Improving Metribuzin Tolerance in Lentil (*Lens culinaris*)

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ABSTRACT

Weeds are a major limitation to lentil (*Lens culinaris* Medik.) production worldwide with grain yield losses of up to 87% from weed competition. In broad-acre mechanized lentil production systems, weed control relies on herbicide application; however, limited options exist. This study identified, characterised and validated novel tolerance in lentil to the photosystem II (PSII) inhibitor herbicide, metribuzin.

Field research involving variable sowing dates, induced shade treatments and metribuzin rate were conducted to understand soil and weather factors responsible for herbicide phytotoxicity in lentil. Analysis of soil and weather factors around the time of herbicide application to the cultivar PBA Flash suggested a combination of factors were involved. Heavy rainfall within 10 days of application, particularly on light textured soils or where soil moisture was low, was most strongly linked to plant damage. A higher level of selective tolerance to metribuzin than that currently present in commercial lentil cultivars is required.

Two methods, germplasm screening using a hydroponic sand assay and field screening of a large mutated population of PBA Flash, were used to identify lines with improved tolerance to metribuzin compared to current cultivars. Dose response experiments found germplasm line SP1333 had GR_{50} (the rate required to reduce dry weight (DW) 50%) values up to four-fold that of PBA Flash. However, GR_{50} values were greater than 25-fold that of PBA Flash in mutant selections M009 and M043. A field study in Canada with 20 Canadian and Australian genotypes confirmed the improved tolerance level of the mutants.

Dose response analysis of five PSII inhibiting herbicides and DNA sequencing of the *psbA* chloroplast gene was undertaken to quantify the spectrum and mechanism of herbicide tolerance in M009 and M043. Compared to PBA Flash, metribuzin tolerance was increased 33-fold in M043 and 10-fold in M009, but no additional tolerance to other herbicides. Nucleotide sequencing of the *psbA* gene of both mutants identified a substitution at position 751 compared to PBA Flash. The resulting

deduced amino acid sequence indicated an $Ala_{251}Thr$ substitution as responsible for the metribuzin tolerance. The substitution is unique in mutagenised higher plants and is the first report of an induced *psbA* target site mutation in higher plants.

Reciprocal F_1, F_2 and F_3 populations developed from M009 and M043 with PBA Flash identified a maternal inheritance pattern, but with paternal leakage in approximately 20% of F_1 phenotypes. Reciprocal BC_1F_2 and BC_1F_3 populations were developed to identify any fitness cost associated with the tolerance. Field experiments identified reductions in net assimilation rate, DW and grain yield (GY) in tolerant lines with a fitness cost of 20 to 40%. This finding is comparable with the fitness cost measured in triazine tolerant (TT) canola due to tolerance to the PSII inhibiting triazine herbicides.

Agronomic field experiments over two years at contrasting sites in South Australia compared the plant growth and GY of M009 and M043 with PBA Flash and SP1333 to post-emergent metribuzin. Clear differences existed in the responses of M009 and M043 compared with PBA Flash and SP1333 to metribuzin rate across sites. This finding confirmed that the mutant genotypes have an agronomically useful level of tolerance to metribuzin in southern Australia. However, DW was generally reduced linearly with metribuzin rate in both M043 and M009 suggesting a level of herbicide sensitivity at higher rates on some soil types.

All three lentil genotypes with improved metribuzin tolerance are in use as parents in Australian breeding programs. The higher level of tolerance and superior agronomic performance of M043 makes it the genotype of choice. Knowledge of the genetic controls of inheritance and associated fitness cost of the target site provided by this study will aid plant breeders in rapid and effective incorporation of the tolerance into agronomically accepted plant types. The potential of developing a metribuzin tolerant lentil industry in Australia, similar to that which has occurred in TT canola, now exists.

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.

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ABBREVIATIONS

AB	Alberta
ABARES	Australian Bureau of Agricultural and Resource Economics and Sciences
ACCase	acetyl coA carboxylase
ACST	Australian central standard time
AHAS	acetohydroxyacid synthase
a.i.	active ingredient
ANOVA	analysis of variance
BC	back cross
CDC	Crop Development Center, University of Saskatchewan
CE	contrast estimate
CER	controlled environment growth room
CTAB	cetyl trimethylammonium bromide
CWFP	critical weed free period
D	duplicate
DA	6-*tert*-butyl-3(methylthio)-1,2,4-triazin-5(4H)-one
DADK	6-*tert*-butyl-1,2,4-triazin-3,5(2H,4H)-dione
DAS	days after sowing
DAT	days after treatment
Dim	dimensions
DK	4-amino-6-*tert*-butyl-1,2,4-triazin-3,5(2H,4H)-dione
DNA	deoxyribonucleic acid
DPIRD	Department of Primary Industries and Regional Development
DRC	dose response curve
DSE	daily solar exposure
Dup	duplicate
DW	dry weight
ED_{50}	dose required for 50% inhibition
EMS	ethyl methane sulfonate
FAO	Food and Agriculture Organization of the United Nations
GR_{50}	the rate required to reduce dry weight (DW) 50%
GRDC	Grains Research and Development Corporation
GY	grain yield
ICARDA	International Center for Agricultural Research in the Dry Areas
IL	Illinois
IMI	imidazolinone
LA	leaf area
KASP	Kompetitive allele specific PCR
M	mutant, mutagenised
M009	PBA Flash-EMS10-11SK-12PAHM009
M043	PBA Flash-EMS10-11SVHM043
NAR	net assimilation rate
NCRIS	National Collaborative Research Infrastructure Strategy

NDSU	North Dakota State University
NS/ns	not significant
NSW	New South Wales
NVT	National Variety Trials
ON	Ontario
P	parents
PBA	Pulse Breeding Australia
PCA	principal component analysis
PCR	polymerase chain reaction
PF	parent PBA Flash
PIRSA	Primary Industries and Regions South Australia
PM	parent mutant
POST	post-emergent
PQ	plastoquinone
PRE	pre-emergent
PSII	photo systems II
QLD/Qld	Queensland
R	R Development Core Team
REML	residual maximum likelihood
RF	resistant factor
RFLP	restriction fragment length polymorphism
RH	relative humidity
RR	reciprocal replicate
RO	reverse osmosis
S	site
SA	South Australia
SARDI	South Australian Research and Development Institute
SE	standard error
SI	selective index
SK	Saskatchewan
SMA	Saskatchewan Ministry of Agriculture
SNP	single nucleotide polymorphism
SX	sex
TAE	tris-acetate buffer
Tris-HCL	2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride
TT	triazine tolerant
UPDG	*N*-glucosyltransferase
UK	United Kingdom
USA/US	United States of America
VIC/Vic	Victoria

Chapter 1.

Introduction and Review of literature

CHAPTER 1

Introduction and Review of Literature

1.1 Introduction

Cultivated lentil (*Lens culinaris* Medikus) is one of the world's oldest food crops. Traditionally it has been an essential part of diets in the semiarid regions of the world, where it is grown under labour intensive production systems including manual weeding (Knott and Halila 1988; Muehlbauer *et al*. 1995). In contrast, lentil is a relatively new crop in the developed countries of Australia, Canada and the United States where mechanised broadacre production systems occur. In these systems, weed management in lentil relies heavily on the application of chemical herbicides (Brand *et al*. 2007; Yenish *et al*. 2009). The Australian lentil industry expanded rapidly following the release of high yielding cultivars suitable for machine harvest in the late 1990's (Materne 2003). However, further industry expansion requires a number of current constraints to production, including limited weed control options, to be addressed (Materne *et al*. 2002; Materne *et al.* 2011).

Lentil is regarded as a poor competitor with weeds due to inherent levels of slow early growth, short stature and a lack of protective canopy development (Knott and Halila 1988; Muehlbauer *et al*. 1995; Hanson and Thill 2001). Further compounding these issues in lentil is a lack of safe and effective herbicides to control broadleaf weeds, particularly once the crop has emerged (Preston 2002). The sensitivity of lentil to commonly used broadleaf herbicides often leads to yield losses, particularly where management practices are sub-optimal or soil and seasonal conditions conducive to damage (Muehlbauer *et al*. 1995; Materne *et al*. 2002). Improvement in tolerance to existing herbicides and/or tolerance to novel herbicides in lentil is required to expand weed control options in this crop.

Metribuzin is an aminotriazinone or asymmetrical triazine compound and an inhibitor of photosynthesis at photosystems II (PSII) (Hatzios and Penner 1988). It is widely used in Australian crop production due to broad-spectrum grass and broadleaf weed control (Frankel 2010). Metribuzin

is applied post-emergent to lentil in North America and to certain cereal and pulse crops in Australia. However, due to low levels of crop safety is only used pre-emergence in lentil in Australia (Frankel 2010). Other PSII inhibitor herbicides used routinely in Australian lentil production include the phenylurea; diuron and the triazines simazine and terbuthylazine, due to their unique and cost-effective control of a range of broadleaf and grass weeds (Frankel 2010). However, as metribuzin is rapidly taken up by plant leaves and roots, has a high water solubility and relatively short persistence in the soil (Webster and Reimer 1976; Hatzios and Penner 1988), it is better suited to post-emergent applications in lentil than these alternatives. To improve the tolerance of Australian lentil cultivars to metribuzin the national breeding program (Pulse Breeding Australia (PBA)) implemented a routine screening of advanced breeding lines to this herbicide (McMurray *et al*. 2009). However, a step-wise improvement in whole plant tolerance to metribuzin, similar to the level that exists in Australian field pea (*Pisum sativum* L.) cultivars, is required in lentil (Materne pers. comm.).

In Australia, metribuzin is widely used as a pre-emergence herbicide in lentil, but is favoured less on soils that are light textured and low in organic matter due to a high risk of herbicide leaching and subsequent crop damage from post-application rainfall events (Gill and Bowran 1990, Frankel 2010). There are no reports quantifying the weather conditions associated with increased phytotoxicity and the extent of any related grain yield loss in lentil from post-emergent metribuzin application in Australia. Grain yield losses of up to 47% from two split post-emergent applications have been reported in lentil in Canada (Friesen and Wall 1986). Furthermore, conditions of cold, cloudy weather or frost within three days of application are listed on the metribuzin label in Canada as being associated with crop phytotoxicity (SMA 2011). An understanding of the weather conditions responsible for crop damage from post-emergent metribuzin application in lentil in Australia will be essential to the process of successfully developing cultivars with improved levels of tolerance to this herbicide. The discovery of genotypes with high levels of metribuzin tolerance along with an understanding of the genetic controls and mechanisms for differential tolerance will also be required.

This literature review describes the research reported and gaps in knowledge on chemical weed control in lentil, metribuzin use and performance in crop production and progress towards the development of improved metribuzin tolerance in crops, at the commencement of the project in 2011. More recent developments in these areas are described in the introduction sections of the subsequent chapters as relevant.

1.2 Lentil

1.2.1 Background and world production

Lentil belongs to the *Leguminosae* family (sub family *Papilionaceae*) and originated in the Near East arc over 7,000 years ago from *L. culinaris* ssp. *orientalis* (Ladizinsky 1979). Lentil is split into two major groups based on seed size and cotyledon colour (Barulina 1930). The "macrosperma" group, also known as green, brown, yellow or Chilean lentils, have large seeds ranging from 6 to 9 mm and cotyledons yellow in colour. The "microsperma" group, also referred to as red or Persian lentils, are typically 2 to 6 mm in diameter with red cotyledons.

Lentil is grown across a wide range of environments and traditionally consumed in regions where it is grown (McNeil *et al.* 2007). It is mainly grown as a dryland crop, as the plant is poorly adapted to systems based on irrigation and high inputs (McNeil *et al.* 2007). It is regarded as one of the more drought tolerant crops. Lentil is adapted to a wide range of soil pH (5.5 to 9.0), but is better suited to well drained neutral to alkaline soils (Andrews and McKenzie 2007; Materne and Siddique 2009). Lentil is poorly suited to tropical areas (McNeil *et al.* 2007).

Traditionally, the majority of lentil production occurs in developing countries, such as India, Turkey, Nepal, Syria and Bangladesh (Erskine, 2009). However, recently significant production of lentil has occurred in developed countries, such as Canada, United States and Australia, where it is grown both as a cash income source and for its rotational benefits. World production increased four-fold from an average of 0.9 Mt in 1961 to 1963 to 3.8 Mt in 2004 to 2006 (Erskine, 2009). Further

increases in production since 2009, most notably in the developed countries, has led to a peak at 7.5 Mt in 2017 (FAOSTAT 2019).

1.2.2 Australian Production

Significant commercial production of lentil in Australia only began in 1994 (Brouwer, 2002). The area sown to the crop increased rapidly to a peak area of 207,000 ha in 2007 before declining by 30 to 40% over the following three years (ABARES 2011). Production consists almost entirely of the red type with the vast majority grown in the southern states of Victoria and South Australia (SA). Within these areas, production is limited to regions characterised by well drained alkaline soils with a winter dominant annual rainfall of 350 to 500 mm (Materne *et al.* 2002). Further increase in area sown to lentil will require expansion away from these growing regions, genetic improvement, reduced production costs and continued high relative grain prices (Materne *et al*. 2002; Materne *et al*. 2011). Materne *et al.* (2002) grouped Australian lentil production into five major regions based on soil type and climate and weed competition was listed as an important constraint in all five regions.

1.2.3 Lentil improvement in Australia

Initial attempts to grow lentil in Australia were based on poorly adapted genotypes introduced from countries such as Canada and Ethiopia (Materne 2003). Production increased following the release of better adapted genotypes from the International Center for Agricultural Research in the Dry Areas (ICARDA) along with the development of agronomic packages aimed at increasing the productivity and reliability of the crop in 1994 (Brouwer 2002). At the same time, a national lentil breeding program was established and focused on incorporating key production traits not present in the introduced material. Initial releases from the program, cultivars Nipper and Boomer, had improvements in disease resistance, seed quality and reliability of production (Materne *et al.* 2011). More recent releases from this program (now named Pulse Breeding Australia: PBA) combine improvements in foliar and seed disease resistance with high grain yield, improved adaptation through phenological and maturity timings and high seed marketability (Materne *et al.* 2011) . Further

genetic improvements are expected over the next one to three years including genotypes with greater adaptation to specific regions and/or dry seasons, improvements for mechanised harvesting and the incorporation of novel imidazolinone (IMI: acetohydroxyacid synthase [AHAS] inhibitors) herbicide tolerance (Materne *et al.* 2011).

The initial concentration by PBA on disease resistance and key plant traits meant few resources were available for other traits, such as herbicide tolerance. Furthermore, there was an initial high use in hybridisations of the North American disease-resistant forage cultivar Indian Head. This cultivar has sensitivity to the broad-spectrum herbicide metribuzin (Materne pers. comm.) and is the likely source of higher levels of metribuzin sensitivity in the disease resistant cultivars, Nipper and PBA Herald XT when compared to cultivar Nugget. The program has expanded in capacity in recent years and now routinely conducts field screening of advanced genotypes to identify and eliminate those with sensitivity to metribuzin and other registered herbicides. Further, improving tolerance to registered herbicides and the development of novel herbicide tolerance in lentil is a current PBA breeding priority (Materne pers. comm.).

1.2.4 Use of wild lentil species in lentil improvement

Wild species are recognised as a valuable resource for increasing the genetic diversity in breeding programs. The *Lens* genus consists of six genetically isolated groups and all species are self-pollinated diploids ($2n = 2x = 14$). Cubero *et al.* (2009) and Muehlbauer *et al.* (1995) summarised the phylogeny of the lens genus using hybridization barriers to differentiate the species. They suggested *L. culinaris* subsp. *culinaris and L. c.* subsp. *orientalis* (Boiss.) Ponert belong to the primary gene pool. *Lens odemensis* (Ladiz.) belongs to the secondary gene pool, *Lens nigricans* (M. Bieb.) Godr. and *Lens ervoides* (Brign.) Grande to the tertiary gene pool and *Lens tomentosus* (Ladiz.) and *Lens lamottei* to either the secondary or tertiary gene pool. Embryo rescue is required for crosses with certain accessions in *L. odemensis* and if used for *L. nigricans* and *L. ervoides* crosses may move these species into the secondary gene pool.

Variation for a number of traits has been observed in the *Lens* wild species and recently summarised by Tullu *et al.* (2011). These include winter hardiness, drought tolerance, tolerance to the parasitic weed broomrape (*Orobanche* spp), diseases including rust, vascular wilt, ascochyta blight (causal agent *Ascochyta lentis*), anthracnose (causal agent *Colletotrichum truncatum*) and variability in a range of morphological characters. However, few examples exist of subsequent introgression of these traits into breeding programs, most probably due to the low success rate in many interspecific crosses. The most advanced example reported is the use of *L. ervoides* in Canada for higher levels of anthracnose resistance. Hybridisation was achieved between *L. ervoides* and *L. culinaris* using ovule and embryo rescue techniques (Fiala *et al.* 2009). A number of high yielding anthracnose resistant lines have been identified following intensive backcrossing into *L. culinaris* and evaluated in the field with cultivar release expected in the next three to four years (Tullu *et al.* 2011). They also suggest there may be further yield gain and higher levels of resistance to stemphylium blight (causal agent *Stemphylium botryosum*) in the back crossed lines. Wild lentil species could be a potential useful source of higher levels of metribuzin herbicide tolerance as found in related *Vigna* species (Harrison 1988) and wild soybean (G*lycine soja* Sieb. & Zucc.) (Kilen and Guohao 1992), although the difficulties, cost and time taken for successful introgression into adapted cultivars need to be considered.

1.3 Weed Competition in Lentil

1.3.1 Weed competition and yield loss

Weed control is considered a major limitation to lentil production worldwide (Trivedi and Tiwari 1986; Knights 1987; Knott and Halila 1988; Sakar *et al.* 1988; Swanton *et al.* 1993; Muehlbauer *et al.* 1995; Materne *et al.* 2002; Brand *et al.* 2007; Yenish *et al.* 2009). Lentil is a poor competitor with weeds due to slow early growth rates, short stature and a lack of protective canopy development (Basler 1981; Knott and Halila 1988; Boerboom and Young 1995; Muehlbauer *et al.* 1995; Mohamed *et al.* 1997; Hanson and Thill 2001; McDonald *et al.* 2007). Brand *et al.* (2007) suggested that weed

competition in lentil is further compounded in situations with low growing season temperatures and Basler (1981) proposed that lentils with a relatively shallow root system were more affected by moisture stress from weed competition than deeper rooted species.

Weed competition in lentil leads to reductions in crop yield and quality through direct competition for moisture, space and nutrients and indirectly through hosting insects and pathogens (Yenish *et al.* 2009). Furthermore, quality losses and increased cost of production can occur through contamination of grain at harvest (Brand *et al.* 2007) and reduced sustainability of the production system due to increasing weed seed banks (Preston 2002).

