frozen in liquid nitrogen and stored at −20°C for further use. An Isolate II Plant DNA kit (Bioline Reagents, Sydney, Australia) was used to extract the DNA from 50–100 mg plant tissue, as per the manufacturer's instructions. Spectrophotometry (NanoDrop ND-1000; Thermo Scientific, Waltham, MA, USA) was used to determine the concentration of the nucleic acids. The DNA then was stored at −20°C for further analysis.

As no full sequence of the *psb*A gene of oriental mustard was available, a pair of primers (TTP1F and TTP2R) was designed, based on the conserved sequences of *psb*A among other species, including *Arabidopsis thaliana* (accession no. X79898), *Bromus tectorum* (accession no. AY744774) and *Oryza sativa* (accession no. M36191). All the gene sequences were obtained from Genbank, the National Center for Biotechnology Information (NCBI). The software Primer 3 Plus (Biomatics, Wageningen University, the Netherlands) and the website of NCBI were used to design and check for the specificity of the primers before use. An ~250 bp fragment of the *psb*A gene of two triazine-resistant (P17 and P18) and one susceptible (S1) genotypes (accession no. MF615263), including the known target sites of the chloroplast *psb*A gene, where a known mutation (Ser-264-Gly) that confers resistance to atrazine had already been reported, was amplified in standard polymerase chain reaction (PCR) conditions with the use of the forward primer, TTP1F (5′- CTCCTGTTGGCAGCTGCTACT'), and the reverse primer, TTP2R (5′-AGATTAGCACGGTT-GATGA-3′). A MyFi DNA polymerase kit (Bioline Reagents, Sydney, Australia) was used to run a PCR

reaction of 25 μL, containing 80–100 ng DNA, 1 × Myfi reaction buffer, 1 μM of each specific primer and one unit of Myfi Taq DNA polymerase. An automated DNA thermal cycler (Master CyclerH Gradient; Eppendorf, Hamburg, Germany) was used for the DNA amplification with PCR conditions as follows: 3 min denaturing at 95°C, 34 cycles of 15 s denaturation at 95°C, 15 s annealing at 57°C, 2 min elongation at 72°C and a final extension for 7 min at 72°C. The PCR products were visualized on 1.5% agarose gel that had been stained with 1 × SYBR® Safe DNA gel stain and prepared with 1 × Ficoll loading dye (15% [w/v] Ficoll 4000, 0.25% [w/v] bromophenol blue and 0.25% [w/v] xylene cyanol FF). The samples were electrophoresed in 1 × TAE Buffer (40 mMTrizma base, 1 mM Na_2EDTA, pH to 8 with glacial acetic acid) at 110 V and photographs were taken under UV light (λ302 nm). The sizes of the DNA fragment were estimated by comparing their mobility to bands of known sizes in an Easy-ladder (Bioline Reagents). The PCR products were sequenced at the Australian Genome Research Facility, Ltd., Melbourne, Australia, by using the same primers that had been used for the DNA amplification. The DNA sequence data were assembled, compared and analyzed by using the Geneious software program (Biomatters, Ltd., Auckland, New Zealand).

Data collection and analysis

In all the experiments, the assessments of plant survival were made at 28 DAT. The plants with new green leaf tissue were recorded as survivors, whereas those that displayed no new growth and severe necrosis were recorded as dead. The above-ground parts of the harvested plants were collected and dried in an oven at 65°C for 72 h and then weighed. The plant biomass data from the dose-response experiment were converted to a percentage of the untreated control before the regression analysis. A two-way ANOVA was conducted on the dose–response data for the repeat experiments (run). There was a significant effect of dose in all populations, but no effect of the experimental run. Therefore, the data from the two experimental runs were pooled. The LD_{50} values (the herbicide doses required for 50% mortality) and their 95% fiducial limits were analyzed by using an all-or-nothing model and a normal distribution function PriProbit v. 1.63 (Sakuma 1998). The probits were back-transformed to percentages for plotting. The dry weight data were analyzed by log-logistic analysis by using GraphPad Prism v. 6.0 (GraphPad Software, La Jolla, CA, USA) and the GR_{50} values (the herbicide doses required for 50% biomass reduction) were calculated. Resistance indices (RIs) were calculated as the ratio between the LD_{50} (or GR_{50}) of each population and the LD_{50} (or GR_{50}) of the mean values of the susceptible populations.

RESULTS

Herbicide response

Among the 40 oriental mustard populations that had been collected, two (5%) populations were identified as resistant to atrazine when treated with 1000 g ha^{-1}, while the other 38 populations were fully controlled (data not shown). The subsequent dose–response studies confirmed that the P17 and P18 populations were highly resistant to atrazine, but susceptible to diuron (Tables 1–2). For the atrazine treatment, the susceptible populations S1 and S2 were fully controlled at the recommended field rate (1000 g ha^{-1}), whereas 100% of the resistant plants (P17 and P18) survived and continued to grow, even at 16,000 g ha^{-1} (Fig. 1). In comparison with the mean values of the LD_{50} of the two susceptible populations (S1 and S2), the P17 and P18

Table 1. Estimated herbicide dose required for 50% mortality (LD_{50}), herbicide dose required for 50% biomass reduction (GR_{50}) and resistance index (RI)† values for the oriental mustard populations that were treated with atrazine

	Atrazine			
Population	LD_{50} (g ha^{-1})	RI	GR_{50}(g ha^{-1})	RI
P17	69,613 (59,532, 81,401)	310.9	41,239 (35,774, 47,539)	278.9
P18	70,642 (60,155, 82,958)	315.5	43,131 (38,357, 48,500)	295.2
S1	224.5 (224.5, 224.5)	–	146.1 (136.9, 155.9)	–
S2	223.3 (223.3, 223.3)	–	149.6 (139.6, 160.4)	–

†Resistance indices were calculated as the ratio between the LD_{50} (or GR_{50}) of each resistant population, compared to the mean value of the LD_{50} (or GR_{50}) of the two susceptible populations. The recommended field rate for the postemergent treatment of oriental mustard in crops in South Australia for atrazine is 1000 g ha^{-1}.

Values in parentheses are the 95% confidence intervals. The data for the two experimental runs were pooled.

Table 2. Estimated herbicide dose required for 50% mortality (LD_{50}), herbicide dose required for 50% biomass reduction (GR_{50}) and resistance index(RI)[†] values for the oriental mustard populations that were treated with diuron

	Diuron			
Population	LD_{50} (g ha ^{1})	RI	GR_{50}(g ha ^{1})	RI
P17	250.8 (220.8, 284.9)	1.14	168.7 (161.8, 176.0)	1.15
P18	272.6 (240.0, 309.6)	1.24	176.9 (169.7, 184.3)	1.22
S1	235.3 (207.2, 267.3)		150.9 (146.4, 155.5)	
S2	205.3 (205.3, 205.3)		140.0 (134.5, 145.5)	

[†]Resistance indices were calculated as the ratio between the LD_{50} (or GR_{50}) of each resistant population, compared to the mean value of the LD_{50} (or GR_{50}) of the two susceptible populations. The recommended field rate for the postemergent treatment of oriental mustard in crops in South Australia for diuron is 500 g ha^{-1}.

Values in parentheses are the 95% confidence intervals. The data for the two experimental runs were pooled.

populations were 311- and 315-fold more resistant to atrazine, respectively. Meanwhile, no cross-resistance to diuron was identified in these populations, as all the studied populations were controlled at the recommended field rate (500 g ha^{-1}). The response of the resistant and susceptible populations to diuron was nearly identical (Fig. 2) and the LD_{50} for diuron of the four populations ranged from 205 to 273 g ha^{-1} (Table 2).

The biomass of the P17 and P18 populations was only slightly reduced by the field rate of atrazine and these populations had a GR_{50} of 41,239 and 43,131 g ha^{-1}, respectively, while the GR_{50} of the S1 and S2 populations was 146 and 150 g ha^{-1}, respectively (Table 1, Fig. 1), making the resistant populations 279- and 295-fold more resistant than the susceptible populations, respectively. The biomass of all four studied populations was equally reduced by the diuron treatment and the GR_{50} for diuron ranged from 140 to 177 g ha^{-1} (Table 2, Fig. 2).

Sequencing of the *psb*A gene

When the sequences that were obtained from the chloroplastic *psb*A gene fragment from the resistant (P17, P18) and susceptible (S1) populations were aligned, a change in a single nucleotide that resulted in an amino acid substitution from serine 264 to glycine (Ser-264-Gly) was identified in all five individuals of the two resistant populations, but not in any individual of the susceptible population (Fig. 3).

DISCUSSION

Triazine and urea herbicides are both PSII-inhibitors. However, triazine-resistant biotypes can be controlled by a substituted urea herbicide due to a difference in binding mechanisms to the D1 protein of the PSII reaction centers. Triazine herbicides act by inhibiting photosynthesis via blocking the electron transport chain at the plastoquinone-binding region of the D1 protein subunit of PSII (Trebst 2008). Urea herbicides, such as diuron, bind at an overlapping, but not identical, site in PSII to that of triazines (Trebst 1996). Binding at this site prevents the transfer of electrons from Q_A (the primary quinone acceptor) to Q_B (the binding pocket that is located in the D1 protein), which limits electron flow in PSII (Haynes *et al.* 2000; Powles & Yu 2010).

The mutation, Ser-264-Gly, modifies the D1 protein in the region of the quinone-binding niche, meaning that triazine herbicides cannot bind effectively to this protein (Bettini *et al.* 1987; Fuerst & Norman 1991), enabling the plants to survive when treated with atrazine. However, due to the difference in binding sites, this mutation does not confer resistance to urea herbicides (Gronwald 1994; Trebst 1996).

Different substitutions in the *psb*A gene; namely, Val219 to Ile and Ser264 to Ala, have been found to confer resistance to diuron (Mengistu *et al.* 2000). These mutations alter the Q_B-binding pocket in the way that allows Q_B-binding, but prevents diuron-binding (Brusslan & Haselkorn 1989; Powles & Yu 2010), enabling the plants to survive when treated with diuron.

Some mutations in the *psbA* gene that give resistance to some PS II-inhibiting herbicides also result in supersensitivity or negative cross-resistance to other PS II-inhibiting herbicides (Oettmeier 1999). The Ser-264-Gly mutation is one of the mutations in which negative cross-resistance occurs. For example, red amaranth (*Amaranthus cruenatus*) and smooth pigweed (*Amaranthus hybridus*) populations that are resistant to atrazine showed negative cross-resistance to bentazone and pyridate (De Prado *et al.* 1992). However, triazine-resistant redroot pigweed (*Amaranthus retroflexus*) (Arntzen *et al.* 1979) and annual

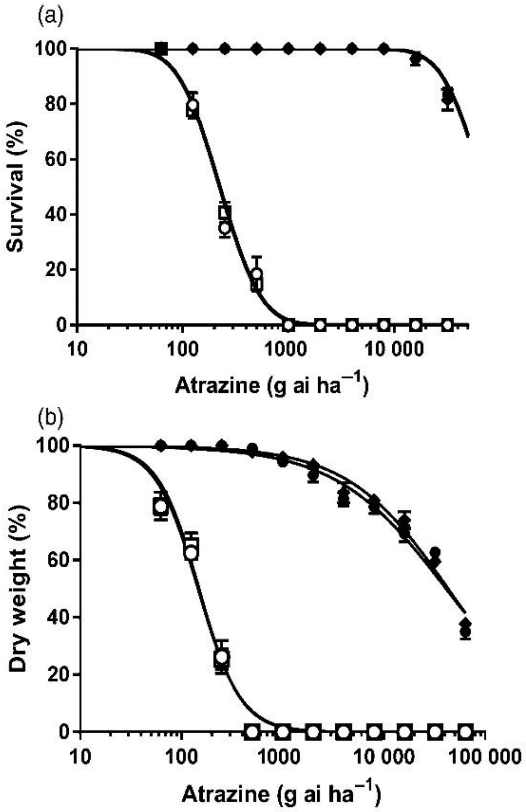


Fig. 1. Dose-response curves for the (a) survival and (b) biomass of the S1 (○), S2 (□) and resistant P17 (●) and P18 (♦) populations of the oriental mustard that was treated with atrazine. The curves for the survival data (LD_{50}) were fitted by using the equation $Y = 100 \times (1 - \text{NORMSDIST } [B + A \times X])$, where X is the log (dose) and Y (% survival) is back-transformed from mortality (expressed as normal equivalent deviates). The curves for the biomass reduction (GR_{50}) were fitted to a non-linear, log-logistic regression model by using GraphPad Prism, v. 6.0 (GraphPad Software). Each data point is the mean of six replicates and the vertical bars are the standard error of the mean.

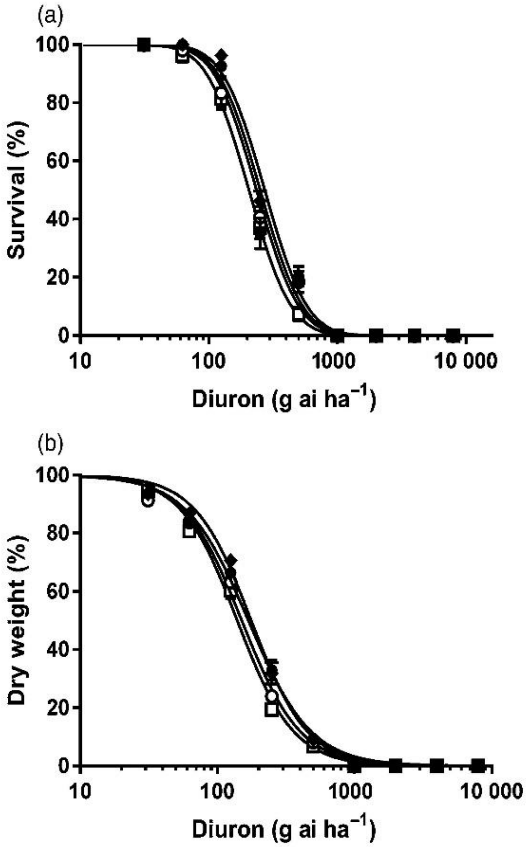


Fig. 2. Dose-response curves for the (a) survival and (b) biomass of the S1 (○), S2 (□), and resistant P17 (●) and P18 (♦) populations of the oriental mustard that was treated with diuron. The curves for the survival data (LD_{50}) were fitted by using the equation $Y = 100 \times (1 - \text{NORMSDIST } [B + A \times X])$, where X is the log (dose) and Y (% survival) is back-transformed from mortality (expressed as normal equivalent deviates). The curves for the biomass reduction (GR_{50}) were fitted to a non-linear, log-logistic regression model by using GraphPad Prism v. 6.0 (GraphPad Software). Each data point is the mean of six replicates and the vertical bars are the standard error of the mean.

bluegrass (*Poa annua*) (Svyantek *et al.* 2015) were equally as susceptible to diuron as the triazine-susceptible populations. In the current study, the two triazine-resistant populations that were investigated (P17 and P18) were equally sensitive to diuron as the two susceptible populations (S1 and S2), which is consistent with the mutation (Ser-264-Gly) (Fig. 3) in the binding site of chloroplast *psb*A being the main cause of atrazine resistance.

Although resistance to triazine herbicides in weeds has been well documented in a number of weed species all over the world, resistance to this herbicidal mode of action is still at a low level in Australia (<10% of the total herbicide-resistant cases) (Heap 2017). In this study, resistance to atrazine was identified in only 5% of the surveyed populations of oriental mustard, which makes triazine resistance in this species less common

Fig. 3. Aligned partial sequences of the *psbA* alleles from the triazine-susceptible (S) and resistant (P17 and P18) oriental mustard biotypes. Mutations in the *psbA* gene of the resistant oriental mustard biotypes are indicated by the bold letters that represent the nucleotide differences, when compared with the susceptible biotypes.

than resistance to other herbicide groups, such as ALS-inhibitors and auxinic herbicides. This is likely to be the result of the limited use of triazine herbicides in Australian crop production systems.

This is the first documented case of field-evolved target-site resistance to triazine herbicides in oriental mustard in Australia. The well-known Ser-264-Gly *psb*A gene mutation is likely to be the main cause of resistance in the oriental mustard populations that were investigated. The evolution of high-level triazine resistance in oriental mustard related to the presence of this target-site mutation has made the management of this weed considerably more difficult in the cropping systems of southern Australia. Therefore, management practices based on increased crop and herbicide diversity and the use of herbicide mixtures (Beckie 2007; Lagator *et al.* 2013), biological options such as maximizing crop competition (Beckie *et al.* 2008) or harvest weed-seed control (Walsh *et al.* 2013) could play an important role in managing herbicide-resistant oriental mustard populations and preventing or delaying the evolution of further triazine-resistant populations.

ACKNOWLEDGMENTS

This research was supported by the Grains Research and Development Corporation (GRDC) and a PhD scholarship offered by the University of Adelaide (UoA) and Vietnamese Government. The authors would like to thank Ruwan Lenorage and Geetha Velappan for technical assistance.

DISCLOSURE STATEMENT

The authors declare no conflict of interest.

REFERENCES

Adkins S., Wills D., Boersma M., Walker S., Robinson G., McLeod R. *et al.* 1997. Weeds resistant to chlorsulfuron and atrazine from the north-east grain region of Australia. *Weed Res.* **37**, 343–349.

Arntzen C.J., Ditto C.L. and Brewer P.E. 1979. Chloroplast membrane alterations in triazine-resistant *Amaranthus retroflexus* biotypes. *Proc. Natl. Acad. Sci. U. S. A.* **76**, 278–282.

Ashworth M.B., Han H., Knell G. and Powles S.B. 2016. Identification of triazine-resistant *Vulpia bromoides*. *Weed Technol.* **30**, 456–463.

Beckie H.J. 2007. Beneficial management practices to combat herbicide-resistant grass weeds in the northern Great Plains. *Weed Technol.* **21**, 290–299.

Beckie H.J., Johnson E.N., Blackshaw R.E. and Gan Y. 2008. Weed suppression by canola and mustard cultivars. *Weed Technol.* **22**, 182–185.

Bettini P., McNally S., Sevignac M., Darmency H., Gasquez J. and Dron M. 1987. Atrazine resistance in *Chenopodium album*: Low and high levels of resistance to the herbicide are related to the same chloroplast *psb*A gene mutation. *Plant Physiol.* **84**, 1442–1446.

Boutsalis P. 1996. Resistance to acetolactate synthase-inhibiting herbicides in *Sonchus oleraceus*, *Sisymbrium orientale* and *Brassica tournefortii* (PhD disertation). The University of Adelaide, Adelaide, Australia.

Boutsalis P., Karotam J. and Powles S.B. 1999. Molecular basis of resistance to acetolactate synthase–inhibiting herbicides in *Sisymbrium orientale and Brassica tournefortii*. *Pest Management Science* **55**, 507–516.

Boutsalis P., Gill G.S. and Preston C. 2012. Incidence of herbicide resistance in rigid ryegrass (*Lolium rigidum*) across southeastern Australia. *Weed Technol.* **26**, 391–398.

Boutsalis P. and Powles S.B. 1995. Resistance of dicot weeds to acetolactate synthase (ALS)-inhibiting herbicides in Australia. *Weed ResearchRes.* **35**, 149–155.

Boutsalis P. and Powles S.B. 1998. Seedbank characteristics of herbicide-resistant and susceptible *Sisymbrium orientale*. *Weed ResearchRes.* **38**, 389–395.

Brusslan J. and Haselkorn R. 1989. Resistance to the photosystem II herbicide diuron is dominant to sensitivity in the cyanobacterium *Synechococcus* sp. PCC7942. *EMBO J.* **8**, 1237–1245.

Burnet M.W., Hart Q., Holtum J.A. and Powles S.B. 1994. Resistance to nine herbicide classes in a population of rigid ryegrass (*Lolium rigidum*). *Weed Sci.* **42**, 369–377.

Chauhan B.S., Gill G. and Preston C. 2006a. Influence of environmental factors on seed germination and seedling emergence of oriental mustard (*Sisymbrium orientale*). *Weed Sci.* **54**, 1025–1031.

Chauhan B.S., Gill G. and Preston C. 2006b. Seedling recruitment pattern and depth of recruitment of 10 weed species in minimum tillage and no-till seeding systems. *Weed Sci.* **54**, 658–668.

De Prado R., Sanchez M., Jorrin J. and Dominguez C. 1992. Negative cross-resistance to bentazone and pyridate in atrazine-resistant *Amaranthus cruentus* and *Amaranthus hybridus* biotypes. *Pestic. Sci.* **35**, 131–136.

Devine M.D. and Shukla A. 2000. Altered target sites as a mechanism of herbicide resistance. *Crop Prot.* **19**, 881–889.

Erickson J.M., Rahire M., Bennoun P., Delepelaire P., Diner B. and Rochaix J.D. 1984. Herbicide resistance in *Chlamydomonas reinhardtii* results from a mutation in the chloroplast gene for the 32-kilodalton protein of photosystem II. *Proc. Natl. Acad. Sci. U. S. A.* **81**, 3617–3621.

Fuerst E.P. and Norman M.A. 1991. Interactions of herbicides with photosynthetic electron transport. *Weed Sci.* **39**, 458–464.

Giacomazzi S. and Cochet N. 2004. Environmental impact of diuron transformation: a review. *Chemosphere* **56**, 1021–1032.

Goloubinoff P., Edelman M. and Hallick R.B. 1984. Chloroplast-coded atrazine resistance in Solanum nigrum: *psb*A loci from susceptible and resistant biotypes are isogenic except for a single codon change. *Nucleic Acids Res.* **12**, 9489–9496.

Gronwald J. 1994. Resistance to photosystem II inhibiting herbicides. In: *In: Herbicide Resistance in Plants: Biology and Biochemistry* (ed. by Holtum J.A.M. and Powles S.B.). Lewis Publishers, Boca Raton, FL, USA, 27–60.

Hashem A., Dhammu H.S., Powles S.B., Bowran D.G., Piper T.J. and Cheam A.H. 2001. Triazine resistance in a biotype of wild radish (*Raphanus raphanistrum*) in Australia. *Weed Technol.* **15**, 636–641.

Haynes D., Ralph P., Prange J. and Dennison B. 2000. The impact of the herbicide diuron on photosynthesis in three species of tropical seagrass. *Mar. Pollut. Bull.* **41**, 288–293.

Heap I. 2017. *The International Survey of Herbicide Resistant Weeds.* [Cited 28 March, 2017.] Available from URL: www.weedscience.org

Hirschberg J., Bleecker A., Kyle D.J., McIntosh L. and Arntzen C.J. 1984. The molecular basis of triazine-herbicide resistance in higher-plant chloroplasts. *Zeitschrift für Naturforschung* **39**, 412–420.

Lagator M., Vogwill T., Mead A., Colegrave N. and Neve P. 2013. Herbicide mixtures at high doses slow the evolution of resistance in experimentally evolving populations of *Chlamydomonas reinhardtii*. *New Phytol.* **198**, 938–945.

LeBaron H.M., McFarland J.E. and Burnside O.C. 2008. The triazine herbicides: A milestone in the development of weed control technology. In: *The Triazine Herbicides* (ed. by LeBaron H.M., McFarland J.E. and Burnside O.C.). Elsevier, San Diego, CA, 1–12.

Lossli R. 1994. Triazines. *Toxicology* **91**, 59–62.

McGillion T. and Storrie A.,.e. 2006. *Integrated Weed Management in Australian Cropping Systems–a Training Resource for Farm Advisors*. CRC for Australian Weed Management, Glen Osmond, Australia.

Mengistu L.W., Mueller-Warrant G.W., Liston A. and Barker R.E. 2000. *psb* A mutation (valine$_{219}$ to isoleucine) in *Poa annua* resistant to metribuzin and diuron. *Pest Manag. Sci.* **56**, 209–217.

Moreland D.E. 1967. Mechanisms of action of herbicides. *Annu. Rev. Plant Physiol.* **18**, 365–386.

Müller G. 2008. History of the discovery and development of triazine herbicides. In: *The Triazine Herbicides*, vol. **50** (ed. by LeBaron H.M., McFarland J.E. and Burnside O.C.). Elsevier, San Diego, CA, 13–29.

Oettmeier W. 1999. Herbicide resistance and supersensitivity in photosystem II. *Cell. Mol. Life Sci.* **55**, 1255–1277.

Powles S.B. and Yu Q. 2010. Evolution in action: plants resistant to herbicides. *Annu. Rev. Plant Biol.* **61**, 317–347.

Preston C., Dolman F.C. and Boutsalis P. 2013. Multiple resistance to acetohydroxyacid synthase-inhibiting and auxinic herbicides in a population of oriental mustard (*Sisymbrium orientale*). *Weed Sci.* **61**, 185–192.

Preston C., Mallory-Smith C.A., Powles S. and Shaner D. 2001. Biochemical mechanisms, inheritance, and molecular genetics of herbicide resistance in weeds. In: *Herbicide Resistance and World Grains* (ed. by Shaner D.L.). CRC Press LLC, Boca Raton, FL, 23–60.

Sakuma M. 1998. Probit analysis of preference data. *Applied Entomology and Zoology.* **33**, 339–347.

Svyantek A.W., Aldahir P., Chen S., Flessner M.L., McCullough P.E., Sidhu S.S. *et al.* 2015. Target and nontarget resistance mechanisms induce annual bluegrass (*Poa annua*) resistance to atrazine, amicarbazone, and diuron. *Weed Technol.* **30**, 773–782.

Trebst A. 1996. *The Molecular Basis of Plant Resistance to Photosystem II Herbicides*. ACS Publications, American Chemical Society, USA.

Trebst A. 2008. The mode of action of triazine herbicides in plants. In: *The Triazine Herbicides*, vol. **50** (ed. by LeBaron H.M., McFarland J.E. and Burnside O.C.). Elsevier, San Diego, CA, 101–110.

Walsh M., Newman P. and Powles S. 2013. Targeting weed seeds in-crop: a new weed control paradigm for global agriculture. *Weed Technol.* **27**, 431–436.

Wilding J.L., Barnett A.G. and Amor R.L. 1986. *Crop Weeds*. Inkata Press, Melbourne.

CHAPTER 3

REDUCED TRANSLOCATION IN 2,4-D RESISTANT ORIENTAL MUSTARD POPULATIONS (*SISYMBRIUM ORIENTALE* L.) FROM AUSTRALIA

Hue T Dang, Jenna M Malone, Peter Boutsalis, Mahima Krishnan

Gurjeet Gill and Christopher Preston

School of Agriculture, Food and Wine, University of Adelaide

PMB1, Glen Osmond, South Australia 5064, Australia

Pest Manag. Sci. First published: 14 February 2018

Early view: http://onlinelibrary.wiley.com/doi/10.1002/ps.4845/full

(with permission from the Pest Management Science and John Wiley and Sons)

JOHN WILEY AND SONS LICENSE
TERMS AND CONDITIONS

Feb 26, 2018

This Agreement between Mrs. Hue Dang ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	4287880907581
License date	Feb 14, 2018
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	Pest Management Science
Licensed Content Title	Reduced translocation in 2,4-D-resistant oriental mustard populations (Sisymbrium orientale L.) from Australia
Licensed Content Author	Hue Thi Dang,Jenna M Malone,Peter Boutsalis,Mahima Krishnan,Gurjeet Gill,Christopher Preston
Licensed Content Date	Feb 14, 2018
Licensed Content Pages	1
Type of Use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Electronic
Portion	Full article
Will you be translating?	No
Title of your thesis / dissertation	Investigation of Herbicide Resistance in Oriental Mustard (Sisymbrium orientale L.) in Australia
Expected completion date	Mar 2018
Expected size (number of pages)	160
Requestor Location	Mrs. Hue Dang School of Agriculture, Food and Wine PMB 1, Glen Osmond Adelaide, South Australia 5064 Australia Attn: Mrs. Hue Dang
Publisher Tax ID	EU826007151
Total	**0.00 USD**

Statement of Authorship

Title of Paper	Reduced Translocation in 2,4-D-Resistant Oriental Mustard Populations (*Sisymbrium orientale* L.) from Australia
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Dang, H. T., Malone, J. M., Boutsalis, P., Krishnan, M., Gill, G. and Preston, C. (2018), Reduced translocation in 2,4-D-resistant oriental mustard populations (*Sisymbrium orientale* L.) from Australia. Pest. Manag. Sci. doi:10.1002/ps.4845.

Principal Author

Name of Principal Author (Candidate)	Hue Thi Dang		
Contribution to the Paper	Planned the study, conducted all experiments, analyzed and interpreted data and wrote manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	23.02.2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Peter Boutsalis		
Contribution to the Paper	Supervised the whole plant studies and helped with data interpretation		
Signature		Date	28 Feb 2018

Name of Co-Author	Jenna Moira Malone		
Contribution to the Paper	Supervised molecular work, data interpretation and edited manuscript		
Signature		Date	26/2/18

Name of Co-Author	Mahima Krishnan		
Contribution to the Paper	Supervised translocation work, helped with data interpretation		
Signature		Date	26/02/18

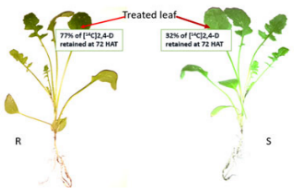
Name of Co-Author	Christopher Preston		
Contribution to the Paper	Designed and supervised development of research, data interpretation and analysis, and edited manuscript		
Signature		Date	23/02/2018
Name of Co-Author	Gurjeet Gill		
Contribution to the Paper	Designed and supervised development of research, data interpretation and analysis, and edited manuscript		
Signature		Date	23.2.2018

Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.4845

Research Article

A high level of resistance to 2,4-D was identified in two oriental mustard populations (P2 and P13). This article confirms that a reduction of 2,4-D translocation is likely the main cause of 2,4-D resistance and the inheritance of resistance is managed by a single gene with a high level of dominance in oriental mustard.



Reduced translocation in 2,4-D-resistant oriental mustard populations (*Sisymbrium orientale* L.) from Australia

Hue Thi Dang, Jenna M Malone, Peter Boutsalis, Mahima Krishnan, Gurjeet Gill and Christopher Preston

Research Article

Received: 30 October 2017 Revised: 15 December 2017 Accepted article published: 29 December 2017 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.4845

Reduced translocation in 2,4-D-resistant oriental mustard populations (*Sisymbrium orientale* L.) from Australia

Hue Thi Dang,* Jenna M Malone, Peter Boutsalis, Mahima Krishnan, Gurjeet Gill and Christopher Preston

Abstract

BACKGROUND: Two oriental mustard populations (P2 and P13) collected from Port Broughton, South Australia were identified as resistant to 2,4-D. The level of resistance, mechanism and the mode of inheritance for 2,4-D resistance in these populations were investigated.

RESULTS: Populations P2 and P13 were confirmed to be resistant to 2,4-D at the field rate (600 g a.e. ha^{-1}). P2 and P13 were 81- and 67-fold more resistant than the susceptible populations (S1 and S2) at the dose required for 50% mortality (LD_{50}), respectively. No predicted amino acid modification was detected in sequences of potential target-site genes (ABP, TIR1 and AFB5). Resistant populations had reduced 2,4-D translocation compared with the susceptible populations, with 77% of [^{14}C]2,4-D retained in the treated leaf versus 32% at 72 h after treatment. Resistance to 2,4-D is encoded on the nuclear genome and is dominant, as the response to 2,4-D of all F_2 individuals were similar to the resistant biotypes. The segregation of F_2 phenotypes fitted a 3: 1 (R: S) inheritance model.

CONCLUSION: Resistance to 2,4-D in oriental mustard is likely due to reduced translocation of 2,4-D out of the treated leaf. Inheritance of 2,4-D resistance is conferred by a single gene with a high level of dominance.