Actual yield loss in lentil due to weed infestation is a function of the weed density, individual weed species involved and the soil fertility and moisture levels (Basler 1981). Grain yield losses in lentil from weed competition have been measured in various countries to be as much as 87% (Ahlawat *et al.* 1979; Basler 1980; Curran *et al.* 1987; Swanton *et al*. 1993; AL Thahabi *et al.* 1994; Boerboom and Young 1995; Mohamed *et al.* 1997; Elkoca *et al.* 2005; Tepe *et al.* 2005; McDonald *et al.* 2007). Many of these studies concluded that the competitive relationship between lentil and the weeds was also influenced by environmental conditions; in particular temperature and rainfall.

1.3.2 Timing of control

Lentil crops need to be kept relatively weed free for their entire lifecycle to maximise grain yield (Basler 1981; Brand *et al.* 2007). Experiments measuring yield loss in lentils at two weed removal dates found high yield loss occurred between 30 and 60 days after sowing in India (Ahlawat *et al.* 1979) and 60 to 90 days in Syria (Saxena and Wassimi 1980). Work conducted in Tunisia suggested lentil had a critical weed free period starting at 4 weeks under severe weed infestation and at 16 weeks where infestation was only low to medium (Knott and Halila 1988). The critical weed free period (CWFP) refers to the period of crop growth during which weeds must be absent to prevent yield loss due to the presence of weeds (Knezevic *et al.* 2002). Identification of this period is important to determine the most appropriate weed control strategy. In particular it can be used to determine the timing of post-emergent herbicides (Knezevic *et al.* 2002). There have been only a few

studies looking at the CWFP in lentil. Under rain fed conditions in Jordan, Singh *et al.* (1996) suggested the CWFP was between 34 to 41 days from emergence until 85 to 99 days after emergence; however, results varied across locations. Experiments under irrigated conditions in Sudan suggested the CWFP for lentil was between 2 and 4 weeks after emergence (Mohamed *et al.* 1997). In a more recent study in Canada the CWFP again differed across environments, but was described as beginning at the five node growth stage and ending at the 10 node growth stage, the latter stage typically coincided with canopy closure in their environment (Fedoruk 2010). Studies using the weed wild oats (*Avena fatua* L.) in North America found that yield loss in lentil started to occur 5 to 7 weeks after sowing (Curran *et al.* 1987). However, the duration of yield loss was not measured and the authors also acknowledged the influence of environment on their results, particularly conditions favouring the presence of disease (plant enation virus). Weed removal during crop pod development had no effect on grain yield in experiments conducted in Australia using canola (*Brassica napus* L.) as a surrogate weed in lentil (McDonald *et al.* 2007).

It is difficult to reliably predict the critical weed free period in lentil in Australia due to the lack of work conducted in this country and the difficulties with extrapolating work conducted under different environments. However, early weed control (4 to 6 weeks after emergence) and maintenance of that control until 10 to 12 weeks, or the onset of canopy closure, appears important for limiting grain yield loss in lentil in most environments.

1.3.3 Weed composition

Weed flora composition is dictated by location, soil type and nutrition and climatic conditions combined with the farming system practiced (Basler 1981; Brand *et al.* 2007; Yenish *et al.* 2009). Weeds found in lentil in all regions are generally grouped into annual and perennial monocot (grasses), annual and perennial dicot (broadleaf) and parasitic groups (Brand *et al.* 2007; Yenish *et al.* 2009). Apart from the parasitic weeds *Orobanche* and *Cascuta* spp., which are partially or completely obligate to lentil (Rubiales *et al.* 2009), weeds in lentil are a function of the crop rotation and region rather than the crop itself (Basler 1981; Knott and Halila 1988; Yenish *et al.* 2009). The

broadleaf weed group is generally recognised as the most difficult to control in lentil with herbicide due to the ineffectiveness of registered herbicides (Wall and McMullan 1994; Young *et al.* 2000; Hanson and Thill 2001; Day *et al.* 2008) and because their ecology and biology are similar to lentil (Yenish *et al.* 2009). Parasitic (Rubiales *et al.* 2009) and herbicide resistant (Preston 2002) weeds are also in this difficult to control category and require management across all phases of the system.

Common broadleaf and grass weeds infesting lentil crops in southern Australia are listed in Table 1.1. There are no reports of parasitic weeds in the lentil cropping regions of southern Australia, although branched broom rape (*Orobanche ramose* L.) has been identified and quarantined in the Murray Mallee region of South Australia (PIRSA 2011). This weed could pose a significant problem to lentil production if it was to spread away from this area. The most problematic weeds in lentil in southern Australia are the broadleaf weeds vetch (cultivated and wild) (*Vicia* spp.), musk (*Myagrum perfoliatum* L.), wild radish (*Raphanus raphanistrum* L.), bedstraw (*Galium tricornutum* Dandy), bifora (*Bifora testiculata* (L.) Spreng.), medic (*Medicago* spp.) and clover (*Melilotus* spp.) due to the absence of effective post-emergent herbicides (Long 2002; Materne *et al.* 2002; Preston 2002; Yeatman *et al.* 2008). Preston (2002) suggests that these weeds need to be managed in other phases of the crop rotation and weed seed bank run down before lentils are grown; however, acknowledges this strategy will reduce the frequency of lentil in the rotation. The availability of IMI tolerant lentil in Australia will provide a herbicide option to control some of these weeds (Materne pers. comm.). However, a heavy reliance on this technology for weed control in southern Australia currently exists, as imidazolinone tolerance is already available in wheat (*Triticum aestivum* L), barley (*Hordeum vulgare* L.) and canola. Overuse of any one herbicide group in a cropping system will greatly increase the risk of developing weeds resistant to that mode of action.

Table 1.1 Common broadleaf and grass weeds of southern Australian lentil crops

(Adapted from: (Day *et al.*, 2008).

South Australian common name	Scientific name	Family
Broadleaf weeds		
Bifora	*Bifora testiculata*	Apiaceae
Bedstraw	*Galium tricornutum*	Rubiaceae
Capeweed	*Arctotheca calendula*	Asteraceae
Charlock	*Sinapis arvensis*	Brassicaceae
Clover[A]	*Melilotus* spp.	Leguminosae
Deadnettle	*Lamium amplexicaule*	Lamiaceae
Dock[A]	*Rumex* spp.	Polygonaceae
Fumitory – common	*Fumaria officinalis*	Papavaraceae
Fumitory – red	*Fumaria densiflora*	Papavaraceae
Fumitory – white	*Fumaria parviflora*	Papavaraceae
Geranium[A]	*Erodium* spp.	Geraniaceae
Hoary cress	*Cardaria draba*	Brassicaceae
Horehound	*Marrubium vulgare*	Lamiaceae
Ice-plant – common	*Gasoul crystallinum*	Aizoaceae
Lettuce - prickly (whip thistle)	*Lactuca serriola*	Asteraceae
Marshmallow[AB]	*Malva parviflora*	Malvaceae
Medic[A]	*Medicago* spp.	Fabaceae
Mignonette – cut leaf[AB]	*Reseda* spp.	Resedaceae
Milkthistle[A]	*Sonchus oleraceus*	Asteraceae
Muskweed	*Myagrum perfoliatum*	Brassicaceae
Mustard – ball	*Nesila paniculata*	Brassicaceae
Mustard - Indian hedge	*Sisymbrium orientale*	Brassicaceae
Mustard - wild[A]	*Sisymbrium arvensis*	Brassicaceae
Poppy – rough	*Papaver hybridum*	Papveraceae
Raddish – wild	*Raphanus raphanistrum*	Brassicaceae
Sheep weed (white iron weed)	*Buglossoides arvensis*	Boraginaceae
Shepherd's purse	*Capsella bursa-pastoris*	Brassicaceae
Sorrel	*Rumex acetosella*	Polygonaceae
Soursob	*Oxalis pes-caprae*	Oxalidaceae
Speedwell - ivy leaf	*Veronica hederifolia*	Scrophulariaceae
Three corner jack (spiny emex)[A]	*Emex* spp.	Polygonaceae
Toadrush	*Juncus bufonius*	Juncaceae
Turnip - long fruited	*Brassica tournefortii*	Brassicaceae
Turnip - short fruited	*Rapistrum rugosum*	Brassicaceae
Vetch - common[A]	*Vicia sativus*	Fabaceae
Vetch – tares[A]	*Vicia* spp.	Fabaceae
Vetch - woolly pod[A]	*Vicia villosa*	Fabaceae
Ward's weed	*Carrichtera annua*	Brassicaceae
Wireweed[A]	*Polygonum* spp.	Polygonaceae
Grass weeds		
Barley grass[A]	*Hordeum* spp.	Poaceae
Brome grass[A]	*Bromus* spp.	Poaceae
Phalaris[A]	*Phalaris* spp.	Poaceae
Rye grass annual	*Lolium rigidum*	Poaceae
Sand fescue	*Vulpia fasciculate*	Poaceae
Silver grasses	*Vulpia bromoides & Vulpia myuros*	Poaceae
Wild oats	*Avena fatua*	Poaceae

[A]Various; [B]Perennial

1.4 Weed control methods

1.4.1 Integrated weed management in lentil

Due to its low level of competiveness with weeds and the lack of effective herbicides, successful production of lentil necessitates some form of integrated weed management (IWM) (Yenish *et al.* 2009). Lentil is generally the recipient of an IWM system and current genotypes have little to offer to it themselves. An IWM concept for food legumes proposed by Young *et al.* (2000) consisted of the five widely accepted weed control methods of prevention, biological, chemical, mechanical and cultural. Furthermore, they suggest that changing only one management practice would have little effect on weed dynamics and that the redesigning of present cropping systems is required for significant advances to be made in cool season food legume weed management. McDonald *et al.* (2007) also suggest that an IWM strategy is required in lentil; however, production of this crop in mechanised systems will continue to rely heavily on chemical weed control.

1.4.2 Non-Chemical Weed Control in lentil

Preventative weed control is based around eliminating or slowing the introduction and/or spread of new weeds into areas currently not infested, generally through strict weed hygiene risk management strategies (Holding and Bowcher 2004). Biological control methods use organisms other than the crop itself to control weeds; however, this method of weed control is currently seen as problematic in most diversified annual cropping systems and few examples exist (Young *et al.* 2000). The traditional method of weed control in developing countries with small farm sizes, low labour costs and where weeds are valued for livestock feed is hand or mechanical weed control (Basler 1981). These methods, along with stubble burning, are also the predominant method in organic lentil production where chemical methods are not permitted (Brand *et al.* 2007). Due to increasing farm size, rising labour costs and the widespread uptake of no or minimum tillage, stubble retained cropping, these methods are generally prohibitive in modern farming systems.

Cultural weed control methods are aimed at increasing the crop's competitive ability with the weeds and can be achieved to some extent through ensuring general plant health. Other techniques include crop rotations, competitive cultivars, intercropping, seed placement, row spacing, plant density, sowing time and irrigation timing (Young *et al.* 2000; Brand *et al.* 2007; Yenish *et al.* 2009). Research in Australia and Turkey suggests there is little useful variation amongst genotypes for competitive ability with weeds (Tepe *et al.* 2005; McDonald *et al.* 2007). This contrasts with field pea (*Pisum sativum* L.) where significant variation in competitiveness amongst genotypes has been identified. In field pea the variation is mainly due to differences in plant height (McDonald 2003). Sowing lentil at soil depths of 3 to 5 cm compared with 8 to 10 cm has shown to increase plant emergence, height, dry weight and grain yield (Brand *et al.* 2007). However, shallower sowing is likely to compromise crop safety in systems where weed control depends on water soluble herbicides with high leaching indices (Muehlbauer *et al.* 1995; Holding and Bowcher 2004).

1.4.3 Chemical Weed Control

Chemical control using herbicides is the major method of weed management used in most large scale intensive lentil production systems in developed countries and is becoming increasingly popular in many developing countries (Young *et al.* 2000; Brand *et al.* 2007; Yenish *et al.* 2009). It allows timely and efficient coverage of large areas of crop and is essential for intensive cropping systems based on no or minimum tillage practices. However, the longevity of systems heavily reliant on chemical weed control is doubtful due to increasing public concern about the effect of pesticides on the environment, government regulation and laws restricting availability and use and the increasing number of cases of herbicide resistance weeds worldwide (Young *et al.* 2000). Complicating this approach in lentil is that it is a minor crop internationally, there are few herbicide manufacturers, limited market potential, and currently few effective and safe herbicides for weed control.

Herbicides inhibit essential physiological or biochemical processes in plants usually through a specific interaction with a target cell or tissue leading to cessation in plant growth or death (Devine and Preston 2000). The choice of herbicide, even within the limited list available to lentil, varies

considerably from country to country and region to region. It is dependent upon many factors including environmental conditions, soil type, weed spectrum, rotational practice, farming system, seeding method, cultivar susceptibility, herbicide resistant status and government regulation and local laws limiting economics and availability. Herbicides registered or under permit in lentil in SA are listed in Table 1.2 and grouped as pre-sowing, pre-emergent, post-emergent and pre-harvest herbicides.

Pre-sowing herbicides are generally non-selective and are used alone or in conjunction with tillage to reduce weed numbers prior to sowing and also to aid sowing operations. This timing is particularly important for control of herbicide resistant weeds or those weeds difficult to control in crop.

The pre-emergent application timing includes herbicides applied pre-seeding, e.g. trifluralin, and those applied post-seeding but before emergence, e.g. metribuzin. These herbicides may directly control selective weeds by contact, but more generally control emerging weeds through the residual life of the chemical. Yenish *et al.* (2009) suggests that the length of time of this residual control often varies due to environmental factors and may not provide longevity of weed control, limiting their usefulness. Residual herbicides are often tank mixed with other residual or non-selective herbicides to broaden and increase weed control. This timing is currently the primary method of weed control in lentil in mechanised crop systems and essential for minimising early weed competition (Brand *et al.* 2007). Many of these herbicides have a narrower level of selectivity in lentil than in other crops, e.g. field pea or narrow leafed-lupin (*Lupinus angustifolius* L.) (Day *et al.* 2008). In these cases, crop safety is improved in lentil by increasing seeding depth to at least 5 cm and up to 10 cm depending upon soil type (Knott and Halila 1988; Holding and Bowcher 2004).

Pre-emergent herbicides require moist soil conditions and adequate rainfall to move into the zone where weed seeds germinate, however under conditions of rapid and excessive rainfall, particularly in lighter soils with low organic matter contents, some may leach into the crop root zone causing injury (Muehlbauer *et al.* 1995). Under dry conditions weed control is often reduced due to

low uptake by the plants (Muehlbauer *et al.* 1995). However, Basler (1981) states that increased damage to both crop and weeds can occur from these herbicides in the low rainfall zones. The author suggests that soils low in moisture have relative higher concentrations of chemical in the resulting solution and an increased amount of open-air spaces that are more favourable for the rapid movement of these herbicides. Increased stubble retention often increases the risk of crop injury, as uniform seeding depth is harder to achieve. In situations of high risk for crop damage, reduced application rates or split application timings between pre-sowing, pre-emergence and even post-emergence are employed to reduce the amount of herbicide directly channelled into the seed row by rainfall (Knott and Halila 1988; Brand *et al.* 2007).

Post-emergent herbicides are used to control weed escapes from earlier control methods and late weed germinations. They also allow a 'wait and see' approach to weed control, providing more controlled and targeted application (Knott and Halila, 1988), potentially reducing the risks of crop damage. Outside of the grass selective herbicides (acetyl coA carboxylase (ACCase) inhibitors), these herbicides are extremely limited in lentil (Knott and Halila 1988; Wall and McMullan 1994; Muehlbauer *et al*. 1995; Preston 2002; Yenish *et al.* 2009). Chemical control of grass weeds including volunteer cereals using post-emergent grass selective herbicides in lentil has been very successful due to high crop safety levels. The evolution and spread of herbicide resistant grass weeds, e.g. annual ryegrass (*Lolium rigidum* Gaud), is reducing the effectiveness of these herbicides in many situations (Day *et al.* 2008). In contrast to grass control in lentil, post-emergent broadleaf weed control is more difficult (Table 1.2). There are only two products with registrations for post-emergent broadleaf control in lentil in Australia. Both have limited weed control spectra and their usefulness is restricted somewhat by the fact they cause crop damage and yield loss under certain conditions (NVT Online 2011).

Pre-harvest weed control is aimed at reducing weed seed set and/or assisting with the harvesting process through the desiccation of green weeds. Despite weed control at this stage having little positive impact on crop grain yield in lentil (McDonald *et al.* 2007), this practice has become

important for managing seed set of herbicide resistant weeds for the following crops and is one of the few ways lentils can contribute to an IWM system. It also is used to reduce grain contamination, particularly from weed seeds similar in size to lentil. The effectiveness of this strategy relies on lentil varieties with earlier maturity than the target weed, otherwise crop yield loss and quality issues will occur (Holding and Bowcher 2004).

Table 1.2 Herbicides registered for weed control in lentil in South Australia
(Adapted from Preston 2002).

Herbicide	Group	Target weeds or weed type	Comments
Pre- sowing			
Glyphosate	M	Non-selective	
Paraquat (+/- diquat)	L	Non-selective	
2,4-D isopropylamine	I	Broadleaf	
Pre-emergent (pre-/post-sowing pre-emergent)			
Trifluralin	D	Annual ryegrass and some broadleaf	Maximum rate 1.25 L/ha lentil
Pendimethalin	D	Annual ryegrass and wire weed	Seed should be sown below chemical band
Cyanazine	C	Some broadleaf and grasses	Still registered
Metribuzin	C	Broadleaf and some grasses	Sow at least 5cm deep, rate varies for soil type, check tolerance ratings of varieties, post-emergent application will result in crop damage
Diuron	C	Broadleaf and some grasses	Permit Do not use on light sandy soils, sow at least 5cm deep
Post-emergent			
Sethoxydim	A	Grasses	
Clethodim	A	Grasses	
Propaquizafop	A	Grasses	
Haloxyfop-r	A	Grasses	
Butroxydim	A	Grasses	Use high rates where some resistance to Group A
Quizalofop-P-tefuryl	A	Grasses	
Quizalofop-P-ethyl	A	Grasses	
Tepraloxydim	A	Grasses	
Fluazifop	A	Grasses	
Flumetsulam	B	Some broadleaf weeds	Yield loss occurs under some conditions
Diflufenican	F	Some Brassica weeds	Do not use on cultivar Northfield
Pre-harvest			
Paraquat	L		For crop topping of annual rye grass. Crop yield loss of more than 25% can occur if seed not fully developed
Diquat	L		For crop and weed desiccation, permit

Other chemical control methods used in lentil include seed coating or soaking with imazapyr herbicide for control of *Orobanche crenata* Forskal (Expósito *et al.* 1997), inter-row spraying with non-selective herbicides (Holding and Bowcher 2004) and the use of weedwipers (sponge or rope wicks containing non-selective herbicides traversed across the top of the crop contacting taller weeds) (Holding and Bowcher 2004). These techniques all have been reported as having various levels of success depending upon seasonal conditions, target weed, weed density and distribution and are likely to be part of an IWM strategy rather than the main component.