Keywords: 2,4-D resistance; non-target-site mechanism (NTSR); reduced translocation; inheritance; single dominant gene; *Sisymbrium orientale* L

1 INTRODUCTION

The synthetic auxin herbicides were discovered in the 1940s and since then three major classes including phenoxyacetic acids (e.g. 2,4-D and MCPA), benzoic acids (e.g. dicamba, chloramben) and pyridine acids (e.g. picloram, fluroxypyr) have been marketed.[1] Among these, 2,4-D has been the most widely used herbicide worldwide for the control of dicot weeds in crop and non-crop situations over the last seven decades.[2–4] The use of 2,4-D for weed control in cereals, especially maize and wheat, created a revolution in agricultural production by allowing effective control of broadleaf weeds.[2,5] 2,4-D interferes with numerous plant pathways and causes symptomatic metabolic abnormalities or disruption of growth processes in susceptible plants leading to plant epinasty, followed by growth inhibition and death.[4,6] To date, three auxin receptors have been identified including auxin-binding protein 1 (ABP),[7,8] auxin-signalling F-box (TIR1/AFB) receptor protein homolouegs[9–11] and, most recently, S-phase kinase-associated protein 2 (SKP2), an auxin-binding protein that connects auxin signalling with cell division in plants.[12]

Even though the first case of resistance to 2,4-D was reported in wild carrot (*Daucus carota*) in 1957,[13] only 34 cases of resistance to auxinic herbicides have been identified worldwide,[14] and the mechanisms of resistance in many cases remain unknown.[15] Several putative target-site resistance (TSR) and non-target-site resistance (NTSR) mechanisms have been reported to confer resistance to this herbicide group.[2] However, due to its complex mode of action, the evolution of resistance to auxinic herbicides in weeds is considered to be more likely associated with NTSR.[16] Mechanisms of NTSR include: (1) reduced herbicide uptake, where absorption of the herbicide into the mesophyll is prevented by the leaf cuticle or other structural barriers;[17] (2) rapid metabolic detoxification or enhanced metabolism, where the plant has the ability to degrade the herbicide to non-toxic forms before it can affect the plant;[18–20] and (3) reduced translocation, where the herbicide remains at its site of application instead of reaching its site of action at a sufficient concentration to cause death.[20] To date, field-evolved auxinic resistance conferred by NTSR mechanism(s) has been reported in several weed species. Resistance conferred by decreased absorption of 2,4-D has been identified in ground ivy (*Glechoma hederacea*);[17] enhanced metabolism has been reported in hemp-nettle (*Galeopsis tetrahit*)[21] and corn poppy (*Papaver*

* *Correspondence to: HT Dang, School of Agriculture, Food and Wine, University of Adelaide, PMB1, Glen Osmond, South Australia 5064, Australia. E-mail: huethi.dang@adelaide.edu.au*

School of Agriculture, Food and Wine, University of Adelaide, Glen Osmond, Australia

rhoeas);[22] and reduced translocation has been confirmed in wild radish (*Raphanus raphanistrum*),[16] hemp-nettle (*G. tetrahit*),[21] corn poppy (*P. rhoeas*)[23] and prickly lettuce (*Lactuca serriola*).[24] Analysis of mutants of *Arabidopsis thaliana*[25,26] selected for resistance to auxinic herbicides mutations in the TIR1 gene and its homologue (AFB5) were responsible for resistance. Likewise, studies on field-evolved resistant wild mustard (*Sinapis arvensis*) suggested resistance was associated with mutant ABP receptor.[27,28] These findings suggested that resistance to auxinic herbicides can also result from TSR mechanisms.

Oriental mustard (*Sisymbrium orientale* L.), a diploid species ($2n = 14$) belonging to the Brassicaceae family, is a problematic broadleaf weed of field crops in southern Australia (South Australia and Victoria). Oriental mustard often infests and competes for essential resources with winter crops such as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), oats (*Avena sativa*), canola (*Brassica napus*) and chickpea (*Cicer arietinum*),[29] and can impede crop harvest.[30] The seeds of oriental mustard have a short seed-bank life and very short dormancy with the majority of seed germinating early in the winter cropping season.[30,31] Oriental mustard has evolved resistance to four different herbicide groups including acetolactate synthase (ALS) inhibitors, phytoene desaturase (PDS) inhibitors, synthetic auxins and triazine herbicides.[14,32–34] A study of inheritance of 2,4-D resistance in oriental mustard found resistance was conferred by a single gene with high levels of dominance.[35] However, the mechanism of resistance to 2,4-D has not been investigated fully.

Understanding the mechanism and mode of inheritance of resistance provides helpful information for the formulation of weed management strategies to reduce the evolution of resistance and aid management of resistant weed species. This study aimed to quantify the level of resistance to 2,4-D and investigate the mechanism(s) and mode of inheritance of 2,4-D resistance in oriental mustard. The study also aimed to determine whether this resistance was associated with mutations in the target genes (ABP, TIR1 and AFB5) or NTSR mechanisms.

2 MATERIALS AND METHODS

2.1 Plant materials

Two 2,4-D-resistant oriental mustard populations P2 and P13 originally collected from wheat fields near Port Broughton, South Australia were used in this study. These populations survived 600 g a.e. ha^{-1} 2,4-D amine (Amicide Advance 700 g a.e. L^{-1}, Nufarm Australia, Melbourne, Australia), the recommended field rate to control oriental mustard in Australia. Two known herbicide-susceptible oriental mustard populations S1 and S2 were also used throughout the study as susceptible controls. The S1 population was collected from an organic field near Roseworthy, South Australia[32] and S2 from near Port Kenny, Eyre Peninsula, South Australia. These populations have been confirmed susceptible to all herbicides commonly used to control oriental mustard in Australia (glyphosate, imazamox, chlorsulfuron, diflufenican, atrazine and 2,4-D).[34]

To generate homogeneous populations for the current study, five healthy plants from the P2 and P13 populations that survived 2,4-D treatment at 1200 g ha^{-1} were transplanted into 25-cm diameter pots containing standard potting mix,[36] with one plant per pot. Furthermore, five untreated plants from each susceptible population (S1 and S2) were also planted into pots as described above. Because oriental mustard is self-pollinating,[37] pots were transferred outdoors and allowed to self-pollinate and produce seeds. Mature seeds from these plants were collected and stored at 10 °C for use in all experiments.

2.2 Seed germination, plant growth and herbicide treatment

Seeds of the four (P2, P13, S1 and S2) populations were germinated on the surface of trays containing standard soil mix. Seedlings at the one- to two-leaf stage were transplanted into pots (8.5 cm × 9.5 cm × 9.5 cm) containing standard potting mix. Nine plants per pot with three replicates for each herbicide dose treatment were planted. Pots were maintained outdoors during the normal growing season (May to October), watered and fertilised as required. At the three- to four-leaf stage, seedlings were treated with herbicide using a laboratory twin-nozzle sprayer with a nozzle height of 40 cm above the seedlings, moving at the speed of 1 m s^{-1}, at 250 kPa pressure producing a volume of 110 L ha^{-1}. The number of seedlings in each pot was counted prior to herbicide application. Control plants were not treated with any herbicide. All experiments were conducted at the Waite Campus, The University of Adelaide, South Australia (34°58′13.5″S, 138°38′22.7″E).

2.3 Whole-plant dose response

In the 2015 growing season, seeds of P2, P13, S1 and S2 populations were germinated and grown in pots as described above. At the three- to four-leaf stage, seedlings were treated with 2,4-D amine at 0, 37.5, 75, 150, 300, 600, 1200, 2400, 4800 and 9600 g ha^{-1}. The experiment was repeated in July 2016.

Plant survival was assessed 28 days after treatment (DAT). Plants with new green leaf tissue were recorded as alive, whereas those that displayed severe epinasty or no new growth were recorded as dead. The above-ground parts of the harvested plants were collected and dried in an oven at 65 °C for 72 h, and then weighed. Plant biomass data from the dose–response experiment were converted to per cent of untreated control before regression analysis. The LD_{50} values (the herbicide dose required for 50% mortality) and their 95% confidence limits were analysed using an all-or-nothing model and a normal distribution function PriProbit v. 1.63.[38] Probits were back transformed to percentages for plotting. Dry weight data were analysed by log-logistic analysis using GraphPad Prism v. 6.0 and GR_{50} values (the herbicide dose required for 50% biomass reduction) calculated. Resistance indices (RI) were calculated as the ratio between the LD_{50} (or GR_{50}) of each population and the mean LD_{50} (or GR_{50}) of the susceptible populations. The experiment was repeated and a two-way analysis of variance (ANOVA) was used to examine the effect of experimental run. Data from the two runs were pooled prior to data analysis if no effect of experimental run was identified.

2.4 Gene amplification and sequencing

Approximately 100 mg of the youngest green leaf tissue of resistant and susceptible plants (five individuals per population) was collected for DNA extraction. For the resistant populations, samples were collected from five survivors of each population at 1200 g 2,4-D ha^{-1} at 28 DAT. For the susceptible population, samples were collected from five plants prior to treatment with 2,4-D at the recommended rate (600 g ha^{-1}) to ensure they were susceptible. An Isolate II Plant DNA kit (Bioline, Eveleigh, Australia) was used to extract DNA and the concentration of nucleic acids measured using a spectrophotometric NanoDrop ND–1000 (Thermo Scientific, Waltham, MA, USA). DNA was then stored at −20 °C for further analysis.

Resistance to auxinic herbicides was reported to be associated with target-site mutations in the genes ABP, TIR1 and its homologue AFB5.[26,27] Primers were designed to amplify partial fragments of these three genes, containing the known mutations sites, from oriental mustard. Because no complete sequences of these genes were available for oriental mustard, primers for ABP and TIR1 genes were designed based on the *Arabidopsis thaliana* sequences of ABP (accession number NM_116532) and TIR1 (accession number NM_116163) genes. The sequences were obtained from GenBank, the National Center for Biotechnology Information (NCBI). The software Primer 3 Plus (Biomatics, Wageningen University, The Netherlands), the Expasy (Bioinformatics Resource Portal) and the website of NCBI were used to design and check for specificity of primers before use. Primers for the AFB5 gene were obtained from the published study on Arabidopsis.[26]

The three primer pairs (ABP–1F 5′-TCATCTCMGATCGYCGYCRT-3′, ABP-1R-5′-ACGTCGTCTTTTTGYGATTCTTG-3′; AFB-5F-5′-CACCAAAATGACACAAGATCGCTCAGAAATGTC-3′, AFB-5R-5′-TCCTACACTTACCCATTTCATTCCCG-3′; and TIR-F2-5′-GTGTGYAARTCRTGGTACGAGAT–3′, TIR-R1-5′-CCTGCAABTRGCAGCRATRGC–3′) were used for polymerase chain reaction (PCR) amplification using Phire Hot Start II DNA polymerase (Thermo Fisher Scientific Australia Pty Ltd, Norwood, Australia). Reactions of 20 µL contained 80–100 ng DNA, 1× Phire kit reaction buffer, 0.5 µM of each specific primer and 1 U of the Phire *Taq* DNA polymerase. An automated DNA thermal cycler (Eppendorf Master CyclerH Gradient, Eppendorf, Hamburg, Germany) was used for DNA amplification with PCR conditions applied as follow: 3 min denaturing at 95 °C; 40 cycles of 15 s denaturation at 95 °C, 15 s annealing at 57–59 °C depending on the primer pair, 2 min elongation at 72 °C and a final extension for 7 min at 72 °C.

PCR products were visualised on 1.5% agarose gels stained with 1× SYBR® Safe DNA gel stain, prepared with 1× Ficoll loading dye [15% (w/v) Ficoll 4000, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF]. Samples were then electrophoresed in 1× TAE buffer [40 mM Trizma base, 1 mM Na_2EDTA, and pH to 8 with glacial acetic acid] at 110 V. Photographs of the samples were taken under UV light ($\lambda = 302$ nm). Fragment sizes amplified from DNA were estimated by comparing their mobility to bands of known sizes in a DNA ladder (Easy Ladder, Bioline). PCR products were sequenced by the Australian Genome Research Facility (AGRF) Ltd. (Parkville, Australia) using the same primers used for DNA amplification. DNA sequence data were assembled, compared and analysed using Geneious version 8.0 (Biomatters Limited, Auckland, New Zealand).

2.5 Absorption and translocation of 2,4-D

Absorption and translocation of 2,4-D in oriental mustard was measured using the method described by Goggin *et al.*,[16] with modifications. Because auxin overdose symptoms in pre-treated susceptible plants created challenges in handling of the plants (e.g. stem and petiole swelling and brittleness, tight leaf curling),[16] experiments were conducted without a 2,4-D pre-treatment. Two resistant (P2 and P13) and two susceptible (S1 and S2) populations were used. Twenty seedlings of each population at the one- to two-leaf stage were transplanted into trays containing standard soil mixture. A solution of 500 g a.e. ha^{-1} of 2,4-D was prepared and [^{14}C]2,4-D (ring ^{14}C[U] American Radiolabelled Chemicals) was added to the 2,4-D solution to obtain radioactivity of ~ 33 Bq μL^{-1}. When seedlings reached the three- to four-leaf stage, they were transferred into hydroponic medium prepared in a black plastic container (26 × 19 × 9 cm) containing Hoagland's nutrient solution[39] with pH adjusted to 6.5–7.0. The experiment was run in a growth chamber maintained at 22 °C (light)/18 °C (dark) day/night cycle; 12 h photoperiod with 550 µmol m^{-2} s^{-1} light intensity during the day phase. The second leaf (one leaf per plant) of 15 plants of similar size from each population was treated with ten 0.5-µL droplets of the [^{14}C]2,4-D solution, avoiding the mid-vein of the leaf.

Five plants of each population were harvested at 24, 48 and 72 h after treatment (HAT). Each harvested plant was divided into three sections: treated leaf plus petiole, untreated leaves plus petioles and stem (the rest of the shoot), and roots. The treated leaf was submerged for 5 s in 5 mL of 0.1% Triton X-100 solution in a 20 mL glass vial (Sigma-Aldrich, Castle Hill, NSW, Australia) to remove unabsorbed [^{14}C]2,4-D. To this wash solution, 8 mL of Ultima Gold XR scintillation fluid (PerkinElmer, Waltham, MA, USA) was added for scintillation counting. Each plant section was transferred into a combustor cone (PerkinElmer, Waltham, MA, USA) and allowed to dry at room temperature for three days. When fully dried, each sample was separately combusted in an automatic preparation sample oxidizer (PerkinElmer, Shelton, CT, USA). The $^{14}CO_2$ emitted from each sample was trapped in 14 mL of a mixture containing 7 mL Carbo-Sorb E and 7 mL Permafluor E+ (PerkinElmer, Shelton, CT, USA). A liquid scintillation analyser (Tri-card 2100TR; Packard Bioscience, Meriden, CT, USA) was used to quantify the radioactivity of the wash solution and each plant section. The percentage of [^{14}C]2,4-D absorbed was expressed as the sum of the total amount absorbed in the plant divided by the sum of the amount recovered in the plant and the treated leaf wash solution. The percentage of [^{14}C]2,4-D in each plant section was expressed as the amount in the plant part divided by the total [^{14}C]2,4-D absorbed. The percentage values were arcsine transformed before ANOVA analysis. The experiment was repeated. Fisher's protected LSD multiple comparisons were applied to differentiate between predicted means, and the means were presented as back-transformed data (GenStat 18; VSN International, Hemel Hempstead, UK).

2.6 Inheritance of resistance to 2,4-D

2.6.1 *Generation of F_1 and F_2 seeds*

Three populations P2, P13 and S1 (hereafter referred as R1, R2 and S) were selected for the inheritance study. Five survivors of each resistant population (R) at 1200 g ha^{-1} and five untreated plants of the susceptible (S) population were transplanted into 25-cm diameter pots as described above, with one plant per pot. The pots were transferred into a temperature-regulated glasshouse (15–22 °C) under natural light. Plants were watered and fertilised as needed. Three days after the first flower opened, from each inflorescence, only two to three young buds were retained and all mature siliques, opened flowers and small buds were removed using fine scissors or forceps. From each selected bud, all immature anthers were removed exposing only the stigma. Each inflorescence was immediately bagged to avoid desiccation or cross-pollination. On the following day, cross-pollination was conducted by tapping the anthers with pollen grains of the susceptible or resistant biotype against the exposed stigma of the other biotype using forceps. On sunny days, crossing was conducted in the morning from 7.00 a.m. to 11.00 a.m. However, on overcast days, flower opening was delayed, therefore crossing was conducted later in the morning (from 10.00 a.m. onwards) to ensure that the flowers of the pollen donors were fully open. The forceps were cleaned using 96% ethanol and dried with a tissue between each cross. The pollen receptor female flowers were

bagged promptly after hand-pollination. Individual flowers of the parental plants were also bagged to promote self-fertilisation. Three days after fertilisation, the bag on each crossed flower was removed and the developing pod marked by a string-tag. At maturity, pods from crossed and selfed flowers were harvested. Control florets were not pollinated. Pods developed from control florets did not produce seeds but turned brown and were shed ~ 1 week after emasculation.

Seeds of pods generated from cross-fertilizations were collected separately from each cross and considered an F_1 family. Seeds from the parents (R1, R2 and S parental plants) and the F_1 seeds were germinated and transplanted into small pots with a density of five plants per pot. Seedlings at the three- to four-leaf stage were treated with 2,4-D at 200 g ha^{-1} to determine resistance status of the progenies. This rate of herbicide controlled all susceptible plants, but all resistant plants survived.[35] Seedlings from the susceptible parent of each cross that survived the herbicide application were considered to be true F_1 plants and were transplanted into larger pots (25 cm in diameter) and allowed to self-pollinate to produce F_2 seeds. Mature seeds (F_2) from each F_1 individual were collected separately and used in the inheritance studies.

2.6.2 *Segregation and dose–response of the F_2 populations*

A phenotypic segregation test was conducted on the F_2 populations to determine whether they all had a similar segregation response to 2,4-D. Seeds of F_2 and parental populations were germinated and a total of 108 seedlings of each population were transplanted into trays. At the three- to four-leaf stage, they were treated with 2,4-D at 200 g ha^{-1}. The homogeneity and segregation of the F_2 phenotypes were tested against a single-gene model with a dominant allele using the *G*-test as described by Preston and Malone [35].

A dose–response experiment was also conducted on F_2 populations and parental populations to determine the number of genes involved in resistance. Susceptible and resistant populations and individuals from 16 F_2 populations were treated with 2,4-D at rates 0, 18.8, 37.5, 75, 150, 300, 600, 1200, 2400, 4800 and 9600 g ha^{-1} at the three- to four-leaf stage as described above. There were three replicate pots (nine plants per pot) for each population at each herbicide rate.

Probit analysis using PriProbit was used to analyse the dose–responses of the parental populations. For the F_2 population, a model of single dominant gene was created by summing 0.75 (equivalent to 75%) × survival of the resistant population and 0.25 (equivalent to 25%) × survival of the susceptible population at each 2,4-D rate as determined by the probit analysis. This model was compared with the real responses of the F_2 populations to test that the dose response fitted to a single-gene model as predicted.[40] All experiments were conducted twice in the main growing seasons of 2015 and 2016. Effect of experimental run was examined using a two-way ANOVA. Data from the two runs were pooled if no effect of the experimental run was identified as described above.

3 RESULTS

3.1 Whole plant dose response

The dose–response studies confirmed that the P2 and P13 populations were highly resistant to 2,4-D. The susceptible populations S1 and S2 were controlled by 2,4-D at the recommended field rate (600 g ha^{-1}), whereas all the plants from resistant populations (P2 and P13) survived and continued to grow, even at higher rates (Fig. 1). In comparison with the mean values of the LD_{50} of the two susceptible populations (S1 and S2), the P13 and P2 populations were 81- and 67-fold more resistant, respectively (Table 1). The recommended field rate of 2,4-D caused a slight reduction in biomass of the P2 and P13 populations. These populations had a GR_{50} of 1968 and 1549 g ha^{-1}, respectively, compared with the mean GR_{50} of the S1 and S2 populations of 30 g ha^{-1}. Resistant populations were 50- to 64-fold more resistant than the susceptible populations based on GR_{50} (Table 1; Fig. 1).

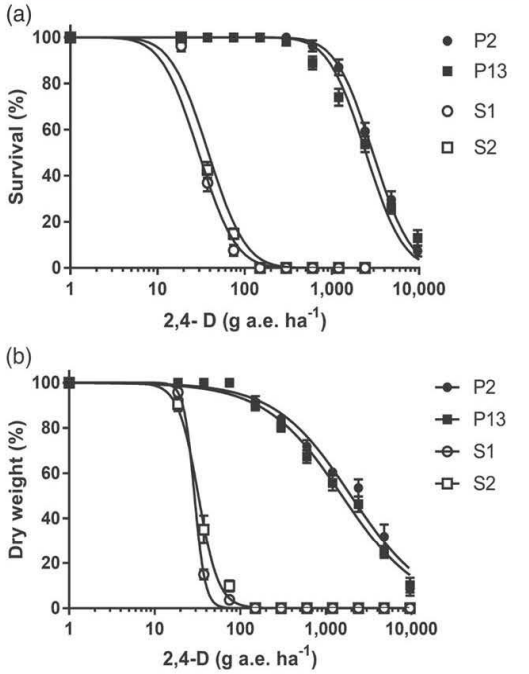


Figure 1. Dose–response curves for the survival (a) and biomass (b) of susceptible S1 (○), S2 (□), and resistant P2 (●) and P13 (■) populations of oriental mustard treated with 2,4-D. Each data point is the mean of six replicates, and the vertical bars are the standard error of the mean (SEM). Curves for survival data were fitted using the equation $Y = 100*[1 - \text{NORMSDIST}(B + A*X)]$, where X is log (dose) and Y (% survival) is back-transformed from mortality (expressed as normal equivalent deviates). Curves for biomass reduction (GR_{50}) were fitted to a non-linear, log-logistic regression model using GraphPad Prism v.6.0. Each data point is the mean of six replicates, and the vertical bars are SEM.

3.2 Gene sequencing and mutation detection in resistant populations

Partial gene fragments of the ABP (accession number MF781078), AFB5 (accession number MF662594) and TIR1 (accession number MF692794) genes of oriental mustard were sequenced and analysed, however, no predicted amino acid differences between sequences from susceptible and resistant plants were observed.

3.3 Absorption and translocation of [^{14}C]2,4-D

As ANOVA showed no difference between experimental runs, the data of two runs were pooled prior to analysis. There was a

Table 1. Estimated LD_{50} (dose required for 50% mortality), GR_{50} (dose required for 50% biomass reduction) and resistance index (RI)* values for oriental mustard populations treated with 2,4-D. Values in parentheses are 95% confidence intervals. Data for the two experimental runs were pooled

Population	Survival		Biomass	
	LD_{50}	RI	GR_{50}	RI
P2	2956 (2549, 3429)	81.0	1968 (1735, 2233)	63.8
P13	2450 (2116, 2837)	67.1	1549 (1391, 1726)	50.3
S1	34.7 (34.7, 34.7)	–	29.1 (28.4, 30.1)	–
S2	38.3 (38.3, 38.3)	–	32.4 (31.0, 34.0)	–

*Resistance indices (RI) were calculated as the ratio between the LD_{50} (or GR_{50}) of the resistant (P3) population compared with the mean LD_{50} (or GR_{50}) values of two susceptible populations (S1 and S2). The recommended field rate of 2,4-D for POST treatment of oriental mustard in crops in South Australia is 600 g a.e. ha^{-1}.

significant difference in absorption of [^{14}C]2,4-D with time after treatment with more absorption at 72 HAT than at 24 HAT (Table 2). Absorption of [^{14}C]2,4-D increased gradually from 50% at 24 HAT to 70% at 48 HAT but did not increase further at 72 HAT. There were no significant differences among populations in absorption of [^{14}C]2,4-D (Table 2).

Unlike absorption, there was a significant difference in the distribution of [^{14}C]2,4-D in resistant populations compared with susceptible populations. The two resistant (P2 and P13) populations retained a significantly greater amount of [^{14}C]2,4-D in treated leaves at all three sampling times than the susceptible (S1 and S2) populations (Table 2). The distribution of 2,4-D was not different between the two resistant (P2 and P13) populations, or between the two susceptible (S1 and S2) populations individually, suggesting that this difference in 2,4-D distribution was correlated with resistance. For the resistant populations (P2 and P13), [^{14}C]2,4-D present in the treated leaf gradually decreased from ~ 90% of that absorbed at 24 HAT to ~ 76% of that absorbed at 72 HAT. By contrast, in the susceptible populations the amount of [^{14}C]2,4-D present in the treated leaf in the susceptible populations reduced sharply from ~ 80% of that absorbed at 24 HAT to <35% of that absorbed at 72 HAT.

The [^{14}C]2,4-D translocated from the treated leaves accumulated mainly in the rest of the shoot with smaller amounts in the roots (Table 2). At all time points, the susceptible populations accumulated significantly more 2,4-D in both the rest of the shoots and the roots than did the resistant populations. As time after treatment increased, the difference between resistant and susceptible populations in the percentage of 2,4-D accumulated in the rest of the shoot and the roots increased.

3.4 Evaluation of F_1 populations and segregation pattern of F_2 populations

Pods from the crosses were harvested from the susceptible S1 and resistant P2 and P13 parent plants. When screened with 2,4-D at 200 g ha^{-1}, all the seedlings from self-pollinated seeds of the susceptible plants showed severe epinasty and died 28 DAT. By contrast, all the seedlings from self-pollinated seeds of the resistant individuals showed little or no visible damage. All F_1 seedlings from seed set collected from the resistant parents survived with little or no damage. Seedlings of the F_1 seed set on the susceptible parent (R♂ × S♀) also survived 2,4-D treatment with little or no damage, showing that crossing had been successful. Sixteen F_1 plants from eight P2♂ × S♀ crosses and eight P13♂ × S♀ crosses were allowed to self-pollinate to produce F_2 families.

The F_2 plants of both populations segregated when treated with 2,4-D at 200 g ha^{-1}. For the P2♂ × S♀ crosses, the response of the eight F_2 families were identical (homogeneity $P = 0.94$; 7 d.f.) (Table 3). Similarly, the response of the eight F_2 families from the P13♂ × S♀ crosses were the same (homogeneity $P = 0.90$; 7 d.f.) (Table 4). These results indicate that the parents of the crosses investigated were likely homozygous for the resistance trait, a feature expected in a highly self-pollinating species such as oriental mustard.[35,37] The pattern of segregation was consistent with the model of a single dominant gene. For the phenotypes, the segregation of F_2 plants with high levels of damage and those with little or no damage fitted the expected 3: 1 ratio for a single dominant allele for both P2♂ × S♀ and P13♂ × S♀ crosses (Tables 3 and 4).

Dose–response experiments with F_2 seedlings showed that the responses of F_2 populations (P2♂ × S♀ and P13♂ × S♀) were intermediate between the resistant and susceptible populations (Fig. 2). The dose–response curve in each F_2 population showed a single step, with survival declining to ~ 75% at 75 g ha^{-1} with

Table 2. [^{14}C]2,4-D absorbed and distribution of absorbed radiolabel from the treated leaf to other plant parts of two susceptible (S1 and S2) and two resistant (P2 and P13) populations of oriental mustard at 24, 48 and 72 h after treatment (HAT). Means (± SE) within a column followed by the same letter are not significantly different (Fisher's protected LSD test, $P \leq 0.05$)

Harvested time (HAT)	Population	Absorption (% applied)	Distribution (% absorbed)		
			Treated leaf	Rest of shoot	Root
24	P2	55.0 ± 3.3 a	88.7 ± 1.9 b	10.7 ± 1.8 a	0.7 ± 0.2 a
	P13	54.8 ± 3.0 a	91.4 ± 1.4 b	7.8 ± 1.3 a	0.8 ± 0.2 a
	S1	61.6 ± 3.4 a	80.6 ± 1.1 a	17.5 ± 1.1 b	1.9 ± 0.4 b
	S2	54.9 ± 3.6 a	76.8 ± 3.0 a	20.7 ± 3.3 b	2.5 ± 0.4 b
48	P2	72.1 ± 0.5 a	78.1 ± 0.9 b	20.5 ± 0.8 a	1.40 ± 0.1 a
	P13	71.8 ± 1.0 a	79.3 ± 0.8 b	19.7 ± 0.8 a	0.96 ± 0.2 a
	S1	69.5 ± 0.6 a	61.2 ± 1.7 a	35.6 ± 1.5 b	3.22 ± 0.6 b
	S2	70.2 ± 0.5 a	59.9 ± 1.1 a	36.9 ± 1.1 b	3.20 ± 0.3 b
72	P2	71.7 ± 1.7 a	77.5 ± 2.4 b	20.5 ± 2.3 a	2.0 ± 0.2 a
	P13	75.3 ± 1.4 a	76.8 ± 2.2 b	21.6 ± 2.2 a	1.6 ± 0.2 a
	S1	71.0 ± 1.8 a	33.6 ± 1.8 a	60.5 ± 2.2 b	5.9 ± 1.5 b
	S2	76.1 ± 3.1 a	31.8 ± 3.9 a	63.6 ± 3.6 b	4.6 ± 0.8 b

Table 3. Segregation for resistance to 200 g ha^{-1} 2,4-D in F_2 populations generated from crosses (P2♂ × S♀) between resistant P2 and susceptible S1 oriental mustard plants

Family	Treated	Dead*	Alive†	*G*-statistic	*P*
P2	203	0	203		
S1	209	209	0		
P2 (1)	198	54	144	0.534	0.465
P2 (2)	209	55	154	0.190	0.663
P2 (3)	203	54	149	0.273	0.601
P2 (4)	209	53	156	0.014	0.905
P2 (5)	211	52	159	0.014	0.905
P2 (6)	207	56	151	0.456	0.499
P2 (7)	203	56	147	0.707	0.401
P2 (8)	210	55	155	0.157	0.692
Total	1650	435	1215	1.617	0.204
Homogeneity				2.345	0.938

*Plants with no new growth and severe epinasty recorded as dead or susceptible.
†Plants with new green leaf tissue recorded as alive or resistant.

Table 4. Segregation for resistance to 200 g ha^{-1} 2,4-D in F_2 populations generated from crosses between resistant P13 and susceptible S1 (P13♂ × S♀) oriental mustard plants

Family	Treated	Dead*	Alive†	*G*-statistic	*P*
P13	204	0	204		
S1	212	212	0		
P13 (1)	202	56	146	0.779	0.378
P13 (2)	211	53	158	0.002	0.968
P13 (3)	213	59	154	0.807	0.369
P13 (4)	200	48	152	0.107	0.743
P13 (5)	209	57	152	0.563	0.453
P13 (6)	206	55	151	0.312	0.577
P13 (7)	205	51	154	0.002	0.968
P13 (8)	195	52	143	0.284	0.594
Total	1641	431	1210	1.384	0.239
Homogeneity				2.856	0.898

*Plants with no new growth and severe epinasty recorded as dead or susceptible.
†Plants with new green leaf tissue recorded as alive or resistant.

no change until the herbicide rate increased above 1200 g ha^{-1} (Fig. 2). This type of response is an indication of segregation for a single dominant allele for resistance in the F_2 populations.[41] To confirm this hypothesis, a model for the response for a single dominant allele was estimated (dotted line in Fig. 2). The F_2 response was similar to this model at most rates of 2,4-D (Fig. 2).

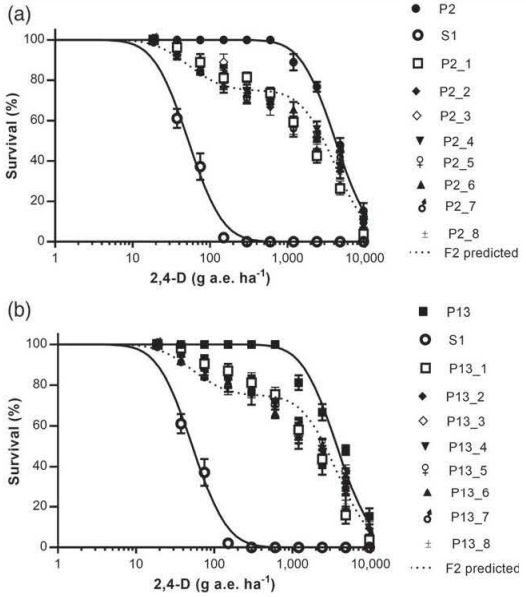


Figure 2. Dose–response of susceptible S1 (○), resistant P2 (●), and eight F_2 of the crosses P2♂ × S♀ (△, ◆, ◇, ▼, ♀, ▲, ♂ and ±) (a), and S1 (○), resistant P13 (■) and eight F_2 of the crosses P13♂ × S♀ (△, ◆, ◇, ▼, ♀, ▲, ♂ and ±) (b) of oriental mustard treated with 2,4-D at different rates (0 to 9600 g ha^{-1}). The dotted line is the predicted response for a single dominant gene at all doses of the 2,4-D. Data points are means ±95% confidence intervals for six replicates (three replicates × two runs) and the vertical bars are standard error of the mean.

4 DISCUSSION

4.1 Whole plant dose response to 2,4-D in oriental mustard

Despite its worldwide use for over 70 years, the evolution of auxinic herbicide resistance has been low in comparison with other herbicide modes of action.[42,43] Since the first case of resistance to auxinic herbicides in *Daucus carota* in 1957, the number of weed species resistant to auxinic herbicides worldwide has remained relatively low (34) compared with the number of weed species that have evolved resistance to ALS-inhibiting herbicides (159).[14,44] Similarly, in Australia, resistance to auxinic herbicides has been confirmed in only a few weed species, including *R. raphanistrum*,[16,45] annual sowthistle (*Sonchus oleraceus*), cape weed (*Arctotheca calendula*)[14] and oriental mustard (*S. orientale*).[32] The findings from the current dose–response experiments showed that the recommended field rate of 2,4-D for the control of broadleaf weeds in Australia (600 g ha^{-1}) is more than adequate for the control of susceptible oriental mustard populations (LD_{50} < 40 g ha^{-1}). By contrast, resistant populations required much higher doses (LD_{50} of 2450 to 2960 g ha^{-1}) than the recommended field rate for effective control, equating to 67- to 81-fold more resistance than the susceptible populations (Table 1). This result confirmed that the P2 and P13 oriental mustard populations in this study are highly resistant to 2,4-D, making this herbicide ineffective for their control.

4.2 Gene sequences, absorption and translocation

A previous study had showed that mutations in the ABP, TIR1 and AFB5, a homologue of the TIR1 gene could provide resistance to auxinic herbicides in *A. thaliana*[26] and *S. arvesis*.[27] However, in the current study, no mutation was detected in the targeted gene fragment of the ABP, AFB5 and TIR1 genes of oriental mustard when gene sequences of the resistant (P2 and P13) and susceptible (S1) populations were compared. These indicate that resistance to 2,4-D in the oriental mustard populations investigated is unlikely to be due to target-site mutations within these genes. Another potential target site is SKP2A, a protein that binds auxin and is involved in cell division in roots.[46] A mutation in SKP2A that

abolishes auxin binding in *A. thaliana* leads to a phenotype with auxin-resistant root growth.[12] We did not sequence the gene for this protein in oriental mustard; however, no differences in root phenotype were observed in the resistant biotypes (data not shown), which suggests that mutations in SPK2A may not be involved.

Resistance to herbicides can also be conferred by non-target site mechanism(s), such as a reduction in herbicide translocation or increased herbicide metabolism.[44] Where resistance is conferred by a reduction in herbicide translocation, the availability of herbicides at the target site in the plant is reduced, enabling an individual plant to survive the herbicide treatment.[44,47] In the current study, there was no difference in absorption of [^{14}C]2,4-D between resistant and susceptible populations. However, there was a significant reduction in the amount of translocation of [^{14}C]2,4-D from the treated leaf in both resistant populations compared with the two susceptible populations (Table 2). Translocation of 2,4-D from the treated leaf was relatively slow in the susceptible populations, with <25% of the absorbed herbicide translocated out of the treated leaves at 24 HAT. By contrast, translocation was even slower in the resistant population with less than 12% of the absorbed herbicide translocated out of the treated leaves at 24 HAT. The differences in herbicide translocation widened further at 72 HAT when more than 66% 2,4-D had been translocated out of the treated leaves in the susceptible populations compared with less than 24% translocation in the resistant populations. Both resistant populations responded similarly, suggesting that this lack of translocation of the herbicide is the likely mechanism of resistance in this species. Previous studies in *R. raphanistrum*,[16] *G. tetrahit*,[21] *P. rhoeas*[23] and *L. serriola*[24] also found the 2,4-D-resistant biotypes had significantly lower herbicide translocation out of the treated leaves.

In 2,4-D studies on *R. raphanistrum*[16] and *P. rhoeas*,[23] most of the herbicide was retained in the treated leaf of resistant biotypes (> 97% at 96 HAT), compared with ~ 77% in oriental mustard, despite these species having lower resistance to 2,4-D. Compared with *P. rhoeas*,[23] more 2,4-D was translocated out of the treated leaf for both susceptible and resistant biotypes of oriental mustard. This suggests there may be differences between species in translocation of 2,4-D. *R. raphanistrum*[16] had higher translocation of 2,4-D in the susceptible plants compared with oriental mustard and virtually none in the resistant plants. Light intensity was much lower for the *R. raphanistrum*[16] study compared with our oriental mustard study (550 versus 90 µmol m^{-2} s^{-1}), which may have influenced translocation of this herbicide. By contrast, 2,4-D-resistant *L. serriola*[24] had similar amounts of translocation of 2,4-D to that found in this study. Overall, these data suggest that there may be differences in 2,4-D translocation patterns in 2,4-D-resistant plants between species and/or between growth conditions. It also suggests that the mechanism for reduced 2,4-D translocation may vary among species.

4.3 Inheritance of 2,4-D resistance in oriental mustard

In herbicide resistance, modes of inheritance include: (1) nuclear inheritance, where the resistance-conferring alleles are transmitted through pollen and ovules; and (2) cytoplasmic inheritance, where the transmission of the chloroplast resistant gene occurs through the ovules.[48] A majority of herbicide resistance cases are inherited via nuclear genes; the main exception being resistance to triazine herbicides, which has maternal or cytoplasmic inheritance.[49,50] Moreover, most resistant traits in weeds are controlled by semi-dominant or dominant alleles.[51,52] Traits controlled by dominant alleles will provide resistance in both homozygous and heterozygous states.[53,54] In the current study, F_1 seeds harvested from susceptible parents survived treatment with 2,4-D at 200 g ha^{-1}, which indicated that resistance to 2,4-D in oriental mustard was transmitted in pollen. In addition, dose–response analysis of the F_2 populations (Fig. 2) showed a high level of dominance over susceptibility, with heterozygotes having a similar response to resistant parents, and the F_2 populations fitted well to the one gene segregation model. In theory, this pattern of resistance means that evolution of resistance to 2,4-D in oriental mustard is likely to be quicker than in cases where resistance is conferred by complex genetic inheritance.[55] Results of this study support findings in *S. arvensis*,[27,55] *L. serriola*,[24] *S. orientale*[35] and *R. raphanistrum*,[57] where inheritance of 2,4-D resistance was managed by a single gene with high levels of dominance.

This study confirmed high levels of resistance to 2,4-D in two oriental mustard populations, which is controlled by a single nuclear gene with high levels of dominance. It is likely that this resistance is the result of reduced herbicide translocation out of the treated leaf. Although the metabolism of 2,4-D was not investigated in this study, the fact that a single gene was responsible for resistance suggests that other mechanisms are not contributing to resistance in these populations of oriental mustard. Because resistance to 2,4-D in the oriental mustard populations resulted in high levels of resistance to 2,4-D and was a dominant trait, higher herbicide rates would be unlikely to improve weed control. Therefore, proactive and integrated measures for resistance management are critical to slow the spread and control these 2,4-D-resistant oriental mustard populations.[58,59] These strategies include changes in the use of herbicide modes of action, and adoption of mechanical weed control, herbicide mixtures[60] in combination with other options such as maximising crop competition.[61,62]

ACKNOWLEDGEMENTS

The authors would like to acknowledge the Grains Research and Development Corporation (GRDC), the University of Adelaide and Vietnamese Government for funding this project. We also thank Weihua Long, Alicia Merriam, Ruwan Lenorage and Geetha Velappan for their technical support.

REFERENCES

1 Kelley KB and Riechers DE, Recent developments in auxin biology and new opportunities for auxinic herbicide research. *Pestic Biochem Physiol* **89**:1–11 (2007).
2 Mithila J, Hall JC, Johnson WG, Kelley KB and Riechers DE, Evolution of resistance to auxinic herbicides: historical perspectives, mechanisms of resistance, and implications for broadleaf weed management in agronomic crops. *Weed Sci* **59**:445–457 (2011).
3 Peterson GE, The discovery and development of 2,4-D. *Agric Hist* **41**:243–254 (1967).
4 Chinalia FA, Regali-Seleghin MH and Correa EM, 2,4-D toxicity: cause, effect and control. *Terr Aquatic Environ Toxicol* **1**:24–33 (2007).
5 Holt JS, Powles SB and Holtum JA, Mechanisms and agronomic aspects of herbicide resistance. *Annu Rev Plant Biol* **44**:203–229 (1993).
6 Grossmann K, Auxin herbicides: current status of mechanism and mode of action. *Pest Manag Sci* **66**:113–120 (2010).
7 Shi J-H and Yang Z-B, Is ABP1 an auxin receptor yet? *Mol Plant* **4**:635–640 (2011).
8 Thomas C, Meyer D, Wolff M, Himber C, Alioua M and Steinmetz A, Molecular characterization and spatial expression of the sunflower ABP1 gene. *Plant Mol Biol* **52**:1025–1036 (2003).
9 Kepinski S and Leyser O, The Arabidopsis F-box protein TIR1 is an auxin receptor. *Nature* **435**:446–451 (2005).

10 Dharmasiri N, Dharmasiri S and Estelle M, The F-box protein TIR1 is an auxin receptor. *Nature* **435**:441–445 (2005).
11 Ruegger M, Dewey E, Gray WM, Hobbie L, Turner J and Estelle M, The TIR1 protein of Arabidopsis functions in auxin response and is related to human SKP2 and yeast Grr1p. *Gene Devel* **12**:198–207 (1998).
12 Jurado S, Abraham Z, Manzano C, López-Torrejón G, Pacios LF and Del Pozo JC, The Arabidopsis cell cycle F-box protein SKP2A binds to auxin. *Plant Cell* **22**:3891–3904 (2010).
13 Hilton H, Herbicide tolerant strains of weeds, in *Hawaiian Sugar Planters Association Annual Report*. Hawaiian Sugar Planters Association, Honolulu, HI, pp. 69–72 (1957).
14 Heap I, *The International Survey of Herbicide Resistant Weeds*. [Online]. Available: http://weedscience.org [18 September 2017].
15 Christoffoleti PJ, Figueiredo MRAd, Peres LEP, Nissen S and Gaines T, Auxinic herbicides, mechanisms of action, and weed resistance: a look into recent plant science advances. *Sci Agricola* **72**:356–362 (2015).
16 Goggin DE, Cawthray GR and Powles SB, 2,4-D resistance in wild radish: reduced herbicide translocation via inhibition of cellular transport. *J Exp Bot* **67**:3223–3235 (2016).
17 Kohler EA, Throssell CS and Reicher ZJ, 2,4-D rate response, absorption, and translocation of two ground ivy (*Glechoma hederacea*) populations. *Weed Technol* **18**:917–923 (2004).
18 Kreuz K, Tommasini R and Martinoia E, Old enzymes for a new job (herbicide detoxification in plants). *Plant Physiol* **111**:349–353 (1996).
19 Preston C, Herbicide resistance in weeds endowed by enhanced detoxification: complications for management. *Weed Sci* **52**:448–453 (2004).
20 Heap I. Herbicide resistant weeds, in *Integrated Pest Management*, ed. by Peshin R and Dhawan AK. Springer, Dordtrecht, pp. 281–301 (2014).
21 Weinberg T, Stephenson GR, McLean MD and Hall JC, MCPA (4-chloro–2-ethylphenoxyacetate) resistance in hemp-nettle (*Galeopsis tetrahit* L.). *J Agric Food Chem* **54**:9126–9134 (2006).
22 Torra J, Rojano-Delgado AM, Rey-Caballero J, Royo-Esnal A, Salas ML and De Prado R, Enhanced 2,4-D metabolism in two resistant *Papaver rhoeas* populations from Spain. *Front Plant Sci* **8**:1584 (2017).
23 Rey-Caballero J, Menendez J, Gine-Bordonaba J, Salas M, Alcantara R and Torra J, Unravelling the resistance mechanisms to 2,4-D (2,4-dichlorophenoxyacetic acid) in corn poppy (*Papaver rhoeas*). *Pest Biochem Physiol* **133**:67–72 (2016).
24 Riar DS, Burke IC, Yenish JP, Bell J and Gill K, Inheritance and physiological basis for 2,4-d resistance in prickly lettuce (*Lactuca serriola* L.). *J Agric Food Chem* **59**:9417–9423 (2011).
25 Gleason C, Foley RC and Singh KB, Mutant analysis in Arabidopsis provides insight into the molecular mode of action of the auxinic herbicide dicamba. *PLoS One* **6**:e17245 (2011).
26 Walsh TA, Neal R, Merlo AO, Honma M, Hicks GR, Wolff K *et al.*, Mutations in an auxin receptor homolog AFB5 and in SGT1b confer resistance to synthetic picolinate auxins and not to 2,4-dichlorophenoxyacetic acid or indole-3-acetic acid in Arabidopsis. *Plant Physiol* **142**:542–552 (2006).
27 Zheng H-g and Hall JC, Understanding auxinic herbicide resistance in wild mustard: physiological, biochemical, and molecular genetic approaches. *Weed Sci* **49**:276–281 (2001).
28 Mithila J and Hall J, Comparison of ABP1 over-expressing Arabidopsis and under-expressing tobacco with an auxinic herbicide-resistant wild mustard (*Brassica kaber*) biotype. *Plant Sci* **169**:21–28 (2005).
29 Chauhan BS, Gill G and Preston C, Influence of environmental factors on seed germination and seedling emergence of oriental mustard (*Sisymbrium orientale*). *Weed Sci* **54**:1025–1031 (2006).
30 McGillion T and Storrie A, *Integrated weed management in Australian cropping systems – a training resource for farm advisors*. CRC for Australian Weed Management, Adelaide, SA (2006).
31 Wilding JL, Barnett AG and Amor RL, *Crop Weeds*. Inkata Press, Melbourne (1986).
32 Preston C, Dolman FC and Boutsalis P, Multiple resistance to acetohydroxyacid synthase–inhibiting and auxinic herbicides in a population of oriental mustard (*Sisymbrium orientale*). *Weed Sci* **61**:185–192 (2013).
33 Boutsalis P and Powles S, Resistance of dicot weeds to acetolactate synthase (ALS)-inhibiting herbicides in Australia. *Weed Res* **35**:149–155 (1995).
34 Dang HT, Malone JM, Boutsalis P, Gill G and Preston C, Identification of a target-site mutation conferring resistance to triazine herbicides in oriental mustard (*Sisymbrium orientale* L.) from Australia. *Weed Biol Manag* **17**:153–160 (2017).
35 Preston C and Malone JM, Inheritance of resistance to 2,4-D and chlorsulfuron in a multiple-resistant population of *Sisymbrium orientale*. *Pest Manag Sci* **71**:1523–1528 (2015).
36 Boutsalis P, Gill GS and Preston C, Incidence of herbicide resistance in rigid ryegrass (*Lolium rigidum*) across southeastern Australia. *Weed Technol* **26**:391–398 (2012).
37 Boutsalis P, *Resistance to acetolactate synthase-inhibiting herbicides in* Sonchus oleraceus, Sisymbrium orientale *and* Brassica tournefortii, PhD dissertation, University of Adelaide (1996).
38 Sakuma M, Probit analysis of preference data. *Appl Entomol Zool* **33**:339–347 (1998).
39 Hoagland DR and Arnon DI, *The water-culture method for growing plants without soil (2nd edn). California Agricultural Experiment Station Circular 347*, University of California, Berkeley (1950).
40 Mantel N, Chi-square tests with one degree of freedom; extensions of the Mantel-Haenszel procedure. *J Am Stat Assoc* **58**:690–700 (1963).
41 Tabashnik BE, Determining the mode of inheritance of pesticide resistance with backcross experiments. *J Econ Entomol* **84**:703–712 (1991).
42 Ashworth MB, Walsh MJ, Flower KC and Powles SB, Recurrent selection with reduced 2,4-D amine doses results in the rapid evolution of 2,4-D herbicide resistance in wild radish (*Raphanus raphanistrum* L.). *Pest Manag Sci* **72**:2091–2098 (2016).
43 Schulz B and Segobye K, 2,4-D transport and herbicide resistance in weeds. *J Exp Bot* **67**:3177–3179 (2016).
44 Powles SB and Yu Q, Evolution in action: plants resistant to herbicides. *Annu Rev Plant Biol* **61**:317–347 (2010).
45 Walsh MJ, Powles SB, Beard BR, Parkin BT and Porter SA, Multiple-herbicide resistance across four modes of action in wild radish (*Raphanus raphanistrum*). *Weed Sci* **52**:8–13 (2004).
46 Jurado S, Díaz-Triviño S, Abraham Z, Manzano C, Gutierrez C and Pozo Cd, SKP2A, an F-box protein that regulates cell division, is degraded via the ubiquitin pathway. *Plant J* **53**:828–841 (2008).
47 Ferreira JF and Reddy KN, Absorption and translocation of glyphosate in *Erythroxylum coca* and *E. novogranatense*. *Weed Sci* **48**:193–199 (2000).
48 Rao VS. *Transgenic Herbicide Resistance in Plants*. CRC Press, Boca Raton, FL (2014).
49 Jasieniuk M, Brule-Babel AL and Morrison IN, The evolution and genetics of herbicide resistance in weeds. *Weed Sci* **44**:176–193 (1996).
50 Gressel J. Model and genetics of herbicide resistace in plants, in *Pesticide Resistance: Strategies and Tactics for Management*. National Academy Press, Washington DC, pp. 54–73 (1986).
51 Darmency H, Genetics of herbicide resistance in weeds and crops, in *Herbicide Resistance in Plants: Biology and Biochemistry*, ed. by Powles SB and Holtum JAM. Lewis, Boca Ratob, FL, pp. 263–298 (1994).
52 Tardif F, Preston C, Holtum J and Powles S, Resistance to acetyl-coenzyme acarboxylase-inhibiting herbicides endowed by a single major gene encoding a resistant target site in a biotype of *Lolium rigidum*. *Funct Plant Biol* **23**:15–23 (1996).
53 Gasquez J, Genetics of herbicide resistance within weeds. Factors of evolution, inheritance and fitness, in *Weed and Crop Resistance to Herbicides*, ed. by De Prado R, Jorrín J and García-Torres L. Springer, Dordtrecht, pp. 181–189 (1997).
54 Christoffers MJ, Genetic aspects of herbicide-resistant weed management. *Weed Technol* **13**:647–652 (1999).
55 Jasieniuk M and Maxwell BD, Population genetics and the evolution of herbicide resistance in weeds. *Phytoprotection* **75**:25–35 (1994).
56 Jugulam M, McLean MD and Hall JC, Inheritance of picloram and 2,4-D resistance in wild mustard (*Brassica kaber*). *Weed Sci* **53**:417–423 (2005).
57 Busi R and Powles SB, Inheritance of 2,4-D resistance traits in multiple herbicide- resistant *Raphanus raphanistrum* populations. *Plant Sci* **257**:1–8 (2017).

58 Rey-Caballero J, Royo-Esnal A, Recasens J, Gonzalez I and Torra J, Management options for multiple herbicide-resistant corn poppy (*Papaver rhoeas*) in Spain. *Weed Sci* **65**:295–304 (2017).
59 Beckie HJ, Beneficial management practices to combat herbicide-resistant grass weeds in the northern Great Plains. *Weed Technol* **21**:290–299 (2007).
60 Lagator M, Vogwill T, Mead A, Colegrave N and Neve P, Herbicide mixtures at high doses slow the evolution of resistance in experimentally evolving populations of *Chlamydomonas reinhardtii*. *New Phytol* **198**:938–945 (2013).
61 Beckie HJ, Johnson EN, Blackshaw RE and Gan Y, Weed suppression by canola and mustard cultivars. *Weed Technol* **22**:182–185 (2008).
62 Walsh M, Newman P and Powles S, Targeting weed seeds in-crop: a new weed control paradigm for global agriculture. *Weed Technol* **27**:431–436 (2013).

CHAPTER 4

THE MECHANISM OF DIFLUFENICAN RESISTANCE AND ITS INHERITANCE IN ORIENTAL MUSTARD (*SISYMBRIUM ORIENTALE* L.) FROM AUSTRALIA

Hue T Dang, Jenna M Malone, Peter Boutsalis, Gurjeet Gill and Christopher Preston

School of Agriculture, Food and Wine, University of Adelaide

PMB1, Glen Osmond, South Australia 5064, Australia

Pest Manag. Sci. First published: 26 February 2018

http://onlinelibrary.wiley.com/doi/10.1002/ps.4858/full

(with permission from the Pest Management Science and John Wiley and Sons)

JOHN WILEY AND SONS LICENSE
TERMS AND CONDITIONS

Feb 26, 2018

This Agreement between Mrs. Hue Dang ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	4296400279778
License date	Feb 26, 2018
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	Pest Management Science
Licensed Content Title	The mechanism of diflufenican resistance and its inheritance in oriental mustard (Sisymbrium orientale L.) from Australia
Licensed Content Author	Hue Thi Dang,Jenna Moira Malone,Peter Boutsalis,Gurjeet Gill,Christopher Preston
Licensed Content Date	Feb 26, 2018
Licensed Content Pages	1
Type of Use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Electronic
Portion	Full article
Will you be translating?	No
Title of your thesis / dissertation	Investigation of Herbicide Resistance in Oriental Mustard (Sisymbrium orientale L.) in Australia
Expected completion date	Mar 2018
Expected size (number of pages)	160
Requestor Location	Mrs. Hue Dang School of Agriculture, Food and Wine PMB 1, Glen Osmond Adelaide, South Australia 5064 Australia Attn: Mrs. Hue Dang
Publisher Tax ID	EU826007151
Total	**0.00 USD**

Statement of Authorship

Title of Paper	The Mechanism of Diflufenican Resistance and its Inheritance in Oriental Mustard (Sisymbrium orientale L.) from Australia
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Dang, H. T., Malone, J. M., Boutsalis, P., Gill, G. and Preston, C. (2018), The Mechanism of Diflufenican Resistance and its Inheritance in Oriental Mustard (*Sisymbrium orientale* L.) from Australia. Pest. Manag. Sci.. Accepted Author Manuscript. doi:10.1002/ps.4858.

Principal Author

Name of Principal Author (Candidate)	Hue Thi Dang		
Contribution to the Paper	Planned the study, conducted all experiments, analyzed and interpreted data and wrote manuscript.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	23/02/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Peter Boutsalis		
Contribution to the Paper	Supervised the whole plant studies and helped with data interpretation		
Signature		Date	27 Feb 2018

Name of Co-Author	Jenna Moira Malone		
Contribution to the Paper	Supervised the molecular work, data interpretation and edited manuscript		
Signature		Date	26/2/18

Name of Co-Author	Christopher Preston		
Contribution to the Paper	Supervised development of research, data interpretation and analysis, and edited manuscript		
Signature		Date	23/02/2018

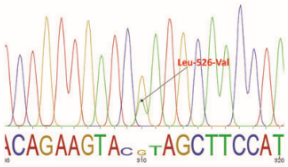
Name of Co-Author	Gurjeet Gill		
Contribution to the Paper	Supervised development of research, data interpretation and analysis, and edited manuscript		
Signature		Date	23. 2. 2018

Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.4858

Research Article

Resistance to diflufenican in oriental mustard is confirmed as being attributable to the Leu-526-Val mutation in phytoene desaturase. The inheritance of resistance is conferred by a highly dominant single gene.



The mechanism of diflufenican resistance and its inheritance in oriental mustard (*Sisymbrium orientale* L.) from Australia

Hue Thi Dang, Jenna Moira Malone, Peter Boutsalis, Gurjeet Gill and Christopher Preston

Research Article

Received: 9 November 2017 Revised: 22 December 2017 Accepted article published: 13 January 2018 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.4858

The mechanism of diflufenican resistance and its inheritance in oriental mustard (*Sisymbrium orientale* L.) from Australia

Hue Thi Dang,* Jenna Moira Malone, Peter Boutsalis, Gurjeet Gill and Christopher Preston

Abstract

BACKGROUND: An oriental mustard population (P3) collected near Quambatook, Victoria was identified as being resistant to diflufenican by screening with the field rate (200 g a.i. ha^{-1}) of the herbicide. The mechanism(s) of diflufenican resistance and its inheritance in this population were therefore investigated.

RESULTS: Dose-response experiments confirmed that population P3 was 140-fold more resistant to diflufenican than susceptible populations, as determined by the comparison of 50% lethal (LD_{50}) values. The phytoene desaturase (*PDS*) gene from five individuals each of the S1 [susceptible (S)] and P3 [resistant (R)] populations was sequenced, and a substitution of valine for leucine at position 526 (Leu-526-Val) was detected in all five individuals of P3, but not in the S1 population. Inheritance studies showed that diflufenican resistance is encoded in the nuclear genome and is dominant, as the response to diflufenican at 200 g a.i. ha^{-1} of F_1 families was equivalent to that of the resistant biotype. The segregation of F_2 phenotypes fitted a 3:1 inheritance model. Segregation of 42 F_2 individuals by genotype sequencing fitted a 1:2:1 (ss:Rs:RR) ratio.

CONCLUSION: Resistance to diflufenican in oriental mustard is conferred by the Leu-526-Val mutation in the *PDS* gene. Inheritance of resistance is managed by a single gene with high levels of dominance.

Keywords: diflufenican; phytoene desaturase; PDS; single dominant gene; *Sisymbrium orientale* L.; target-site mutation

1 INTRODUCTION

The intensive use of herbicides in the last five decades has resulted in widespread evolution of herbicide resistance in many weed species in Australian agriculture. Worldwide, resistance has been confirmed in 485 biotypes of 252 different weed species (146 dicots and 106 monocots) occurring in 92 crops in 69 countries.[1] Resistance has been confirmed to 163 different herbicides with 23 of the 26 different modes of action, according to the Weed Science Society of America (WSSA) classification.

Oriental mustard (*Sisymbrium orientale*) is a troublesome broadleaf weed of field crops in Australia, especially winter crops such as cereals, chickpea (*Cicer arietinum*), canola (*Brassica napus*) and field pea (*Pisum sativum*).[2,3] Oriental mustard causes crop yield loss as a result of competition for resources with the crops, and can also cause difficulties in crop harvest.[2] The seed of oriental mustard has short dormancy and can germinate at any time of the year under favorable conditions.[4,5] However, oriental mustard seed requires light exposure for germination and is therefore unable to geminate if buried.[3] Recently, populations of oriental mustard have been confirmed to be resistant to several herbicides including acetolactate synthase (ALS) inhibitors, synthetic auxins, carotenoid biosynthesis inhibitors and photosystem II inhibitors.[6–9]

Diflufenican is a pyridinecarboxamide herbicide that inhibits phytoene desaturase (PDS) in the carotenoid pathway. It was developed in 1979 and has been widely used in agriculture since the mid-1980s.[10] Diflufenican has been used as both a pre- and early post-emergent herbicide for the selective control of certain broadleaf weeds, especially Brassicaceae, in winter cereals.[11,12] The main target for diflufenican in plants is the enzyme PDS, a nuclear-encoded protein that is active in the chloroplasts, at the site of carotenoid synthesis.[13,14] When enzymes involved in carotenoid biosynthesis are inhibited, degradation of chlorophyll and the destruction of the chloroplast membranes occur. This causes pronounced bleaching symptoms and necrosis of the tissues of susceptible plants, leading to plant death.[15,16]

The first case of resistance to PDS inhibitors in higher plants was confirmed in hydrilla (*Hydrilla verticillata*) when some biotypes were identified as being resistant to fluridone in the USA in 2002.[17] Resistance to fluridone in hydrilla populations was conferred by one of three independent somatic mutations in the *PDS* gene at the arginine 304 (Arg304) codon.[17,18] Resistance has also been confirmed in some other weed species such as eastern groundsel

* *Correspondence to: HT Dang, School of Agriculture, Food and Wine, University of Adelaide, PMB1, Glen Osmond, South Australia 5064, Australia. E-mail: huethi.dang@adelaide.edu.au*

School of Agriculture, Food and Wine, University of Adelaide, PMB1, Glen Osmond, South Australia, Australia

(*Senecio vernalis*) and oriental mustard (*Sisymbrium orientale*); however, the mechanism of resistance to the PDS-inhibiting herbicides in these species is not known.[9]

Resistance to ALS-inhibiting herbicides in oriental mustard in Australia was first reported in the 1990s.[7] Since then, farmers have relied on PDS inhibitors and phenoxy herbicides to control this weed in crops. Despite increasing recent concern about resistance to PDS inhibitors in oriental mustard,[9] there is little information available about the mechanism(s) and inheritance of resistance to carotenoid biosynthesis inhibitors. This study aimed to quantify the level of resistance to diflufenican, and the mechanism(s) as well as the mode of inheritance of diflufenican resistance in oriental mustard. The study also aimed to determine whether diflufenican resistance in oriental mustard was associated with any known mutation in the *PDS* gene.

2 MATERIALS AND METHODS

2.1 Plant material

A population of oriental mustard (P3) collected from a crop of field peas near Quambatook, Victoria in 2011 was suspected to be resistant to diflufenican. In a screening experiment conducted in 2013 (data not shown), this population survived diflufenican at 200 g a.i. ha^{-1} (Brodal 500 g a.i. L^{-1}; Bayer Crop Science, Victoria, Australia), the field rate to control oriental mustard in Australia. Two known susceptible populations of oriental mustard, S1 and S2, were used in this study as susceptible controls. S1 is a known standard susceptible population collected from an organic field near Roseworthy, South Australia as described in Preston *et al.*[19] The S2 population was collected near Port Kenny, Eyre Peninsula, South Australia. These populations (S1 and S2) were confirmed to be susceptible to all herbicides tested including diflufenican, glyphosate, imazamox, chlorsulfuron, atrazine and 2,4-D at the recommended field rates for post-emergent control of oriental mustard in Australia (data not shown).

To generate homogeneous populations for the current study, five healthy plants of the P3 population that survived diflufenican treatment at 400 g ha^{-1} were transplanted into large pots (25 cm diameter) containing standard potting mix,[20] with one plant per pot. In addition, five non-treated plants from each susceptible population (S1 and S2) were also planted into pots as described above. As oriental mustard tends to self-pollinate,[21] the pots were transferred outdoors, watered and fertilized as required. The plants were placed 50 cm apart and allowed to self-pollinate to produce seeds. Mature seeds from these plants were collected and stored at 10 °C for use in all experiments.

2.2 Seed germination, plant growth and herbicide treatment

Seeds of each population were sown into a seedling nursery tray containing standard potting mix. Seedlings at the one to two true leaf stage were transplanted into small punnet pots (8.5 cm x 9.5 cm x 9.5 cm) (Masrac Plastics, Dry Creek, Australia) containing standard potting mix, with three replicates of nine seedlings per pot for each herbicide treatment. The seedling pots were maintained outdoors during the normal growing season (May to October), watered and fertilized as required. Seedlings were treated with herbicides at the three to four true leaf stage, using a moving-boom laboratory twin nozzle sprayer. The nozzle height of the sprayer was 40 cm above the seedlings with a water volume of 110 L ha^{-1} at a pressure of 250 kPa and speed of 1 m s^{-1}. The number of seedlings in each pot was counted before each herbicide treatment. Control plants were not treated with any herbicide. All experiments were conducted at the Waite Campus, the University of Adelaide, South Australia (34°58′13.5″ S, 138°38′22.7″ E).

2.3 Whole-plant dose–response to diflufenican

In July 2015, a dose–response experiment was conducted on the three oriental mustard populations (P3, S1 and S2). Diflufenican was applied to plants at the three- to four-leaf stage. Seedlings were treated with diflufenican at 0, 6.25, 12.5, 25, 50, 100, 200, 400, 800, 1600 and 3200 g ha^{-1}. Non-ionic surfactant [0.2% (v/v) alcohol alkoxylate; BS1000; Crop Care, Murarrie, Australia] was added to the herbicide solution before spraying. The experiment was repeated in July 2016.

Plant survival was assessed 28 days after treatment (DAT). Plants with new green leaf tissue were recorded as alive, whereas those that displayed no new growth and severe necrosis were recorded as dead. The aboveground parts of the harvested plants were collected and dried in an oven at 65 °C for 72 h, and then weighed. Plant biomass data from the dose–response experiment were converted to percent of non-treated control before regression analysis. The herbicide dose required for 50% mortality [50% lethal dose (LD_{50})] values and their 95% confidence limits were analyzed using an all-or-nothing model and a normal distribution function in PriProbit v.1.63.[22] Probits were back-transformed to percentages for plotting. Dry weight data were analyzed by log-logistic analysis using GraphPad Prism v.6.0 (GraphPad, San Diego, CA, USA) and the herbicide dose required for 50% biomass reduction [50% growth reduction (GR_{50})] values were calculated. Resistance indices (RIs) were calculated as the ratio between the LD_{50} (or GR_{50}) of each population and the mean LD_{50} (or GR_{50}) of the susceptible populations. The experiment was repeated and a two-way analysis of variance (ANOVA) was used to examine the effect of experimental run. Data from the two runs were pooled prior to data analysis if no effect of experimental run was identified.