Herbicide tolerant crops developed by transgenic procedures or induced mutation are appealing as they allow effective post-emergent control of weeds without crop injury; e.g. glyphosate tolerant canola (Duke 2005). The interest in applying this technology to minor crops, such as lentil, has increased in recent years, although actual commercial development is low (Devine 2005). There are no examples of genetically modified herbicide tolerant lentil being used in the world despite the technology existing (Ford *et al.* 2009). However IMI tolerant lentil is widely grown in Canada and was developed through induced mutation using the chemical ethyl methane sulfonate (EMS) (Muehlbauer *et al.* 2009). This technology allows growers to use higher rates of IMI herbicide post-emergent for broadleaf weed control. Registration of new herbicides in internationally minor crops like lentil is always problematic due to the high costs involved relative to the size of the industry. Further complicating this is the issue of increasing herbicide resistant weeds, which can dramatically shorten the life of the technology and the return on investment (Yenish *et al.* 2009). This is particularly an issue in herbicides rated as high risk for resistance, e.g. AHAS inhibitors (Hall *et al.* 1999).

Genetic variation in lentil to registered herbicides such as MCPB (Morrison and Slinkard 1983), trifluralin (Basler, 1981) and metribuzin (McMurray *et al.* 2009) has been reported. An alternative approach to developing new herbicide tolerance is to develop increased tolerance to one or more registered herbicides. This would allow higher rates and alternative application timings of these herbicides to be used. Further to this, the pyramiding of multiple improved herbicide tolerances

into the one genotype could provide multiple chemical weed control strategies, potentially reducing the reliance on one herbicide and delaying the onset of herbicide resistance. This strategy would still need to be part of an IWM system for sustained and effective weed control in lentil, but could strengthen the current frail chemical component of it.

1.5 Metribuzin application to control weeds

1.5.1 Metribuzin properties and use

Metribuzin (4-amino-6-t-butyl-3-(methylthio)-1,2,4-triazin-5(4H)-one) was first reported in 1968 and is one of a group of compounds known as the substituted aminotriazinones or asymmetrical triazines (Hatzios and Penner 1988). In Australia it is a member of herbicide Group C, which are inhibitors of photosynthesis at PSII (Devine *et al.* 1992). Metribuzin is a heterocyclic, basic organic molecule (Fig. 1.1) with an empirical formula of $C_8H_{14}N_4OS$, a molecular weight of 214.3 and a relatively high solubility in water of 1200 ppm at 20 °C (Barrentine *et al*. 1976; Hatzios and Penner 1988). It is protonated and ionizes in acidic aqueous solutions forming cations and molecular species depending on the pH of the solution (Weber 1980). The pK_a of metribuzin was determined as 1.1 (Weber 1980) and 1.0 (Albro *et al.* 1984) by ultra violet spectrophotometry. However, the latter also obtained a reading of 7.1 when the pK_a was determined by potentiometric titrimetry and suggested that the value obtained spectrophotometrically may be associated with acid-catalysed decomposition during protonation of the molecule.

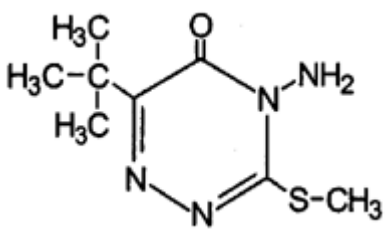

Fig. 1.1 Chemical structure of metribuzin

Metribuzin comes in various formulations. It can be applied as a wettable powder where the metribuzin active ingredient makes up 50 to 75% of the product, as dry-flowable and water-dispersible granular formulations with up to 75% metribuzin and a flowable suspension with 42.1% metribuzin (Hatzios and Penner 1988).

1.5.2 Metribuzin use

Metribuzin is registered for use as a selective herbicide for control of either or both grass and broadleaf weeds in a range of crops worldwide including soybean (*Glycine max* L.), potato (*Solanum tuberosum* L.), tomato (*Lycopersicon esculentum* Mill.), wheat, lentil, barley, field pea, narrow-leafed lupin, faba bean (*Vicia faba* L.) and chickpea (*Cicer arietinum* L.). It is applied in the field either as a pre-emergent application incorporated by sowing, post-sowing pre-emergent or post-emergent (Frankel 2010). In Australia metribuzin is used post-sowing pre-emergent in wheat, lentil, soybean, faba bean, post-emergent in barley, narrow-leafed lupin, potato, and pre- or early post-emergent in field pea (Frankel 2010). However, due to a narrow range of selectivity in the above crops, many restrictions on its use exist based on regional location, crop and weed growth stage, genotypes, cropping system, soil type, texture and pH, ambient temperature, crop disease status or potential, rainfall before and after application, soil moisture status, soil surface condition, retained stubble amount and sowing depth (Day *et al.* 2008; Frankel 2010).

1.5.3 Metribuzin use in lentil

Metribuzin is a recommended herbicide in lentil in a number of countries for control of a range of grass and broadleaf weeds. Within Australia, weeds controlled by metribuzin vary with herbicide rate and location, but include: capeweed (*Arctotheca calendula* L.), charlock (*Sinapis arvensis* L.), sheep weed (*Buglossoides arvensis* L.), ivy leaf speedwell (*Veronica hederifolia* L.), deadnettle (*Lamium amplexicaule* L.), fumitory (*Fumaria spp.*), heliotrope (*Heliotropium amplexicaule* Vahl), horehound (*Marrubium vulgare* L.), oriental mustard (*Sisymbrium orientale* L.), sowthistle (*Sonchus oleraceus* L.), wild radish, African mustard (*Brassica tournefortii* Gouan), annual ryegrass, barley

grass (*Hordeum* spp.), brome grass (*Bromus* spp.), doublegee (*Emex australis* Steinh.), geranium (*Erodium* spp.), and wire weed (*Polygonum* spp.) (Frankel, 2010). It also reportedly gives control of medic, wild vetch and bifora under some conditions (Day *et al.* 2008).

Metribuzin application methods and rates vary across regions due to problems with severe crop phytotoxicity under some conditions (Friesen and Wall 1986; Wall and McMullan 1994; Muehlbauer *et al.* 1995; Yasin *et al.* 1995; Elkoca *et al.* 2005). For example, it is used as a pre- or post-emergent application in lentil in the United States (NDSU 2011) and a pre- or early post-emergent application in Canada (SMA 2011). However, in Australia it is only used as a pre-emergent treatment, as crop injury will occur from post-emergent application (Frankel 2010). In all cases, a number of recommendations are listed to achieve crop safety, many of which require additional management operations and/or compromise optimum weed control (Gosheh and El-Shatnawi 2003). Within Australia it is widely used, but favoured less on light textured soils low in organic matter content due to high risk of leaching and crop damage with heavy rainfall after application (Day *et al.* 2008; Frankel 2010). To avoid this risk, it is recommended for growers to sow lentil at uniform depths greater than 5 cm and apply metribuzin to moist soil before crop emergence (in some situations this can be as little as five days), but not before significant rain events. The combination of these requirements is often difficult to achieve in dry land broadacre, no-till stubble retained systems. Furthermore varietal herbicide tolerance experiments in South Australia have shown that cultivar Nipper has a lower safety margin to metribuzin than other cultivars (NVT Online 2011).

The reasons for higher crop damage from post-emergent metribuzin application in Australia (winter crop) compared with the northern hemisphere countries (summer crop) are not reported in the literature, but could be environmental and or genetic. Growing conditions listed as favourable for crop damage in Canada from post-emergent metribuzin application include cold, cloudy weather or frost within three days of application (SMA 2011). These conditions are commonly experienced during early crop growth in winter grown lentils in Australia.

1.6 Metribuzin in the plant and soil

1.6.1 Mode of action

Metribuzin inhibits photosynthesis through interfering with the reducing side of PSII (Oettmeier *et al.* 1984; Devine *et al.* 1992). Photosynthesis is inhibited through metribuzin binding to the plastoquinone (PQ) binding site on the D1 protein in place of native PQ in the PSII complex of the chloroplast (Devine *et al.* 1992). In doing so, the transfer of electrons from the donor Q_A to the mobile electron carrier, Q_B is disrupted (Devine *et al.* 1992). This is the same target binding site for the triazine herbicide atrazine and the phenylurea herbicide diuron and is encoded by the maternally inherited chloroplast *psbA* gene (Hatzios and Penner 1988; Powles and Yu 2010). The inhibition of photosynthesis reduces the carbohydrate supply to the plant, but it is unlikely that starvation would explain the phytotoxicity effects of metribuzin, as the toxicity symptoms develop very rapidly (Hatzios and Penner 1988). The inhibition of photosynthetic electron transport leads to excessive radiative excitation in the blocked photosynthetic pigment system and in turn results in maximum fluorescence emission, energy spillover to oxygen and other nearby molecules, photooxidation and eventually phytotoxicity at the organelle, cell and tissue level (Devine *et al.* 1992).

Specific effects induced by metribuzin on the chloroplast anatomy and morphology are summarised in (Hatzios and Penner 1988) as dilated thylakoids, decrease of starch grains, decrease of plastoglobuli, decrease of chlorophyll a/b, decrease and/or increase of chlorophyll (a+b) and decrease of carotenoids. Most of these changes are in common with those found in carbohydrate stressed plants due to a decreased rate of photosynthetic electron transport from treatment with low light intensities or chemicals inhibiting photosynthesis (Hatzios and Penner 1988).

A number of secondary or indirect effects of metribuzin on treated plants have also been reported. Among these, interference with nitrogen metabolism resulting in increase of soluble nitrogen, ammonia and amino acids has been reported in soybeans, maize (*Zea mays* V.S.C.) and wheat (Fedtke 1979; Nemat Alla *et al.* 2008), and growth inhibition of dark-grown non-

photosynthetic cultured tissue from tomato and soybean occurred following very high metribuzin applications (Ellis 1978; Oswald *et al.* 1978).

1.6.2 Metribuzin visual symptoms

Visual symptoms of metribuzin toxicity on plants are typical of those caused by the slow-acting photosynthesis-inhibiting herbicides and appear late after inhibition of photosynthesis (Hatzios and Penner 1988). Symptom expression in treated susceptible plants are leaf chlorosis, bleaching and browning of leaf tissue along the margins, leaf veins and petioles, necrotic tissue progressing from leaflet margin, loss of flowers (if in flower) and plant death (Smith and Wilkinson 1974; Graf and Ogg 1976; Gawronski 1983; Gill and Bowran 1990). Moderately susceptible and moderately tolerant crop cultivars show symptoms of damage as petiole chlorosis, leaf interveinal chlorosis and necrosis and slowed growth (Smith and Wilkinson 1974; Gawronski 1983; Gaul *et al.* 1995).

1.6.3 Metribuzin uptake and translocation

Metribuzin is absorbed by the roots and translocated apoplastically to the stems and leaves in a range of plants (Smith and Wilkinson 1974). Absorption is rapid and directly proportional to the rate of transpiration after an initial period of uptake associated with saturation of the root adsorptive sites (Jensen 1982). This process is passive and not influenced by metabolic inhibitors, although it is affected by factors that affect transpiration, such as temperature, humidity, light intensity and stomatal aperture (Jensen 1982). Foliar applied metribuzin is absorbed into the leaf through the hydrated cuticle and primarily transported toward the leaf apex with little herbicide translocated basipetally from the treated area in barley and tomato (Fortino and Splittstoesser 1974a; Gawronski *et al.* 1986). However Frank and Beste (1983) suggested that metribuzin applied to basal leaves of jimsonweed (*Datura stramonium* L.) and tomato moved both basipetally and acropetally in the former and basipetally only in the latter. Hatzios and Penner (1988) summarised metribuzin movement in plants as predominantly, but not exclusively, in the apoplast. Penetration of root or leaf

symplasm can occur but due to the inability of the symplasm to retain it for long, it is leached into the apoplast and carried away with the transpiration stream.

1.6.4 Soil factors affecting metribuzin availability and persistence

Metribuzin has a relative short persistence in the soil (Webster and Reimer 1976). A field study by Sharom and Stephenson (1976) found a half-life ranging from 2.5 to 4 months in a Guelph loam soil while Bouchard *et al.* (1982) measured the half-life at 2.6 months in a Taloka silt loam. The latter also found soil persistence was less as incubated soil temperatures increased from 7 to 37°C and Ladlie *et al.* (1976a) showed that the half-life decreased as soil pH increased in a Hillsdale sandy loam. Mobility of metribuzin in soil is inversely correlated with organic matter, soil texture (clay content) and increasing soil pH of the soil, but highly correlated with soil water content at 0.033 MPa tension and soil surface area (Ladlie *et al.* 1976b; Savage 1976; Sharom and Stephenson 1976; Peter and Weber 1985). The low adsorptive capacity and organic content of acidic sandy soils in Western Australia is suggested as the reason for good activity of metribuzin on brome grass at low rates (100 to 200 g ha^{-1}) compared to the higher rates (300-600 g ha^{-1}) required for a similar level of brome grass control on soils in the USA with higher clay and organic matter contents (Gill and Bowran 1990). Leaching of metribuzin can occur readily due to its high water solubility measure, but is dependent upon soil adsorption capacity, the amount of rainfall, rate of plant uptake and metabolism and microbial breakdown (Sharom and Stephenson, 1976; Barrentine *et al.*, 1982; Peter and Weber, 1985; Allen and Walker, 1987; Kim and Feagley 1998).

1.6.5 Factors affecting phytotoxicity

Soil conditions conducive to high levels of metribuzin injury are generally well defined and have been discussed above. A number of other factors are suggested to affect metribuzin phytotoxicity in plants and are less understood due to variation being found across species and experimental methods. The effect of ambient temperature on metribuzin induced plant damage appears two-fold. Pot experiments with metribuzin applied pre-emergence in field pea (Al-Khatib *et al.* 1997) and post-

emergence in tomato (Phatak and Stephenson 1973; Fortino and Splittstoesser 1974a; Fortino and Splittstoesser 1974b) found that herbicide injury increased as day and night temperatures were increased above 16 to 21°C and 13 to 16°C respectively. Conversely increased metribuzin damage occurred in a susceptible barley cultivar when applied post-emergent at a temperature of 0°C compared with when applied at 30°C in growth cabinet studies (Caldwell and O'Sullivan 1985). However, there was no effect on the tolerant cultivar used in this study. Schroeder *et al.* (1985) suggested differences in environmental conditions post-application, including the presence of air temperatures below freezing, were responsible for increased levels of metribuzin damage from post-emergent field applications to wheat across seasons. Temperatures at the lower end of these ranges are common in southern Australia at the time metribuzin is applied to winter-grown lentil.

Reduced light intensities up to three days before treatment with post-emergent metribuzin in tomato in growth chamber and field experiments increased crop damage (Phatak and Stephenson 1973; Fortino and Splittstoesser 1974a; Da Silva and Warren 1976; Friesen and Hamill 1978; Pritchard and Warren 1980). Arsenault and Ivany (2001) also attributed reduced light intensity to increased levels of post-emergent damage in potatoes grown under field conditions across seasons. In growth room studies with barley, both susceptible and tolerant cultivars incurred greater levels of damage when exposed to increasing periods of dark treatment following post-emergent application of metribuzin (Caldwell and O'Sullivan 1985). The tolerant cultivar also incurred higher levels of herbicide damage when subjected to a pre-spraying regime of 8 hours of darkness compared with 8 hours of light. The opposite result occurred with a susceptible cultivar and was unexplained.

Tomato and barley plants grown in growth cabinets and treated with post-emergent metribuzin under conditions of higher relative humidity incurred higher levels of plant damage than those grown at lower levels (Fortino and Splittstoesser 1974a; Caldwell and O'Sullivan 1985). It was suggested that an environment with higher moisture content would increase absorption through retaining the chemical in solution on the leaf surface for longer (Fortino and Splittstoesser 1974a).

In pot experiments, pre-emergent applications of metribuzin to field pea and post-emergent applications to barley (sensitive cultivar only) were more damaging when applied to plants grown in soil held at field capacity than at lower levels (Caldwell and O'Sullivan 1985; Al-Khatib *et al.* 1997). However, in field experiments Runyan and McNeil (1982), attributed a lack of expected yield reduction and cultivar response at only 3 of 13 sites where post-emergent metribuzin was applied to various red wheat cultivars, to the soils being saturated at the time of application. It was thought that these conditions would reduce herbicide penetration into the soil. In tomato, damage from metribuzin was greater following post-emergent applications at earlier growth stages, with the addition of surfactants and to abraded leaves. Growth stage timing was also reported as being important to the level of damage incurred in wheat as cited by Gill and Bowran (1990), a susceptible barley cultivar (Caldwell and O'Sullivan 1985) and potato (Gawronski *et al.* 1977).

Metribuzin is often applied in combination with other pesticides and experiments have been conducted to identify interactions between metribuzin and the other pesticides. A phytotoxic interaction occurred between metribuzin and the grass herbicide tridiphane in soybean and a metribuzin susceptible, but not tolerant tomato cultivar, and the order and timing of herbicide applications were important in determining the extent of injury (Gaul *et al.* 1995). Ladlie *et al.* (1977a) found soil applied atrazine and metribuzin combinations acted synergistically to reduce soybean growth in field and green house experiments and that these reductions were greater as soil pH increased. In a different set of field and green house experiments these authors found that combination applications of trifluralin and metribuzin to the soil protected soybeans from metribuzin-induced injury (Ladlie *et al.* 1977b). This was attributed to the trifluralin treatment reducing root development and subsequent metribuzin uptake. Field experiments in Canada with lentil showed tank mixes of trifluralin and metribuzin and metolachlor and metribuzin applied pre-planting did not increase phytotoxicity to the crop compared with single applications (Friesen and Wall 1984). When metribuzin was applied sequentially and post-emergent with these treatments, slightly increased transient phytotoxicity occurred in the trifluralin treatment and significant increases in phytotoxicity

and yield reduction in the metolachlor treatment over both the combination pre-planting treatments and the singular post-emergent metribuzin treatment (Friesen and Wall 1986). The application of trifluralin pre-sowing followed by a post-sowing pre-emergent application of metribuzin is widely practiced in southern Australian lentil production systems.

1.7 Metribuzin tolerance

1.7.1 Interspecific variation in tolerance

A number of studies on the variation in tolerance to metribuzin between various crop-weed combinations have been undertaken including soybean and hemp sesbania (*Sesbania exaltata* L.) (Hargroder and Rogers 1974), soybean and common cocklebur (*Xanthium strumarium* L.) (Salzman *et al.* 1992), tomato and jimsonweed (Frank and Beste 1983), winter wheat and downy brome (*Bromus tectorum* L.) (Devlin *et al.* 1987) and lentil and tumble mustard (*Sisymbrium altissimum* L.) and may weed (*Anthemis cotula* L.) (Hassanein *et al.* 1984). The mechanisms attributed to the differences in selectivity of metribuzin in these cases were differences in one or a combination of absorption (retention or uptake), translocation and metabolism of the herbicide, although differential absorption appears to be generally of minor significance (Hatzios and Penner 1988). In the only report involving lentil it is suggested a combination of both difference in retention and metabolism are responsible for the selectivity between the crop and the weed species (Hassanein *et al.* 1984). Conversely differential translocation was suggested as a major reason for selectivity of metribuzin between tomato and jimsonweed when applied to the leaves (Frank and Beste 1983).

1.7.2 Intraspecific variation in tolerance

Variation in tolerance to metribuzin within species grown in various media has been reported in soybean (Hardcastle 1974; Mangeot *et al.* 1979; Barrentine *et al.* 1982), tomato (Gawronski *et al.* 1983; Souza Machado *et al.* 1978), potato (Ivany 1979), sweet potato (*Ipomoea batatas* (L.) Lam.) (Harrison *et al.* 1985; Motsenbocker and Monaco 1993), southernpea (*Vigna unguiculate* (L.) Walp.)

(Harrison 1988), field pea (AL Thahabi *et al.* 1994), barley (Caldwell and O'Sullivan 1985; Gawronski *et al.* 1986; Gawronski *et al.* 1987), wheat (Runyan and McNeil 1982; Schroeder *et al.* 1985; Kleemann and Gill 2007), lentil (McMurray *et al.* 2009) and narrow-leafed lupin (Si *et al.* 2006). Where a difference between tolerant and susceptible cultivars was given, an increase of up to three-fold as estimated by ED_{50} (dose required for 50% inhibition) was reported in these studies; however, a ten-fold difference was reported in nutrient solution experiments with potato (Gawronski *et al.* 1985). Two separate induced mutants of narrow-leafed lupin were identified with four and six times the tolerance to metribuzin over the original genotype (Si *et al.* 2009). The authors in a separate pot study showed a further five-fold increase in tolerance was achieved in F_2 plants from a cross between these tolerant mutants (Si *et al.* 2011).

Differences in either the metabolic pathways and/or the rate of metabolism of metribuzin are reported as the major mechanism conferring genetic variation in tolerance within soybean (Smith and Wilkinson 1974; Falb and Smith Jr 1984; Frear *et al.* 1985), tomato (Stephenson *et al.* 1976; Frear *et al.* 1983), barley (Gawronski *et al.* 1987) and potato (Gawronski *et al.* 1986). Tolerant cultivars in these species more rapidly conjugate the metribuzin parent compound or, in the case of soybean, deaminated metribuzin (DA) into less toxic products. It was thought initially in soybeans that metribuzin was metabolised to a diketo- form and then conjugated to glucose (Smith and Wilkinson 1974; Oswald *et al.* 1978; Mangeot *et al.* 1979). However, Frear *et al.* (1985) proposed that the major pathway of metabolism involves the initial oxidation of the methylthio- group to a reactive sulfoxide intermediate. This intermediate may then form a homoglutathione conjugate or be hydrolysed to a second intermediate (DK) which may be incorporated into insoluble residue, malonated to a malonic acid conjugate or deaminated to DADK. They also proposed an alternative minor pathway where the metribuzin forms an intermediate *N*-glucoside conjugate followed by acylation to form a malonyl *N*-glucoside conjugate. Conversely, in tomato metribuzin is initially enzymatically metabolised by UPDG: *N*-glucosyltransferase to a B-D-(*N*-glucoside) conjugate followed by the rapid acylation through a Malonyl-CoA transferase to a malonyl B-D-(*N*-glucoside) conjugate (Frear *et al.* 1983). A

correlation between foliar UPDG: *N*-glucosyltransferase levels in leaves of tomato seedlings and seedling tolerance was also suggested. Further to this, leaves of older tomato plants possessed higher enzyme activities than leaves of seedlings, a finding in agreement with that of Da Silva and Warren (1976), who suggested that sensitivity of tomato to metribuzin decreased with increasing age.

Due to the rapid uptake of metribuzin occurring in both tolerant and susceptible genotypes of various species, variation in absorption appears to have a minor role in intraspecific variation in tolerance (Frear *et al.* 1983; Smith and Wilkinson 1974; Gawronski *et al.* 1987). However, translocation appears to have some role in intraspecific variation in tolerance in potato and soybean (Smith and Wilkinson 1974; Gawronski *et al.* 1985). Tolerant genotypes had higher concentrations of radiolabelled metribuzin in their petioles, stems or major veins compared with higher levels in the interveinal leaf tissue of susceptible genotypes. However, translocation differences were not considered responsible for any genetic variation in tolerance in barley genotypes (Gawronski *et al.* 1987).

A target site-based tolerance mechanism for metribuzin has not been reported in a crop species. This contrasts with TT canola where a chloroplastic *psbA* gene mutation encoding the PSII D1 protein leads to a $Ser_{264}Gly$ amino acid substitution, and is responsible for a high level of tolerance to the triazine herbicide, atrazine (Shukla and Devine 2008; Powles and Yu 2010). An $Ala_{251}Val$ target site substitution of the D1 protein was recently identified in a field population of *Chenopodium album* L. from Sweden where repeated use of the triazinone herbicides metamitron and metribuzin had occurred (Mechant *et al*. 2008). The C. *album* biotype exhibited a high level of tolerance to metribuzin but a lack of cross tolerance to atrazine.

1.7.3 Cross tolerance

Cross tolerance or cross resistance is used to describe the tolerance obtained in a plant to a distinct herbicide class after selection to another distinct class of herbicide has occurred. This differs to multiple herbicide tolerance which is the term given to plants which have tolerance to two or more herbicides through selection by each individual herbicide. Cross resistance to metribuzin and a

number of other Group C herbicides has been identified in a biotype of annual ryegrass in Australia through repeated exposure over a 10 year period to amitrole and atrazine (Burnet *et al.* 1991). The authors concluded through oxygen evolution studies with isolated thylakoids and herbicide metabolism studies on whole plants that the basis of resistance was enhanced metabolism or sequestration of the herbicide in the leaf rather than an alteration to the target site.

1.7.4 Genetics of tolerance

Where within species variation exists for a particular herbicide an understanding of the type of inheritance involved in tolerance is essential to allow the effective incorporation of this trait into superior germplasm. There has been some investigation into the genetic controls of metribuzin in various crops. Monogenic recessive inheritance of tolerance has been reported in soybean, wild soybean and potato (Edwards *et al.* 1976; Kilen and Barrentine 1983; DeJong, 1983; Kilen and Guohao 1992). However, in sweet potato a polygenic inheritance was identified (Harrison *et al.* 1987). Limited research in tomato suggested metribuzin response was controlled by one major gene; however, some doubt existed due to phenotyping difficulties (Souza Machado *et al.* 1982). In all these studies, a cytoplasmic source was suggested as not being responsible for the phenotype.

Ratliff *et al.* (1991) reported that sensitivity was partly dominant in wheat and claimed that both nuclear and cytoplasmic inheritances were involved. A study on *T. durum* cultivars using plant weight as the measure of response suggested that cytoplasmic inheritance was not involved in metribuzin tolerance and genetic control was semi-dominant and complex, involving many alleles (Villarroya *et al.* 2000). The authors also suggested mass selection would be a sufficient breeding method for cultivar improvement. In a genetic study using two different metribuzin tolerant narrow-leafed lupin mutants a single semi-dominant gene conferred tolerance over the original cultivar and in both cases nuclear genes, not a cytoplasmic source, were implicated (Si *et al.* 2011).

1.7.5 Screening methods

A number of screening methods for identifying metribuzin tolerance in plants have been attempted, with the method varying with crop type, genotype number and herbicide application method. Post-emergent applications to narrow leafed lupin and barley grown in pots were applied using motorised cabinet sprayers with plants being grown in controlled conditions before and after application (Caldwell and O'Sullivan 1985; Si *et al.* 2006). This method was also used for pre-emergent applications in soybean and wheat genotypes (Barrentine *et al.* 1982; Kleemann and Gill 2007). In all these cases, the maximum number of genotypes screened was 19 indicating the relative low throughput capacity of this method. A sinking leaf disk assay was used for identifying tolerance in potato (Gawronski *et al.* 1977) and tomato (Gawronski 1983), but required the donor plants to be grown under tightly controlled uniform conditions to reduce variability in results.

Various hydroponic pot methods have been used across a range of crops, particularly where a pre-emergent herbicide field application is practiced and larger numbers are being attempted. A sand growing medium with metribuzin added via an irrigated nutrient solution was used in potato (DeJong 1983); however, in field pea the herbicide was pre-mixed in a sand/silt loam mix prior to planting the seed (Al-Khatib *et al.* 1997). Harrison (1988) rapidly screened over 1200 accessions of *Vigna* spp. in a glass house using a similar technique to Al-Khatib *et al.* (1997) and detected significant levels of variation. However, they concluded the use of more precise herbicide application and controlled environment facilities would give a greater sensitivity of separation between genotypes. Genotypes of soybean, tomato and wheat have all been successfully screened in various nutrient solution assays with the herbicide being introduced at the seedling stage (Barrentine *et al*. 1976; Souza Machado *et al.* 1978; Villarroya *et al*. 2000). Plant cell cultures have also been used to identify metribuzin tolerance. Differential tolerance was detected in two cell suspensions of soybean and findings correlated with field observations (Oswald *et al.* 1978); however, in tomato genotypes either no variation in tolerance was detected or results were variable (Ellis 1978; Harrison *et al.* 1983).

Field screening has been used to quantify metribuzin tolerance, but generally only where a small number of genotypes are required to be evaluated. However, it has the additional benefit of allowing validation in the target environment. McMurray *et al.* (2009) identified improved levels of metribuzin tolerance in two lentil genotypes through field screening single plant rows of approximately 100 genotypes; however, this method was primarily used to detect genotypes with a high level of herbicide sensitivity. Field selection was recently successfully used to identify two highly metribuzin tolerant genotypes from an estimated 79,000 M_2 seeds of a mutated narrow-leafed lupin genotype (Si *et al.* 2009).

1.7.6 Breeding for herbicide tolerance

Classical plant breeding methods have been used to develop herbicide tolerant cultivars and to improve relative herbicide tolerance in cultivars. Numerous TT canola cultivars have been developed through crossing *Brassica napus* L. with a triazine-tolerant biotype of birdsrape mustard, *B. rapa* L (Beversdorf and Kott 1987). The latter had evolved cytoplasmically-inherited tolerance through a point mutation in the *psb*A gene. Commercial varieties were successfully developed through a combination of backcrossing and cytogenetic selection, however grain yields are lower in tolerant varieties due to the cytoplasmic herbicide tolerance (Beversdorf and Kott 1987). The soybean cultivar Tracy-M has tolerance to rates of metribuzin that result in unacceptable damage in other cultivars (Barrentine *et al.* 1982). It was developed by screening several hundred seedlings of the parent cultivar, Tracy in a hydroponic solution and surviving plants were transplanted to pots and grown to maturity (Hartwig, 1987).

Induced mutants with tolerance to imidazolinone herbicides in canola (Swanson *et al.* 1989), wheat (Newhouse *et al.* 1992) and other crops have been identified and developed into numerous herbicide tolerant cultivars worldwide (Tan *et al.* 2005). More recently this process has been used in lentil leading to the release of imidazolinone tolerant cultivars in Canada (Muehlbauer *et al*. 2009) and Australia (Materne *et al*. 2011).

Finally the development of cultivars with tolerance to the broad-spectrum herbicides glyphosate (soybean, canola, cotton, maize) and glufosinate (canola, cotton and corn) has been achieved through the use of transgenes (Duke 2005). It is unlikely that this approach would ever lead to commercial cultivars in a minor crop like lentil due to low return on investment and international trade issues (Devine 2005).

1.8 Summary and aims of research

Lentil is a significant and expanding pulse crop in southern Australian broadacre dryland cropping systems. However, it is inherently a poor competitor with weeds and an integrated weed management system based on multiple chemical control methods will be required for continued production and industry expansion. Few herbicides are registered for weed control in lentil, particularly for safe and effective post-emergent broadleaf weed control.

The recent development of lentil cultivars tolerant to IMI herbicides has occurred in North America (Muehlbauer *et al*. 2009) and Australia (Materne *et al*. 2011) and will increase weed control options. However, the AHAS herbicides have a relatively high risk of herbicide resistance development. Furthermore, they are already widely used in Australia in IMI tolerant crops grown in rotation with lentil, such as canola, wheat and barley. Alternative and/or complimentary in-crop chemical weed control strategies to the AHAS herbicides will be required in lentil to allow sustainable and effective weed control and maintain production of this crop.

Metribuzin is a PSII inhibitor herbicide controlling a wide range of grass and broadleaf weeds. It is currently registered for post-sowing pre-emergent use in lentil in Australia, but causes high levels of crop phytotoxicity if applied post-emergent. Metribuzin is not recommended for post-emergent application in Australia; however is applied this way in the northern hemisphere. Despite its registration in Canada, it is known to be harmful to lentil and can cause stunting, yellowing, leaf drop and subsequent yield loss (Friesan and Wall 1986). Weather conditions of low temperatures and low light intensities around the time of herbicide application have been linked to increased crop

phytotoxicity from post-emergence metribuzin in barley, tomato and soybean and could be responsible for the sensitivity in lentil in Australia. An understanding of the weather factors associated with crop phytotoxicity from post-emergent metribuzin application to lentil in southern Australia will be important in any attempt to develop germplasm with improved tolerance to this herbicide.

Some level of genetic variation for tolerance to metribuzin has been identified using various screening methods in a number of crop species. Generally, only up to a three-fold increase in metribuzin tolerance between tolerant and sensitive cultivars was identified, and in the case of lentil, no cultivar with an agronomically useful level of tolerance has been reported. The highest level of herbicide tolerance improvement reported in a crop and evaluated under field conditions was a four- to six-fold improvement developed in narrow-leafed lupin though induced mutation with sodium azide and subsequent field selection under herbicide pressure (Si *et al*. 2009). Given there is some level of tolerance to metribuzin in advanced Australian lentil breeding lines (McMurray *et al*. 2009) and germplasm with improved levels of tolerance has been identified in other crop species, agronomically useful levels of tolerance could be developed in lentil.

Increased plant metabolism is the major mechanism suggested as being responsible for intraspecific variation in tolerance to metribuzin. The genetic controls of metribuzin tolerance appears to vary according to crop species, however, a semi-dominant nuclear inheritance was the most common control reported.

A target site-based tolerance mechanism has not been reported for metribuzin in any crop species unlike for atrazine tolerance in TT canola. However, a chloroplastic *psbA* gene mutation encoding a target site substitution in the PSII D1 protein was reported for *C. album* after repeated field exposure to metamitron and metribuzin herbicides (Mechant *et al*. 2008). This finding suggests that the potential for developing a target site based metribuzin tolerance in a crop species exists and the most likely mode of development is through mutation breeding and field selection.

The aims of the research presented in this thesis were to:

i) identify the major weather factors responsible for post-emergent metribuzin damage in lentil in southern Australia;

ii) identify an agronomically useful level of tolerance to metribuzin in lentil, and

iii) understand the mechanisms and genetic controls of any identified metribuzin tolerance in lentil.

1.9 Linking statement

This thesis has been prepared according to the University of Adelaide's specification for 'PhD by publications' format. Research in this thesis is presented in seven chapters, including five research chapters, two of which have been published in peer reviewed journals, one (Chapter 2) that has been accepted for publication and, two (Chapters 5 and 6) which have been submitted for publication. Each manuscript is presented in either published or submitted form according to the instructions to author of the specific journal, leading to some overlap between the literature review and the introduction sections of the manuscripts. The manuscripts are presented in chronological order of the research.

In herbicide tolerance research globally, the terms tolerance and resistance or tolerant and resistant are often interchanged depending upon region and interpretation. In Australia, generally resistance is used when referencing the response of weeds to herbicides and tolerance when referencing differences within crops; this is the interpretation used in this thesis. The exception is Chapter 4, where the associate editor and anonymous reviewers of the journal requested the term resistance be used instead of tolerance.

Chapter 1 consists of the introduction to the research area and a review of relevant literature on lentil and the herbicide metribuzin. The chapter concludes with a summary of the review and the aims of the study.

Chapter 2 presents research that quantifies the level of grain yield loss from post-emergent metribuzin application to lentil in southern Australia. The soil and weather factors associated with post-emergent metribuzin plant damage in lentil are quantified and reported. This chapter provides a detailed understanding of how metribuzin interacts with lentil under Australian conditions and underpins all subsequent research, providing the rationale for its positioning as the first research chapter.

Chapter 3 describes the use of both controlled environment germplasm screening and induced mutagenesis field selection approaches for developing metribuzin tolerance in lentil. Two separate controlled environment dose response experiments and a preliminary field study in Canada confirmed a germplasm line with an intermediate level of tolerance and two mutation derived lines with high levels of tolerance. The three identified lines and the dose response methods derived in this chapter are used in the research detailed in subsequent chapters. Additional information on the germplasm lines screened in this chapter are included as supplementary information in the appendix section of the thesis (Appendices 1 to 6).

Chapter 4 details the findings from dose response experiments that characterise the cross tolerance profile of the mutant genotypes. It also details the nucleotide and resulting deduced amino acid sequencing of the chloroplastic *psbA* gene of both mutants that identified a unique $Ala_{251}Thr$ substitution in higher plants. The sequencing data and subsequent molecular marker developments from this research are used in the genetic studies in the following chapter. The intermediate tolerant germplasm line discovered in Chapter 3 (SP1333), is not included in the research reported in this Chapter nor in Chapter 5. The research in both these chapters focuses on identifying and characterising the target site mutation tolerance present only in the mutant lines. Agronomic performance of SP1333 is evaluated in Chapter 6 and its value as a source of herbicide tolerance for lentil improvement is discussed in the general discussion chapter.

In Chapter 5 the genetic controls of metribuzin tolerance in the mutant lentils were identified using the phenotyping and genotyping methods determined in Chapters 3 and 4. Field experiments

with reciprocal BC_1F_2 and BC_1F_3 populations identified that a fitness cost of 20 to 40% was associated with the target site tolerance in both mutants.

Chapter 6 reports on the field performance of the three lines identified in Chapter 3 to agronomically useful and higher application rates of post-emergent metribuzin on contrasting soil types over two years in southern Australia.