2.4 Inheritance of resistance to diflufenican

2.4.1 *Generation of F_1 and F_2 seeds*

F_1 families were generated using the method described by Preston and Malone[19,21] with modifications. Two populations, the diflufenican-resistant P3 and the susceptible S1, were selected for the inheritance study. Five survivors of the P3 population at 800 g ha^{-1} and five non-treated plants of the S1 population were transplanted into 25-cm-diameter pots containing standard potting mix, with one plant per pot. The pots were transferred into a heated/cooled (minimum 15 °C; maximum 25 °C) glasshouse under natural light and placed 40 cm apart. They were watered and fertilized as needed. Once flowering had occurred each morning, reciprocal crosses were made by tapping the anthers with pollen of the susceptible or resistant biotype against the stigma of the other biotype using a small paintbrush. Crossing was conducted in the morning from 7:00 am onwards, when the flowers of the pollen donors were fully open. The pollen receptors were bagged immediately after each hand-pollination. Individual flowers of the parental plants were also bagged and allowed to self-pollinate. Three days after fertilization, the bag from each crossed flower was removed and the developing pod was marked by a string-tag. At maturity, pods from crossed and selfed flowers were harvested. Seeds generated from cross-fertilizations were collected separately from each cross and considered a new F_1 family or F_1 population. Seeds from the parents [resistant (R) and susceptible (S) parental plants] and the F_1 seeds were germinated, and

then transplanted into small pots with a density of 5 plants/pot. Seedlings were sprayed with diflufenican at 200 g ha^{-1} to determine the resistance status of the progeny. This rate of herbicide controlled all susceptible plants, but all resistant plants survived. Seedlings from the susceptible parent of each cross that survived the herbicide application were considered to be true F_1 plants and were transplanted into larger pots (25 cm in diameter) and allowed to self-pollinate to produce F_2 seeds. Mature seeds (F_2) from each F_1 individual were collected separately, and these seeds were used in the inheritance studies.

2.4.2 *Segregation and dose–response of the F_2 populations*

To determine whether all F_2 populations investigated had a similar segregation response to diflufenican, a phenotypic segregation test was conducted on the F_2 populations. Seeds of F_2 and parental populations were germinated. A total of 108 seedlings of each population were transplanted into a tray containing potting mix and treated with diflufenican at 200 g ha^{-1} at the three- to four-leaf stage. The homogeneity and segregation of the F_2 phenotypes were tested against a single-gene model with a dominant allele using the *G*-test with Williams's correction as described by Preston and Malone.[19]

A dose–response experiment was also conducted on F_2 populations and parental populations to help determine the number of genes involved in resistance. Susceptible and resistant populations and individuals from six F_2 populations were treated with diflufenican at rates of 0, 6.25, 12.5, 25, 50, 100, 200, 400, 800, 1600 and 3200 g ha^{-1} at the three- to four-leaf stage as described above. There were three replicates for each population at each herbicide rate.

PriProbit[22] and GraphPad Prism v6 were used to analyze the dose–responses of the parental populations and the F_2 populations, respectively. A model of a single dominant gene was created by summing 0.75 (equivalent to 75%) × survival of the resistant population and 0.25 (equivalent to 25%) × survival of the susceptible population. This model was compared with the real responses of the F_2 populations to determine whether the dose–response fitted a single-gene model as predicted. All experiments were conducted twice in the winter growing seasons of 2015 and 2016. As there was no difference between experimental runs, data were pooled prior to analysis.

2.5 DNA and RNA extraction and cDNA synthesis

For DNA extraction, plant material (~100 mg) from the youngest green leaf tissue of resistant P3 and susceptible S1 plants (five individuals per population) was collected. For the resistant population, samples were collected from the five survivors of diflufenican at 800 g ha^{-1} at 28 days after treatment (DAT). For the susceptible population, samples were collected from five plants prior to treatment with diflufenican at the recommended rate to ensure that they were susceptible. In addition, leaf tissue was collected from 42 non-treated individuals of an F_2 population which had clear segregation in the segregation tests. Leaf samples obtained were snap-frozen in liquid nitrogen and stored at −20 °C for further use. An Isolate II Plant DNA kit (Bioline, Alexandria, Australia) was used to extract DNA from 50–100 mg of plant tissue as per the manufacturer's instructions. A spectrophotometric NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, USA) was used to determine the concentration of nucleic acids. DNA was then stored at -20 °C for further analysis.

For RNA extraction, samples of the susceptible and resistant plants were collected as described above. However, in this case, fresh samples were obtained, snap-frozen in liquid nitrogen and used immediately for RNA extraction. Total RNA was isolated using an Isolate II Plant RNA kit (Bioline) according to the manufacturer's protocol. To check the integrity of the RNA extracted, 3 to 5 μg of total RNA was loaded on 1% agarose gels stained with 1 × SYBR® Safe DNA gel stain (Bioline). Samples were prepared with 1 x Ficoll loading dye [15% (w/v) Ficoll 4000, 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanol FF]. Samples were then electrophoresed in 1 x TAE buffer (40 mM Trizma base and 1 mM Na_2EDTA, pH to 8 with glacial acetic acid) at 110 V. Photographs of the samples were taken under ultraviolet (UV) light (λ 302 nm).

The Tetro cDNA synthesis kit (Bioline) was used to synthesize cDNA. The reaction mixture of 20 μl contained 5 μg of RNA, 0.4 μM oligo $(dT)_{18}$ primer mix, 0.5 mM dNTP mix, 1 × reverse transcriptase buffer, 10 U RiboSafe RNase inhibitor (Bioline), 200 U Tetro reverse transcriptase and diethylpyrocarbonate (DEPC)-treated water. The reaction was incubated at 45 °C for 30 min and then terminated at 85 °C for 5 min. RNA and cDNA were stored at -20 °C until further use.

2.6 Primer design

Two primer pairs were designed to amplify two target regions of the genomic DNA and cDNA of the *PDS* gene which contained the amino acids Arg288 and Leu526, equivalent to the position Arg304 in hydrilla[17] and Leu504 in green algae,[23] respectively. As no full sequence of the *PDS* gene of oriental mustard was available, primers were designed based on the *PDS* gene sequences of five other species, namely *Arabidopsis thaliana* [National Center for Biotechnology Information (NCBI) accession number NM117498], *Brassica napus* (NCBI accession number NM001316208), *Brassica rapa* (NCBI accession number GQ200741), *Hydrilla verticillata* (NCBI accession number AY639658.1) and *Haematococcus pluvialis* (NCBI accession number AY768691.1). All gene sequences were obtained from GenBank (NCBI). The software Primer 3 Plus (Biomatics, Wageningen University, the Netherlands), Expaxy (Swiss Institute of Bioinformatics, Lausanne, Switzerland) and the NCBI website were used to design and check for specificity of the primers before use.

2.7 Polymerase chain reaction (PCR) amplification and sequencing of the *PDS* gene

In order to align the sequences of oriental mustard with database sequences, cDNA was used to amplify the *PDS* gene fragment containing amino acid Arg288. The primers Arg288F (5′-TGGAARGATGAWGATGGWGAYTGGTA-3′) and Arg288R (5′-GAC ATGTCNGCATANACRRCTYA-3′) were used to amplify an approximately 450-bp fragment of the *PDS* gene from cDNA extracted from five individuals of the resistant (P3) and susceptible (S1) populations (NCBI accession number MF593463). Phire Hot Start II DNA polymerase (Thermo Fisher Scientific Australia Pty Ltd, Scoresby, Australia) was used for amplification. Reactions of 20 μl contained 80–100 ng cDNA, 1 x Phire kit reaction buffer, 0.5 μM of each specific primer and 1 U of the Phire Taq DNA polymerase. Thermo cycling conditions were as follows: 3 min denaturing at 95 °C; 40 cycles of 15 s of denaturation at 95 °C, 15 s of annealing at 58 °C and 2 min of elongation at 72 °C, and a final extension for 7 min at 72 °C.

Once *PDS* gene sequences of oriental mustard had been obtained from cDNA to allow for sequence comparison to database mRNA sequences and identification of the amino acid positions of interest, further amplification and sequencing were

conducted on genomic DNA. The primers Arg288F and Arg288R were used to amplify an approximately 850-bp fragment of the *PDS* gene from genomic DNA extracted from five individuals of the resistant (P3) and susceptible (S1) populations (NCBI accession number MF593464) using Phire Hot Start II DNA polymerase (Thermo Fisher Scientific Australia Pty Ltd) as described above.

The forward primer Leu526F (5′-GCACCWGCAGAGGAATGGRT-3′) and reverse primer Leu526R (5′-AGACTGAGAGCAGAATTTGCC-3′) were used to amplify an approximately 370-bp fragment of the *PDS* gene containing the Leu526 codon from genomic DNA (NCBI accession number MF593462) using Phire Hot Start II DNA polymerase as described above. These primers were also used to screen 42 F_2 individuals for the presence of the Leu-526-Val mutation.

The PCR products were visualized on 1.5% agarose gels as described for RNA visualization above. Fragment sizes amplified from DNA or cDNA were estimated by comparing their mobility to that of bands of known sizes in a DNA ladder (Easy Ladder; Bioline). PCR products were sequenced by the Australian Genome Research Facility (AGRF) Ltd (Adelaide, Australia) using the same primers as used for amplification. DNA or cDNA sequence data were assembled, compared and analyzed using Geneious version 8.0 (Biomatters Limited, Auckland, New Zealand). In view of its high sequence similarity to oriental mustard, the *PDS* gene sequence of *A. thaliana* (NCBI accession number NM117498) was used as a reference gene to determine the equivalent amino acids to amino acids Arg304 in hydrilla and Leu504 in green algae. Multiple sequence alignment (Clustal Omega; www.ebi.ac.uk/Tools/msa/clustalo/) of the partial *PDS* gene sequence of oriental mustard with the *PDS* gene sequences of 21 other species was conducted to ensure that the mutation identified in the resistant oriental mustard population was not a natural variant present in other weed or plant species.

A *G*-test of goodness of fit with Williams' correction[24] was conducted to test the hypothesis that the genotypes of the F_2 populations had segregated in a 1:2:1 ratio, as expected for the one dominant gene model. A model of a single dominant gene was created by summing 0.25 (equivalent to 25%) × homozygous susceptible: 0.5 (equivalent to 50%) × heterozygous: 0.25 (equivalent to 25%) × homozygous resistant individuals (0.25ss:0.5Rs:0.25RR). This model was compared with the real genotype segregation of the 42 F_2 individuals to determine whether the genotype segregation fitted a single-gene model as predicted.

3 RESULTS

3.1 Whole-plant dose–response to diflufenican

Dose–response studies confirmed that the P3 population was highly resistant to diflufenican. The susceptible populations S1 and S2 were completely controlled by diflufenican at the recommended field rate (200 g ha^{-1}), whereas all of the P3 plants survived and continued to grow, even at higher rates (Fig 1). In comparison with the mean values of the LD_{50} of the two susceptible populations (S1 and S2), the P3 population was 140-fold more resistant to diflufenican (Table 1). The biomass of the P3 plants was only slightly reduced by the field rate of diflufenican and this population had a GR_{50} of 3165 g ha^{-1}, while the GR_{50} of the S1 and S2 populations was about 24 g ha^{-1}, making the resistant population 131-fold more resistant than the susceptible populations (Table 1 and Fig. 1).

3.2 Sequencing of the *PDS* gene

When the sequences obtained from the partial *PDS* gene fragments of the resistant (P3) and susceptible (S1) populations were compared, a single nucleotide change resulting in an amino acid substitution at codon 526 from leucine (TTA) to valine (GTA) (Leu-526-Val) was identified in all the individuals of the resistant population but not in any individual of the susceptible population (Fig. 2). In addition, when compared with the *PDS* gene sequences of 21 other species, it was found that a leucine at the equivalent position to Leu526 in oriental mustard was conserved in all other species and only the diflufenican-resistant biotype contained the Val526 substitution. (Fig 2). However, no substitution was detected at Arg288 in any individuals of both resistant and susceptible populations.

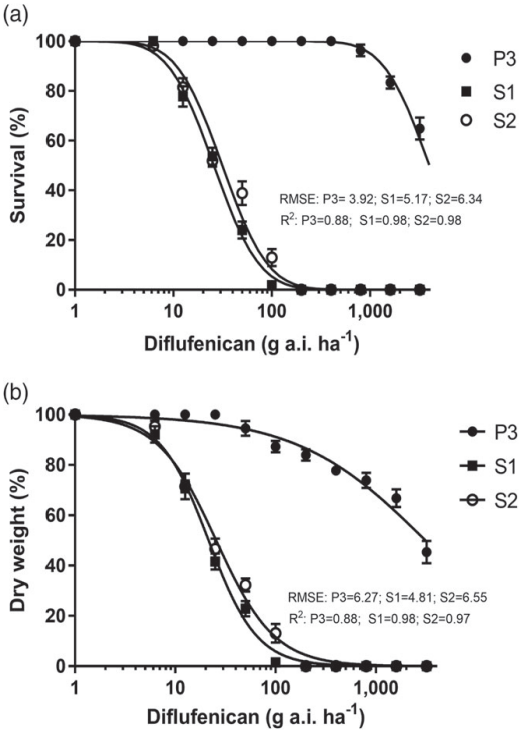


Figure 1. Dose–response curves for the survival (a) and biomass (b) of the S1 (■), S2 (○), and resistant P3 (●) populations of oriental mustard treated with diflufenican. The curves for the survival data (LD_{50}) were fitted using the equation $Y = 100 \times [1 - \text{NORMSDIST}(B + AX)]$, where X is log(dose), and Y (% survival) is back-transformed from mortality (expressed as normal equivalent deviates). The curves for biomass reduction (GR_{50}) were fitted to a nonlinear, log-logistic regression model using GraphPad Prism v.6.0. Each data point is the mean of six replicates, and the vertical bars are standard error of the mean (SEM).

3.3 Evaluation of F_1 populations and segregation pattern of F_2 populations

Crosses (pods) were harvested from the susceptible S1 and resistant P3 parent plants. When screened with diflufenican at 200 g ha^{-1}, all the seedlings from self-pollinated seeds of the susceptible plants showed severe damage and died 28 DAT. Meanwhile, all the seedlings from self-pollinated seeds of the resistant individuals showed very little or no damage. All F_1 seedlings raised from seed set on the resistant parent survived with little or no damage. Some F_1 seedlings raised from seed set on the susceptible parent (R♂ × S♀) also survived diflufenican treatment with little or

Table 1. Estimated LD_{50} (the dose required for 50% mortality), GR_{50} (the dose required for 50% biomass reduction) and resistance index[a] (RI) values for oriental mustard populations treated with diflufenican

	Survival		Biomass	
Population	LD_{50}	RI	GR_{50}	RI
P3	4003 (3244, 5031)	139.7	3165 (2514, 3985)	130.5
S1	25.8 (22.1, 30.1)	-	22.0 (20.7, 23.3)	-
S2	31.5 (27.0, 36.6)	-	26.5 (24.4, 28.9)	-

Values in parentheses are 95% confidence intervals. Data for the two experimental runs were pooled.
[a] RIs were calculated as the ratio between the LD_{50} (or GR_{50}) of the resistant population (P3) and the mean LD_{50} (or GR_{50}) value of the two susceptible populations (S1 and S2). The recommended field rate of diflufenican for post-treatment of oriental mustard in crops in South Australia is 200 g a.i. ha^{-1}.

no damage. Six surviving F_1 plants from six R♂ × S♀ crosses were allowed to self-pollinate to produce F_2 families.

The F_2 plants segregated when treated with diflufenican at 200 g ha^{-1}. The responses of the six F_2 families from the R♂ × S♀ crosses were similar (homogeneity $P = 0.99$; 5 df) (Table 2). This indicates that the parents investigated were homozygous for the resistance trait, which is as expected for a highly self-pollinated species such as oriental mustard. The pattern of segregation was consistent with a model of a single dominant gene. For the phenotypes, the segregation of plants with high levels of damage and those with little or no effect was not different from a 3:1 ratio expected for a single dominant allele (Table 2).

The dose–response experiments on F_2 seedlings showed that the response of F_2 populations was intermediate between those of the resistant and susceptible populations (Fig. 3). The dose–response curve in each F_2 population showed a single step, with survival declining to about 75% at 100 g ha^{-1} and remaining at this level until the herbicide rate increased above 1600 g ha^{-1} (Fig. 3). This type of response is an indication of segregation for resistance in the F_2 population.[25] To confirm this hypothesis, a model for a single dominant allele was calculated (dotted line). The F_2 response fitted the model for a single dominant allele at 100 g diflufenican ha^{-1} and continued to follow this model until the highest dose of diflufenican (3200 g ha^{-1}) (Fig. 3).

When 42 individuals of the F_2 populations were screened for the presence of the Leu-526-Val mutation, the genotypes could be classified into 8 homozygous susceptible: 25 heterozygous: 9 homozygous resistant individuals. This observed segregation ratio is consistent with a 1:2:1 ratio when tested with the *G*-test of goodness of fit at $G = 1.57$ and $P = 0.455$.

4 DISCUSSION

PDS inhibitors were first commercialized in the early 1980s and have been used in agriculture and aquaculture for a number of years. To date, the evolution of resistance to this herbicide group has been reported in only a few weed species,[9] including hydrilla,[17] eastern groundsel and oriental mustard.[9] Even though resistance to fluridone in hydrilla is relatively low (3- to 6-fold), it has made management of this aquatic weed more difficult in the USA.[17,26] In the current study, the level of resistance to diflufenican in oriental mustard population P3 was very high (140-fold) in comparison to the susceptible populations. Therefore, diflufenican is likely to be completely ineffective on resistant populations, which will make their management much more difficult.

Carotenoids are essential pigments which play important roles in plants, especially in photosynthesis.[27,28] The desaturation

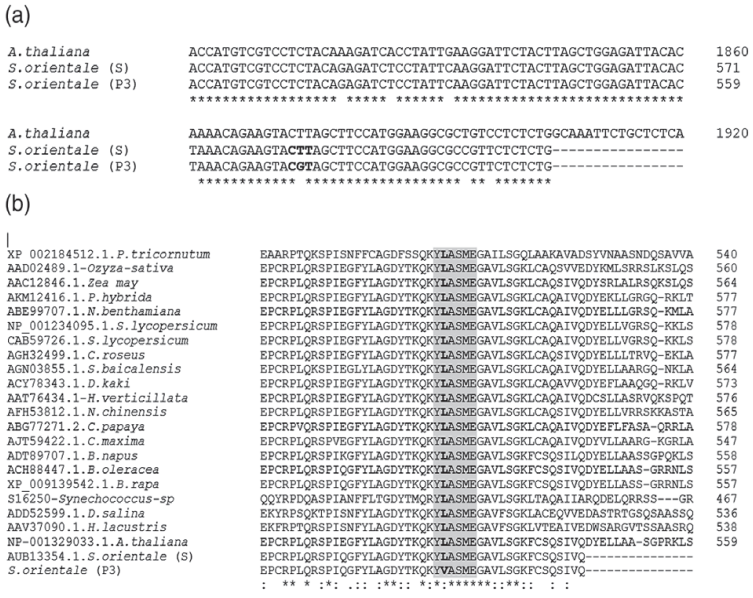


Figure 2. Aligned partial sequences of phytoene desaturase (*PDS*) alleles from diflufenican-susceptible (S) and resistant (P3) oriental mustard biotypes with the partial sequence of the *PDS* gene from *A. thaliana* (a) and 21 other plant species (b). A mutation in the resistant oriental mustard biotype *PDS* gene is indicated by a bold letter representing the nucleotide differences when compared with the susceptible oriental mustard or the wild type of other species.

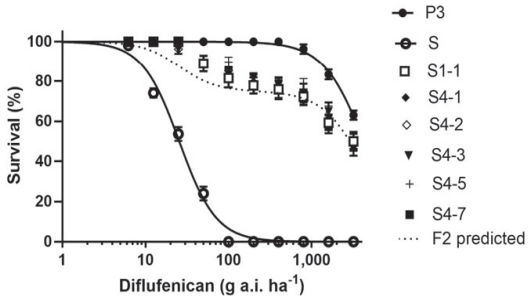


Figure 3. Dose–response of susceptible S1 (○) and resistant P3 (●) populations, and six F_2 populations (□, ◆, ◇, ▼, +, and ■) of oriental mustard treated with diflufenican. The dotted line is the predicted response for a single dominant gene at all doses of diflufenican. Data points are means ±95% confidence intervals for six replicates (three replicates × two runs).

Table 2. Segregation for resistance to 200 g ha^{-1} diflufenican in F_2 populations generated from crosses between resistant P3 and susceptible S1 oriental mustard plants

Family	Treated	Dead[a]	Alive[b]	*G*-statistic	*P*
S1	214	214	0		
P3	214	0	214		
S1-1	210	51	159	0.057	0.811
S1-2	216	56	160	0.098	0.755
S1-3	210	54	156	0.057	0.812
S1-4	212	52	160	0.026	0.874
S1-5	216	55	161	0.025	0.876
S1-6	214	53	161	0.006	0.937
Total	1278	321	957	0.009	0.923
Homogeneity				0.268	0.998

[a] Plants displaying no new growth and severe necrosis were recorded as dead or susceptible.
[b] Plants with new green leaf tissue were recorded as alive or resistant.

sequence starting from phytoene in carotenoid biosynthesis has become the target of many bleaching herbicides.[29,30] In previous studies on hydrilla, the presence of a mutation at Arg304 in the *PDS* gene resulted in the expression of a low level of resistance to fluridone (4- to 6-fold).[17,18] No mutation, however, was present at this position in oriental mustard in this study.

In a study by Steinbrenner and Sandmann[23] on the alga *H. pluvialis*, substitution of leucine (CTG) to arginine (CGC) at codon 504 increased carotenoid biosynthesis in the alga and conferred a 43-fold higher resistance to norflurazon (a PDS-inhibiting herbicide) compared with the susceptible biotypes. In previous studies, Leu538 mutated to Phe or Arg has been reported in *Chlorella zofingiensis*[31],[32] *Chlamydomonas reinhardtii*,[33] and *Synechococcus* sp. PCC 7942.[34] This position is equivalent to Leu538 in *Oryza sativa*. The Leu538 mutations were found to affect the size of the binding pocket (Ala539) of PDS inhibitors in the plant.[35] In addition, the Leu-256-Val substitution is a unique and natural mutation in oriental mustard, which has not been identified in the *PDS* gene of any other species. This clearly shows a critical role of Leu538 in resistance to PDS inhibitors and suggests that the Leu-526-Val (equivalent to Leu538) substitution in the *PDS* gene identified in this study is likely to be responsible for resistance to diflufenican in the P3 population of oriental mustard.

Most cases of herbicide resistance are inherited as nuclear genes, except for resistance to triazine herbicides, which has maternal inheritance.[36] In the current study, resistance to diflufenican was passed by pollen, as some F_1 seed harvested from the susceptible parents survived when treated with diflufenican at 200 g ha^{-1}. Resistance traits in weeds are often controlled by semidominant or dominant genes.[37,38] This also means that the traits managed by dominant genes will be expressed in both homozygous and heterozygous states.[39] Resistance to diflufenican was largely controlled by a single dominant allele (Table 2). The dose–response analysis of the F_2 population (Fig. 3) showed a high level of dominance over susceptibility, with heterozygotes having a similar response to that of the resistant parent. In addition, the segregation rate of F_2 genotypes conformed to a 1:2:1 ratio at $G = 1.57$ and $P = 0.455$, indicating that the resistance to diflufenican is correlated with the Leu-526-Val substitution in the *PDS* gene and that this mutation in oriental mustard provides a high level of resistance to diflufenican. However, the F_2 response deviated slightly from the model at rates <100 g ha^{-1}, which suggests that the contribution of a minor gene or genes to diflufenican resistance at low rates of diflufenican in oriental mustard cannot be ruled out.

This study has confirmed diflufenican resistance in one oriental mustard population where substitution of Leu526 to Val in the *PDS* gene was identified as the target-site mechanism of resistance. The study has also demonstrated that resistance to diflufenican in oriental mustard is controlled by a single gene with high levels of dominance. The mutation observed at Leu526 in the *PDS* gene of oriental mustard could be used as a marker associated with diflufenican resistance, which allows genotyping of segregating populations and rapid screening of other populations for target-site resistance. In addition, this mutation could be used for generating diflufenican-tolerant/resistant crops by genetic engineering techniques in the future.

As resistance to PDS inhibitors in the oriental mustard populations resulted in high levels of resistance to diflufenican and was a dominant trait, increasing the herbicide dose is unlikely to significantly improve weed control. Weed control strategies for effective management of diflufenican-resistant oriental mustard populations will need to include an alternation of herbicide modes of action,[40] mechanical control,[41] herbicide mixtures[42] and other options such as maximizing crop competition.[43]

ACKNOWLEDGEMENTS

This research was funded by the Grains Research and Development Corporation (GRDC), a PhD scholarship from the Vietnam Government and the University of Adelaide. The authors also would like to thank Ruwan Lenorage and Geetha Velappan from the Weed Science Group at the University of Adelaide for their technical support.

DISCLOSURES

The authors declare no conflict of interest.

REFERENCES

1 Heap IM, *The International Survey of Herbicide Resistant Weeds* [Online]. Available: http://weedscience.org/ [9 November 2017].
2 McGillion T and Storrie A, *Integrated weed management in Australian cropping systems: a training resource for farm advisors*. CRC for Australian Weed Management, Adelaide SA (2006).

3 Chauhan BS, Gill G and Preston C, Influence of environmental factors on seed germination and seedling emergence of oriental mustard (*Sisymbrium orientale*). *Weed Sci* **54**:1025–1031 (2006).
4 Boutsalis P and Powles SB, Seedbank characteristics of herbicide-resistant and susceptible *Sisymbrium orientale*. *Weed Res* **38**: 389–395 (1998).
5 Chauhan BS, Gill G and Preston C, Seedling recruitment pattern and depth of recruitment of 10 weed species in minimum tillage and no-till seeding systems. *Weed Sci* **54**:658–668 (2006).
6 Preston C, Dolman FC and Boutsalis P, Multiple resistance to acetohydroxyacid synthase–inhibiting and auxinic herbicides in a population of oriental mustard (*Sisymbrium orientale*). *Weed Sci* **61**:185–192 (2013).
7 Dayan FE, Owens DK, Tranel PJ, Preston C and Duke SO, Evolution of resistance to phytoene desaturase and protoporphyrinogen oxidase inhibitors - state of knowledge. *Pest Manag Sci* **70**:1358–1366 (2014).
8 Dang HT, Malone JM, Boutsalis P, Gill G and Preston C, Identification of a target-site mutation conferring resistance to triazine herbicides in oriental mustard (*Sisymbrium orientale* L.) from Australia. *Weed Biol Manag* **17**:153–160 (2017).
9 Dayan FE, Owens DK, Tranel PJ, Preston C and Duke SO, Evolution of resistance to phytoene desaturase and protoporphyrinogen oxidase inhibitors--state of knowledge. *Pest Manag Sci* **70**:1358–1366 (2014).
10 Rouchaud J, Gustin F, Van Himme M, Bulcke R, Benoit F and Maddens K, Metabolism of the herbicide diflufenican in the soil of field wheat crops. *J Agric Food Chem* **39**:968–976 (1991).
11 Ashton IA, Abulnaja KO, Pallett KE, Cole DJ and Harwood JL, The mechanism of inhibition of fatty acid synthase by the herbicide diflufenican. *Phytochemistry* **35**:587–590 (1994).
12 Haynes C and Kirkwood RC, Studies on the mode of action of diflufenican in selected crop and weed species: Basis of selectivity of pre-and early post-emergence applications. *Pest Manag Sci* **35**:161–165 (1992).
13 Corona V, Aracri B, Kosturkova G, Bartley GE, Pitto L, Giorgetti L *et al.*, Regulation of a carotenoid biosynthesis gene promoter during plant development. *The Plant Journal* **9**: 505–512 (1996)
14 Sandmann G, Schmidt A, Linden H and Boger P, Phytoene desaturase, the essential target for bleaching herbicides. *Weed Sci* **39**:474–479 (1991).
15 Armel GR, Rardon PL, McComrick MC and Ferry NM, Differential response of several carotenoid biosynthesis inhibitors in mixtures with atrazine. *Weed Technol* **21**:947–953 (2007).
16 Sandmann G, Schneider C and Boger P, A new non-radioactive assay of phytoene desaturase to evaluate bleaching herbicides. *J Biosci* **51**:534–538 (1996).
17 Michel A, Arias RS, Scheffler BE, Duke SO, Netherland M and Dayan FE, Somatic mutation-mediated evolution of herbicide resistance in the nonindigenous invasive plant hydrilla (*Hydrilla verticillata*). *Mol Ecol* **13**:3229–3237 (2004).
18 Benoit LK and Les DH, Rapid identification and molecular characterization of phytoene desaturase mutations in fluridone-resistant hydrilla (*Hydrilla verticillata*). *Weed Sci* **61**:32–40 (2013).
19 Preston C and Malone JM, Inheritance of resistance to 2,4-D and chlorsulfuron in a multiple-resistant population of *Sisymbrium orientale*. *Pest Manag Sci* **71**:1523–1528 (2015).
20 Boutsalis P, Gill GS and Preston C, Incidence of herbicide resistance in rigid ryegrass (Lolium rigidum) across southeastern Australia. *Weed Technol* **26**:391–398 (2012).
21 Boutsalis P, Karotam J and Powles SB, Molecular basis of resistance to acetolactate synthase-inhibiting herbicides in *Sisymbrium orientale* and *Brassica tournefortii*. *Pest Manag Sci* **55**: 507–516 (1999).
22 Sakuma M, Probit analysis of preference data. *Appl Entomol Zool* **33**:339–347(1998).
23 Steinbrenner J and Sandmann G, Transformation of the green algae *Haematococcus pluvialis* with a phytoene desaturase for accelerated astaxanthin biosynthesis. *J Appl Environ Microbiol* **72**:7477–7484 (2006).
24 Sokal R and Rohlf F, *Biometry*. Second ed. WH Freeman and Co, New York, NY, p. 859 (1981).
25 Tabashnik BE, Determining the mode of inheritance of pesticide resistance with backcross experiments. *J Econ Entomol* **84**:703–712 (1991).
26 Netherland MD and Jones D, Fluridone-resistant hydrilla (*Hydrilla verticillata*) is still dominant in the Kissimmee chain of lakes FL., *Invasive Plant Sci Manag* **8**:212–218 (2015).
27 Mathews-Roth MM, Carotenoids and photoprotection. *J Photochem Photobiol* **65**:148–151 (1997).
28 Hashimoto H, Uragami C and Cogdell RJ, Carotenoids and Photosynthesis, in *Carotenoids in Nature: Biosynthesis, Regulation and Function*, ed. by Stange C. Springer International Publishing, Cham, pp. 111–139 (2016).
29 Misawa N, Masamoto K, Hori T, Ohtani T, Böger P and Sandmann G, Expression of an Erwinia phytoene desaturase gene not only confers multiple resistance to herbicides interfering with carotenoid biosynthesis but also alters xanthophyll metabolism in transgenic plants. *The Plant Journal* **6**:481–489 (1994).
30 Ohki S, Miller-Sulger R, Wakabayashi K, Pfleiderer W and Boger P, Phytoene desaturase inhibition by O-(2-phenoxy)ethyl-N-aralkyl carbamates. *J Agric Food Chem* **51**:3049–3055 (2003).
31 Huang JC, Liu J, Li YT and Chen F, Isolation and characterization of the phytoene desaturase gene as a potential selective marker for genetic engineering of the astaxanthin-producing green alga Chlorella zofingiensis (*Chlorophyta*). *J Phycol* **44**:684–690 (2008).
32 Liu J, Zhong Y, Sun Z, Huang J, Sandmann G and Chen F, One amino acid substitution in phytoene desaturase makes Chlorella zofingiensis resistant to norflurazon and enhances the biosynthesis of astaxanthin. *Planta* **232**:61–67 (2010).
33 Liu J, Gerken H, Huang J and Chen F, Engineering of an endogenous phytoene desaturase gene as a dominant selectable marker for *Chlamydomonas reinhardtii* transformation and enhanced biosynthesis of carotenoids. *Process Biochem* **48**:788–795 (2013).
34 Chamovitz D, Sandmann G and Hirschberg J, Molecular and biochemical characterization of herbicide-resistant mutants of cyanobacteria reveals that phytoene desaturation is a rate-limiting step in carotenoid biosynthesis. *J Biol Chem* **268**:17348–17353 (1993).
35 Brausemann A, Gemmecker S, Koschmieder J, Ghisla S, Beyer P and Einsle O, Structure of phytoene desaturase provides insights into herbicide binding and reaction mechanisms involved in carotene desaturation. *Structure* **25**:1222–1232 (2017).
36 Jasieniuk M, Brule-Babel AL and Morrison IN, The evolution and genetics of herbicide resistance in weeds. *Weed sci* **44**:176–193 (1996).
37 Darmency H, Genetics of herbicide resistance in weeds and crops. *Herbicide Resistance in Plants: Biology and Biochemistry*. pp. 263–298 (1994).
38 Tardif F, Preston C, Holtum J and Powles S, Resistance to acetyl-coenzyme A carboxylase-inhibiting herbicides endowed by a single major gene encoding a resistant target site in a biotype of *Lolium rigidum*. *Funct Plant Biol* **23**:15–23 (1996).
39 Christoffers MJ, Genetic aspects of herbicide-resistant weed management. *Weed Technol* **13**:647–652 (1999).
40 Beckie HJ, Beneficial management practices to combat herbicide-resistant grass weeds in the northern Great Plains. *Weed Technol* **21**:290–299 (2007).
41 Walsh M, Newman P and Powles S, Targeting weed seeds in-crop: a new weed control paradigm for global agriculture. *Weed Technol* **27**:431–436 (2013).
42 Lagator M, Vogwill T, Mead A, Colegrave N and Neve P, Herbicide mixtures at high doses slow the evolution of resistance in experimentally evolving populations of Chlamydomonas reinhardtii. *New Phytologist* **198**:938–945 (2013).
43 Beckie HJ, Johnson EN, Blackshaw RE and Gan Y, Weed suppression by canola and mustard cultivars. *Weed Technol* **22**:182–185 (2008).