A general discussion of the research in this thesis is presented in Chapter 7. The discussion incorporates findings from all research chapters and highlights the overall significance of the work and its contribution to lentil and herbicide research. It also discusses potential future research directions and needs, particularly in relation to effectively introgressing the target site tolerance into lentil breeding programs.

Chapter 2.

Soil and weather factors associated with plant damage from post-emergent metribuzin in lentil (*Lens culinaris*) in southern Australia.

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Name of Principal Author (Candidate)	Larn S McMurray		
Contribution to the Paper	Designed, conducted all research experiments, performed data analysis and interpreted all data, and drafted and constructed the manuscript and was corresponding author.		
Overall percentage (%)	75%		
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.		
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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.

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Soil and weather factors associated with plant damage from post-emergent metribuzin in lentil (*Lens culinaris*) in southern Australia

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Abstract. Multiple field experiments and a controlled environment temperature study were conducted to investigate soil and weather factors responsible for herbicide phytotoxicity in lentil (*Lens culinaris* Medik.) from post-emergent metribuzin application. A linear relationship was observed between plant injury (% necrosis) and metribuzin rate at all 12 environments, but at only 11 environments for anthesis DW and at nine environments for both plant density and grain yield. Grain yield reductions from label metribuzin rates of 135 (sand) and 285 (clay) g a.i. ha^{-1} ranged from 0 to 32% and 0 to 67%, respectively across all environments. A principal component analysis of soil and weather factors around the time of herbicide application suggested that metribuzin induced plant damage in lentil was due to a combination of multiple soil and weather factors. However, rainfall events within 10 days of herbicide application, particularly on light textured soils or where soil moisture was low, was most strongly correlated to plant damage. Experiments targeting the impact of reductions in temperature post- and light intensities pre- and post-metribuzin application had no and low effects, respectively, on plant damage measures. As rainfall in the 10 days after application is a major determinant of metribuzin damage in winter grown lentil in southern Australia, a higher level of selective tolerance to metribuzin than that present in commercial cultivars is needed for its safe post-emergent use. Early and late measures of plant damage will be required to accurately assess plant tolerance to post-emergent metribuzin application in lentil.

Additional keywords: yield loss, phytotoxicity, plant injury, environmental conditions, tolerance

Introduction

Lentil (*Lens culinaris* Medik.) production area in southern Australia has increased from less than 500 ha in 1993 to over 350,000 ha in 2017 (Brouwer 2002; ABARES 2018). Production is limited by an inability to control broadleaf weeds, due in part to a lack of safe and effective post-emergent herbicides (Brand *et al*. 2007). The development of cultivars with tolerance to imidazolinone (IMI: acetohydroxyacid synthase [AHAS] inhibitors) herbicides has increased weed control options (Materne *et al*. 2011). However, an over-reliance on IMI herbicides in lentil and other rotation crops such as barley, wheat and canola has led to the development of IMI resistant broadleaf weed species including oriental mustard (*Sisymbrium orientale* L.), African mustard (*Brassica tournefortii* Gouan), and wild radish (*Raphanus raphanistrum* L.) (Boutsalis *et al*. 2016).

Metribuzin is a photosystem II inhibitor herbicide which has a lower risk of herbicide resistance development than the AHAS inhibitors. It provides control and suppression of a range of grass and broadleaf weeds, including IMI resistant, and problematic weeds in lentil, such as milk thistle (*Sonchus oleraceus* L.) and prickly lettuce (*Lactuca serriola* L.) (Davey 2014). Metribuzin is a registered herbicide in lentil in a number of countries although its application method and use rate vary depending on the ecosystem. For example, it is applied pre- or post-emergent in lentil in the United States (NDSU 2014), pre- or early post-emergent in Canada (SMA 2014), but only pre-emergent in Australia due to severe crop damage from post-emergent application (White 2015).

Metribuzin is widely used in Australia, but favoured less on soils that are light textured and low in organic matter due to a high risk of herbicide leaching and subsequent crop damage from post-application rainfall events (Gill and Bowran 1990). Leaching of metribuzin can occur readily due to high water solubility (1200 ppm), but is dependent upon factors such as soil adsorption capacity, soil moisture, the amount of rainfall, and rate of microbial breakdown (Sharom and Stephenson 1976; Peter and Weber 1985; Allen and Walker 1987; Kim and Feagley 1998). To avoid this risk in

Australia recommended application rates are 135 (sand), 210 (loam) and 285 g a.i. ha^{-1} (clay) (White 2015). Additionally, lentil should be sown at a uniform depth greater than 5 cm and metribuzin applied post-sowing pre-emergence to a moist, level soil surface, but not before significant rain events. Achieving this combination of requirements is difficult in dry land broadacre, stubble retained systems particularly when autumn sown lentil can emerge five days after sowing. Reduced herbicide rates are often used to lower the risk of crop damage but can lead to inadequate weed control (Gosheh and El-Shatnawi 2003).

A post-emergent application of metribuzin in lentil would improve control of late germinating weeds and reduce off target plant damage through expanding application timing. Reasons for the apparent higher level of plant damage from post-emergent metribuzin application in Australia (winter crop) compared with North America (summer crop) are unclear. Growing conditions listed on the metribuzin label as risk factors for lentil crop phytotoxicity in Canada include cold, cloudy weather or frost within three days of application (SMA 2014). These growing conditions are frequently observed in Australia at the time of post-emergent herbicide application in lentil. Conditions of reduced light intensity and increased relative humidity (RH) can increase plant phytotoxicity from metribuzin in barley and tomato (Fortino and Splittstoesser 1974; Pritchard and Warren 1980; Caldwell and O'Sullivan 1985).

Pot experiments with metribuzin applied pre-emergence in field pea (Al-Khatib *et al*. 1997) and post-emergent in tomato (Fortino and Splittstoesser 1974) found that herbicide damage increased with day/night temperatures above 20/15 and 16/16°C, respectively. Conversely, increased plant damage occurred from post-emergent metribuzin application to a sensitive barley cultivar grown at 0 compared to at 30°C (Caldwell and O'Sullivan 1985). In one of the few field studies reported, Schroeder *et al*. (1985) suggested the presence of air temperatures below freezing may have been responsible for increased levels of plant damage from post-emergent metribuzin in wheat.

The development of lentil genotypes with improved tolerance to post-emergent metribuzin is a current objective of Australian lentil breeding programs (McMurray *et al*. 2019). However, there

are no reports on the extent of plant damage and associated grain yield loss from this application timing in Australia, nor of the field conditions that regularly lead to plant damage from post-emergent metribuzin applications. Therefore, field experiments, on varying soil types in lentil growing regions of southern Australia, and a controlled environment room temperature experiment were conducted to i) quantify the extent of plant damage and grain yield loss from post-emergent metribuzin applications and ii) identify soil and weather conditions that induce post-emergent metribuzin plant damage in lentil.

Materials and methods

Two field experiments were located at Pinery in the Mid North and near Arthurton on the Yorke Peninsula, South Australia in 2011 and 2012. The first examined the impact of weather and soil factors through changes in sowing date, and the second, the effect of differences in light intensity on post-emergent metribuzin application in lentil. These locations represented a light and medium textured soil type respectively, in key lentil growing regions. In 2012, a third site on a heavy textured soil in a higher rainfall region at Riverton in the Mid North was included in the sowing date study. Details of the sites and management of the experiments are presented in Table 2.1.

A common source of certified seed of lentil cultivar PBA Flash was used in all experiments. PBA Flash was released in 2009 and was a widely grown cultivar in Australia due to its broad adaptation, earlier maturity, improved salinity and boron tolerance and greater harvestability than other cultivars available at the time. It also has a level of metribuzin tolerance that is representative of most Australian cultivars but more tolerant than the sensitive cultivar Nipper (GRDC, 2015). PBA Flash seed was adjusted for germination percentage and seed weight and sown at a density of 120 seeds m^{-2} in plots measuring 1.35 by 10 m using a small plot cone seeder with six narrow tynes on 0.225 m row spacing. All experiments were sown into retained straw residue at a depth of 50 to 60 mm to avoid herbicide damage from shallow sowing (White 2015). Basal fertilizer at a rate of 7 kg N ha^{-1}, 15 kg P ha^{-1} and 1.8 kg Zn ha^{-1} was banded below the seed. A steel roller trailed the seeder

and provided a level and uniform surface for herbicide application. General insecticide and fungicide application in all experiments followed local agronomic practice for lentil. Hand weeding was employed where needed to remove weed competition and eliminate the use of additional herbicides.

Metribuzin (Mentor®, 750 g kg^{-1}, Farm Oz Pty Ltd, St Leonards, NSW, Australia) at rates of 0, 135, 210 and 285 g a.i. ha^{-1}, representing label post-emergent rates for sand, loam and clay soil types in Australia was applied between the fourth and sixth-node above ground stage of development (V6 to V8; Erskine *et al*. 1990) with a hand-held sprayer at 107 L ha^{-1} using four Air Mix 110-015 low-pressure nozzles on 0.5 m spacing at an operating pressure of 240 kPa (Table 2.2). All herbicide treatments, except for the dawn treatments in the light intensity experiments, occurred between 15:15 and 17:00 Australian Central Standard Time (ACST) to reduce any effect of application timing (Table 2.2).

An automatic weather station was located at each site and relative humidity, temperature and rainfall readings were logged twice hourly. Daily solar exposure (DSE) data was accessed through the Australian Bureau of Meteorology (Bureau of Meteorology 2013). The nearest recording sites were Agery station for Arthurton, (4 km west of site), Owen station for Pinery (8 km east of site) and Saddleworth station for Riverton (5 km north of site). Soil samples for soil moisture estimates were taken at the time of each herbicide application by sampling soil across the sites in a W pattern at depths of 0 to 2, 2 to 10 and 10 to 30 cm. All samples were weighed upon collection and then oven dried at 80°C for 72 hours (h) prior to re-weighing.

Plant injury scores were recorded on a plot basis at 14 and 42 days after treatment (DAT) by estimating the percentage of necrotic tissue in the plot. Lentil plant density was assessed 42 DAT by counting the number of alive plants in four 1 m sections of the internal rows at two locations in each plot. Plant dry weight (DW) was estimated when plants in the nil metribuzin control were at the anthesis stage. Cuts of 0.5 m by the four internal rows were taken at two locations in each plot, the two sub-samples were combined and oven-dried at 80°C for 48 h. Grain yield was estimated by harvesting the lentils with a small plot harvester at maturity.

Sowing date experiments

Sowing date experiments were set up at two locations in 2011 and three in 2012 (Table 2.1) and arranged in split-plot, randomized complete block designs with four replicates. Sowing date was the main plot for logistic management reasons, and herbicide rate the subplot. Buffer strips were sown between all sowing date treatments at each sowing date to prevent spray drift and shading effects from earlier sown plots onto later sown plots. There were three sowing dates at Pinery and two at Arthurton and Riverton, with three to four weeks separation between treatments (Table 2.1). The Pinery site is located in a region considered less reliable for lentil production due to light textured soils and low growing season rainfall, therefore an additional early sowing date, close to the season break, was included. To reduce excessive plant growth production and related disease issues the first sowing date at Arthurton and Riverton occurred about three to four weeks after the opening break of the season.

Light intensities

Light intensity experiments were sown at Arthurton and Pinery in 2011 and 2012. In 2011 three treatments varying the amount of light available to lentil prior to post-emergent metribuzin application were applied. These were spraying at dawn (approximately 07:30 ACST), spraying at dusk (approximately 16:30 ACST) and spraying at dusk following 72 h of induced shade (Table 2.2). An additional treatment of spraying at dusk prior to 72 h of induced shade was included in the 2012 experiment since an effect of the dusk pre-shade treatment was observed in 2011. Shade was imposed with 75% polypropylene shade cloth supported 0.6 m above the ground by a wire frame for either 72 h immediately prior or after metribuzin treatment as per Pritchard and Warren (1980). The experimental design was a split-plot, randomized complete block with four replicates. Light intensity was the main plot due to logistic management reasons associated with the induced shade treatment and herbicide rate the subplot.

Indoor temperature experiment

Experiments to investigate the impact of ambient temperature for 72 h following post-emergent metribuzin treatment in lentil were located in controlled environment growth room (CER) facilities at the Waite Research Precinct, Urrbrae, South Australia. The experimental design was a split-plot, randomized complete block. Two temperature treatments, of either 20/10 or 20/4°C day/night for the 72 h immediately post-treatment with metribuzin, were the main plot and metribuzin rate the subplot. The temperature regimes were chosen to represent average and cold overnight conditions typically experienced in southern Australia during early winter. Metribuzin application rates were 0, 9.4, 18.8, 37.5, 75, 150 g a.i. ha^{-1}. Treatments were replicated six times and the experiment was repeated in time. Pots, 8 cm x 10 cm x 8 cm were filled with steam sterilized coarse sand [Waikerie sand (> than 90% sand) from Berri, South Australia] and suspended in customised racks to avoid contamination from herbicide solution leaching out of adjacent pots.

Four seeds per pot of PBA Flash were sown at a depth of 2 cm. Pots were watered to field capacity and placed in the main CER with conditions of 14/10 h day/night 20/10°C day/night temperatures, light intensity of 1,100 μmol m^2 s^{-1} and RH maintained at 90%. Seeds for each temperature treatment were sown three days apart to allow plants to be moved consecutively post-spraying to a separate small CER for application of the temperature treatment. Pots were watered with 25 ml of 25% Hoagland's nutrient solution thrice weekly. Seven days after sowing (DAS) seedlings were thinned to two uniform seedlings per pot. At either 13 or 14 DAS, all pots were watered with 25 ml of 25% Hoagland's nutrient solution and were treated with metribuzin using a laboratory track applicator with a twin nozzle (110° flat fan) moving boom situated 40 cm above the top of the plants and delivering 103 L ha^{-1} at 1 m s^{-1} and 250 kPa. Immediately following herbicide application plants were moved to the small CER for the post-spraying temperature treatment before being returned to the main CER after 72 h. Conditions of the small CER were set identical to that of the main CER except for night temperatures of the 20/4°C treatment. All pots received 25 ml of water

post-herbicide treatment, and then received thrice weekly waterings with 25 ml of 25% Hoagland's nutrient solution. Above ground plant material was harvested at 14 days after herbicide treatment. Plants were combined within experimental units, dried at 80°C for 48 h in a laboratory oven and DW determined.

Data analysis

All data were initially analysed using linear mixed models conducted in the R environment (R Core Team, 2014) using the ASReml-R software (ASReml. Release 4.1. VSN International Ltd 2014) (Butler *et al*. 2009). There was reasonable treatment concurrence between sites for all field experiments, and data was combined across sites for analysis. To examine the effect of sowing date on metribuzin rate, each site by year (site_year) was considered a separate environment and a multi-environment trial analysis undertaken for all plant damage variables measured. Raw data for plant injury and DW in the sowing date experiment underwent square root transformations to meet model assumptions. The model was composed of a treatment (sowing date) and linear rate response to metribuzin (LRATE) for each environment, a random rate by environment interaction term to model the effect of the rate in each environment and random model terms to account for the two-way blocking structure. Additional site specific extraneous fixed and random terms were included in the analysis as required. The residual errors for each site were modelled using spatial methods. The method of residual maximum likelihood (REML) was used for variance parameter estimation.

Multiple linear regression with groups using GenStat 14.1 (VSN International Ltd.) was used to estimate the response of each environment to metribuzin rate for all variables. Slopes were compared to the environment with the lowest estimate for each variable using the reference level function in GenStat. The association between environments and their soil and weather measurements at the time of herbicide application (Table 2.3) was explored through Principal Component Analysis (PCA) using the FactoMineR package (Lê *et al*. 2008) in the R environment. The PCA was based on a Pearsons correlation matrix and the biplot was constructed from the two principal components which explained the largest percentages of variance. The relationship between variates and herbicide

rate in the light intensity experiment was examined using linear regression in GraphPad PRISM Version 7.03.

Initial analysis of plant DW data from both CER temperature experimental runs found no effect of run, and data was pooled prior to further analysis with non-linear regression using the Dose Response Curve (DRC) package in the statistical analysis package R (R Development Core Team, 2014). Estimates of growth reduction (GR_{50}), the effective dose of metribuzin required to reduce the growth of the dependent variable by 50%, were obtained from all the models and the selective index (SI) command of the DRC package was used to compare the relative differences of the GR_{50} values of the temperature treatments.

Results

Sowing date experiments

Analysis of metribuzin plant injury at 14 and 42 DAT, plant density, anthesis DW and grain yield data identified significant ($P \leq 0.001$) three-way interactions between site_year, sowing date and metribuzin rate. Therefore, each sowing date within sites and years was considered as a separate environment in order to understand the soil and weather conditions that promote metribuzin plant damage in lentil. Regression analysis of all five variables showed clear differences in the parameter estimates *b* (slope) of PBA Flash to post-emergent metribuzin applications across environments (Table 2.4).

Plant injury (14 DAT) showed poor relationships with the other four variables (data not presented). However, linear responses to herbicide rate occurred at all environments with estimates ranging from low levels at Pinery12_2 (0.0089) to higher levels at Arthurton12_1 (0.0231), Pinery12_1 (0.0207), Arthurton11_1 (0.0207) and Riverton12_2 (0.0022).

A linear response to herbicide rate also occurred at all 12 environments for plant injury (42 DAT) and at the majority of environments for the other three measures of plant damage. Strong relationships between these four variables occurred with plant injury (42 DAT) negatively correlating

with plant density (-0.81, $P \leq 0.01$), anthesis DW (-0.77, $P \leq 0.01$) and grain yield (-0.85, $P \leq 0.001$). Environments consistently showing high parameter estimates and a strong linear relationship with herbicide rate across all of these variables were Pinery12_1, Pinery11_3, Pinery11_2 and Arthurton12_1. These sites had parameter estimates which were always significantly greater than the environment with the lowest parameter estimate for each measure of plant damage (Table 2.4). Conversely, environments consistently exhibiting low parameter estimates and a poor relationship with herbicide rate were Riverton12_1, Pinery12_3, Arthurton11_2, Arthurton12_2 and to a lesser extent Pinery12_2. The environments of Arthurton11_1, Pinery 11_1 and Riverton12_2 showed differing responses across the variables suggesting a moderate level of herbicide induced plant damage and possible plant recovery during the growing season.

Site mean grain yields across all herbicide rates and sowing dates were lower at Pinery (1.67 t ha^{-1} 2011, 1.02 t ha^{-1} 2012) than Arthurton (3.89 t ha^{-1} 2011, 2.4 t ha^{-1} 2012) and Riverton (2.89 t ha^{-1} 2012). This result reflected both the harsher growing environment of Pinery and the increased susceptibility of light textured soils to metribuzin leaching and subsequent plant damage.

Soil and weather conditions around the time of post-emergent herbicide application

Soil moisture and weather measurements from each environment are presented in Table 2.3. Daily minimum and maximum temperatures were averaged for the seven days, and DSE for the three days pre- and post-metribuzin application. The relative humidity data was averaged for the 2 h immediately post-metribuzin application and rainfall amount was totaled for the 10 days post.

The outputs of the PCA of environment by the soil and weather variables measured around time of post-emergent metribuzin application to PBA Flash are displayed in the biplot (Fig. 2.1). Principal component scores of the first two dimensions (Dim) explained 68% of the variability across the 12 environments. Component Dim1 had significant loadings for the three soil moisture variables [0 to 2 cm (0.91, $P \leq 0.001$), 2 to 10 cm (0.95, $P \leq 0.001$) and 10 to 30 cm (0.95, $P \leq 0.001$)], soil type (0.8, $P \leq 0.01$), RH (0.77, $P \leq 0.01$) and seven day ambient temperature prior to herbicide application

(-0.59, $P \leq 0.05$). Component Dim2 was driven positively by the variables of 10 day post-application rainfall (0.95, $P \leq 0.001$) and negatively by three day post-application DSE (-0.74, $P \leq 0.01$).

Environments of Pinery 12_3, Arthurton11_1 and _2 clustered together and along with Riverton12_1 and Pinery11_1 had relatively high values of pre- and post-application DSE, but low levels of post-application rainfall. These environments generally had lower levels of parameter estimates for the measures of herbicide damage (Table 2.4). The two Riverton environments positioned closely together and were characterised by high and similar levels of soil moisture, soil texture and RH but lower temperatures pre- and post-application. These two environments were directly opposite the highly damaged Pinery11_2 and _3 environments, which clustered tightly and had low values for the soil characteristics and RH. Pinery11_1, which had lower plant damage levels than the other two Pinery11 environments, also had low values for the soil characteristics. However, it differed through having higher loadings for DSE but a lower loading for post-application rainfall.

The highly damaging Arthurton12_1 environment displayed as an outlier on the biplot and incurred very high levels of post-application rainfall, including 36 mm in one rainfall event within 72 h of herbicide application, and very low levels of post-application DSE. It also had a high loading for pre-application temperature. The Pinery 12_1 environment also had high levels of parameter estimates for plant damage, relatively high loadings for post-application rainfall and post-DSE, but also relatively high values for soil moisture and RH. Of the remaining environments, Arthurton12_2 was on the Dim2 origin and close to the Dim1 origin suggesting it represented average values for all variables, and Pinery12_2 was on the origin for Dim1 with average values for the soil characteristics and RH but a slightly higher value for post-application rainfall and lower loading for post-DSE. Both environments had relatively low levels of parameter estimates for the measures of metribuzin induced plant damage.

Shade experiments

The three-way interaction between environment (site_year), treatment and rate was significant for plant injury (14 DAT) and grain yield. Metribuzin plant injury (14 DAT) increased linearly with

herbicide rate at all environments (Table 2.5). The highest mean parameter estimates for plant injury (14 DAT) were at Pinery 2011 (0.029) and the lowest at Arthurton 2011 (0.018). Despite significant difference between shade and application timing treatments occurring across years and sites, there was no significant difference in the slope of treatments within any site_year. However, at all sites the pre- and post-shade treatments had higher values for *b* slope than the dusk treatment (Table 2.5).

The mean grain yields were 4.1 t ha^{-1} Arthurton 2011, 1.6 t ha^{-1} Pinery 2011, 3.2 t ha^{-1} Arthurton 2012 and 1.3 t ha^{-1} Pinery 2012. Grain yield was less responsive to rate of metribuzin than plant injury (14 DAT). Near-zero values for *b* slope and low regression coefficient values (R^2 = 0.29 to 0.69) at Arthurton 2012 indicated a poor relationship between herbicide rate and grain yield at this environment. Despite slightly higher parameter estimates for *b* slope at the other environments there was no significant difference between the slopes of individual shade or time of day treatments. However, the pre- and post-shade treatment at Pinery 2012 and to lesser extent the pre-shade treatment at Arthurton 2011, had higher parameter estimates for *b* slope than their respective dusk treatments (Table 2.5).

A significant interaction between treatment and herbicide rate occurred for plant density and anthesis DW in 2012 but there was no effect of treatment in 2011. In 2012, plant density estimates had a linear response for the dusk and dawn treatments but a curvilinear relationship for the pre- and post-shade treatments with metribuzin rates (Fig. 2.2A). At the highest herbicide application rate, mean plant density estimates were 40 and 36% lower than those at the nil rates for the pre- and post-shade treatments respectively, compared to a non-significant response in the dawn and dusk treatments. Plant anthesis DW was reduced linearly with herbicide rate in 2012 (Fig. 2.2B) and parameter estimates for *b* slope were greater in the pre- and post-shade treatments than for the dusk treatment.

Indoor temperature experiment

Logistic dose response curves were fitted for plant DW to explain the response of lentil to metribuzin rate under two temperature regimes post-herbicide application. A four-parameter log-logistic model

best explained the relationship between metribuzin rate and plant DW and the model converged in DRC with a non-significant lack of fit test achieved (0.98). Large reductions in plant DW to post-emergent metribuzin herbicide occurred at both temperature regimes (Fig. 2.3), however, there was no significant difference in GR_{50} estimates between lentil plants exposed to the 10°C (26 g ha^{-1}) or 4°C (28 g ha^{-1}) treatment.

Discussion

Despite acceptance in a number of countries that post-emergent metribuzin application in lentil can lead to crop phytotoxicity and subsequent grain yield reductions, few reports quantify the yield loss under field conditions. In field studies where metribuzin has been applied as a standalone post-emergent treatment, interference from uncontrolled weeds has complicated yield loss assessments (Friesen and Wall 1986; Fedoruk and Shirtliffe 2011). However, yield losses of 31% from a single metribuzin application of 210 g ha^{-1} and up to 47% from two split applications of 140 g ha^{-1} were reported in Canada (Wall and McMullan 1994). The current study highlights the susceptibility of lentil, as represented by cultivar PBA Flash, to post-emergent applications of metribuzin across soil types and seasons in southern Australia. Based on the linear regression estimates in Table 2.4, average plant injury (% necrosis) at 42 DAT across all environments was 11% at the lowest label rate (135 g ha^{-1}) and 43% at the highest label rate (285 g ha^{-1}). Grain yield reductions averaged 11% (135 g ha^{-1}) and 23% (285 g ha^{-1}) across all environments, but ranged from 0 to 32% and 0 to 67% respectively, similar to those reported in the Canadian study. This suggests that a higher level of tolerance to metribuzin is required for this herbicide to be used safely and effectively post-emergent on lentil.

Plant uptake of metribuzin occurs through both the foliage and roots (Hatzios and Penner 1988). Once metribuzin has reached the plant roots and saturation of the root adsorptive sites occur, uptake and translocation is rapid and directly proportional to the rate of transpiration (Jensen 1982). Symptoms of metribuzin phytotoxicity from foliar applications typically express themselves 7 to 30 days after application, depending upon conditions. Despite a significant relationship between

herbicide rate and plant injury (14 and 42 DAT) at all environments, only the 42 DAT plant injury measurement correlated with plant density, anthesis DW and grain yield estimates. The environments of Arthurton12_1 and Pinery12_1 incurred the highest parameter estimate for plant injury (14 DAT) and also had relatively high levels of damage for the four other variables measured. However, similar levels of plant injury (14 DAT) occurred at Arthurton11_1, but only low to moderate parameter estimates for the other measures of plant damage. A favourable growing season occurred at Arthurton in 2011 and likely aided surviving plants to recover from initial plant damage.

Conversely, the three Pinery11 environments all had low parameter estimates for 14 DAT plant injury, but moderate (Pinery11_1) and very high (Pinery11_2 and _3) for all other variables. These environments were characterised by light textured soils low in both organic carbon and moisture. The mobility of metribuzin in soil is inversely correlated with organic matter, clay content and increasing pH but highly correlated with soil water content (Sharom and Stephenson 1976; Savage 1976; Ladlie *et al*. 1976; Peter and Weber 1985). Herbicide application at the Pinery11_2 and _3 environments was followed by rainfall totals of 24 and 26 mm respectively, over the next 10 days compared with just 5 mm at Pinery11_1. It is likely that the higher rainfall would have increased herbicide leaching and led to the increased levels of plant damage at Pinery11_2 and _3 as suggested by Gill and Bowran (1990) and Muehlbauer *et al*. (1995). At these environments early estimates of herbicide damage were poor indicators of plant tolerance to metribuzin. These results suggest that the level of metribuzin induced plant damage, and the time taken for its expression, will vary across environments and soil types in lentil in southern Australia. Early and late assessments of plant damage from post-emergent metribuzin applications will be required by plant breeders interested in developing lentil cultivars with improved herbicide tolerance.

All environments with high levels of metribuzin damage failed to group together in the PCA of soil and weather factors. This result suggests that the conditions associated with post-emergent metribuzin damage in field grown lentil in southern Australia were complex and, in most cases, likely to be due to a combination of factors. The light textured soils at Pinery, and in particular those low

in soil moisture at Pinery11, were more prone to metribuzin induced plant damage than the heavier textured soils of Arthurton and Riverton. However, some of the highest levels of metribuzin induced plant damage measures occurred at Arthurton12_1 and Riverton12_2, suggesting other factors were important. Furthermore, Pinery12_3 (low damage measures) contrasted with Pinery12_1 (high), Riverton12_1 (low) with Riverton12_2 (moderate) and Arthurton12_2 (low) with Arthurton12_1 (high) despite these paired environments being sown on the same soil type in the same year. In these cases, the environments with higher plant damage parameter estimates had higher post-application rainfall (Arthurton12_1, Pinery12_1), lower post-DSE (all three), higher RH (Pinery12_1), higher pre-application temperatures (Riverton12_2, Arthurton12_1) and lower post-application temperatures (Riverton12_2) when compared directly with their partner environment. These findings support the suggestion that a combination of weather and soil factors are responsible for post-emergent metribuzin damage in lentil. Furthermore, they, along with the results from Pinery11_2 and 11_3 highlight the major role heavy rainfall events within 10 days of application have in determining the extent of this damage.

The majority of Australian lentil production occurs on free draining soils, sandy loam to clay loam in texture and neutral to alkaline in pH, primarily to avoid waterlogging damage (Materne *et al*. 2002). Therefore, the favoured soils for lentil production have a relatively high risk for metribuzin herbicide leaching. This is further exacerbated by seeding occurring after the hot and dry summer period, when soil moisture levels are low, but prior to the wet winter period. This contrasts with North America where lentil is sown in late spring on stored soil moisture after winter rain and/or snow melt (Muehlbauer *et al*. 1995; Materne and Siddique 2009).

Previous research from pot and field studies of crops in North America suggested that weather factors outside of rainfall are important. Increases in RH from 40 to 100% in barley and 58 to 80% in tomato resulted in higher levels of metribuzin-induced plant damage in indoor pot studies (Fortino and Splittstoesser 1974; Caldwell and O'Sullivan 1985). The latter suggested that an environment with higher moisture content would increase leaf absorption of metribuzin through retaining the

chemical in solution on the leaf surface longer, potentially explaining the high level of plant damage incurred at the Pinery12_1 environment.

Increases in day/night growing temperatures from 20/15 to 25/20 and 30/25°C increased metribuzin induced plant damage in pot studies of field pea (Al-Khatib *et al*. 1997) and a similar response was observed in tomato (Fortino and Splittstoesser 1974). Increased growing temperatures (10, 15 or 20°C) for three days prior to herbicide treatment increased root absorption of metribuzin in a hydroponic study involving wheat (Buman *et al*. 1992). In all of these reports, the upper temperature ranges would be higher than those experienced in early winter in southern Australia. Pre-application temperature was negatively correlated with Dim1 in the PCA analysis and generally environments with low relative levels of plant damage had lower average temperatures pre-application. The reason for this apparent temperature response is unclear but may be related to increased plant growth rates or altered leaf morphological characteristics which increased herbicide absorption rates and translocation to shoots under warmer growing conditions (Buman *et al*. 1992; Riethmuller-Haage *et al*. 2007).

Cold temperatures, including frosts, in post-metribuzin application have been associated with higher levels of metribuzin-induced plant damage in barley and wheat. Frosts within three days of application are linked to herbicide damage in lentil in Canada (Caldwell and O'Sullivan 1985; Schroeder *et al*, 1985; SMA 2014). Lower temperature post-spraying was not strongly correlated with environments with high levels of plant damage in lentil in this research. The only environment to incur frosts within three days of application was Pinery12_3, however, only low levels of plant damage were measured. Riverton12_2 and Pinery12_1 had the lowest average minimum temperatures in the seven days post-spraying and incurred moderate and high levels of plant damage, however, similar temperatures occurred at Pinery12_3. In the former two environments it appears likely that a combination of more than one adverse factor, such as high RH at Pinery12_2 and low post-DSE at Riverton12_2, was responsible for the high level of plant damage. This suggestion is also supported by the results of the CER experiment where there was a lack of a response in lentil to

lower overnight minimum temperatures following post-emergent metribuzin herbicide application. Further research is required to understand if frost events are linked to plant damage from post-emergent metribuzin applications in lentil in southern Australia.

A number of reports indicate that reduced light intensities increase post-emergent metribuzin related plant damage in a range of crop species. Field experiments with tomato found that artificial shading of plants for up to three days before treatment with post-emergent metribuzin increased plant damage (Pritchard and Warren 1980). Similar observations were reported in growth room studies with a tolerant barley cultivar that had been exposed to 8 h of darkness compared with 8 h of light following post-emergent application of metribuzin (Caldwell and O'Sullivan 1985). However, the authors reported no effect to a sensitive cultivar. In this current study, a number of the environments with the lowest levels of plant damage had high relative levels of post-application DSE. In contrast, moderate to high levels of plant damage occurred at Pinery12_1, Pinery12_2 and Arthurton12_1, which had the lowest levels of post-application DSE, and support the findings in the tolerant barley cultivar. However, during winter in southern Australia low levels of DSE are likely to occur with rainfall events, conversely higher levels will occur during dry periods, potentially suggesting that the correlation between DSE and metribuzin related plant damage could be in part due to rainfall. Furthermore, there was only relatively low and inconsistent levels of plant damage and grain yield reduction in the shaded treatments of the light intensity experiment, when the factor of rainfall was removed.

Some evidence in this research suggested that weather characteristics of reduced light intensities, increased RH and changes in ambient temperature may have a role in post-emergent metribuzin induced plant damage in lentil in the field in southern Australia. However, it is clear that rainfall events within 10 days of metribuzin application, particularly when on light textured soils or where soil moisture is low, is a major determinant of the extent of plant damage. Finally, a higher level of selective plant tolerance to metribuzin than that which currently exists in commercial cultivars is required in Australian lentils to allow a post-emergent application of this herbicide.

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Table 2.1. Details of sites and trial management of the lentil post-emergent metribuzin spray application experiments conducted in 2011 and 2012 at three field sites in South Australia

		Pinery		Arthurton		Riverton
		2011	2012	2011	2012	2012
Soil type[A]		Lithocalcic Calcaresol		Hypercalcic Calcaresol		Red Chromosol
Soil texture		Light sandy loam/sandy clay loam		Clay loam/light clay		Clay loam/medium heavy clay
pH ($CaCl_2$)	0-10cm	7.9	7.8	7.3	6.6	7.1
	10-60cm	8.3	8.2	7.6	7.6	7.7
Organic carbon (%)	0-10cm	1.43	1.64	1.84	1.74	2.5
	10-60cm	0.32	0.39	0.96	1.25	-
Rainfall (mm)	Annual	433.3	325.7	484.6	328.4	417.8
	Apr.-Oct.	250.6	232.9	282.2	254.4	305.6
Sow date (environment)	_1	6 May	3 May	17 May	15 May	10 June
	_2	27 May	28 May	14 June	15 June	27 June
	_3	24 June	26 June			
	Light intensity	24 June	28 May	4 June	7 June	
Basal fertiliser		7 kg N ha^{-1}, 15 kg P ha^{-1}, 1.8 kg Zn ha^{-1}				

[A](Isbell 2016)

Table 2.2. Details of the post-emergent metribuzin spray application timing, conditions and crop growth stage of the lentil sowing date and light intensity experiments conducted in 2011 and 2012 at three field sites in South Australia

Year	Site	Environment	Application date	Application time	Cloud cover (%)	Soil surface wetness	Leaf wetness	Lentil growth stage (nodes)	Time to 5 node (days)
2011	Pinery	11_1	6 June	15:30	100	Dry	Dry	5	31
		11_2	30 June	16:45	90	Dry	Dry	5	34
		11_3	2 August	17:00	5	Dry	Dry	5-6	39
		Dawn	8 August	7:45	75	Wet	Wet	6	39
		Dusk/Pre-shade	8 August	16:45/17:00	60	Dry	Dry		
	Arthurton	11_1	22 June	15:30	100	Dry	Moist	5	36
		11_2	26 July	16:30	10	Dry	Dry	5-6	39
		Dawn	28 July	7:30	0	Dry	Moist	5-6	39
		Dusk/Pre-shade	28 July	16:30/17:00	20	Dry	Dry		
2012	Pinery	12_1	1 June	15:30	100	Dry	Dry	5-6	27
		12_2	27 June	16:40	5	Moist	Dry	4-5	32
		12_3	31 July	16:50	30	Dry	Dry	5	36
		Dawn	2 July	7:40	95	Moist	Dry	5-6	32
		Dusk & Post-shade/Pre-shade	2 July	15:20/16:20	50	Dry	Dry		
	Arthurton	12_1	18 June	15:30	5	Dry	Dry	5	34
		12_2	20 July	16:40	5	Dry	Dry	4-5	37
		Dawn	16 July	7:15	95	Wet	Wet	5-6	36
		Dusk & Post-shade/Pre-shade	16 July	15:15/16:20	0	Dry	Dry		
	Riverton	12_1	27 June	16:50	80	Dry	Dry	5	42
		12_2	9 August	16:55	20	Dry	Dry	5	48

Table 2.3. Soil type rating, soil moisture and weather factors around the time of spraying post-emergent metribuzin on PBA Flash lentil across two years, three field sites and multiple sowing dates (environment) in South Australia