CHAPTER 5

CROSS-RESISTANCE TO DIFLUFENICAN AND PICOLINAFEN, AND ITS INHERITANCE IN ORIENTAL MUSTARD (*SISYMBRIUM ORIENTALE* L.)

Hue Thi Dang, Jenna M Malone, Gurjeet Gill and Christopher Preston

School of Agriculture, Food and Wine, University of Adelaide

PMB1, Glen Osmond, South Australia 5064, Australia

Pest Management Science: Submitted paper

Statement of Authorship

Title of Paper	Resistance to Diflufenican and Picolinafen and Its Inheritance in Oriental Mustard (*Sisymbrium orientale* L.)
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Dang, H. T., Malone, J. M., Gill, G. and Preston, C. (2018), Resistance to diflufenican and picolinafen and its inheritance in oriental mustard (*Sisymbrium orientale* L.). Pest. Manag. Sci. Submitted paper.

Principal Author

Name of Principal Author (Candidate)	Hue Thi Dang		
Contribution to the Paper	Planned the study, conducted all experiments, analyzed and interpreted data and wrote manuscript.		
Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	23.02.2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Jenna Moira Malone		
Contribution to the Paper	Supervised the molecular work, data interpretation and edited manuscript		
Signature		Date	26/2/18

Name of Co-Author	Christopher Preston		
Contribution to the Paper	Supervised development of research, data interpretation and analysis, and edited manuscript		
Signature		Date	23/02/2018

Name of Co-Author	Gurjeet Gill		
Contribution to the Paper	Supervised development of research, data interpretation and analysis, and edited manuscript		
Signature		Date	23.2.2018

ABSTRACT

BACKGROUND: An oriental mustard population (P40) was identified as resistant to diflufenican by screening at the field rate. As diflufenican and picolinafen both target phytoene desaturase (PDS), cross-resistance to picolinafen was suspected. The mechanism of resistance and its inheritance to diflufenican and picolinafen were investigated.

RESULTS: At the LD_{50} level, population P40 was 237-fold more resistant to diflufenican and 7-fold more resistant to picolinafen than two susceptible populations. Population P40 also had a significantly higher resistance to diflufenican (237-fold) than a previously described P3 population (143-fold). In addition to the Leu-498-Val mutation in PDS identified in all individuals of the P3 and P40 populations, a Glu-425-Asp mutation was also found in P40. Neither mutation was detected in any individuals of the susceptible population. Resistance to both diflufenican and picolinafen is encoded on the nuclear genome and is dominant, as the segregation of phenotype and genotype of the F_2 individuals fitted the model for a single dominant allele.

CONCLUSION: Resistance to diflufenican and picolinafen in the P40 population is likely conferred by Leu-498-Val and Glu-425-Asp mutations in the PDS gene. Inheritance of resistance to these herbicides is managed by a single dominant gene.

Keywords: Diflufenican, picolinafen, phytoene desaturase, PDS, *Sisymbrium orientale* L., cross-resistance, single dominant gene, target-site mutations.

1 INTRODUCTION

Oriental mustard (*Sisymbrium orientale* L.) is an important cruciferous weed of field crops and pastures in Australia [1]. Plants of this weed species produce a large number of small seeds, which can readily germinate during the year under favorable conditions due to a relatively short dormancy period [2, 3]. In southern Australia, the life cycle of oriental mustard is closely aligned with winter crops and therefore it competes with crops for resources causing yield loss [1, 4]. Oriental mustard is usually easy to control with herbicides, but has recently evolved resistance in Australia to several herbicide modes of action that include acetolactate synthase (ALS) inhibitors, synthetic auxins, phytoene desaturase (PDS) inhibitors and photosystem II inhibitors [5-9].

Diflufenican and picolinafen are pyridinecarboxamide herbicides belonging to Group 15 (WSSA designation), which inhibit carotenoid biosynthesis. Diflufenican was developed in 1979 and has been used as a PRE- and early POST-emergent herbicide for the selective control of certain broadleaf weeds, especially Brassicaceae family, in winter cereals since the mid-1980s [10-12]. Picolinafen is a selective, post-emergence herbicide, which has been used to control annual broadleaf weeds in winter crops in Australia since 2001. Carotenoids are essential pigments of the photosynthetic apparatus that play important roles in plants, especially in photosynthesis [13, 14]. They not only participate in light harvesting, but also protect the chloroplasts from the harmful effects of singlet oxygen formed during photosynthesis [15]. The enzyme phytoene desaturase (PDS), which initiates the desaturation sequence starting from phytoene in carotenoid biosynthesis, has been the target of many bleaching herbicides such as diflufenican and picolinafen [16, 17]. These herbicides inhibit the formation of carotenoids [13, 18, 19] to cause a lack of carotenoids, destruction of chloroplast membranes and degradation of chlorophyll. This results in pronounced bleaching symptoms and necrosis of the tissues of susceptible plants, leading to plant death [14, 20].

Even though the PDS-inhibiting herbicides have been used for many years to control weeds in agriculture and aquaculture, only few cases of field-evolved resistance have been recorded worldwide [9, 21]. These include the hydrilla (*Hydrilla verticillata*) [22], wild radish (*Raphanus raphanistrum*) [23], annual bluegrass (*Poa annua*), loose silky-bent (*Apera spica-venti*) and oriental mustard (*Sisymbrium orientale*) [9, 21]. However, the mechanisms of resistance in most cases remain unknown [9], the exceptions are for resistance to fluridone in *H. verticillata* [22, 24] and diflufenican in oriental mustard [25]. In these species, mutations at the Arg304 (*H. verticillata* numbering system) and Leu526 (*A. thaliana* numbering system) in the PDS gene were confirmed the main causes of resistance to fluridone in *H. verticillata* and diflufenican in oriental mustard, respectively [25]. Transgenic studies conducted in some lower plants (e.g. *Haematococcus pluvialis*, *Chlorella zofingiensis* and *Chlamydomonas reinhardtii*) have also confirmed that mutations within the PDS gene could result in resistance to PDS-inhibiting herbicides. The substitution from Leu504 to Arg in the PDS gene of the *H. pluvialis* [26] or from Leu516 to Phe in *C. zofingiensis* and *C. reinhardtii* [27, 28] enable these species to exhibit resistance to norflurazon. These studies demonstrated that resistance to PDS-inhibitors is likely associated with target-site resistance mechanisms.

In weeds, cross-resistance occurs when a species develops simultaneous resistance to more than one herbicide following selection with a single herbicide [29]. Cross-resistance within a mode of action is expected to occur due to target site (TSR) resistance mechanisms. It can also occur across herbicide modes of action due to non-target site based mechanisms [30, 31]. Many weed species evolve cross-resistance to herbicides, some examples are: resistance to imazamox and imazethapyr in red rice (*Oryza sativa*) [32]; cloransulam, chlorimuron, imazethapyr and bispyribac resistance in horseweed (*Conyza canadensis*) [33]; prosulfocarb, triallate and pyroxasulfone-resistance in ryegrass (*Lolium rigidum*) [34]; or tribenuron-resistant *Papaver rhoeas* biotypes that displayed cross-resistance to mesosulfuron, chlorsulfuron and

triasulfuron [35]. However, cross-resistance to PDS-inhibiting herbicides has not been reported in any weed species.

In a recent study, a Leu-526-Val substitution in the PDS gene was identified in diflufenican resistant oriental mustard from Quambatook, Victoria, Australia [25]. Since then, another population of oriental mustard (P40) with a higher level of resistance to diflufenican has been identified. This population showed no leaf bleaching symptoms or growth reduction when treated with the recommended field rate of diflufenican. Research was conducted to compare the level of diflufenican resistance and cross-resistance to another PDS inhibiting herbicide, picolinafen, as well as the mechanism(s) of resistance in these two populations. The mode of inheritance of resistance to diflufenican and picolinafen was also investigated in the P40 population.

2 EXPERIMENTAL METHODS

2.1 Plant materials

An oriental mustard population (P40) collected from a wheat field near Kunat, Victoria was used in this study. The P40 population was confirmed as resistant to diflufenican when screened with 200 g a.i. ha^{-1} diflufenican (Brodal 500 g a.i. L^{-1}, Bayer Crop Science, Australia), the recommended field rate to control oriental mustard in Australia. Two known herbicide-susceptible populations S1 and S2 were also used throughout the study as susceptible controls. The S1 population was collected from an organic crop near Roseworthy, South Australia [36] and the S2 population was collected near Port Kenny, Eyre Peninsula, South Australia. The S1 and S2 populations have been confirmed susceptible to all herbicides commonly used to control oriental mustard in Australia (glyphosate, imazamox, chlorsulfuron, diflufenican, picolinafen, atrazine and 2,4-D) [7]. A previously characterised diflufenican-resistant population (P3) collected from Quambatook, Victoria was included in

some experiments for comparison [25]. Homogeneous resistant (P3 and P40) and susceptible (S1 and S2) populations were generated as described in Dang et al. [25] and the resultant seed used in this study.

2.2 Seed germination, plant growth and herbicide treatment

Seeds from the four (P3, P40, S1 and S2) populations were germinated on the surface of trays containing a standard soil mix [37]. At the 1 to 2-leaf stages, seedlings were transplanted into small punnet pots (Masrac Plastics, South Australia) containing standard potting mix, with nine seedlings per pot. The number of replicates varied in each experiment. The plants were maintained outdoors during the normal growing season (May to October), watered and fertilised as required. At the 3 to 4-leaf stages, seedlings were treated with herbicides using a moving-boom laboratory twin nozzle sprayer as described in previous studies [25]. The number of seedlings in each pot was counted before each herbicide treatment. Control plants were not treated with any herbicide. All experiments were conducted at the Waite campus, The University of Adelaide, South Australia (34°58'13.5"S 138°38'22.7"E).

2.3 Whole-plant dose response to diflufenican and picolinafen

Dose response experiments were conducted on P3, P40, S1 and S2 populations as described in Dang et al. [25] using either diflufenican (Brodal 500 g a.i. L^{-1}, Bayer Crop Science, Victoria, Australia) at rates of 0, 6.25, 12.5, 25, 50, 100, 200, 400, 800, 1600 and 3200 g ha^{-1} or picolinafen (Sniper 750g a.i. kg^{-1} picolinafen, BASF Australia Ltd) at 0, 0.5, 1.0, 2.1, 4.1, 8.3, 16.5, 33, 66, 132, 264 and 528 g ha^{-1}. In Australia, the recommended field rate of diflufenican for post-emergent control of oriental mustard in crops is 200 g a.i. ha^{-1} and the recommended field rate of picolinafen varies from 33 to 50 g a.i. ha^{-1}. Non-ionic surfactant (alcohol alkoxylate, BS1000, Crop Care) 0.2% (v/v), was added to the diflufenican solution, but no

surfactant was added to the picolinafen solution as recommended on product labels. The number of plants were counted before herbicide treatment and the experiment was repeated.

Assessments of plant survival were made at 28 days after treatment (DAT). Plants with new green leaf tissues were recorded as survivors, whereas those that displayed no new growth and severe necrosis were recorded as dead. The above ground parts of the plants were harvested and dried in an oven at 65^{o}C to a constant weight when dry weight was determined. Plant biomass data from the dose-response experiment were converted to percent of untreated control before regression analysis. The LD_{50} values (the herbicide dose required for 50% mortality) and their 95% confidence limits were analysed using an all-or-nothing model and a normal distribution function PriProbit v.1.63 [38]. Probits were back transformed to percentages for plotting. Dry weight data was analysed by log-logistic analysis using GraphPad Prism v.6.0 and GR_{50} values (the herbicide dose required for 50% biomass reduction) calculated. Resistance indices (RI) were calculated as the ratio between the LD_{50} (or GR_{50}) of each population and the mean LD_{50} (or GR_{50}) of the susceptible populations. A two-way ANOVA was used to examine the effect of experimental run. Data from the two runs were pooled prior to data analysis if no effect of experimental run was identified.

2.4 Sequencing of phytoene desaturase

2.4.1 RNA, DNA extraction and cDNA synthesis

Plant material (about 100 mg) from the youngest green leaf tissue of resistant (P3 and P40) and susceptible (S1) plants (five individuals per population) was collected for DNA extraction. For the resistant populations, samples were collected from the five survivors at 800 g diflufenican ha^{-1} at 28 DAT. The susceptible samples were collected from plants before herbicide treatment. These plants were then included in the dose-response experiment and sprayed with diflufenican and picolinafen at the recommended rate to ensure they were

susceptible. For the F_2 populations, leaf tissue was collected from 48 untreated individuals of a population that was confirmed as having clear segregation in the phenotype segregation tests. Samples obtained were snap frozen in liquid nitrogen and stored at –20^{o}C for further use.

For RNA extraction for cDNA synthesis, samples of the susceptible (S1) and resistant plants (P40) were collected as described earlier. However, in this case, fresh samples were obtained, snap frozen in liquid nitrogen and used immediately for RNA extraction. Total RNA was isolated, its quality checked via gel electrophoresis and cDNA synthesised as described in Dang et al. [25].

2.4.2 Sequencing of the PDS gene

Amplification and sequencing of two target regions in the genomic DNA of the PDS gene that contained the amino acids Arg260 (equivalent to the positions Arg304 in *H. verticillata*) and Leu498 (equivalent to Leu504 in *H. pluvialis*) were amplified according to Dang et al. [25]. These fragments were amplified from genomic DNA of five individuals of the resistant (P3 and P40) and susceptible (S1) populations.

In order to investigate whether any other mutations were present in the PDS gene of the P40 population, fragments of the PDS gene of oriental mustard covering 1552 bp of the gene (NCBI accession number MG493466.1) were amplified using cDNA synthesized from the RNA of 5 individuals of the resistant (P40) and susceptible (S1) populations. Six primers were used including (1) PDS-01F-5'-CTGCRGCGAATTTGCCTTA-3'; (2) PDS-02F2-5'-GATGGCVTTCTTRGATGGTA-3'; (3) Arg288F (4) Arg288R; (5) Leu526F and (6) Leu526R. Primers 3, 4, 5 and 6 was obtained from Dang et al. [25], whereas primers 1 and 2 which were designed based on the PDS gene sequences of *A. thaliana* (NCBI accession number NM117498), *Brassica napus* (NCBI accession number NM001316208) and *Brassica*

rapa (NCBI accession number GQ200741) as described in Dang et al. [25]. A multiple sequence alignment of the partial PDS gene sequence of oriental mustard with the PDS gene sequences of 25 other species was conducted to ensure that the mutation identified in the resistant oriental mustard population was not a natural variant present in other weed or plant species, using the Clustal Omega program (www.ebi.ac.uk/Tools/msa/clustalo/).

2.5 Inheritance of resistance to diflufenican

2.5.1 Generation of F_1 and F_2 seeds

Two populations P40 and S1 were selected for the inheritance study. Generation of F_1 and F_2 seeds were conducted using the method described in Dang et al. [8]. Briefly, five survivors of P40 at 800 g ha^{-1} diflufenican and five untreated plants of the S1 were grown in individual 8.5L pots in a glasshouse (15 to 22°C), watered and fertilised as required. Three days after the first flower opened, all mature siliques, opened flowers and small buds were removed from each inflorescence and only 2-3 young buds retained. All immature anthers were removed from each bud, exposing only the stigma and each inflorescence was immediately bagged. On the following day when the pollen donors flowers were fully open, cross-pollination was conducted by tapping the anthers with pollen grains of the susceptible or resistant biotype against the exposed stigma of the other biotype. The pollen receptor female flowers were bagged promptly after hand-pollination. Three days after fertilisation, the bag on each crossed flower was removed and the developing pod marked by a string-tag. Pods were harvested at maturity. Seeds from pods generated from cross-fertilizations were collected separately from each cross and considered an F_1 family. Seeds from the parents (P40 and S1 parental plants) and the F_1 (P40♂ x S1♀) seeds were germinated and transplanted into small pots at a density of 5 plants pot^{-1}. At the 3 to 4-leaf stage, seedlings were treated with diflufenican at 200 g ha^{-1} or picolinafen at 20 g ha^{-1} to determine the resistance status of each progeny. These rates of

herbicide controlled all susceptible plants, but all resistant plants survived. Seedlings from the susceptible parent of each cross that survived the herbicide application were considered to be true F_1 plants. They were transplanted into 8.5 L pots and allowed to self-pollinate to produce F_2 seeds. Mature seeds (F_2) from each F_1 individual were collected separately and used in the inheritance studies.

2.5.2 Segregation and dose-response of the F_2 populations

The phenotypic segregation of the F_2 populations were determined by testing their responses to diflufenican and picolinafen at 200 and 20 g ha^{-1}, respectively, as described above for the F_1 populations. A total of 108 seedlings from each population (P40♂ x S1♀) F_2 population and the parental populations (P40 and S1) were screened and the homogeneity and segregation of the F_2 phenotypes tested against a single-gene model using the *G*-test as described by Preston and Malone [36].

A dose-response experiment was also conducted on F_2 populations and parental populations to determine the number of genes involved in resistance to diflufenican and picolinafen. Dose response experiments were conducted as described earlier. There were three replicates (3 pots with of nine plants per pot) for each parental population and two replicates (2 pots with nine plants per pot) for each F_2 population at each herbicide rate.

Probit analysis was used to analyse the dose-responses of the parental populations. For the F_2 population, a model of single dominant gene was created by summing 0.75 (equivalent to 75%) × survival of the resistant population and 0.25 (equivalent to 25%) × survival of the susceptible population. This model was compared with the real responses of the F_2 populations to ensure that the dose response fitted to a single-gene model as predicted [39]. All experiments were conducted twice in the main growing season in 2017. The effect of experimental run was examined using a two-way ANOVA. Data from the two runs were pooled if no effect of the

experimental run was identified. *G*-test of goodness-of- fit with Williams' correction was conducted to test the hypothesis that the genotypes of the F_2 populations had segregated as 1:2:1 ratio, as expected for the one dominant gene model.

3 RESULTS

3.1 Whole-plant dose response

Dose response studies confirmed that the P40 population was resistant to both diflufenican and picolinafen while the P3 was only resistant to diflufenican. At the recommended field rate (200 g ha^{-1}), diflufenican completely controlled the susceptible populations S1 and S2, whereas, all of the P3 and P40 plants survived and continued to grow, even at the higher rates. The survival rate of the resistant biotypes remained high (60 and 80% survival for P3 and P40, respectively) at the highest herbicide rate (3200 g ha^{-1}) (Fig 1).

In addition, the symptoms of leaf bleaching were clearly observed in the susceptible and the resistant control (P3) populations when treated at the field rate (200 g ha^{-1}), but not in the resistant population P40 when treated with diflufenican at rates lower than 600 g ha^{-1} (data not shown). Based on the mean values of the LD_{50} of the two susceptible populations (S1 and S2), the P3 and P40 populations were 143 and 237-fold more resistant to diflufenican (Table 1).

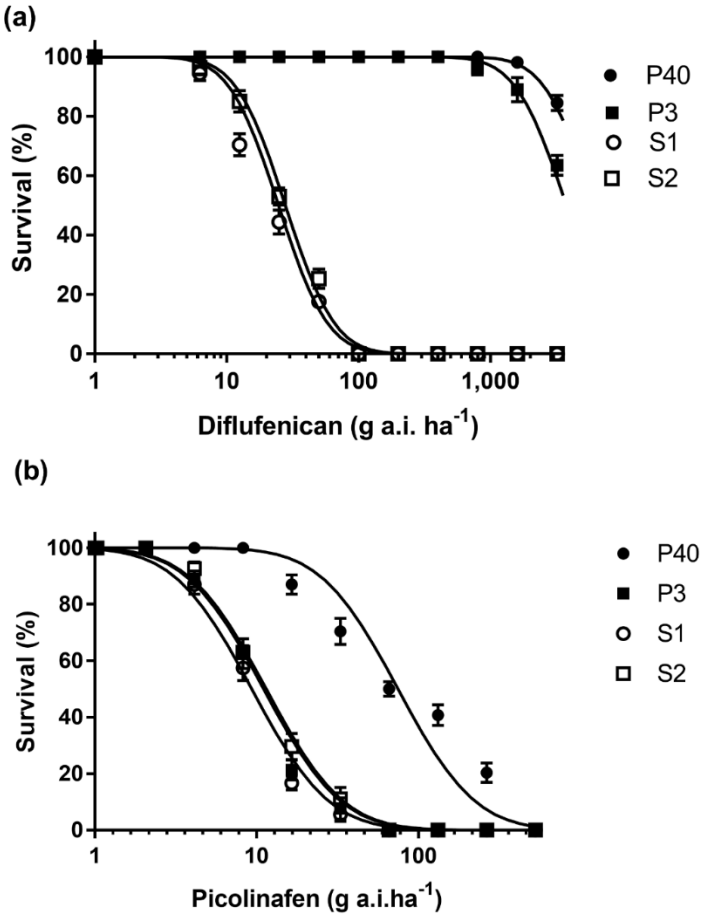


Fig 1: Dose-response curves for the survival of the susceptible S1 (○), S2 (□), resistant P3 (■) and P40 (●) populations of oriental mustard treated with diflufenican (a) and picolinafen (b). The curves for survival data were fitted using the equation Y =100*[1−NORMSDIST (B+A*X)], where X is log (dose), and Y (% survival) is back-transformed from mortality (expressed as normal equivalent deviates). Each data point is the mean of six replicates, and the vertical bars are standard error of the mean (SEM).

The biomass of the resistant plants was only slightly reduced (~10%) by the recommended field rate of diflufenican. The resistant populations P3 and P40 had a GR_{50} of 2922 and 4533 g ha^{-1}, respectively, while the GR_{50} of the S1 and S2 populations was about 23 g ha^{-1}, making them 126 and 195-fold, respectively, more resistant to diflufenican than the susceptible populations (Table 1).

Table 1. Estimated LD_{50} (the dose required for 50% mortality), GR_{50} (the dose required for 50% biomass reduction) and resistance index[a] (RI) values for oriental mustard populations treated with diflufenican. Values in parentheses are 95% confidence intervals. Data for the two experimental runs were pooled.

Population	Survival		Biomass	
	LD_{50} (g a.i. ha^{-1})	RI	GR_{50} (g a.i. ha^{-1})	RI
P40	6244 (4897, 7963)	237.4	4533 (3517, 5844)	195.3
P3	3770 (3154, 4506)	143.3	2922 (2521, 3386)	126.0
S1	24.7 (24.7, 24.7)	-	21.6 (20.2, 23.1)	-
S2	27.9 (27.9, 27.9)	-	24.8 (22.9, 26.9)	-

[a] Resistance indices (RI) were calculated as the ratio between the LD_{50} (or GR_{50}) of the resistant P40 and P3 populations compared with the mean LD_{50} (or GR_{50}) values of two susceptible populations (S1 and S2). The recommended field rate of diflufenican for POST treatment of oriental mustard in crops in South Australia is 200 g a.i. ha^{-1}.

Picolinafen completely killed all plants of both the susceptible populations and the known diflufenican-resistant control population (P3) at 33g ha^{-1}, the lowest recommended field rate in Australia. The resistant population (P40) showed less mortality at this rate (Fig 1). The picolinafen rate causing 50% mortality (LD_{50}) for the susceptible populations (S1 and S2) was about 10 g ha^{-1}, whereas, the LD_{50} for the resistant population P40 was 75 g ha^{-1} making it 7-fold more resistant to picolinafen than the susceptible populations (Fig 1, Table 2).

Table 2. Estimated LD_{50} (the dose required for 50% mortality), GR_{50} (the dose required for 50% biomass reduction) and resistance index[a] (RI) values for oriental mustard populations

treated with picolinafen. Values in parentheses are 95% confidence intervals. Data for the two experimental runs were pooled.

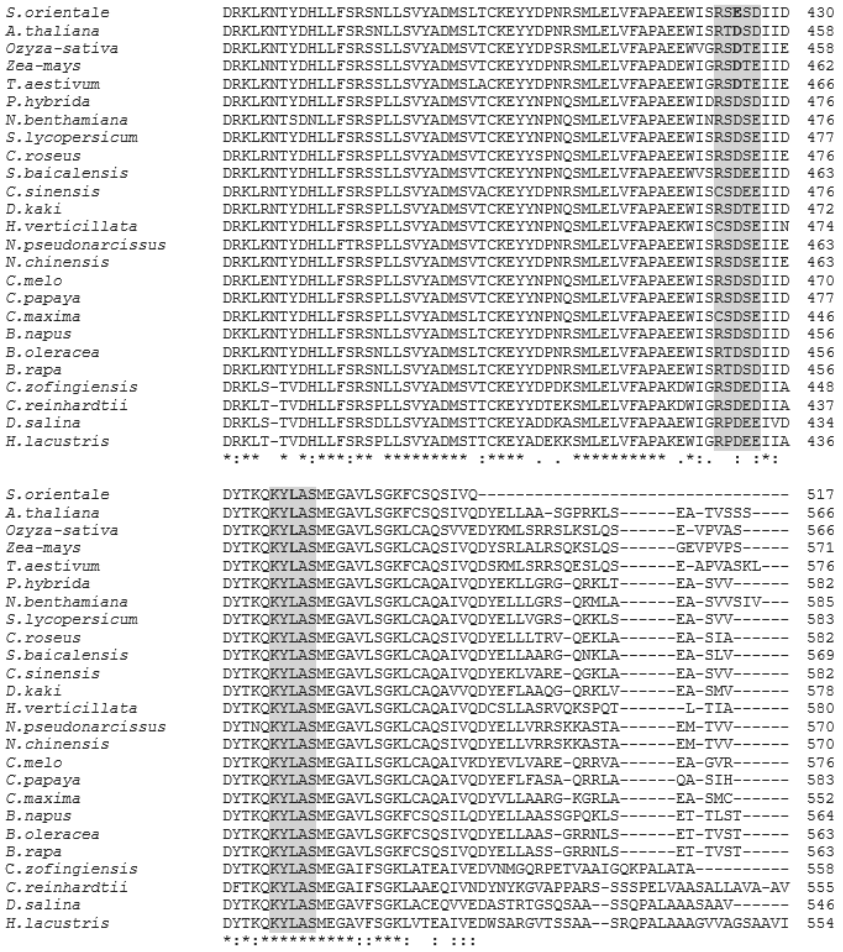
Population	Survival		Biomass	
	LD_{50} (g a.i. ha^{-1})	RI	GR_{50} (g a.i. ha^{-1})	RI
P40	74.7 (64.0, 87.4)	7.2	45.5 (40.6, 51.1)	4.9
P3	11.0 (9.5, 12.9)	1.0	10.5 (9.7, 11.5)	1.0
S1	9.3 (8.0, 10.9)	-	8.5 (8.1, 8.9)	-
S2	11.5 (9.8, 13.4)	-	10.0 (9.7, 10.4)	-

[a] Resistance indices (RI) were calculated as the ratio between the LD_{50} (or GR_{50}) of the resistant (P40) population compared with the mean LD_{50} (or GR_{50}) values of two susceptible populations (S1 and S2). The recommended field rate of picolinafen for POST treatment of oriental mustard in crops in South Australia is 33 to 50 g a.i. ha^{-1}.

3.2 Sequencing of the PDS gene

Unless otherwise specified, the numbering in the PDS gene sequence of oriental mustard (NCBI accession number MG493466.1) was used. When the sequences obtained from the partial PDS gene fragments of the resistant (P40) and susceptible (S1) populations were compared, the change from leucine (TTA) to valine (GTA) at position Leu498 (Leu-498-Val) (Fig 2) was identified in all individuals of both resistant populations but not in any individual of the susceptible population. Meanwhile, the change of amino acid from glutamine (GAG) to asparagine (GAC) at position Asp425 (Glu-425-Asp) (Fig 2) was detected only in population P40, but it was not found in populations P3 and S1.

(a)



(b)

Fig 2: Aligned partial sequence of PDS gene of *S. orientale* with other species containing of two investigated amino acids (Glu425 and Leu498) and aligned sequences of the susceptible (S) and resistant (P3 and P40) biotypes of the oriental mustard. Mutations in the resistant biotypes PDS gene are indicated by shaded and bold letters representing the nucleotide differences when compared with susceptible biotype.

No substitution was detected at the Arg260 (equivalent to Arg304 numbering in *H. verticillata*) in any individuals of either resistant or susceptible populations investigated. In addition, no other substitution was identified in the rest of the PDS gene when aligned with the mRNA obtained from resistant and susceptible individuals of the oriental mustard populations (P40 and S1) investigated in the current study.

3.3 Evaluation of F_1 populations and segregation pattern of F_2 populations

Crosses (pods) harvested from the susceptible S1 and resistant P40 parent plants were screened with diflufenican and picolinafen at 200 and 20 g ha^{-1}, respectively. All seedlings from self-pollinated seeds of the susceptible plants showed severe damage and died 28 DAT. Meanwhile, all the seedlings from self-pollinated seeds of the resistant plants showed little or no visible damage. All F_1 seedlings from seeds collected from the resistant parents survived with little or no damage. Seedlings of the F_1 seed set on the susceptible parent (P40♂ x S1♀) also survived diflufenican and picolinafen treatments with little or no damage showing that cross-pollination had been successful. Ten F_1 plants from ten P40♂ x S1♀ crosses were allowed to self-pollinate to produce F_2 families.

The F_2 plants segregated when treated with diflufenican and picolinafen at 200 and 20 g ha^{-1}, respectively. For diflufenican treatment, the response of the ten F_2 families were not significantly different (homogeneity P=0.944; 9df) (Table 3).

Table 3. Segregation for resistance to 200 g ha^{-1} diflufenican in F_2 populations generated from crosses (P40♂×S1♀) between resistant P40 and susceptible S1 oriental mustard plants.

Family	Treated	Dead*	Alive**	*G*-statistic	*P*
S1	210	210	0		
P40	212	0	212		
P40 (1)	207	56	151	0.456	0.499
P40 (2)	210	56	154	0.306	0.580
P40 (3)	208	55	153	0.227	0.633
P40 (4)	202	56	146	0.779	0.378
P40 (5)	209	57	152	0.563	0.453
P40 (6)	215	56	159	0.124	0.725
P40 (7)	208	56	152	0.403	0.526
P40 (8)	212	57	155	0.395	0.530
P40 (9)	208	53	155	0.025	0.873
P40 (10)	206	49	157	0.163	0.686
Total	2085	551	1534	2.236	0.135
Homogeneity				3.442	0.944

* Plants with no new growth and severe necrosis were recorded as dead

** Plants with new green leaf tissues were recorded as alive

Likewise, the response of these F_2 families were not significantly different (homogeneity *P*=0.872; 9df) when treated with picolinafen (Table 4).

Table 4. Segregation for resistance to 20 g ha^{-1} picolinafen in F_2 populations generated from crosses (P40♂×S1♀) between resistant P40 and susceptible S1 oriental mustard plants.

Family	Treated	Dead*	Alive**	*G*-statistic	*P*
S1	213	213	0		
P40	205	0	205		
P40 (1)	214	60	154	1.024	0.312
P40 (2)	208	51	157	0.026	0.873
P40 (3)	210	59	151	1.043	0.307
P40 (4)	206	56	150	0.513	0.474
P40 (5)	205	55	150	0.359	0.549
P40 (6)	211	55	156	0.126	0.722
P40 (7)	210	54	156	0.057	0.812
P40 (8)	209	58	151	0.822	0.364
P40 (9)	211	51	160	0.078	0.780
P40 (10)	214	58	156	0.495	0.482
Total	2098	557	1541	2.649	0.104
Homogeneity				4.543	0.872

* Plants with no new growth and severe necrosis were recorded as dead

** Plants with new green leaf tissues were recorded as alive

The results show that the parents of the crosses were likely homozygous for the resistance trait, which is a common feature expected in a highly self-pollinating species such as oriental mustard [36]. In both diflufenican and picolinafen treatments, the pattern of segregation was consistent with the model of a single dominant gene. The segregation of F_2 plants with high levels of damage and those with little or no visible damage fitted the expected ratio (3:1) for a single dominant allele for both herbicide treatments (Table 3 and 4).

The dose-response experiments on F_2 seedlings showed that the response of F_2 populations to both herbicides was intermediate between the resistant and susceptible parents (Fig 3). For diflufenican, the dose-response curve for each F_2 population showed a single step down, with survival declining to about 75% at 100 g ha^{-1} and remained at this level until the highest rate (3200 g ha^{-1}) (Fig 3). For picolinafen, the dose-response curve for each F_2 population also showed a single step, with survival declining to about 75% at 8.25 g ha^{-1} and remained at this level until the dose reached 33 g ha^{-1} (Fig 3). This type of response is an indication of segregation for resistance in the F_2 population for a single dominant allele [36]. To confirm this hypothesis, a model for a single dominant allele was calculated (dotted line). The F_2 response was similar to the model prediction for a single dominant allele at most of the rates of diflufenican and picolinafen (Fig 3).

When 48 individuals of the F_2 populations were screened for the presence of the Leu-498-Val and Glu-425-Asp mutations, the genotypes were classified as 9 homozygous susceptible (Glu425/Glu425, Leu498/Leu498): 24 heterozygous (Glu425/Asp425, Leu498/Val498): 15 homozygous resistant (Asp425/Asp425, Val498/Val498) individuals. This observed segregation ratio of genotypes is consistent with a 1:2:1 ratio when tested with the *G*-test of the goodness-of-fit at G=1.50 and P =0.472 (data not shown).

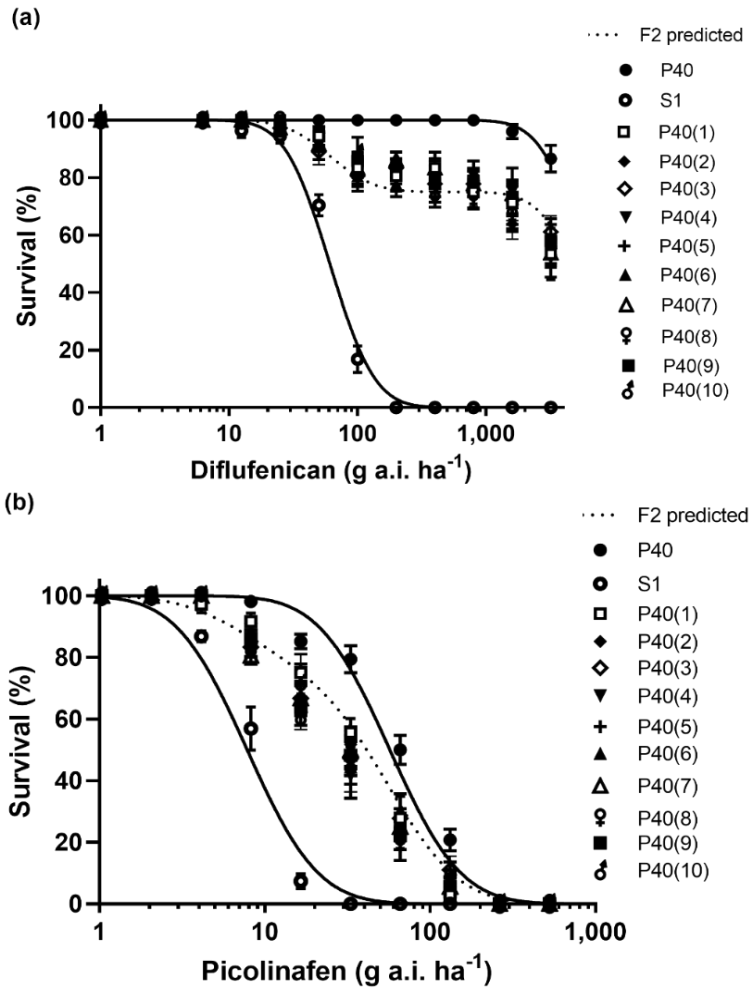


Fig.3. Dose-response of susceptible S1 (○), resistant P40 (●), and ten F_2 populations (□, ♦, ◊, ▼, +, ▲, Δ, ♀, ■ and ♂) of oriental mustard treated with diflufenican (a) and picolinafen (b). The dotted lines is the predicted response for a single dominant gene at all doses of diflufenican or picolinafen. Data points are means ± 95% confidence intervals for six replicates (three replicates x two runs) for parental populations (P40 and S1), and four replicates (two replicates x two runs) for each F_2 population.

4. DISCUSSION

4.1 Whole plant dose-response

The use of herbicides has been the most effective and economic solution for oriental mustard control in Australian cropping systems. However, this option has become less effective over time due to evolution of herbicide resistance in this weed species. Since the first case of resistance to ALS-inhibitors was confirmed in early 1990s [6], resistance to other herbicide groups such as photosystem II-inhibitors [7], synthetic auxins and PDS inhibitors [5, 8, 9] have been reported. Moreover, cross-resistance and multiple resistance in oriental mustard populations has also been confirmed in recent years [5] which has added more challenges to the management of this weed species in the grains industry in southern Australia.

In comparison with other herbicide modes of action, the evolution of resistance to the PDS inhibiting herbicides is at a lower level [9]. To date, only a few weed species have evolved resistance to PDS inhibitors. In most of these species, resistance is limited to a single member of this herbicide group. These examples include resistance to norflurazon in *Poa annua*, fluridone in *H. pluvialis* [22] and *Apera spica-venti* [9], diflufenican in *R. raphanistrum* [23] and oriental mustard [25]. Even though resistance to fluridone in *H. pluvialis* is expressed at a low level (3 to 6-fold), it has caused significant challenges in the control of this weed species in the United States [22, 40]. The findings from the current study showed that the recommended field rate of diflufenican used for the control of broadleaf weeds in Australia (200 g ha^{-1}) is more than adequate for the control of susceptible oriental mustard populations (LD_{50} <30 g ha^{-1}). In contrast, the resistant population P40 required much higher doses (LD_{50}> 6000 g ha^{-1}) than the recommended field rate for weed control, equating to 237-fold greater resistance than the susceptible populations. In comparison with the previously characterized diflufenican-resistant population P3, P40 exhibited a higher level of resistance to diflufenican at the LD_{50} level (237 vs 143-fold) (Table 1 and 2). For picolinafen, plants of both susceptible and the diflufenican-resistant P3 populations required relatively low rates to be controlled (LD_{50} ~ 10 g ha^{-1}), but P40 population required considerably more than the recommended

field rate to be killed (LD_{50} >70 g ha^{-1}) (Table 2, Fig 1). These findings imply that the use of diflufenican and picolinafen, will no longer be effective to control oriental mustard population P40. Furthermore, in the future oriental mustard populations suspected of resistance to diflufenican should also be screened against other PDS inhibitors, such as picolinafen, to determine which herbicides might still be useful for control.

4.2 Mechanisms of resistance to diflufenican and picolinafen

Previous studies [22, 26-28] have demonstrated that resistance to PDS-inhibitors is likely due to point mutations in the PDS gene. In field-evolved fluridone-resistant *H. verticillata*, amino acid substitutions (Ser, Cys, or His) at Arg304 (equivalent to Arg260 numbering in the *S. orientale* sequence) within the PDS gene resulted in the expression of a relatively low-level (3 to 6-fold) resistance to fluridone (4 to 6-fold) [22, 24, 41]. However, no mutation was present at this position in resistant oriental mustard population P40 investigated in this study.

In transgenic studies conducted on *H. pluvialis*, the substitution Leu-504-Arg not only improved the yield of carotenoid biosynthesis, it also exhibited a high level (43-fold) of resistance to norflurazon in comparison with the susceptible biotypes [26]. Similarly, the Leu538 mutated to Phe or Arg in the PDS gene of *C. zofingiensis* [27] and *C. reinhardtii* [28] enhanced biosynthesis of astaxanthin in *C. zofingiensis* and carotenoids in *C. reinhardtii* as well as 28 to 31-fold, respectively, greater resistance to norflurazon. Likewise, a mutation was also identified at the same amino acid of the PDS gene in oriental mustard [25]. This position is equivalent to the Leu538 in *Oryza sativa*, which was found to affect the size of the binding pocket (Ala 539) of PDS-inhibitors in plants (Fig 2) [42] (discussed in Dang et al.[25]). The Leu-498-Val substitution in the PDS gene detected in both P3 and P40 populations appears to confer a high-level resistance to diflufenican but no resistance to picolinafen. In addition to

Leu538 mutation, P40 population also had Glu425 mutation in the PDS gene, which was not present in P3 (Fig 2). The Glu425 (Glu-425-Asp) (NCBI accession number MG493466.1) mutation in the PDS gene of the resistant population P40 identified in the current study has not been reported elsewhere in any organism. Asp at this position in the PDS gene is found in other species (Fig 2). However, in combination with the Leu-498-Val mutation in the PDS gene, the substitution Glu-425-Asp appears to enhance the level of resistance to diflufenican (237-fold) and causes moderate cross-resistance to picolinafen (7-fold) in the oriental mustard population investigated (P40).

4.3 Inheritance of resistance to diflufenican and picolinafen

With the exception of triazine resistance, which has maternal inheritance, herbicide resistance in most cases is inherited as nuclear genes [29]. Resistance traits managed by dominant genes will display resistance in both homozygous and heterozygous states [43]. In a previous study, resistance to diflufenican in oriental mustard was conferred by a single gene with high level of dominance [25]. All F_1 seeds harvested from the susceptible parents showed a similar response to the resistant parent (P40) when treated with diflufenican and picolinafen at 200 and 20 g ha^{-1}, respectively, which indicates a high level of dominance of the resistance gene. Based on the phenotype segregation ratio of each F_2 population when treated with a single rate of the two herbicides, resistance to both diflufenican (P=0.135) and picolinafen (P=0.104) (Table 3 and 4) was likely conferred by a single dominant allele in all families. Analysis of the dose-response of the F_2 plants also showed a high level of dominance over susceptibility as the response of F_2 populations fitted well to the one gene segregation model for both herbicides (Fig 3). Furthermore, segregation rate of the F_2 genotypes based on the PDS mutations fitted to a 1:2:1 ratio at G=1.50 and P = 0.472, indicating that the resistance to diflufenican and picolinafen is correlated with the Leu-498-Val and Glu-425-Asp substitutions in the PDS

gene. As the inheritance pattern was for a single gene trait, the higher level of resistance and the cross-resistance to picolinafen must be the result of a further amino acid substitution within PDS in the P40 population. The Glu-425-Asp substitution was the only difference found between the sequences of the P3 and P40 populations, suggesting that despite it being present normally in other species, this substitution has a role in reducing efficacy of diflufenican and picolinafen in oriental mustard.

A few previous studies have shown that NTSR mechanisms provide tolerance to PDS-inhibitors in crops and other plants. Such examples include reduced translocation of norflurazon in corn (*Zea mays*) and soybean (*Glycine max*) [44] and flurtamone metabolism in sicklepod (*Cassia obtusifolia*) and peanut (*Arachis hypogea*) [45]. Although NTSR mechanisms were not investigated in the current study, the patterns of genotype and phenotype segregation of the F_2 plants observed in the inheritance study are consistent with a single gene effect and imply that NTSR mechanisms are unlikely to be contributing to resistance in this population (P40) of oriental mustard.

This is the first reported case of field-evolved cross-resistance to diflufenican and picolinafen in any plant species. Given the high level of resistance to diflufenican and cross-resistance to picolinafen associated with Leu-498-Val and Glu-425-Asp mutations in the PDS gene, increasing the herbicide dose is unlikely to improve the control of such oriental mustard populations. Therefore, strategies based on the integration of herbicides with non-chemical weed control tactics are needed to achieve an effective level of weed control and slowdown the evolution of resistance to PDS-inhibitors in this weed species [46-48].

ACKNOWLEDGEMENTS

The authors are thankful to the Vietnam Government and the University of Adelaide for a PhD scholarship and the Grains Research and Development Corporation for financial support. We also would like to acknowledge Dr. Peter Boutsalis for providing research samples and Ruwan Lenorage, Geetha Velappan for technical assistance.

REFERENCES

1. Amor R and Francisco T, Survey of weeds in field peas, chickpeas and rapeseed in the Victorian Wimmera. *Plant Protection Quarterly* **2**: 124-127 (1987).
2. Boutsalis and Powles, Seedbank characteristics of herbicide-resistant and susceptible *Sisymbrium orientale*. *Weed Res* **38**: 389-395 (1998).
3. Chauhan BS, Gill G and Preston C, Influence of environmental factors on seed germination and seedling emergence of oriental mustard (*Sisymbrium orientale*). *Weed Science* **54**: 1025-1031 (2006).
4. McGillion T and Storrie A, Integrated weed management in Australian cropping systems–a training resource for farm advisors. *CRC for Australian Weed Management: Adelaide* (2006).
5. Preston C, Dolman FC and Boutsalis P, Multiple resistance to acetohydroxyacid synthase–inhibiting and auxinic herbicides in a population of oriental mustard (*Sisymbrium orientale*). *Weed Sci* **61**: 185-192 (2013).
6. Boutsalis P and Powles S, Resistance of dicot weeds to acetolactate synthase (ALS)-inhibiting herbicides in Australia. *Weed Res* **35**: 149-155 (1995).
7. Dang HT, Malone JM, Boutsalis P, Gill G and Preston C, Identification of a target-site mutation conferring resistance to triazine herbicides in oriental mustard (*Sisymbrium orientale* L.) from Australia. *Weed Biol Manag* **17**: 153-160 (2017).
8. Dang HT, Malone JM, Boutsalis P, Krishnan M, Gill G and Preston C, Reduced translocation in 2,4-D resistant oriental mustard populations (*Sisymbrium orientale* L.) from Australia. *Pest Manag Sci* (2017). *In press*
9. Dayan FE, Owens DK, Tranel PJ, Preston C and Duke SO, Evolution of resistance to phytoene desaturase and protoporphyrinogen oxidase inhibitors - state of knowledge. *Pest Manag Sci* **70**: 1358-1366 (2014).

10. Ashton IA, Abulnaja KO, Pallett KE, Cole DJ and Harwood JL, The mechanism of inhibition of fatty acid synthase by the herbicide diflufenican. *Phytochemistry* **35**: 587-590 (1994).

11. Haynes C and Kirkwood RC, Studies on the mode of action of diflufenican in selected crop and weed species: Basis of selectivity of pre-and early post-emergence applications. *Pest Manag Sci* **35**: 161-165 (1992).

12. Rouchaud J, Gustin F, Van Himme M, Bulcke R, Benoit F and Maddens K, Metabolism of the herbicide diflufenican in the soil of field wheat crops. *J Agric Food Chem Journal* **39**: 968-976 (1991).

13. Hashimoto H, Uragami C and Cogdell RJ. Carotenoids and Photosynthesis. In *Carotenoids in Nature: Biosynthesis, Regulation and Function*, ed. by Stange C. Springer International Publishing: Cham, pp. 111-139 (2016).

14. Armel GR, Rardon PL, McComrick MC and Ferry NM, Differential response of several carotenoid biosynthesis inhibitors in mixtures with atrazine. *Weed Technol* **21**: 947-953 (2007).

15. Boger P, Mode of action of herbicides affecting carotenogenesis. *J Pestic Sci* **21**: 473-478 (1996).

16. Misawa N, Masamoto K, Hori T, Ohtani T, Böger P and Sandmann G, Expression of an Erwinia phytoene desaturase gene not only confers multiple resistance to herbicides interfering with carotenoid biosynthesis but also alters xanthophyll metabolism in transgenic plants. *The Plant Journal* **6**: 481-489 (1994).

17. Ohki S, Miller-Sulger R, Wakabayashi K, Pfleiderer W and Boger P, Phytoene desaturase inhibition by O-(2-phenoxy)ethyl-N-aralkylcarbamates. *J Agric Food Chem* **51**: 3049-3055 (2003).

18. Bartley GE, Viitanen PV, Pecker I, Chamovitz D, Hirschberg J and Scolnik PA, Molecular cloning and expression in photosynthetic bacteria of a soybean cDNA coding for phytoene desaturase, an enzyme of the carotenoid biosynthesis pathway. *Proc Natl Acad Sci* **88**: 6532-6536 (1991).

19. Sandmann G, Schmidt A, Linden H and Boger P, Phytoene desaturase, the essential target for bleaching herbicides. *Weed Sci* **39**: 474-479 (1991).

20. Boger P and Sandmann G, Carotenoid biosynthesis inhibitor herbicides-mode of action and resistance mechanisms. *Pesticide Outlook (United Kingdom)* (1998).

21. Heap I, *The International Survey of Herbicide Resistant Weeds*, http://weedscience.org (acessed 30 Dec 2017).

22. Michel A, Arias RS, Scheffler BE, Duke SO, Netherland M and Dayan FE, Somatic mutation-mediated evolution of herbicide resistance in the nonindigenous invasive plant hydrilla (*Hydrilla verticillata*). *Molecular Ecology* **13**: 3229-3237 (2004).

23. Walsh MJ, Powles SB, Beard BR, Parkin BT and Porter SA, Multiple-herbicide resistance across four modes of action in wild radish (*Raphanus raphanistrum*). *Weed Sci* **52**: 8-13 (2004).

24. Benoit LK and Les DH, Rapid identification and molecular characterization of phytoene desaturase mutations in fluridone-resistant hydrilla (*Hydrilla verticillata*). *Weed Science*; **61**(1): 32-40 DOI Electronic Resource Number (2013).

25. Dang HT, Malone JM, Boutsalis P, Gill G and Preston C, The Mechanism of Diflufenican Resistance and its Inheritance in Oriental Mustard (*Sisymbrium orientale* L.) from Australia. *Pest Manag Sci* (2018). *In press*

26. Steinbrenner J and Sandmann G, Transformation of the green alga Haematococcus pluvialis with a phytoene desaturase for accelerated astaxanthin biosynthesis. *Appl. Environ. Microbiol* **72**: 7477-7484 (2006).

27. Liu J, Zhong Y, Sun Z, Huang J, Sandmann G and Chen F, One amino acid substitution in phytoene desaturase makes *Chlorella zofingiensis* resistant to norflurazon and enhances the biosynthesis of astaxanthin. *Planta* **232**: 61-67 (2010).

28. Liu J, Gerken H, Huang JC and Chen F, Engineering of an endogenous phytoene desaturase gene as a dominant selectable marker for *Chlamydomonas reinhardtii* transformation and enhanced biosynthesis of carotenoids. *Process Biochemistry* **48**: 788-795 (2013).

29. Rao VS. *Transgenic herbicide resistance in plants*. Crc Press (2014).

30. Beckie HJ and Tardif FJ, Herbicide cross resistance in weeds. *Crop Protection* **35**:15-28 (2012).

31. Kaloumenos NS, Chatzilazaridou SL, Mylona PV, Polidoros AN and Eleftherohorinos IG, Target-site mutation associated with cross-resistance to ALS-inhibiting herbicides in late watergrass (*Echinochloa oryzicola* Vasing.). *Pest Manag Sci* **69**: 865-873 (2013).

32. Burgos NR, Norsworthy JK, Scott RC and Smith KL, Red rice (*Oryza sativa*) status after 5 years of imidazolinone-resistant rice technology in Arkansas. *Weed Technol* **22**: 200-208 (2008).

33. Zheng D, Kruger GR, Singh S, Davis VM, Tranel PJ, Weller SC and Johnson WG, Cross-resistance of horseweed (*Conyza canadensis*) populations with three different ALS mutations. *Pest Manag Sci* **67**: 1486-1492 (2011).

34. Busi R and Powles SB, Cross-resistance to prosulfocarb and triallate in pyroxasulfone-resistant *Lolium rigidum*. *Pest Manag Sci* **69**: 1379-1384 (2013).

35. Kaloumenos NS, Adamouli VN, Dordas CA and Eleftherohorinos IG, Corn poppy (*Papaver rhoeas*) cross-resistance to ALS-inhibiting herbicides. *Pest Manag Sci* **67**: 574-585 (2011).

36. Preston C and Malone JM, Inheritance of resistance to 2,4-D and chlorsulfuron in a multiple-resistant population of *Sisymbrium orientale*. *Pest Manag Sci* **71**: 1523-1528 (2015).

37. Boutsalis P, Gill GS and Preston C, Incidence of herbicide resistance in rigid ryegrass (*Lolium rigidum*) across southeastern Australia. *Weed Technol* **26**: 391-398 (2012).

38. Sakuma M, Probit analysis of preference data. *Appl Entomo Zool* **33**: 339-347 (1998).

30. Mantel N, Chi-square tests with one degree of freedom; extensions of the Mantel-Haenszel procedure. *Journal of the American Statistical Association* **58**: 690-700 (1963).

40. Netherland MD and Jones D, Fluridone-resistant hydrilla (*Hydrilla verticillata*) is still dominant in the kissimmee chain of lakes, FL. *J Invas Plant Sci Mana* **8**: 212-218 (2015).

41. Puri A, MacDonald GE, Altpeter F and Haller WT, Mutations in phytoene desaturase gene in fluridone-resistant Hydrilla (*Hydrilla verticillata*) biotypes in Florida. *Weed Sci* **55**: 412-420 (2007).

42. Brausemann A, Gemmecker S, Koschmieder J, Ghisla S, Beyer P and Einsle O, Structure of phytoene desaturase provides insights into herbicide binding and reaction mechanisms involved in carotene desaturation. *Structure* **25**: 1222-1232 (2017).

43. Gasquez J. Genetics of Herbicide Resistance within Weeds. Factors of Evolution, Inheritance and Fitness. In *Weed and Crop Resistance to Herbicides*, ed. by De Prado R, Jorrín J and García-Torres L. *Springer Netherlands*, pp. 181-189 (1997).

44. Strang RH and Rogers RL, Behavior and fate of two phenylpyridazinone herbicides in cotton, corn, and soybean. J Agric Food Chem 22: 1119-1125 (1974).

45. Vencill WK, Absorption, translocation, and metabolism of flurtamone in sicklepod (Cassia obtusifolia) and peanut (Arachis hypogea). *J Plant Growth Regul* **13**: 11-14 (1994).

46. Rey-Caballero J, Royo-Esnal A, Recasens J, Gonzalez I and Torra J, Management options for multiple herbicide-resistant corn poppy (*Papaver rhoeas*) in Spain. *Weed Sci* **65**: 295-304 (2017).

47. Beckie HJ, Beneficial management practices to combat herbicide-resistant grass weeds in the northern Great Plains. *Weed Technol* **21**: 290-299 (2007).

48. Lagator M, Vogwill T, Mead A, Colegrave N and Neve P, Herbicide mixtures at high doses slow the evolution of resistance in experimentally evolving populations of *Chlamydomonas reinhardtii*. *New Phytologist* **198**: 938-945 (2013).

CHAPTER 6

NO APPARENT FITNESS COSTS ASSOCIATED WITH MUTATIONS IN PHYTOENE DESATURASE PROVIDING RESISTANCE TO DIFLUFENICAN AND PICOLINAFEN IN ORIENTAL MUSTARD (*SISYMBRIUM ORIENTALE* L.)

Hue Thi Dang, Weihua Long, Jenna M Malone, Gurjeet Gill and Christopher Preston

School of Agriculture, Food and Wine, University of Adelaide

PMB1, Glen Osmond, South Australia 5064, Australia

Pest Management Science: Submitted paper

Statement of Authorship

Title of Paper	No Apparent Fitness Costs Associated With Mutations in Phytoene Desaturase Providing Resistance to Diflufenican and Picolinafen in Oriental Mustard (*Sisymbrium orientale* L.)
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Dang, H. T., Long, W., Malone, J. M., Preston, C. and Gill, G. (2018), No apparent fitness costs associated with mutations in phytoene desaturase providing resistance to diflufenican and picolinafen in oriental mustard (*Sisymbrium orientale* L.). Pest. Manag. Sci. Submitted paper

Principal Author

Name of Principal Author (Candidate)	Hue Thi Dang		
Contribution to the Paper	Planned the study, conducted all experiments, analyzed and interpreted data and wrote manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	27.02.2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Weihua Long		
Contribution to the Paper	Helped with conducting experiments and data collection		
Signature		Date	28/2/2018

Name of Co-Author	Jenna Moira Malone		
Contribution to the Paper	Supervised the molecular work, data interpretation and edited manuscript		
Signature		Date	27/2/18

Name of Co-Author	Christopher Preston		
Contribution to the Paper	Supervised development of research, data interpretation and analysis, and edited manuscript		
Signature		Date	02/03/2018

Name of Co-Author	Gurjeet Gill		
Contribution to the Paper	Supervised development of research, data interpretation and analysis, and edited manuscript		
Signature		Date	27.2.2018

ABSTRACT

BACKGROUND: Two populations of oriental mustard with mutations at Leu498 or Leu498 and Glu425 in the PDS gene have resistance to diflufenican or to diflufenican and picolinafen, respectively. As mutations in plant genes can affect the fitness of a resistant biotype, this study was designed to investigate presence of fitness penalty associated with these PDS gene mutations.

RESULTS: Genotypes of 435 out of 448 F_2 individuals used in the fitness study were determined based on a cleaved amplified polymorphic sequence (CAPS) marker developed for diagnostic determination of resistance alleles using the restriction enzyme HpyCH4IV. Regression analysis confirmed no significant difference in seed and biomass production of homozygous resistant (RR), heterozygous resistant (RS) and susceptible (SS) biotypes of the two oriental mustard populations (P40.5 and P3.2) either in monoculture or in the presence of wheat competition.

CONCLUSION: In the absence of herbicide selection pressure, the Leu498 or Leu498 and Glu425 mutations in the PDS gene do not reduce the fitness of diflufenican or diflufenican and picolinafen-resistant oriental mustard.

Keywords: diflufenican resistance; picolinafen, CAPS, fitness cost, phytoene desaturase.

1 INTRODUCTION

One of the more critical factors influencing persistence and development of a herbicide resistant biotype is its fitness, which includes its ability to establish, survive, and reproduce in the environment [1, 2]. It is generally expected that in a stress-free environment, mutations conferring herbicide resistance in weeds may result in a fitness penalty [3-5] relative to the susceptible (S) biotypes. In the absence of a fitness penalty, the resistant (R) biotype could grow and reproduce equally to the S biotype. Whereas, if the R population suffers a fitness penalty, resistant biotypes will slowly be replaced by susceptible biotypes [6]. Therefore, investigation of the fitness costs of resistant biotypes is important in predicting population dynamics, as well as for managing herbicide-resistant populations [7, 8].

Previous research has shown that the genetic background of a resistant biotype is important in quantifying the fitness costs associated with resistance [9]. As different genes may be associated with the expression of different fitness costs, it is necessary to identify the genes conferring resistance [10, 11]. If the genetic background of the biotypes has not been controlled, it is difficult to quantify the fitness penalty associated with resistance as numerous other factors may influence growth and reproduction [12]. Many previous studies of plant fitness in weeds have used plant material of different origin, which exhibits genetic variability and can confound the results [4]. Therefore, the use of similar genetic background materials is important in any fitness study [11].

The cleaved amplified polymorphic sequence (CAPS) method is a technique used for the analysis of genetic markers, which combines polymerase chain reaction (PCR) amplification and restriction digestion [13]. The technique involves PCR amplification of a target DNA sequence, followed by restriction digestion using endonuclease [14]. CAPS markers can detect the polymorphisms within a gene that are responsible for the variation in a

phenotypic trait [14]. Due to its high yield, accuracy and low cost, CAPS markers have been used widely in studying functional genetic diversity or identification of microorganisms [15].

Phytoene desaturase (PDS) inhibiting herbicides diflufenican and picolinafen are commonly used for the control of certain broadleaf weeds in Australian cropping systems [16-18]. Resistance to PDS inhibitors due to target-site point mutations has been reported in a few weed species [19, 20]. Oriental mustard (*Sisymbrium orientale* L.) is a widespread broadleaf weed of field crops in southern Australia, which often causes crop yield loss due to high infestations in winter crops (reviewed in Dang et al. [21]). This species has evolved resistance to most of the commonly used herbicides for its control, such as synthetic auxinic herbicides [21, 22], ALS (acetolactate synthase) [23], PDS inhibitors [20, 24] and triazines [25]. In recent studies, the mutation Leu498 (Leu-498-Val) alone in the PDS gene was found to confer a high level of resistance to diflufenican in oriental mustard [20]. In addition, the combination of Leu498 (Leu-498-Val) and Glu425 (Glu-425-Asp) was found to confer resistance to diflufenican and picolinafen in a second population of oriental mustard (Dang et al. unpublished).

In the presence of herbicides, the fitness advantage to resistant plants carrying these mutations (Leu498 or Leu498+Glu425) is obvious [20] (Dang et al. unpublished paper). However, the fitness consequences for the resistant plants in the absence of herbicides in crop competition are unknown. Therefore, this study was designed to investigate the effect of target-site point mutations (Leu498 or Leu498+Glu425) in the PDS gene to the fitness of oriental mustard in the absence of herbicides. We compared growth and fecundity (seed and biomass production) of plants possessing the single mutation (Leu-498-Val) [20] and double mutations (Leu-498-Val and Glu-425-Asp) (Dang et al. unpublished) in the PDS gene with susceptible plants in the F_2 generation of the two crosses P3♂ x S♀ and P40♂ x S♀.

2 EXPERIMENT METHODS

2.1 Plant materials and competition experiment

Seeds of two F_2 families/populations, P3.2 (P3♂ x S♀) and P40.5 (P40♂ x S♀) generated from previous studies [20] and Dang et al. (unpublished) were used. A neighbourhood model was applied as described by Vila-Aiub, Neve and Powles [4], with modification, in which all oriental mustard F_2 progenies were evaluated as the target plants while wheat at different densities was the neighbour providing interspecific competition. Seeds of oriental mustard were sown into trays containing standard potting mix [26]. At the 3-4 leaf stage, seedlings of similar size were transplanted in the middle of a 8.5L round pot with one plant per pot, which equates to a constant density of 20 plants m^{-2}. Seven densities (0, 20, 40, 60, 120, 200 and 400 plants m^{-2}) of wheat seeds (neighbour) were sown and spatially arranged as shown in Fig 1, with each neighbour equidistant from the target plant.

The experiments on diflufenican resistant population P3.2 were conducted in the main growing seasons of 2016 and 2017 (May to Nov) at the Waite campus (34°58'13.5"S 138°38'22.7"E). The experiments on diflufenican and picolinafen-resistant population P40.5 were conducted in the main growing season of 2017 at Waite (34°58'13.5"S 138°38'22.7"E) and the Roseworthy (34°31'28.8"S 138°41'10.2"E) campuses, The University of Adelaide, South Australia. These two sites are about 60 km apart. There were 16 target plants (16 replicates) for each neighbour density treatment, with seven densities, making 112 experimental units at each experimental site. For the experiment at the Waite campus, plant were grown in an outdoor net house, while plant were grown outdoors in a field at the Roseworthy campus. Pots were arranged in a completely randomised design and watered and fertilised as required.