Year	Site	Environment	Soil type[A]	Soil moisture (%)			Daily solar exposure (MJ m^{-2} d^{-2})		Temperature (^{O}C)		RH (%)	Rainfall (mm)
				0-2 cm	2-10 cm	10-20 cm	Avg. 3 day pre	Avg. 3 day post	Avg. of daily min 7 day pre	Avg. of daily min 7 day post	Avg. 2 h post	10 day post
2011	Arthurton	11_1	2	25.6	21.9	19.4	3.0	3.1	6.6	6.6	85.2	7.1
		11_2	2	17.5	19.4	21.8	2.7	2.8	4.1	6.7	65.9	21.2
	Pinery	11_1	1	11.2	11.5	12.2	3.1	3.0	5.9	6.3	64.1	4.5
		11_2	1	7.7	13.0	14.4	2.9	2.6	7.1	7.8	44.9	24.1
		11_3	1	2.9	9.5	11.7	3.5	2.3	6.5	4.9	52.9	25.7
2012	Arthurton	12_1	2	15.8	15.7	19.6	2.2	1.8	7.8	6.1	79.3	42.8
		12_2	2	15.6	16.9	19.8	2.4	2.6	5.4	6.8	81.2	15.7
	Pinery	12_1	1	21.5	19.0	20.8	2.3	2.3	6.3	4.1	90.7	9.6
		12_2	1	16.8	18.8	21.9	2.3	2.2	6.1	4.9	68.9	11.8
		12_3	1	6.9	20.2	19.0	2.5	2.9	5.5	4.5	72.0	1.4
	Riverton	12_1	3	28.2	30.0	28.2	2.5	2.8	3.3	5.8	79.5	17.1
		12_2	3	31.5	32.1	24.5	2.8	2.6	5.5	3.1	82.3	20.1

[A]Soil type category based on soil type, soil texture, pH and organic carbon status (Table 2.1)

Table 2.4. Estimates of regression parameter *b* (slope ± SE) for plant injury, plant density, anthesis dry weight and grain yield of PBA Flash lentil treated with metribuzin herbicide (0, 135, 210 and 285 g a.i. ha $^{-1}$) across 12 field environments in South Australia, 2011 and 2012

Environment	Plant injury (14 DAT)		Plant injury (42 DAT)		Plant density		Anthesis DM		Grain yield	
	b	*P*-value	*b*	*P*-value	*b*	*P*-value	*b*	*P*-value	*b*	*P*-value
Arth11_1	0.02066 ± 0.0022***	<.001	0.01816 ± 0.0034	<.001	-0.1077 ± 0.0328*	0.003	-0.00202 ± 0.0003**	<.001	-0.000772 ± 0.0005	0.099
Arth11-2	0.0173 ± 0.0022*	<.001	**0.01515 ± 0.0034**	<.001	-0.072 ± 0.0328	0.038	-0.001387 ± 0.0003	<.001	-0.001495 ± 0.0005	0.003
Pine11_1	0.00984 ± 0.0022	<.001	0.02282 ± 0.0034	<.001	-0.1797 ± 0.0328***	<.001	-0.002156 ± 0.0003***	<.001	-0.001703 ± 0.0005*	<.001
Pine11_2	0.01195 ± 0.0022	<.001	0.02559 ± 0.0034*	<.001	-0.2586 ± 0.0328***	<.001	-0.001886 ± 0.0003**	<.001	-0.003314 ± 0.0005***	<.001
Pine11_3	0.01719 ± 0.0022*	<.001	0.02505 ± 0.0034*	<.001	-0.2648 ± 0.0328***	<.001	-0.002358 ± 0.0003***	<.001	-0.003977 ± 0.0005***	<.001
Arth12_1	0.02309 ± 0.0022***	<.001	0.02639 ± 0.0034*	<.001	-0.1501 ± 0.0328**	<.001	-0.002516 ± 0.0003***	<.001	-0.003386 ± 0.0005***	<.001
Arth12_2	0.01676 ± 0.0022*	<.001	0.01971 ± 0.0034	<.001	-0.0696 ± 0.0328	0.044	-0.000877 ± 0.0003	0.009	-0.000466 ± 0.0005	0.311
Pine12_1	0.02073 ± 0.0022***	<.001	0.02709 ± 0.0034*	<.001	-0.2373 ± 0.0328***	<.001	-0.002606 ± 0.0003***	<.001	-0.004155 ± 0.0005***	<.001
Pine12_2	**0.0089 ± 0.0022**	<.001	0.02059 ± 0.0034	<.001	-0.0932 ± 0.0328*	0.009	-0.001108 ± 0.0003	0.001	-0.001246 ± 0.0005	0.011
Pine12_3	0.01681 ± 0.0022*	<.001	0.01641 ± 0.0034	<.001	-0.0234 ± 0.0328	0.483	**-0.000566 ± 0.0003**	0.078	**-0.00034 ± 0.0005**	0.457
Riv12_1	0.01218 ± 0.0022	<.001	0.01619 ± 0.0034	<.001	**0.0069 ± 0.0328**	0.835	-0.000825 ± 0.0003	0.013	-0.001091 ± 0.0005	0.023
Riv12_2	0.01916 ± 0.0022**	<.001	0.02293 ± 0.0034	<.001	-0.0459 ± 0.0328	0.174	-0.001372 ± 0.0003	<.001	-0.003682 ± 0.0005***	<.001

*$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$ for significance compared with the environment with the lowest estimate for each parameter (bolded) using reference level function in Genstat

Table 2.5. Estimates of regression parameter *b* (slope ± SE) for plant injury and grain yield of PBA Flash lentil under differing light intensities pre- or post-emergent metribuzin application (0, 135, 210 and 285 g a.i. ha $^{-1}$) at two field sites in South Australia, 2011 and 2012

Year	Site	Light treatment	Plant injury 14 DAT		Grain yield	
			b	R^2	*b*	R^2
2011	Arthurton	Dawn	0.0176 ± 0.0023	0.97	-0.0011 ± 0.0002	0.94
		Dusk	0.0162 ± 0.0027	0.95	-0.0011 ± 0.0003	0.87
		Pre-shade	0.0211 ± 0.0036	0.95	-0.0014 ± 0.0006	0.76
	Pinery	Dawn	0.0282 ± 0.0036	0.97	-0.0035 ± 0.0004	0.98
		Dusk	0.0272 ± 0.0016	0.99	-0.0048 ± 0.0008	0.95
		Pre-shade	0.0304 ± 0.0048	0.95	-0.0041 ± 0.0008	0.93
2012	Arthurton	Dawn	0.0259 ± 0.003	0.97	-0.0006 ± 0.0004	0.51
		Dusk	0.0193 ± 0.0037	0.93	0.0006 ± 0.0006	0.29
		Pre-shade	0.0252 ± 0.0051	0.92	-0.0008 ± 0.0004	0.69
		Post-shade	0.0235 ± 0.0056	0.90	0.0003 ± 0.0002	0.53
	Pinery	Dawn	0.0167 ± 0.0011	0.99	-0.002 ± 0.0004	0.87
		Dusk	0.0166 ± 0.0019	0.97	-0.0017 ± 0.0004	0.91
		Pre-shade	0.0204 ± 0.003	0.96	-0.002 ± 0.0001	1.00
		Post-shade	0.0235 ± 0.0037	0.95	-0.0026 ± 0.0004	0.95

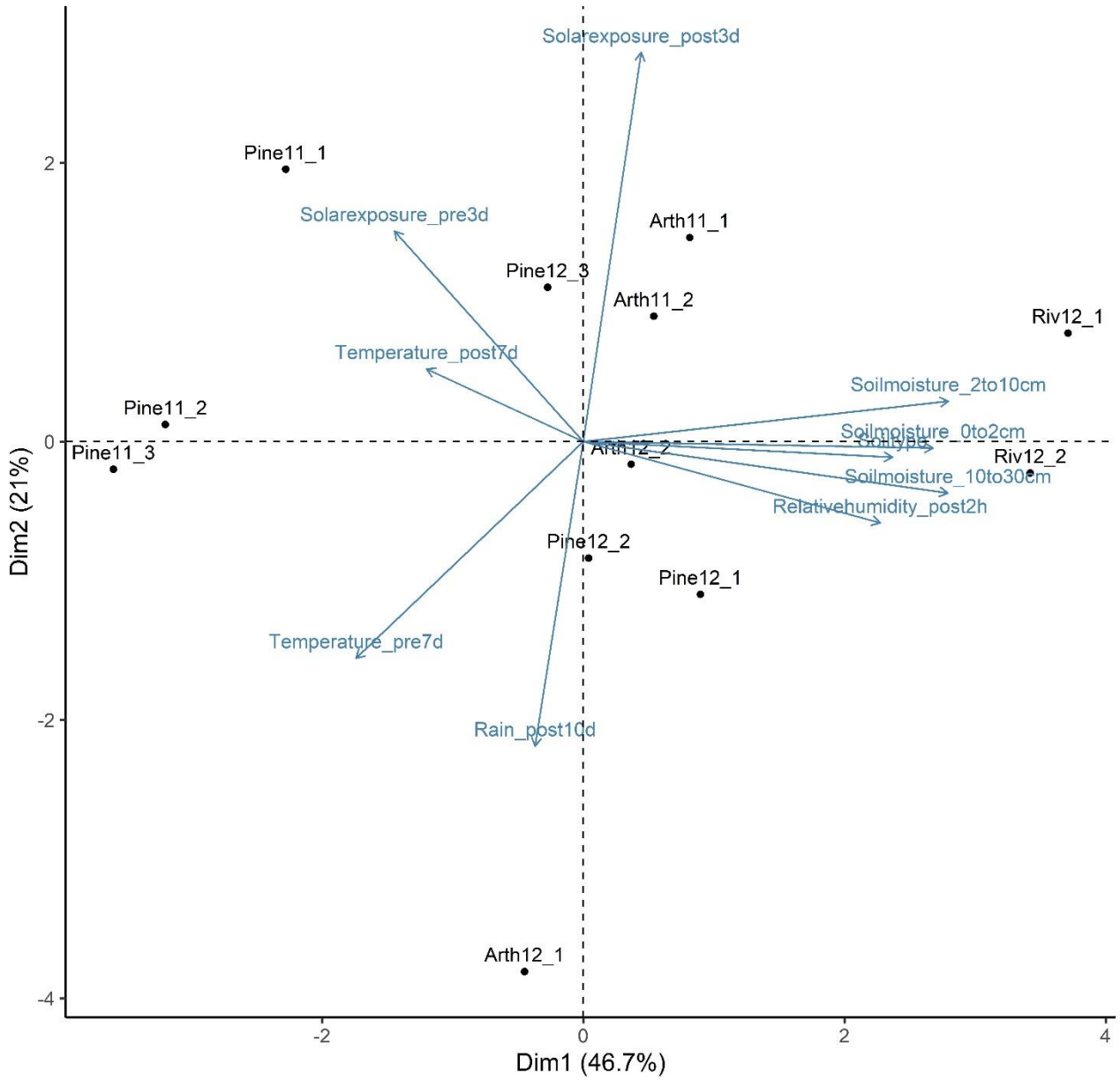

Fig. 2.1. Biplot from the principal component analysis of the association between environments and their soil and weather conditions at the time of post-emergent herbicide application in PBA Flash lentil at three field sites in South Australia, 2011 and 2012.

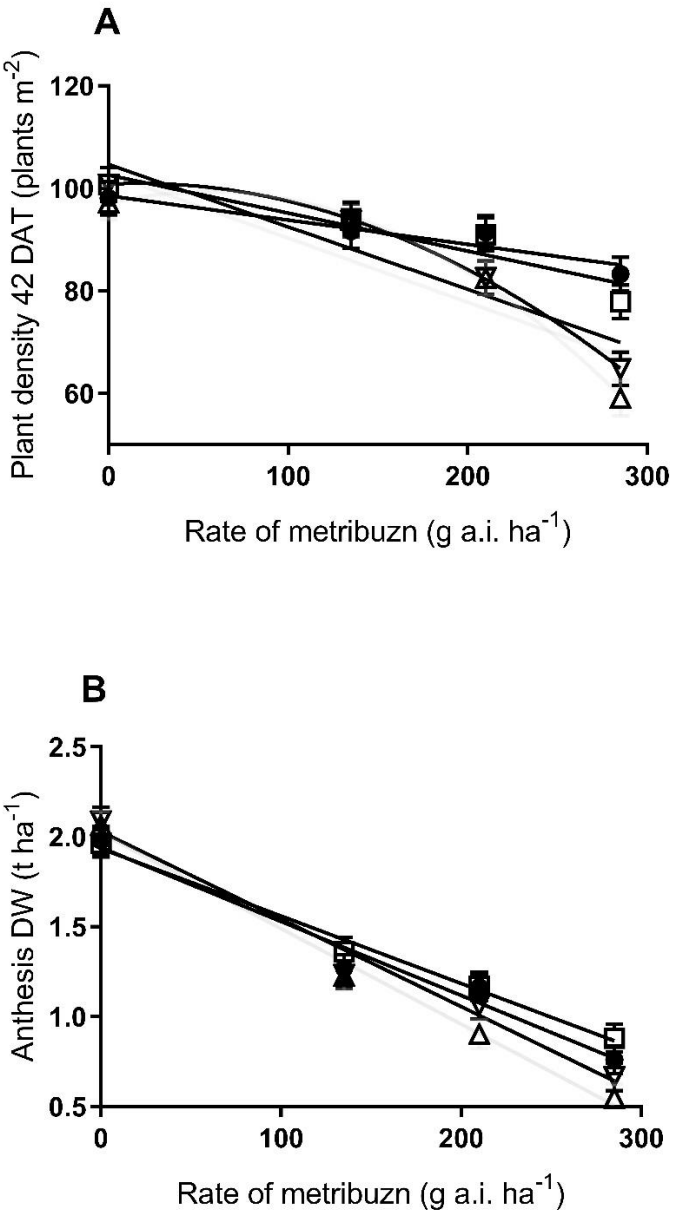

Fig. 2.2. The effect of metribuzin rate on (A) plant density and (B) anthesis DW of PBA Flash lentil under light intensity regimes of dawn (●), dusk (□), post-shade (△) and pre-shade (▽) at the time of post-emergent herbicide application at two field sites in South Australia, 2012. The equations of the lines for plant density are: dawn: Y =98.6 - 0.0473X (R^2 = 0.898; P = NS); dusk: Y = 102.6 - 0.0742X (R^2 = 0.783; P = NS); post-shade: Y = 97.0 - 0.00076X^2 + 0.0848X (R^2 = 0.997; P < 0.001); pre-shade: Y = 100.8 - 0.0005X^2 + 0.0172X (R^2 = 1.00; P < 0.001). The equations of the lines for anthesis DW are: dawn: Y =1.94 - 0.0041X (R^2 = 0.971; P < 0.05); dusk: Y = 1.935 - 0.0038X (R^2 = 0.99; P < 0.01); post-shade: Y = 2.021 - 0.0053X (R^2 = 0.993; P < 0.01); pre-shade: Y =2.03 - 0.0049X (R^2 = 0.975; P < 0.05).

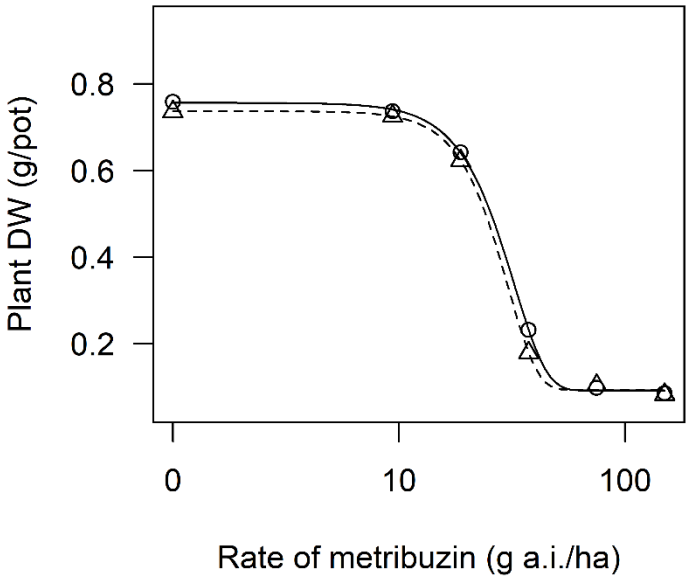

Fig. 2.3. The effect of metribuzin rate on the DW of PBA Flash lentil 14 days after treatment with post-emergent metribuzin herbicide at overnight temperature regimes of 4°C (○) and 10°C (△) imposed for three days immediately post-herbicide treatment in controlled environment conditions.

Chapter 3.

Development of high levels of metribuzin tolerance in lentil.

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Contribution to the Paper	Designed, conducted all research experiments, analysed and interpreted all data, and drafted and constructed the manuscript and was corresponding author.		
Overall percentage (%)	75%		
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.		
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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

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Development of High Levels of Metribuzin Tolerance in Lentil

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Abstract

Lentil (*Lens culinaris* Medik.) is an important and expanding crop in southern Australia and a significant crop in western Canada. Currently, production in both countries is limited by an inability to effectively control weeds, due in part to a lack of registered safe and effective herbicides. Metribuzin is a broad-spectrum herbicide providing an alternative weed control option to the imidazolinones, but it has low crop safety in lentil. Two methods, germplasm screening using a hydroponic sand assay and field screening of a large mutated population of the Australian cultivar 'PBA Flash' were initially used to identify lines with putative metribuzin tolerance over current cultivars. Dose–response experiments showed the germplasm line SP1333 had GR_{50} (the rate required to reduce dry weight 50%) values up to four times higher than PBA Flash. However, the mutation selections M043 and M009 had GR_{50} values more than 25 times higher than PBA Flash. A field study in Canada, under conditions of induced shade and no shade 72 h before POST application of metribuzin, confirmed the intermediate level of tolerance in SP1333 and the high level in the two mutant lines compared with 20 Canadian and Australian genotypes. This relative increase in metribuzin tolerance of the two mutant lines over the parent cultivar is higher than all previous reports in a range of crop species. The development of large mutant populations combined with large M_2 field screens was a successful method for developing high levels of metribuzin tolerance in lentil. The estimated mutation rate of the mutant lines was 9.4×10^{-8}. All three lines are currently being used as parents in lentil breeding programs.

Introduction

Lentil (*Lens culinaris* Medik.) is a food legume grown across a wide range of environments and traditionally consumed where it was grown (McNeil et al. 2007). World production has increased from less than 1,000 million kg in the early 1960s to 4,800 million kg in 2014, with approximately 40% from the developed countries of Canada, Australia, and the United States (FAO 2015). Weed competition is a major limitation to lentil production worldwide due to its slow early growth rate, short stature, and lack of protective canopy development (Knott and Halila 1988; Muehlbauer et al. 1995; Hanson and Thill 2001). Grain yield losses of up to 84% have been attributed to weed competition in lentil (Swanton et al. 1993; Mohamed et al. 1997; Elkoca et al. 2005; McDonald et al. 2007). Chemical control using herbicides is the major method of weed management in lentil in developed countries and is becoming increasingly popular in many developing countries (Brand et al. 2007; Yenish et al. 2009). Until the release of imidazolinone (IMI; acetohydroxyacid synthase [AHAS] inhibitors) herbicide–tolerant lentil cultivars in North America in 2006 (Muehlbauer et al. 2009) and Australia in 2012 (Materne et al. 2011), limited safe and effective in-crop herbicide options were available for controlling broadleaf weeds. However, an overreliance on these herbicides both in lentil and other IMI-tolerant crops grown in rotation such as canola (*Brassica napus* L.), wheat (*Triticum aestivum* L.), and in Australia, barley (*Hordeum vulgare* L.), has resulted in the evolution of IMI-tolerant weeds in these systems (Beckie and Tardif 2012; Boutsalis et al. 2016). For lentil to remain a viable pulse crop in these systems, alternative chemical weed control strategies are required as part of a sustainable integrated weed management system.