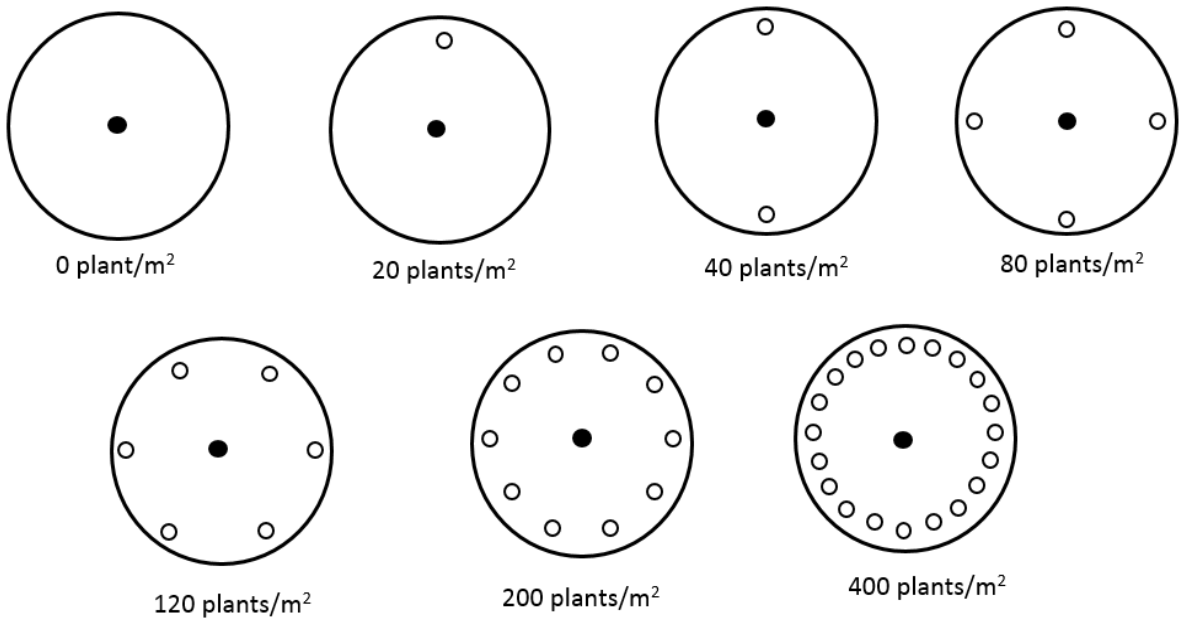


Figure 1. Target-neighbourhood model used to assess the competitive effects and responses of segregated genotype (F_2) in comparison with a crop (wheat). The closed symbols (●) represent target plants (F_2 genotypes) at a constant density of 20 plants per m^{-2} and open symbols (○) represent neighbour plants (wheat) at different densities.

2.2 Data collection and analysis

At maturity, the aboveground biomass of target plants (oriental mustard) was harvested and oven-dried at 65^{o}C until reaching a constant weight. Total biomass and total seed weight produced by each target plant was recorded. Data for seed and biomass production of target plants in the presence of neighbours (wheat) were expressed as a percentage of seed weight or shoot dry weight for that genotype in the absence of crop competition [27] (i.e. per-individual competitive responses). A hyperbolic nonlinear model was used to analyse the data to describe the response of the oriental mustard plants to increasing density of the neighbour plants [28] using GenStat 15.0.

$$Y = a + \frac{b}{(1+dx)}$$

Where *Y* represents the seed or biomass of the target plant at neighbour density *x*, *a* is the seed weight or biomass of the target plant in the absence of competitors (neighbours) (x=0) and *b* the slope of the regression. Data from each experiment was analysed separately.

2.3 Identification of F_2 genotypes

DNA extraction

Leaf tissue of each oriental mustard individual (target plant) was collected for DNA extraction during the vegetative phase of growth. Genomic DNA was extracted using methodology described by Dellaporta et al. [29] and Davis et al. [30], with modifications. Briefly, ~100 mg of young leaf tissue of individual plants was harvested and placed in a single micro-tube of a 96-tube plate (1.2ml, cluster tubes, Corning, Salt Lake City, USA). Plates containing the leaf tissues were stored at - 80°C for a couple of hours before freeze-drying overnight in a Freeze Dryer (DINAVAC, Belmont, WA, Australia). To grind the samples, one stainless steel ball bearing was added to each well and the tissues were ground using a shaking grinder (Retsch MM301, Mixer Tissue Lyser, Qiagen, Chadstone, VIC, Australia) for 2.5 minutes at a frequency of 25 s^{-1}. To each well, 600 µl extraction buffer (100 mM Tris-HCl, pH7.5, 250 mM NaCl, 25mM EDTA pH 8.0, 5gL^{-1} SDS) was added and the samples incubated at 65^{o}C in a water bath for 30 minutes. The plate was cooled at 4^{o}C for 15 minutes before adding 300µl of 6 M $C_2H_7NO_2$ (stored at 4^{o}C) and incubated at 4^{o}C for another 15 minutes. The extract was centrifuged at 4000 rpm for 15 minutes to precipitate proteins and plant tissues, and 400 µl supernatant was collected and mixed with 400 µl isopropanol. The mixture was centrifuged for 15 minutes at 4000 rpm, and the resultant pellet was washed with 200 µl 70%

ethanol. The pellet was vacuum dried, re-suspended in 100 µl MiQ water and stored at -20^{o}C for further use.

PDS cleaved amplified polymorphic sequence (CAPS) marker amplification

As resistant genotypes of populations P3.2 and P40.5 carry the same mutation, Leu-498-Val, genotyping of each F_2 individual of these two populations was based on the presence of the Leu498 mutation in the PDS gene. Specific primers (CAPSF3 and CAPSR4) were designed based on the PDS gene sequence of oriental mustard (NCBI accession number MG493466.1) as described in Dang et al. [21]. Briefly, the gene sequence was obtained from Genbank, the National Center for Biotechnology Information (NCBI) and the software Primer 3 Plus (Biomatics, Wageningen University, the Netherlands) and the website of NCBI were used to design and check for specificity of primers before use. An approximately 425-bp fragment of the PDS gene of each F_2 individual (448 samples) was amplified with the use of forward primer CAPS3F (5'-TGTCGTAAAAACACCAAGGTTAGA-3') and reverse primer CAPS4R (5'- GTCCAGACGCAGCCAHTAGCT- 3'). MyFi DNA polymerase kit (Bioline Australia Pty. Ltd., Alexandria, NSW, Australia) was used to run a PCR reaction of 25 µl, containing 80-100 ng DNA, 1× Myfi reaction buffer, 1 µM of each specific primer and 1 unit of Myfi Taq DNA Polymerase. An automated DNA thermal cycler (Eppendorf Master CyclerH Gradient, Germany) was used for DNA amplification with PCR conditions as follows: 3 min denaturing at 95°C; 38 cycles of 15 s denaturation at 95°C, 15 s annealing at 58°C and 2 min elongation at 72°C, and a final extension for 7 min at 72°C.

The PCR products were visualised on 1.5% agarose gels stained with 1 × SYBR® Safe DNA gel stain and prepared with 1 x Ficoll loading dye [15% (w/v) Ficoll 4000, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF]. Samples were electrophoresed in 1 x TAE Buffer [40 mM Trizma base, 1 mM Na_2EDTA, pH to 8 with glacial acetic acid] at 110 volts

and photographs were taken under UV light (λ302 nm). The sizes of DNA fragment were estimated by comparing their mobility to bands of known sizes in an Easy-ladder (Bioline Australia Pty. Ltd., Alexandria, NSW, Australia). To check the sequence quality, PCR products of 16 randomly selected F_2 samples were sent for DNA sequencing at the Australian Genome Research Facility (AGRF) Ltd., Australia using the same primers that were used for DNA amplification. The DNA sequence of each genotype was analysed and tested to ensure that the developed CAPS maker could be used to differentiate between the three genotypes (RR, RS and SS) of the F_2 populations investigated.

Restriction endonuclease digestion and agarose gel electrophoresis

Amplified DNA fragments were digested using the restriction enzyme HpyCH4IV (New England Biolabs, Massachusetts, USA), in a final volume of 15 µl containing 1 × specific NEB-buffer (1.5 µl), 5 unit endonuclease (0.5 µl) and 5 µl PCR product. The reaction was incubated in a water bath for 1 h at 37°C. The digests were resolved and visualised by electrophoresis in 1.5% agarose gels as described for DNA visualisation above. Photographs of the samples were taken under UV light (λ302 nm). The sizes of DNA fragments were estimated by comparing their mobility to bands of known sizes in an Easy-ladder (Bioline Australia Pty. Ltd). Genotype of each F_2 plant was determined based on patterns of DNA bands of each sample.

3. RESULTS

3.1 Genotyping of the plant

The restriction endonuclease recognition site of HpyCH4IV is A^CGT. This recognition site was found to be present in the PDS gene of oriental mustard at the Leu498 mutation site. The mutation at Lue498 is due to a single nucleotide polymorphism of T to G at the first base position of leucine (TTA), causing an amino acid change to valine (GTA). The first two

nucleotides of codon Leu498 are the last two nucleotides of the HpyCH4IV recognition site. Therefore, in wild type susceptible individuals with no mutation, the sequence at the recognition site is A^C*T*T, and the enzyme does not cut. However in case of resistant individuals, the sequence is A^C*G*T, and the enzyme recognition site is present, and the enzyme does cut. As any mutation (sense or non-sense) could result in loss of the restriction site, it was carefully checked for presence of any additional single nucleotide polymorphisms in the proposed gene fragment. Results showed that DNA sequences from R and S plants were identical around the restriction site. This makes it possible to differentiate RR, RS and SS plants.

The DNA of homozygous resistant (RR) plants displayed two fragments of 157 and 265 bp and the susceptible (SS) plant showed one undigested band of 425 bp, while DNA of the heterozygous resistant (RS) plants displayed a combination of three bands (Fig 2).

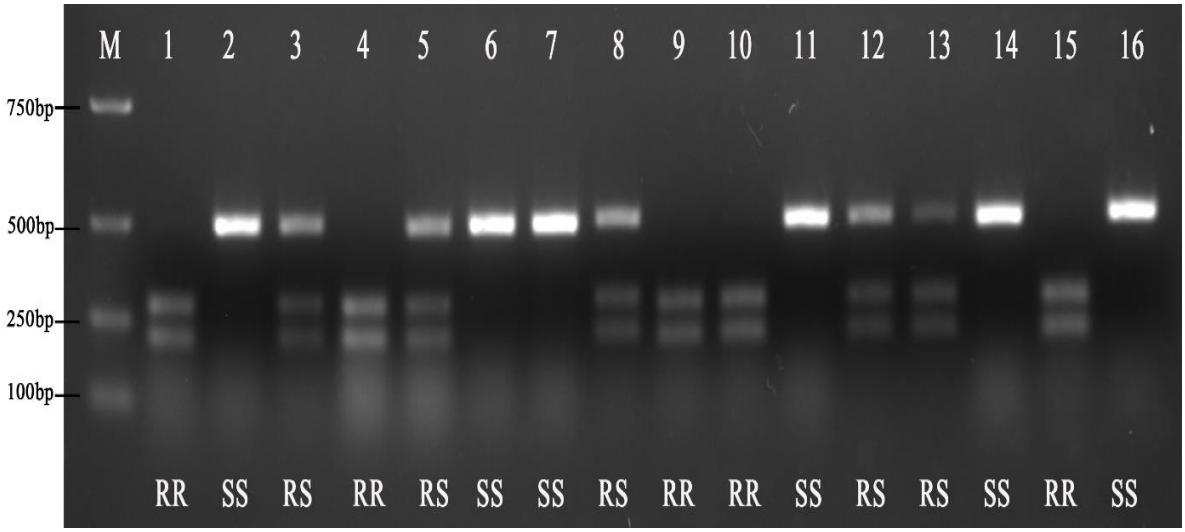


Fig 2. Electrophoresis profile of PDS cleaved amplified polymorphic sequence (CAPS) marker digest. The genotype of each F_2 individual was determined by the banding fingerprint as being either homozygous resistant (RR-double bands), heterozygous resistant (RS-triple bands) and susceptible (SS-a single band).

Except for 13 samples that were undeterminable, genotypes of 435 out of 448 samples (97%) were confirmed based on the patterns of DNA bands. Based on this analyses, 219 samples of population P40.5 could be classified into 56 homozygous resistant: 106 heterozygous resistant: 57 susceptible individuals. For population P3.2, the genotypes 216 samples were classified into 56 homozygous resistant: 114 heterozygous resistant: 46 susceptible individuals.

3.2 Competitive responses of oriental mustard to wheat

Data were analysed separately for each year for population P3.2 or location for population P40.5 as significant differences were found in the parameters between years or locations of the two populations. In the absence of crop competition, the oriental mustard produced the highest amount of seed and dry weight per plant, but there was no significant difference between the three genotypes (Table 1 and 2). The rectangular hyperbola fitted the data well and accounted for 86-97% of the variation in shoot biomass and 81-91% of the variation for seed set of target plants of P40.5 (Table 1). Similarly, hyperbolic model accounted for 75-94% of the variation in biomass and 75-89% of the variation in seed set in P3.2 in 2016. However, the same model accounted for 60-72% variation in shoot biomass and 63-77% of the variation in seed set ($P<0.001$) for P3.2 in 2017. The seed and biomass production of the three genotypes (RR, RS and SS) decreased gradually with the increase in wheat density (Fig 3, 4, 5 and 6). Student's *t*-test (GraphPad Prism v6, GraphPad software, San Diego, CA) showed there were no significant differences in the response of the three genotypes of both populations (P3.2 and P40.5) to wheat competition ($P > 0.05$) (Table 1 and 2).

4. DISCUSSION

A number of studies have been conducted to investigate the fitness cost of TSR and NTSR mechanisms of herbicide resistance. It has been argued that similarity of genetic background of R and S plants would improve the probability of identifying fitness costs of resistant biotypes [31]. The values of the fitness-cost in resistant plants could be affected by intraspecific genetic diversity [11]. In most of the early work, the genetic background of the R and S plants tended to differ, which may have led to flawed conclusions [4]. In the current study, fitness of the individuals was investigated in segregating genotypes within the same F_2 populations [20]. This enabled us to discount the bias that can arise from variable genetic background of different resistant genotypes.

As expected, suppression of oriental mustard increased with increasing densities of the neighbour (wheat) (Fig 3, 4, 5 and 6). This lead to a significant reduction in shoot biomass and seed set in both oriental mustard populations. For the diflufenican-resistant P3.2, there was no significant difference in seed set and shoot dry weight between homozygous resistant (RR), heterozygous resistant (RS) and susceptible (SS) genotypes (Table 2), which indicates no fitness penalty associated with the mutation Leu498 in the PDS gene. Population P40.5 has resistance to both diflufenican and picolinafen conferred by the presence of Leu498 and Glu425 mutations in the PDS gene (Dang et al. unpublished). As compared to the susceptible biotypes, the homozygous resistant (RR) and heterozygous resistant genotypes (RS) of population P40.5 carrying two mutations also did not show any fitness cost, as there was no significant difference in seed and biomass production among the three genotypes (Table 1). Overall analysis of competitive responses across different experiments revealed no significant difference in the competitive response of the three genotypes of both populations (P3.2 and P40.5) to the neighbour plants (wheat).

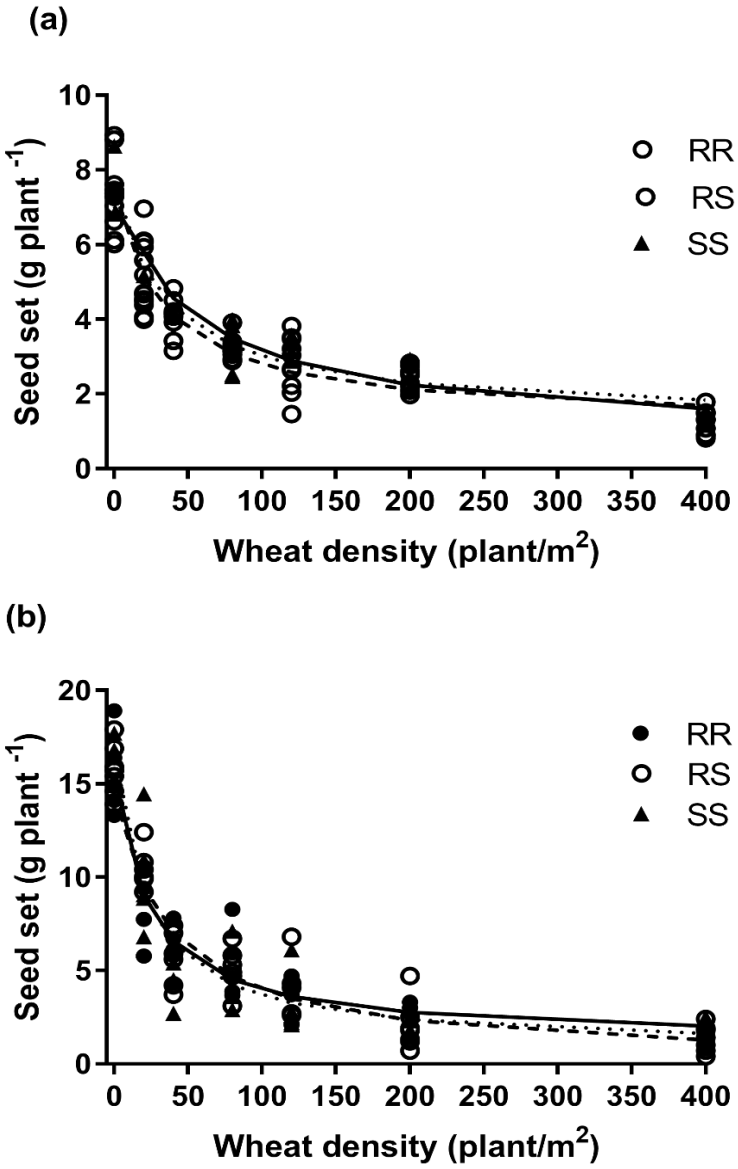


Fig 3. Response of seed set of the homozygous resistant (RR, ●), heterozygous resistant (RS, ○) and susceptible (SS, ▲) plants from the F_2 population of P40.5 to increasing density of wheat neighbour plants at Waite (a) and Roseworthy (b) in 2017. Coefficients of determination (R^2, all data sets $P < 0.001$) after regression to fit hyperbolic model ($y=a+b/(1+dx)$) are presented. Comparison of regression slopes (RR —; RS ---- and SS ⋯) determines the hierarchies in overall per plant competitive responses of oriental mustard plants to neighbour plants (wheat) (Table 1).

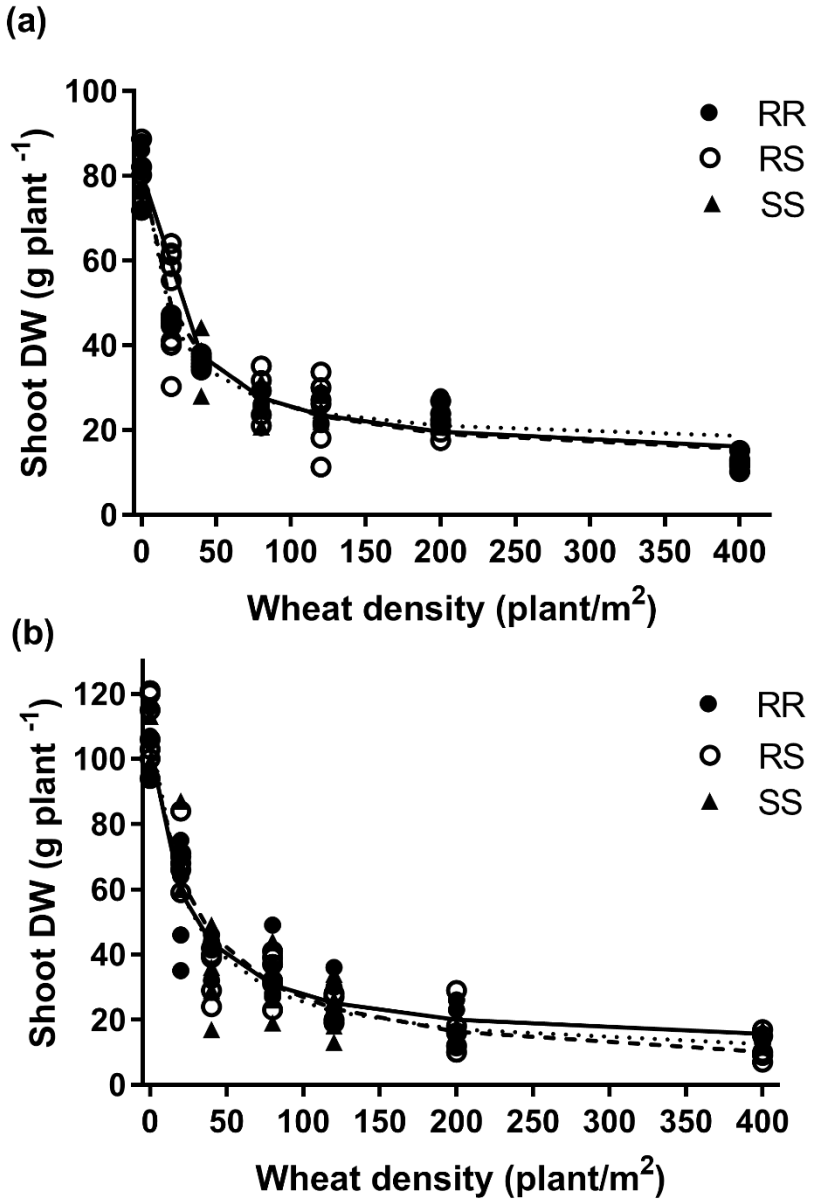


Fig 4. Response of dry weight of the homozygous resistant (RR, ●), heterozygous resistant (RS, ○) and susceptible (SS, ▲) plants from the F_2 population of P40.5 to increasing density of wheat neighbour plants at Waite (a) and Roseworthy (b) in 2017. Coefficients of determination (R^2, all data sets $P < 0.001$) after regression to fit hyperbolic model ($y=a+b/(1+dx)$) are presented. Comparison of regression slopes (RR —; RS ---- and SS ⋯⋯) determines the hierarchies in overall per plant competitive responses of oriental mustard plants to neighbour plants (wheat) (Table 1).

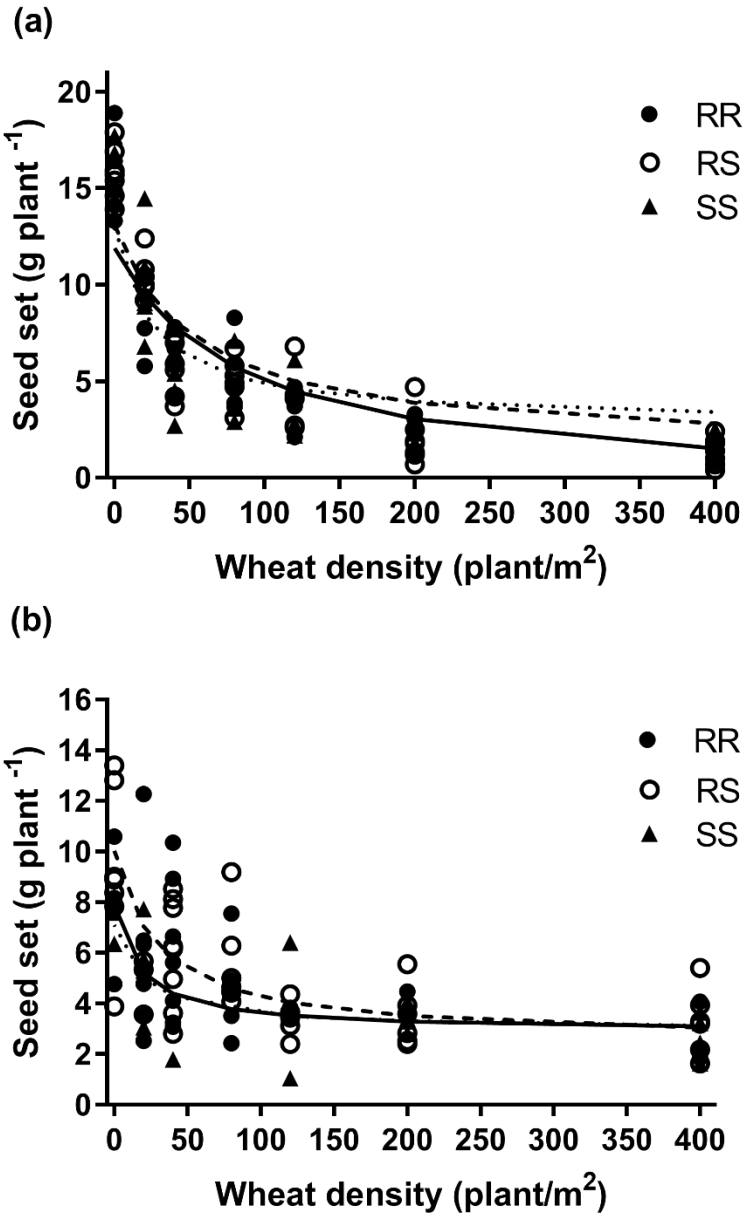


Fig 5. Response of seed set of the homozygous resistant (RR, ●), heterozygous resistant (RS, ○) and susceptible (SS, ▲) plants from the F_2 population of P3.2 to increasing density of wheat neighbour plants at Waite in 2016 (a) and 2017 (b). Coefficients of determination (R^2, all data sets $P < 0.001$) after regression to fit hyperbolic model ($y=a+ b/(1+dx)$) are presented. Comparison of regression slopes (RR —; RS ---- and SS ⋯) determines the hierarchies in overall per plant competitive responses of oriental mustard plants to neighbour plants (wheat) (Table 2).

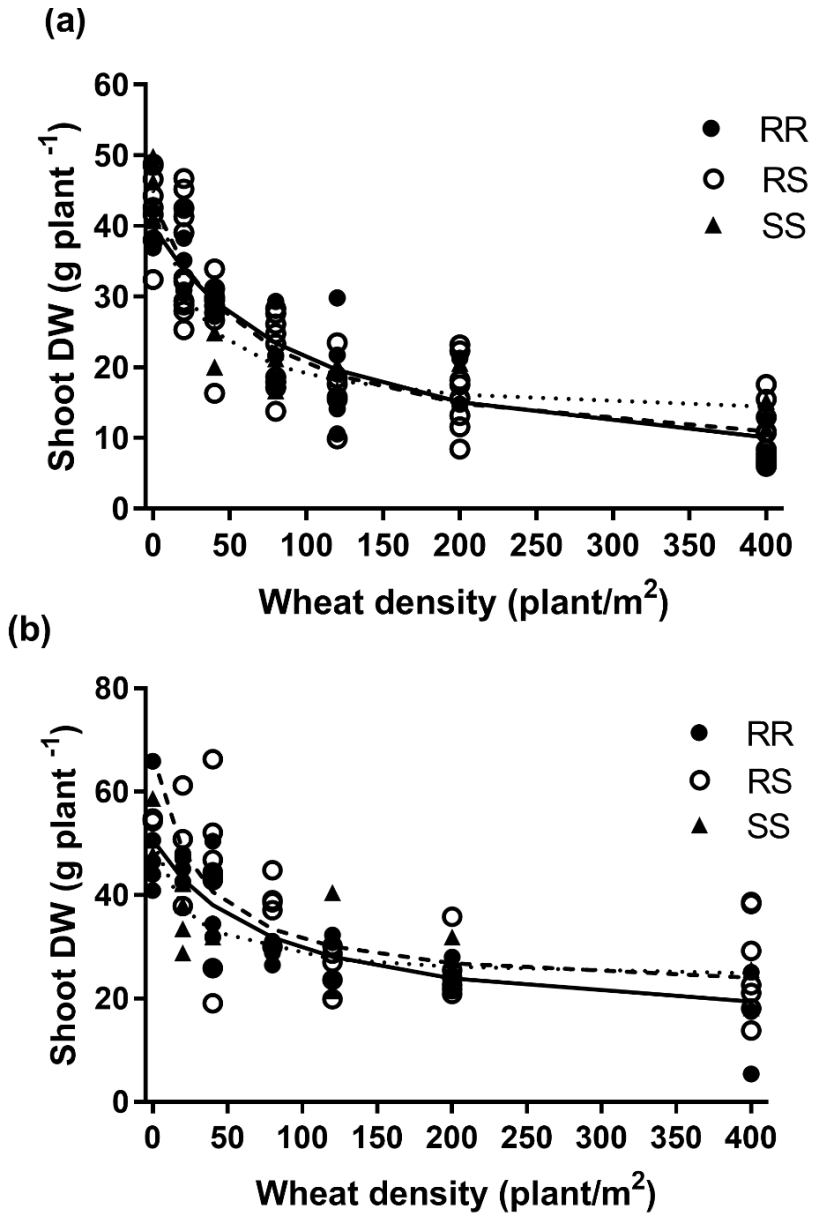


Fig 6. Response of dry weight of the homozygous resistant (RR, ●), heterozygous resistant (RS, ○) and susceptible (SS, ▲) plants from the F_2 population of P3.2 to increasing density of wheat neighbour plants at Waite in 2016 (a) and 2017 (b). Coefficients of determination (R^2, all data sets $P < 0.001$) after regression to fit hyperbolic model ($y=a+ b/(1+dx)$) are presented. Comparison of regression slopes (RR —; RS ---- and SS ⋯⋯) determines the hierarchies in overall per plant competitive responses of oriental mustard plants to neighbour plants (wheat) (see Table 2).

Table 1: Comparisons of competitive responses (seed set and whole plant biomass) of the homozygous resistant (RR), heterozygous resistant (RS) and susceptible (SS) plants of oriental mustard population P40.5 to the neighbour (wheat) plants. Values present the mean estimate of slopes (*b* parameter) derived from the rectangular hyperbolic regression ($y=a+ b/(1+dx)$). Competitive response coefficients of the target genotypes to wheat are compared within each row. Means (± SE) within a row followed by the same letter are not significantly (P=0.05) different.

Location	**Parameter**	**Seed (plant^{-1})**			**Biomass (plant^{-1})**		
		RR	RS	SS	RR	RS	SS
Waite	*d*	0.016 ± 0.005 a	0.029 ± 0.005 a	0.027 ± 0.008 a	0.043 ± 0.009 a	0.041 ± 0.006 a	0.061 ± 0.016 a
	b	6.26 ± 0.51 a	6.25 ± 0.32 a	6.27 ± 0.53 a	68.4 ± 2.95 a	68.75 ± 3.28 a	66.02 ± 3.65 a
	R^2	0.91	0.88	0.91	0.97	0.89	0.96
RW	*d*	0.042 ± 0.011 a	0.026 ± 0.007 a	0.046 ± 0.010 a	0.046 ± 0.011 a	0.032 ± 0.007 a	0.047 ± 0.010 a
	b	14.24 ± 1.07 a	14.37 ± 1.08 a	15.86 ± 1.09 a	92.42 ± 6.31 a	98.53 ± 6.09 a	100.15 ± 6.53 a
	R^2	0.88	0.81	0.87	0.90	0.86	0.88

Table 2: Comparisons of competitive responses (seed set and whole plant biomass) of the homozygous resistant (RR), heterozygous resistant (RS) and susceptible (SS) plants of oriental mustard population P3.2 to the neighbour (wheat) plants. Values present the mean estimate of slopes (*b* parameter) derived from the rectangular hyperbolic regression ($y=a+ b/(1+dx)$). Competitive response coefficients of the target genotypes to wheat are compared within each row. Means (± SE) within a row followed by the same letter are not significantly (P=0.05) different.

Time	**Parameter**	**Seed (plant^{-1})**			**Biomass (plant^{-1})**		
		RR	RS	SS	RR	RS	SS
2016	*d*	0.012 ± 0.004 a	0.019 ± 0.005 a	0.033 ± 0.009 a	0.010 ± 0.005 a	0.015 ± 0.004 a	0.040 ± 0.011 a
	b	12.50 ± 1.23 a	11.59 ± 0.86 a	10.11 ± 0.93 a	37.40 ± 6.06 a	37.66 ± 2.59 a	32.46 ± 2.22 a
	R^2	0.87	0.75	0.89	0.75	0.79	0.94
2017	*d*	0.058 ± 0.034 a	0.033 ± 0.013 a	0.049 ± 0.023 a	0.013 ± 0.006 a	0.033 ± 0.016 a	0.046 ± 0.023 a
	b	5.00 ± 0.81 a	7.51 ± 0.82 b	4.18 ± 0.50 a	37.36 ± 6.10 a	46.25 ± 6.22 b	26.85 ± 3.57 a
	R^2	0.63	0.68	0.77	0.70	0.60	0.72

As fitness costs of a resistant plant can be influenced by environmental factors [32], the expression of fitness costs under controlled conditions (e.g. laboratory or glasshouse) may be different from that grown in the field [32]. In addition, as mutant alleles could impair the ability of the plant to efficiently capture or utilize the captured resources, the fitness costs could be more evident under resource competition [4]. In the current study, there was 26 to 34% lower biomass and 56 to 60% lower seed set in oriental mustard target plants of P40.5 in the net-house at the Waite than outdoors at Roseworthy (Table 1). Even under such contrasting conditions, there were no fitness differences detected among the three genotypes at either site (Table 1). These large differences between the two sites in seed and biomass production of the population P40.5 are likely due to reduced light intensity in the net-house at the Waite as there was no significant difference in rainfall and temperature between the two experimental sites (Table 3). Even though there were some differences in the seed set of resistant genotypes of P3.2 in 2016 and 2017, there were no significant differences in their competitive response to neighbour competition from wheat (Table 2).

CAPS markers have been widely used in molecular biology [33], especially in genotyping and detecting mutations in a number of weed and plant species. In weeds, CAPS markers have been successfully used in determination of genetic diversity in *A. thaliana* [34, 35], *Cryptomeria japonica* [36], and detection of ALS-resistant alleles in *Lolium rigidum* populations [37]. The CAPS marker developed in the current study enabled accurate differentiation of herbicide-resistant genotypes of a large number (435) of individuals at a relatively low cost. This reliable and cost-effective marker could be used for the detection of Leu498 mutation in the PDS gene in resistant biotypes of other weed species.

Table 3: Average rainfall and temperature during experimental times (May to Dec) at Waite (W) (34°58'13.5"S 138°38'22.7"E) and Roseworthy (RW) (34°31'28.8"S 138°41'10.2"E) campuses.

Parameter	**Rainfall (mm)**			**Temperature (ºC)**		
Time	2016 (W)	2017 (W)	2017 (RW)	2016 (W)	2017 (W)	2017 (RW)
May	99.4	50.8	19.2	19.8	19.2	19.9
June	104.6	13.4	10.4	16.0	17.0	17.3
July	133.2	123.6	65.8	15.3	16.4	16.5
Aug	66.2	97.4	70.6	17.7	15.9	15.8
Sept	159.6	73.0	0.0	17.4	19.4	19.2
Oct	0.0	39.8	21.8	21.0	24.3	24.9
Nov	32.2	29.2	0.0	24.7	28.0	29.8
Dec	80.0	0.0	0.0	28.7	27.9	29.5

A high fitness cost is considered an advantage in the management of herbicide resistant weeds as the fitness cost can affect possible destiny of resistance alleles [11]. The higher the fitness cost, the sooner the replacement of resistance with susceptibility will occur leading to a decrease in the frequency of resistant traits [6]. However, many previous studies have identified no fitness penalty in herbicide-resistant biotypes. These include absence of vegetative and seed germinability fitness in ALS-TSR black nightshade (*Solanum ptychanthum* Dunal) [38, 39], no vegetative or fecundity fitness penalty in Leu-1781 and Asn-2041 mutations in ACCase-TSR black-grass (*Alopecurus myosuroides*) [40] and no fitness cost identified in ALS-resistant silky bentgrass (*Apera spica-venti*) [41]. One of the reasons for failure to identify fitness penalties in

experimental studies could be the fitness penalty being less than the resolution of the experiment. For example, the fitness penalty against target-site resistance to ALS-inhibiting herbicides in *L. rigidum* is 0.01% [37], which would be impossible to measure in an experimental set up, such as the one used here. A low fitness penalty associated with mutations in PDS will mean these mutations will be relatively easily selected due to a high initial frequency of the resistance allele [7].

This is the first study of the impact of Leu498 or Leu498+Glu425 mutations in the PDS gene on the fitness cost of diflufenican and cross-resistance to diflufenican and picolinafen in any plant. We compared plant vegetative growth and reproduction of the three genotypes of oriental mustard plants in F_2 segregating progenies. Therefore, resistant genotypes within each population shared the same genetic background. The amino acid substitutions that confer herbicide resistance may impose fitness costs, which would place the mutant plants at a competitive disadvantage with susceptible plants. The amino acid substitutions at Leu498 and Glu425 positions of PDS gene did not impose any negative effects on total biomass, and seed production in the mutant plants relative to the susceptible genotype. The absence of measurable negative effects on plant growth and fecundity associated with Leu498 and Leu498+Glu425 mutations in oriental mustard suggests that the frequency of these alleles is unlikely to decline even in the absence of selection pressure of PDS-inhibitors.

ACKNOWLEDGEMENTS

The authors are thankful to the Vietnam Government, The University of Adelaide for a Ph.D scholarship and the Grains Research and Development Corporation for financial support. We would like to acknowledge Dr. Peter Boutsalis for providing research samples. We also would like to thank all staff of the Weed Science group, The University of Adelaide, especially Benjamin Fleet, Dr. Samuel Kleemann, Malinee Thongmee, Alicia Merriam, David John Brunton, Ruwan Lenorage and Geetha Velappan for providing technical support.

REFERENCES

1. Orr HA, Fitness and its role in evolutionary genetics. *Nature Reviews Genetics* **10**: 531-539 (2009).
2. Gressel J and Segel L, The paucity of plants evolving genetic resistance to herbicides: possible reasons and implications. *J. Theor. Biol.* **75**: 349-371 (1978).
3. Powles SB and Yu Q, Evolution in action: plants resistant to herbicides. *Annu Rev Plant Biol* **61:** 317-347 (2010).
4. Vila-Aiub MM, Neve P and Powles SB, Fitness costs associated with evolved herbicide resistance alleles in plants. *New Phytol* **184**: 751-767 (2009).
5. Coustau C and Chevillon C, Resistance to xenobiotics and parasites: can we count the cost? *Trends Ecol Evol* **15**: 378-383 (2000).
6. Anderson DD, Higley LG, Martin AR and Roeth FW, Competition between triazine-resistant and-susceptible common waterhemp (*Amaranthus rudis*). *Weed Sci*: 853-859 (1996).
7. Jasieniuk M, Brule-Babel AL and Morrison IN, The evolution and genetics of herbicide resistance in weeds. *Weed Sci* **44**: 176-193 (1996).
8. Maxwell BD, Roush ML and Radosevich SR, Predicting the evolution and dynamics of herbicide resistance in weed populations. *Weed Technol* **4**: 2-13 (1990).
9. Delye C, Jasieniuk M and Le Corre V, Deciphering the evolution of herbicide resistance in weeds. *Trends in Genetics* **29**: 649-658 (2013).
10. Roux F, Gasquez J and Reboud X, The dominance of the herbicide resistance cost in several *Arabidopsis thaliana* mutant lines. *Genetics* **166**: 449-460 (2004).
11. Paris M, Roux F, Berard A and Reboud X, The effects of the genetic background on herbicide resistance fitness cost and its associated dominance in *Arabidopsis thaliana*. *Heredity* **101**: 499-506 (2008).

12. Purba E, Preston C and Powles S, Growth and competitiveness of paraquat-resistant and-susceptible biotypes of *Hordeum leporinum*. *Weed Research* **36**: 311-317 (1996).

13. Lexer C. *Molecular Markers in Plant Genetics and Biotechnology* (Book Review). *Kew Bulletin* **59**: 334-334 (2004)

14. Konieczny A and Ausubel FM, A procedure for mapping Arabidopsis mutations using co-dominant ecotype-specific PCR-based markers. *The Plant Journal* **4**: 403-410 (1993).

15. Singh BPe and Gupta VKe. *Molecular markers in mycology: diagnostics and marker developments*. *Springer Science* Business Media (2017).

16. Ashton IA, Abulnaja KO, Pallett KE, Cole DJ and Harwood JL, The mechanism of inhibition of fatty acid synthase by the herbicide diflufenican. *Phytochemistry* **35**: 587-590 (1994).

17. Haynes C and Kirkwood RC, Studies on the mode of action of diflufenican in selected crop and weed species: Basis of selectivity of pre-and early post-emergence applications. *Pest Manag Sci* **35**: 161-165 (1992).

18. Rouchaud J, Gustin F, Van Himme M, Bulcke R, Benoit F and Maddens K, Metabolism of the herbicide diflufenican in the soil of field wheat crops. *J Agri Food Chemis* **39**: 968-976 (1991).

19. Michel A, Arias RS, Scheffler BE, Duke SO, Netherland M and Dayan FE, Somatic mutation-mediated evolution of herbicide resistance in the nonindigenous invasive plant hydrilla (*Hydrilla verticillata*). *Molecular Ecology* **13**: 3229-3237 (2004).

20. Dang HT, Malone JM, Boutsalis P, Gill G and Preston C, The mechanism of diflufenican resistance and its inheritance in oriental mustard (*Sisymbrium orientale* L.) from Australia. *Pest Manag Sci* (2018). *In press*.

21. Dang HT, Malone JM, Boutsalis P, Krishnan M, Gill G and Preston C, Reduced translocation in 2,4-D resistant oriental mustard populations (*Sisymbrium orientale* L.) from Australia *Pest Manag Sci* (2017). *In press*

21. Preston C, Dolman FC and Boutsalis P, Multiple resistance to acetohydroxyacid synthase–inhibiting and auxinic herbicides in a population of oriental mustard (*Sisymbrium orientale*). *Weed Sci* **61**: 185-192 (2013).

23. Boutsalis P and Powles SB, Resistance of dicot weeds to acetolactate synthase (ALS)-inhibiting herbicides in Australia. *Weed Research* **35**: 149-155 (1995).

24. Dayan FE, Owens DK, Tranel PJ, Preston C and Duke SO, Evolution of resistance to phytoene desaturase and protoporphyrinogen oxidase inhibitors - state of knowledge. *Pest Manag Sci* **70**: 1358-1366 (2014).

25. Dang HT, Malone JM, Boutsalis P, Gill G and Preston C, Identification of a target-site mutation conferring resistance to triazine herbicides in oriental mustard (*Sisymbrium orientale* L.) from Australia. *Weed Biol Manag* **17**: 153-160 (2017).

26. Boutsalis P, Gill GS and Preston C, Incidence of herbicide resistance in rigid ryegrass (*Lolium rigidum*) across southeastern Australia. *Weed Technol* **26**: 391-398 (2012).

27. Goldberg DE and Scheiner SM, ANOVA and ANCOVA: field competition experiments. *Design and Analysis of Ecological Experiments* **2**: 69-93 (2001).

28. Goldberg DE, Neighborhood competition in an old-field plant commcay. *Ecology* **68**: 1211-1223 (1987).

29. Dellaporta SL, Wood J and Hicks JB, A plant DNA minipreparation: version II. *Plant Mol Biol Report* **1**: 19-21 (1983).

30. Davis RW, Thomas M, Cameron J, John TPS, Scherer S and Padgett RA. Rapid DNA isolations for enzymatic and hybridization analysis. In *Methods in Enzymology*. *Elsevier*, pp. 404-411 (1980).

31. Bergelson J and Purrington CB, Surveying patterns in the cost of resistance in plants. *The American Naturalist* **148**: 536-558 (1996).

32. Neve P, Challenges for herbicide resistance evolution and management: 50 years after Harper. *Weed Research* **47**: 365-369 (2007).

33. Mccleary TS, Robichaud RL, Nuanes S, Anagnostakis SL, Schlarbaum SE and Romero-severson J, Four cleaved amplified polymorphic sequence (CAPS) markers for the detection of the Juglans ailantifolia chloroplast in putatively native J. cinerea populations. *Mol Ecol Resour* **9**: 525-527 (2009).

34. Hardtke CS, Müller J and Berleth T, Genetic similarity among *Arabidopsis thaliana* ecotypes estimated by DNA sequence comparison. *Plant Molecular Biology* **32**: 915-922 (1996).

35. Barth S, Melchinger A and Lübberstedt T, Genetic diversity in *Arabidopsis thaliana* L. Heynh. investigated by cleaved amplified polymorphic sequence (CAPS) and inter-simple sequence repeat (ISSR) markers. *Molecular Ecology* **11**: 495-505 (2002).

36. Tsumura Y and Tomaru N, Genetic diversity of Cryptomeria japonica using co-dominant DNA markers based on sequenced-tagged sites. *Theor Appl Genet* **98**: (1999).

37. Yu Q, Han HP and Powles SB, Mutations of the ALS gene endowing resistance to ALS-inhibiting herbicides in *Lolium rigidum* populations. *Pest Manag Sci* **64**: 1229-1236 (2008).

38. Ashigh J and Tardif F, An amino acid substitution at position 205 of acetohydroxyacid synthase reduces fitness under optimal light in resistant populations of *Solanum ptychanthum*. *Weed Research* **49**: 479-489 (2009).

39. Ashigh J and Tardif FJ, Water and temperature stress impact fitness of acetohydroxyacid synthase–inhibiting herbicide-resistant populations of eastern black nightshade (*Solanum ptychanthum*). *Weed Sci* **59**: 341-348 (2011).

40. Menchari Y, Chauvel B, Darmency H and Délye C, Fitness costs associated with three mutant acetyl-coenzyme A carboxylase alleles endowing herbicide resistance in black-grass Alopecurus myosuroides. *J Appl Ecol* **45**: 939-947 (2008).

41. Babineau M, Mathiassen SK, Kristensen M and Kudsk P, Fitness of ALS-inhibitors herbicide resistant population of loose silky bentgrass (*Apera spica-venti*). *Front Plant Sci* **8**: DOI: 10.3389/fpls.2017.01660 (2017).

CHAPTER 7: GENERAL DISCUSSION AND CONCLUSION

7.1 General discussion

The study has investigated the level of resistance, its mechanism, inheritance and fitness cost associated with resistance to three major herbicide groups, which have been widely used to control oriental mustard in Australia. These include the PS II inhibitors, PDS-inhibitors and synthetic auxins. A substitution (Ser-264-Gly) in the *psb*A gene, a TSR mechanism, was confirmed the main cause of a relatively high level (>300-fold) of resistance to atrazine in two oriental mustard populations (P17 and P18) (Chapter 2). Likewise, resistance to PDS-inhibitors in oriental mustard was also conferred by a TSR mechanism (Chapters 4 and 5), where the mutation Leu498 alone was the main cause of high level (140-fold) of resistance to diflufenican in population P3 (Chapter 4). Meanwhile, a combination of Leu498 and Glu425 mutations in the PDS gene conferred an even greater resistance (240-fold) to diflufenican as well as cross-resistance to picolinafen in the population P40 (Chapter 5). In contrast, resistance to 2,4-D was conferred by a NTSR mechanism where a reduction of herbicide translocation out of the treated leaf was the main cause of resistance to 2,4-D in two oriental mustard populations P2 and P13 (Chapter 3). Studies on inheritance of resistance to 2,4-D in populations P2 and P13 (Chapter 3) and PDS-inhibitors in populations P3 and P40 (Chapters 4 and 5) confirmed that resistance to these herbicides in oriental mustard are managed by a single gene with a high level of dominance. In the absence of diflufenican treatment, no apparent fitness cost was identified in the resistant plants carrying mutation Leu498 or mutations Leu498+Glu425 in the F_2 generation of the two crosses P3.2 (P3♂ x S♀) and P40.5 (P40♂ x S♀) (Chapter 6).

Oriental mustard is a problematic weed in field crops in the southern grain belt of Australia (McGillion and Storrie, 2006). In Australia, oriental mustard is often easily controlled by herbicides that include synthetic auxins (e.g. 2,4-D, MCPA) and inhibitors of photosystem II

(PSII inhibitors, i.e. atrazine and diuron), acetolactate synthase (ALS inhibitors, i.e. chlorsulfuron and imazamox), phytoene desaturase (PDS inhibitors, i.e. diflufenican and picolinafen) and 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS* inhibitors, i.e. glyphosate) (Chapter 1). However, the frequent use of these herbicides has caused evolution of resistance to these herbicide modes of action in oriental mustard populations (Dayan et al., 2014; Preston et al., 2013).

Since the first case of herbicide resistance to ALS inhibitors was reported in South Australia in early 1990s (Boutsalis and Powles, 1995), the number of herbicide resistant biotypes has increased gradually all over Australia (Heap, 2017). Resistance to other herbicide mode of actions such as *psb*A inhibitors (Heap, 2017), PDS inhibitors (Dayan et al., 2014), and especially multiple resistance to synthetic auxins and ALS inhibitors has also been confirmed (Preston et al., 2013). With the exception of resistance to ALS-inhibitors (Boutsalis and Powles, 1995), the mechanisms of resistance to other herbicide modes of action have not been fully understood. Similarly, information on the mode of inheritance of resistance is limited to 2,4-D and ALS-inhibitors (Preston and Malone, 2015). Likewise, fitness cost associated with resistance is also a knowledge gap, which needs to be investigated. Therefore, there was an urgent need to undertake a comprehensive study on the level of resistance, the mechanism (s), inheritance as well as the fitness penalty associated with resistance to some major herbicide groups in this weed species.

Initially, 75 oriental mustard populations collected from the southern grain belt of Australia during 2010 to 2015 were screened for resistance to six herbicides (glyphosate, diflufenican, imazamox, chlorsulfuron, atrazine and 2,4-D), which are commonly used to control oriental mustard in Australia. Six representative populations (P2, P3, P13, P17, P18 and P40) resistant to the 3 herbicide groups (PS II inhibitors, PDS inhibitors and 2,4-D) and two known susceptible populations (S1 and S2) were then selected for further studies to investigate the level

of resistance, its mechanism, inheritance and fitness cost associated with resistance in oriental mustard.

The intensive use of triazine herbicides in weed control has resulted in widespread evolution of resistance to this herbicide group in many weed species worldwide. However, resistance to triazine herbicides is relatively rare in Australia, where resistance to this herbicide group accounted for less than 10% of the total herbicide resistance cases. In the current study, resistance to atrazine was identified in only 5% of the surveyed populations (Chapter 2). In comparison to two known susceptible oriental mustard populations (S1 and S2), the populations P17 and P18 evolved a high level of resistance (>300-fold) to atrazine as determined by comparison of LD_{50} values. However, they are not resistant to diuron, a PS II inhibitor with a different chemistry to atrazine. The substitution Ser-264-Gly was identified the main cause conferring resistance to atrazine in the populations P17 and P18. This target-site mutation has been detected in several atrazine resistant biotypes (Goloubinoff et al., 1984; Powles and Yu, 2010). Resistance to triazine herbicides and its mechanism of resistance has been well documented in a number of weed species over the world. However, identification of triazine resistance in oriental mustard provides useful information to assist farmers in the management this species in the future.

Chapter 3 examined the physiological and molecular basis of 2,4-D resistance in oriental mustard. Whole plant experiments, inheritance studies and gene sequencing of candidate target genes (ABP, TIR 1, AFB 5) were performed. Results showed there was no difference in absorption of [^{14}C] 2,4-D between resistant (P2 and P13) and susceptible (S1 and S2) populations. However, a significant reduction in the amount of [^{14}C] 2,4-D translocated from the treated leaf was detected in both resistant populations (P2 and P13) compared to the two susceptible populations (S1 and S2). As no potential target-site mutations were found in the genes for ABP, TIR 1, and AFB 5, reduced translocation of 2,4-D is considered the mechanism

of resistance to 2,4-D in this weed species. Resistance to herbicides in weeds can be conferred by NTSR such as reduction of herbicide translocation or increased herbicide metabolism (Dayan et al., 2014; Powles and Yu, 2010). Where resistance is conferred by reduction of herbicide translocation, the availability of herbicides at the target site in the plant is reduced enabling an individual plant to survive the treatment (Ferreira and Reddy, 2000; Powles and Yu, 2010). Results of this study support the findings in previous studies where the 2,4-D resistant biotypes had significantly lower herbicide translocation out of the treated leaf such as *R. raphanistrum* (Goggin et al., 2016), *G. tetrahit* (Weinberg et al., 2006), *P. rhoeas* (Rey-Caballero et al., 2016) and *L. serriola* (Riar et al., 2011).

As greater knowledge of inheritance patterns of resistance traits provides better knowledge on the evolution and spread of resistance (Maxwell and Mortimer, 1994), the mode of inheritance of resistance to 2,4-D in oriental mustard were also examined. Initially, the resistant plants (pollen donor) were crossed with susceptible plants (pollen acceptor) to generate an F_1 generation. The F_1 individual was confirmed to be heterozygous by screening with 2,4-D at 200 g ha^{-1}, indicating that the inheritance of resistance is nuclear encoded. The F_1 hybrid was self-pollinated to produce the F_2 generation. A detailed dose-response analysis of the F_2 populations to 2,4-D confirmed that resistance to 2,4-D in oriental mustard is conferred by a single gene with partially-dominant allele. The F_2 plants segregated in a 3:1 ratio when treated with 200 g ha^{-1} of 2,4-D, which is consistent with a single major gene model (Chapter 3). The majority of previous studies have shown that resistance to 2,4-D is conferred by a single dominant nuclear gene, either dominant or partially-dominant (Busi and Powles, 2017; Jugulam et al., 2005; Preston and Malone, 2015; Riar et al., 2011; Zheng and Hall, 2001). The current study also demonstrated that mode of inheritance of resistance to 2,4-D in highly resistant populations (> 65-fold to 2,4-D) due to reduced herbicide translocation, was managed by a single gene with dominant allele.

Following the confirmation of resistance to triazines and 2,4-D, extensive work was conducted to study the mechanism(s) and inheritance of resistance to inhibitors of phytoene desaturase (PDS inhibitors) (Chapters 4 and 5). Dose-experiments conducted on two oriental populations (P3 and P40) collected from Quambatook and Kunat, Victoria confirmed that the mutation Leu-498-Val alone in the PDS gene could cause high level (140-fold) of resistance to diflufenican. Meanwhile, the combination of Leu-498-Val and Glu-425-Asp mutations in PDS gene confers an even greater level (240-fold) of resistance to diflufenican and evolves cross-resistance to another member of PDS inhibitors, picolinafen. To have greater understanding of the evolution and spread of resistance to diflufenican and picolinafen in oriental mustard, research on inheritance patterns of resistance traits was also conducted. The F_1 and F_2 seeds were generated by hand crossing using the method as described in the Chapter 3. The F_1 and F_2 seeds were generated by hand-crossing using the method described in Chapter 3. The F_1 individuals were confirmed to be heterozygous by screening with diflufenican at 200 g ha^{-1}, indicating that the inheritance of resistance is nuclear encoded. Analysis of the results of the segregation tests and detailed dose-response experiments on F_2 populations showed that the F_2 plants segregated in a 3:1 ratio when treated with 200 g diflufenican ha^{-1}, which is consistent with a single major gene model. In addition, sequencing of the PDS gene in individuals of the F_2 population also confirmed that resistance alleles segregated in 1:2:1 ratio, as expected for single-gene inheritance. This study documented the first known cases of field evolved diflufenican resistance and cross-resistance to diflufenican and picolinafen in weeds due to target-site resistance mechanism.

In Chapter 6, the effect of two target–site point mutations of PDS gene: Leu-498-Val/or Leu-498-Val and Glu-425-Asp on plant growth and fecundity of oriental mustard biotypes grown in monoculture and in competition with wheat was investigated. As the two resistant genotypes of F_2 populations (P3.2 and P40.5) carried the same mutation, Leu-498-Val, a CAPS marker was

developed to differentiate the genotype of F_2 plants based on the presence of the Leu498 mutation in the PDS gene. In weeds, evolution of herbicide resistance is suspected to involve a fitness costs (Powles and Yu, 2010; Vila-Aiub et al., 2009), which plays a vital role in natural selection and adaptation of a resistant plants. A high level of fitness penalty of a resistant trait is considered an advantage in the management of resistance alleles (Paris et al., 2008) as the higher the fitness cost, the sooner the replacement of resistance with susceptible biotypes will occur leading to a reduction of resistant traits (Anderson et al., 1996). However, overall analysis of competitive responses across different experiments in this study revealed no significant difference in the competitive response of the three genotypes of both populations (P3.2 and P40.5) to the neighbour plants (wheat). This means, the amino acid substitutions at Leu498 and Glu425 positions of PDS gene did not impose any measurable negative effects on the fitness of the mutant plants relative to the susceptible genotype. No fitness penalty in herbicide-resistant biotypes has been confirmed in a number of weed species with different herbicide modes of action such as ALS-resistant *S. ptychanthum* (Ashigh and Tardif, 2009; Ashigh and Tardif, 2011), ACCas-resistant *A. myosuroides* (Menchari et al., 2008) and ALS-resistant *A. spica-venti* (Babineau et al., 2017). The absence of fitness cost associated with Leu498 and Leu498+Glu425 mutations in these oriental mustard populations means that the frequency of these alleles is unlikely to decline even in the absence of selection pressure of PDS-inhibitors.

As the expression of resistance to PDS-inhibitors and 2,4-D in oriental mustard was very strong (67 to 240 -fold), the use of higher rates of the same herbicide mode of action is unlikely to improve control of these resistant populations. In order to slowdown the spread and achieve effective control of PDS-inhibitors and 2,4-D resistant oriental mustard populations, proactive integrated measures for resistance management would be required (Beckie, 2007; Rey-Caballero et al., 2017). These strategies could include the use of alternative herbicide modes of action or application of herbicide mixtures (Lagator et al., 2013) in combination with other tactics such as

increased crop competition, crop rotations, delayed sowing and mechanical weed control (Beckie et al., 2008; Rey-Caballero et al., 2017; Walsh et al., 2013).

7.2 Conclusion

This research provides comprehensive insights into resistance to some major herbicide groups in oriental mustard in Australia including triazines, PDS-inhibitors and 2,4-D. It is clear from results that resistance to triazines in oriental mustard is conferred by a well-known mutation (Ser-264-Gly) in the *psb*A gene, which has been reported in a number of weed species worldwide. Resistance to 2,4-D is due to NTSR mechanism when impaired transport of herbicide is likely the main mechanism involved in the resistance response to 2,4-D in oriental mustard. Meanwhile, resistance to PDS-inhibitors in oriental mustard is due to TSR mechanism when mutations (Leu498/Leu+Glu425) in the PDS gene are likely the main factors enabling oriental mustard populations survive diflufenican (P3), and diflufenican and picolinafen (P40) treatment. The findings that inheritance of resistance to 2,4-D and PDS-inhibitors in oriental mustard populations is controlled by a single dominant gene imply that once resistant biotypes to these herbicides occur in the field, they will spread faster than the cases conferred by a complex genetic inheritance (Jasieniuk and Maxwell, 1994). Furthermore, the frequency of PDS-resistant alleles is unlikely to decline even in the absence of selection pressure of PDS-inhibitors due to the absence of apparent fitness cost associated with Leu498 and Leu498+Glu425 mutations in oriental mustard.

Based on the findings presented in this thesis, further research is required to identify the fitness cost associated with reduced translocation in 2,4-D-resistant populations, as well as management practices to control oriental mustard populations with evolved resistance to triazines, PDS-inhibiting herbicides and 2,4-D.

REFERENCES

Anderson DD, Higley LG, Martin AR, Roeth FW (1996) Competition between triazine-resistant and-susceptible common waterhemp (*Amaranthus rudis*). Weed Sci 44: 853-859

Ashigh J, Tardif F (2009) An amino acid substitution at position 205 of acetohydroxyacid synthase reduces fitness under optimal light in resistant populations of *Solanum ptychanthum*. Weed Research 49:479-489

Ashigh J, Tardif FJ (2011) Water and temperature stress impact fitness of acetohydroxyacid synthase–inhibiting herbicide-resistant populations of eastern black nightshade (*Solanum ptychanthum*). Weed Sci 59:341-348

Babineau M, Mathiassen SK, Kristensen M, Kudsk P (2017) Fitness of ALS-inhibitors herbicide resistant population of loose silky bentgrass (*Apera spica-venti*). Frontiers in Plant Science. Doi: 10.3389/fpls.2017.01660.

Beckie HJ (2007) Beneficial management practices to combat herbicide-resistant grass weeds in the northern Great Plains. Weed Technology 21:290-299

Beckie HJ, Johnson EN, Blackshaw RE, Gan Y (2008) Weed suppression by canola and mustard cultivars. Weed Technology 22:182-185

Boutsalis P, Powles SB (1995) Resistance of dicot weeds to acetolactate synthase (ALS)-inhibiting herbicides in Australia. Weed Research 35:149-155

Busi R, Powles SB (2017) Inheritance of 2,4-D resistance traits in multiple herbicide- resistant *Raphanus raphanistrum* populations. Plant Sci 257:1-8

Dayan FE, Owens DK, Tranel PJ, Preston C, Duke SO (2014) Evolution of resistance to phytoene desaturase and protoporphyrinogen oxidase inhibitors state of knowledge. Pest Manag Sci 70:1358-66

Ferreira JF, Reddy KN (2000) Absorption and translocation of glyphosate in *Erythroxylum coca* and *E. novogranatense*. Weed Science 48:193-199.

Goggin DE, Cawthray GR, Powles SB (2016) 2,4-D resistance in wild radish: reduced herbicide translocation via inhibition of cellular transport. Journal of Experimental Botany 67:3223-3235

Goloubinoff P, Edelman M, Hallick RB (1984) Chloroplast-coded atrazine resistance in *Solanum nigrum*: *psb*A loci from susceptible and resistant biotypes are isogenic except for a single codon change. Nucleic Acids Res 12:9489-96

Heap I (2017) The International Survey of Herbicide Resistant Weeds. http://weedscience.org (acessed 20 Feb 2017).

Jasieniuk M, Maxwell BD (1994) Population genetics and the evolution of herbicide resistance in weeds. Phytoprotection 75:25-35

Jugulam M, McLean MD, Hall JC (2005) Inheritance of picloram and 2,4-D resistance in wild mustard (*Brassica kaber*). Weed Science 53:417-423

Lagator M, Vogwill T, Mead A, Colegrave N, Neve P (2013) Herbicide mixtures at high doses slow the evolution of resistance in experimentally evolving populations of *Chlamydomonas reinhardtii*. New Phytologist 198:938-945

Maxwell BD, Mortimer AM (1994) Selection for herbicide resistance. Pages 1-26 *in* Powles SB, Holtum JAM, eds. Herbicide Resistance in Plants: Biology and Biochemistry. Boca Raton, FL: Lewis Publishers

McGillion T, Storrie A (2006) Integrated weed management in Australian cropping systems–a training resource for farm advisors. CRC for Australian Weed Management: Adelaide

Menchari Y, Chauvel B, Darmency H, Délye C (2008) Fitness costs associated with three mutant acetyl-coenzyme A carboxylase alleles endowing herbicide resistance in black-grass *Alopecurus myosuroides*. Journal of Applied Ecology 45:939-947

Paris M, Roux F, Berard A, Reboud X (2008) The effects of the genetic background on herbicide resistance fitness cost and its associated dominance in *Arabidopsis thaliana*. Heredity 101:499-506

Powles SB, Yu Q (2010) Evolution in action: plants resistant to herbicides. Annu Rev Plant Biol 61:317-47

Preston C, Dolman FC, Boutsalis P (2013) Multiple resistance to acetohydroxyacid synthase–inhibiting and auxinic herbicides in a population of oriental mustard (*Sisymbrium orientale*). Weed Science 61:185-192

Preston C, Malone JM (2015) Inheritance of resistance to 2,4-D and chlorsulfuron in a multiple-resistant population of *Sisymbrium orientale*. Pest Manag Sci 71:1523-8

Rey-Caballero J, Menendez J, Gine-Bordonaba J, Salas M, Alcantara R, Torra J (2016) Unravelling the resistance mechanisms to 2,4-D (2,4-dichlorophenoxyacetic acid) in corn poppy (*Papaver rhoeas*). Pesticide Biochemistry and Physiology 133:67-72

Rey-Caballero J, Royo-Esnal A, Recasens J, Gonzalez I, Torra J (2017) management options for multiple herbicide-resistant corn poppy (*Papaver rhoeas*) in Spain. Weed Science 65:295-304

Riar DS, Burke IC, Yenish JP, Bell J, Gill K (2011) Inheritance and physiological basis for 2,4-d resistance in prickly lettuce (*Lactuca serriola* L.). J. Agric Food Chem 59:9417-9423

Vila-Aiub MM, Neve P, Powles SB (2009) Fitness costs associated with evolved herbicide resistance alleles in plants. New Phytol 184:751-67

Walsh M, Newman P, Powles S (2013) Targeting weed seeds in-crop: a new weed control paradigm for global agriculture. Weed Technology 27:431-436

Weinberg T, Stephenson GR, McLean MD, Hall JC (2006) MCPA (4-chloro-2-ethylphenoxyacetate) resistance in hemp-nettle (*Galeopsis tetrahit* L.). J. Agric Food Chem 54:9126-9134

Zheng H-g, Hall JC (2001) Understanding auxinic herbicide resistance in wild mustard: physiological, biochemical, and molecular genetic approaches. Weed Science 49:276-281