Development and registration of new herbicides in minor crops like lentil are likely to be problematic due to the high costs involved relative to the size of the industry (Devine 2005; Duke 2005). An alternative approach is to improve crop tolerance to existing herbicides where a lack of selectivity exists between crop and weeds. Metribuzin is a broad-spectrum herbicide recommended in many countries for controlling a range of grass and broadleaf weeds in lentil. It is an aminotriazinone or asymmetrical triazine (Hatzios and Penner 1988) and a photosystem II inhibitor (Devine et al. 1992). The rates and timing of metribuzin application to lentil vary across countries due to the potential for severe crop phytotoxicity on soils low in organic matter, under continued wet soil conditions, or where application of metribuzin to dry soil is followed by heavy rainfall (Muehlbauer et al. 1995; Yasin et al. 1995; Elkoca et al. 2005). Metribuzin is used as a PRE or POST application in the United States (North Dakota State University 2014) and as a PRE or early-POST application in Canada (Saskatchewan Ministry of Agriculture 2014). In Australia, it is only used PRE, as severe crop phytotoxicity and subsequent yield loss occurs from POST application (White 2015). In all these cases, there are many application regulations surrounding crop safety. These include restrictions around soil type, clod and crop residue level, soil moisture content, ambient temperature, rainfall postapplication, light intensity, crop growth stage, and sowing depth. Furthermore, some recently released Australian lentil cultivars exhibit increased sensitivity to metribuzin when compared with existing cultivars and incur grain yield losses of up to 35% PRE and 52% POST to label application rates in herbicide tolerance response trials (GRDC 2015). The development of lentil germplasm with higher levels of metribuzin tolerance would reduce crop phytotoxicity, improve crop safety, and provide an alternative broadleaf weed control option to the AHAS inhibitors, alleviating herbicide-resistant weed issues.

Some level of genetic variation for tolerance to metribuzin has been identified using various screening methods in many crop species. These include soybean [*Glycine max* (L.) Merr.] (Hardcastle 1974; Mangeot et al. 1979; Barrentine et al. 1982), tomato (*Solanum lycopersicum* L.) (Souza Machado et al. 1978; Gawronski 1983), potato (*Solanum tuberosum* L.) (Ivany 1979; Gawronski et al. 1985), sweetpotato [*Ipomoea batatas* (L.) Lam.] (Harrison et al. 1985; Motsenbocker and Monaco 1993), cowpea [*Vigna unguiculata* (L.) Walp.] (Harrison 1988), field pea (*Pisum sativum* L.) (Al Khatib et al. 1997), barley (Caldwell and O'Sullivan 1985; Gawronski et al. 1987), wheat (Runyan and McNeil 1982; Schroeder et al. 1985; Kleemann and Gill 2007; Bhoite et al. 2017), lentil (McMurray et al. 2009; Meier 2016; Sharma et al. 2017), and narrowleaf lupin (*Lupinus angustifolius* L.) (Si et al. 2006). Generally, only up to a 3-fold increase in metribuzin tolerance between tolerant and susceptible cultivars was identified, and in the case of lentil, no cultivar with an agronomically useful level of tolerance has been reported. A higher level of improved metribuzin tolerance (4- to 6-fold) was also developed through induced mutation and subsequent field selection in the presence of the herbicide in narrowleaf lupin (Si et al. 2009). As genetic variability to metribuzin tolerance in lentil exists, and previous research has identified improved levels in other crop species, agronomically useful levels of tolerance could be developed in lentil. This study aimed to identify and validate lentil germplasm with improved tolerance to metribuzin compared with existing Australian and Canadian cultivars through using both controlled-environment germplasm screening and induced-mutagenesis field-selection approaches.

Materials and Methods

Germplasm Screening

Approximately 750 lentil accessions were obtained from the Australian Grains Genebank and Pulse Breeding Australia (PBA), Horsham, VIC, for comparison of tolerance to metribuzin against the standard cultivar 'PBA Flash.' The PBA lentil genotypes 99-088L*02H037 and 96-047L*99R099, which have the highest and lowest reported tolerance to metribuzin in Australia, respectively (McMurray et al. 2009), were used as controls. Six growth room experiments using randomized block designs and two replicates and consisting of between 71 and 266 accessions each, depending upon availability of growth room space, were conducted using a sand-pot assay derived from DeJong (1983). Eight seeds of each line were scarified with a knife and imbibed in water for a period of 24 h to promote even germination. Pots (4.5-cm diameter, 9-cm depth) were filled with 30 g of prewashed blue metal stone on the bottom followed by 50 g of coarse Waikerie sand (more than 90% sand) and watered to field capacity. Four seeds of each line were sown in each pot at a depth of 2 cm. Pots were randomized and suspended in customized racks to allow free drainage without contamination of solution leaching from the bottom of each pot and placed in a growth room at a day/night temperature setting of 20/5 C with 16-h daylight. Pots were watered with 25% Hoagland's nutrient solution to 100% of water-holding capacity every 2 or 3 d. At 7 d after sowing (DAS), seedlings were thinned to 2 uniform seedlings pot^{-1}. At 12 DAS (2- to 3-aboveground node stage), all pots were watered to 100% of water-holding capacity with deionized water. Immediately following this, all pots were treated with 20 ml of metribuzin (750 g ai kg^{-1}, Mentor®, Farm Oz, St Leonards, NSW, Australia) at 3,900 µg L^{-1}, which allowed the solution to leach out of the bottom of pots. This rate of metribuzin applied as a soil drench was found to repeatedly and reliably lead to high levels of damage in the tolerant control under controlled-environment conditions. Metribuzin solution was applied using a wide-mouthed beaker to rapidly flood the entire soil surface, avoiding contact with leaves but enabling complete saturation of the sand medium. Pots were irrigated with 40 ml of 25% strength Hoagland's solution at 24 h after herbicide treatment to leach all metribuzin from pots. Complete removal of metribuzin from the medium was confirmed through the subsequent growing of susceptible control plants in pots without any herbicide-damage symptoms appearing. Normal watering resumed 3 d after the herbicide flushing, and plants were assessed 12 to 14 d after treatment (DAT) for herbicide injury or plant death. Plant injury was scored as the percentage of necrotic plant tissue, with a score of 90% or greater considered as plant death.

Development of Mutagenized Population

Approximately 670,000 seeds of lentil PBA Flash were mutagenized with ethyl-methanesulfonate (EMS) in 2010. Seeds were first rinsed twice in water to remove dust particles, then soaked for 12 h in reverse osmosis (RO) water inside four sealable 20-L containers and shaken periodically. Seeds were decanted and rinsed in RO water, returned to containers, and soaked in 0.04% EMS solution for a further 12 h in darkness and shaken gently.

Sodium thiocyanate 100 g L^{-1} was then added to seed solution for 30 min before seed was rinsed with water eight times. Mutagenized (M) M_1 seed was sown in a field near Maitland, SA, Australia (34.433°S, 137.632°E) with an experimental small plot cone seeder at a targeted rate of 100 seeds m^{-2} over approximately 0.7 ha. The resulting M_1 plants were bulk harvested at maturity with an experimental small plot harvester. A clean M_2 seed yield of 1,350 kg was achieved and mixed thoroughly using a small commercial cement mixer before being separated into two 600-kg lots and two 60-kg lots. One 60-kg M_2 seed lot was multiplied near Maitland in the 2011 growing season as per the previous year, returning 2,800 kg of cleaned M_3 seed.

Mutagenized Population Screening

Initial mass field and large glasshouse progeny screens were used to identify putative metribuzin-tolerant lines from the large mutagenized population. An estimated 9,500,000 M_2 PBA Flash seeds from one 600-kg retained seed lot were sown in 2011 on a Calcic Calcaresol (Isbell 2016) soil type near Sunnyvale, SA, Australia (34.145°S, 137.797°E) using a field-selection method based on Si et al. (2009). A commercial seeder was used to sow the seed at a targeted rate of 100 viable seeds m^{-2} over approximately 14 ha. Plants were sprayed POST at the 5- to 6-node stage with metribuzin at an aggregated rate of 1,300 g ha^{-1}, greater than six times the recommended PRE rate to achieve a high level of crop phytotoxicity. The herbicide was applied by a commercial self-propelled spray rig in two passes, each at a water application rate of 100 L ha^{-1}, but from opposite directions to improve plant coverage. At 21 DAT, surviving plants were identified and transplanted from the field to pots. Plants were grown through to seed either in a glasshouse at SARDI Field Crop Centre, Clare, SA, Australia (33.835°S, 138.614°E) or a shade house at the Waite Research Precinct, Urrbrae, SA, Australia (34.965°S, 138.634°E). A total of 48 plants set viable seed and were harvested individually and multiplied in a glasshouse over the summer. The field screen was repeated near the 2011 site in 2012 with an estimated 10,000,000 M_3 PBA Flash seeds from the 2011 M_2 multiplication, with 47 M_3 plant selections collected.

A progeny metribuzin screen was conducted in a glasshouse at Waite Research Precinct to allow further identification of putatively tolerant lines. The glasshouse was maintained at day/night temperatures of 25/10 C. Four scarified seeds per pot were sown into 10 pots (10-cm diameter) filled with >90% coarse sand (Waikerie sand) for each line. The 10 pots were arranged into four by three 12-pot trays with two additional pots, located in the center of each tray, sown to PBA Flash as a susceptible check. All pots received 100 ml of water after sowing and then twice weekly. Plants were thinned to 2 uniform seedlings pot^{-1} before POST metribuzin treatment with a single rate of 112.5 g ha^{-1} at 14 DAS at the 4- to 5-node growth stage. This rate of metribuzin resulted in greater than 90% plant injury in PBA Flash when applied postemergence under glasshouse conditions (L McMurray, D Mao, C Preston, J Paull unpublished data). Herbicide was applied using a laboratory track applicator with a twin nozzle (110° flat fan) moving boom situated 30 cm above the top of the plants and delivering 103 L ha^{-1} at 1 m s^{-1} and 250 kPa. Plants were watered immediately before herbicide treatment and 24 h after with 50 ml of water, and then twice-weekly watering with 25% Hoagland's nutrient solution was resumed. Plant injury was recorded on individual plants at 21 DAT as for the germplasm screen. Surviving lines were initially harvested as single plants but later bulked as M_2- and M_3-derived lines.

Dose–Response Experiments: Australia and Canada

Experiments were located in controlled-environment growth room facilities at the Waite Research Precinct and the University of Saskatchewan, Saskatoon, SK, Canada in 2014 to enable comparisons with elite germplasm from both countries. Day length was 14/10-h day/night in both countries; however, other conditions varied. In Australia, temperatures were 20/10 C day/night, light intensity of 1,100 µmol m^{-2} s^{-1}, and relative humidity (RH) was maintained at 90%. In Canada, temperatures were 21/15 C day/night, light intensity was 540 µmol m^{-2} s^{-1}, and RH was 40%. Both experiments were designed as a randomized complete block design with four replicates of six plants in Australia; however, three replicates of two plants each were used in Canada, due to space constraints. The Australian experiment consisted of the four most putatively tolerant mutant lines and the two most putatively tolerant germplasm lines from the progeny and germplasm screens, respectively. PBA Flash was included as the control cultivar. All seeds were scarified and sown into pots and watered as for glasshouse screening, except that a potting mix of 90% composted pine bark and 10% sand was used due to its improved suitability for growing lentils compared with the sand medium under growth room conditions. Metribuzin treatment was as for glasshouse screening, with rates of 0, 142.5, 285, 570, 855, 1,140, 2,280, and 4,560 g ha^{-1} applied. Plant damage (as for germplasm screen) was recorded at 7, 14, and 21 DAT, and all plants were harvested for dry weight (DW) after the final recording. Plant DW samples were combined within experimental units and dried at 80 C for 48 h in a laboratory oven.

The Canadian study followed the procedure used in Australia with the following modifications. The Australian genotypes were reduced to PBA Flash, the two highly tolerant mutants, and SP1333. The cultivar 'CDC Maxim,' which is currently Canada's most widely grown red lentil, and 'CDC Greenstar,' a recently released large-seeded green lentil, were included for comparison. A custom soil mix of 60% Sunshine 3 (Sun Gro Horticulture, Seba Beach, AB, Canada) and 40% coarse sand was used. Pots were watered with 25% Hoagland's nutrient solution from sowing onward. Metribuzin (Sencor®, 750 g ai kg^{-1}, Bayer Crop Science, Calgary, AB, Canada) was applied using a laboratory track applicator with a single even flat spray nozzle (Lechler, St Charles, IL, USA) 8001 EVS delivering 108 L ha^{-1} at 240 kPa. Eight doses were applied at rates of 0, 100, 150, 225, 337, 506, 759, 1,138, 1,707, and 2,560 g ha^{-1} in all genotypes except the two mutant lines, for which the rates of 100, 150, and 225 were replaced with rates of 3,840, 5,761, and 8,641 g ha^{-1}, respectively, based on the Australian dose–response findings.

Field Validation: Canada

A field experiment was conducted near Saskatoon, SK, Canada (52.135°N, 106.621°W) in 2014 to validate the metribuzin-tolerant lines identified in the dose–response experiments against Australian and Canadian genotypes. The trial site was in the Dark Brown Chernozemic soil zone with a typical soil organic matter content of 3.5% to 4.5%. Rainfall received at the field site from May to September in 2014 was 313 mm, compared with the long-term average of 236 mm. The experiment was sown on

June 4 with a row-seeder into cultivated soil with no crop residue present. Twenty-four lentil genotypes representing diverse commercial germplasm from Australia and Canada, known metribuzin-sensitive types, and the lines from the Canadian dose–response trial, were sown in a split-split-plot randomized complete block design with three replicates. Plots were first blocked by shade or no shade and then by metribuzin rate with genotype as the sub-subplot. Shade was applied with 75% polypropylene shade cloth supported 0.6 m above the ground by a wire frame for 72 h before metribuzin treatment to decrease the plant tolerance to metribuzin (Pritchard and Warren 1980). All genotypes except for the two mutants were sown at 40 seeds linear m^{-1} of row at 0.3-m row spacing. Due to limited seed supply, the two mutant lines were sown at 20 seeds 0.5 m^{-1} row, and interplant spacing was kept consistent in all rows. Group F granular inoculum was applied with seed, which was sown at a depth of 3 to 4 cm. Metribuzin at rates of 0, 150, 300, 600, and 1,200 g ha^{-1} was applied at the 4- to 5-node stage at 20 d POST with a handheld sprayer at 100 L ha^{-1} using four Air Mix 110-015 low-pressure nozzles on 0.5-m spacing at an operating pressure of 240 kPa. Plant damage scores were recorded on a row basis at 10 and 20 DAT by estimating the percentage of necrotic tissue in the row. Plant DW estimates at 20 DAT occurred by sampling 10 random plants in each row and oven-drying them at 80 C for 48 h.

Statistical Analysis

Germplasm screens were analyzed with ANOVA using GenStat v. 14.1 (VSN International, Hemel Hempstead, Hertfordshire, UK). Initial general linear mixed-model ANOVA of square-root-transformed plant DW data from the field validation trial identified a significant three-way interaction among shade, metribuzin rate, and genotype and was further analyzed within shade environments as described later. Plant DWs obtained from dose–response analysis and the field validation experiment were analyzed with nonlinear regression using the dose–response curve DRC package in the statistical analysis software R (R Development Core Team 2014). Estimates of growth reduction (GR_{50}), the effective dose of metribuzin required to reduce the growth of the dependent variable by 50%, were obtained from all models, and the selective index *SI* command of the DRC package was used to compare the relative differences of the GR_{50} values from the control genotype PBA Flash.

Results and Discussion

Germplasm Screening

High levels of metribuzin injury occurred in all germplasm screens, allowing the identification of a small number of lines with improved metribuzin tolerance. The control line 99-088L*H037 had greater than 50% necrotic damage in all screens and performed similarly to PBA Flash and better than the known sensitive line 96-047L*99R099, which averaged greater than 85% necrosis in all screens (Table 1). Across all screens, less than 5% of lines showed plant necrosis of 15% or less, a figure considered agronomically acceptable. The green lentil line SP1333 from Argentina consistently showed a lower level of plant damage than 99-088L*H037, and along with a reselected line from USSR-05-05, which segregated for expression of tolerance, was used in subsequent dose–response experiments.

Initial Line Screening of Mutant Field Selections

The four PBA Flash check plants incurred plant damage scores of 100% necrosis in all 95 trays of the glasshouse progeny screen (unpublished data). The two mutant lines, PBA Flash-EMS10-11SVHM043 (M043) from the 2011 M_2 screen and PBA Flash-EMS10-11SK-12PAHM009 (M009) from the 2012 M_3 screen exhibited no symptoms of damage (0%) on all 20 plants within their respective trays. All other mutant lines tested showed severe plant damage (greater than 80% necrosis) on all 20 plants. PBA Flash-EMS10-11SVHM062 (M062) and PBA Flash-EMS10-11SVHM091 (METD) showed improved levels of visual plant recovery posttreatment compared with all other mutant lines, except for M043 and M009. These four mutant lines were therefore examined further in dose–response experiments.

Dose–Response Experiments

Logistic dose–response curves were fit for plant DW to explain the response of lentil genotypes to metribuzin rate in both experiments. A three-parameter log-logistic model best explained the relationship between metribuzin rate and plant biomass in the Australian experiment and was required to fix the lower asymptote at zero. The model converged in DRC with a nonsignificant lack-of-fit test achieved (0.33). All lines incurred reductions in plant DW to applied metribuzin as measured by GR_{50} (Table 2).

Table 1. Number of lentil genotypes screened and plant damage (% necrosis) of selected lines from six metribuzin herbicide sand-pot assays.

	Mean % plant necrosis[a]					
Genotype	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6
'PBA Flash'	4.5[b]	83	26	70	85	91
96-047L*99R099	5.3[b]	100	95	100	86	91
99-088L*02H037	4.3[b]	72	56	81	84	80
SP1333	—	—	—	8	6	17
Screen mean	4.6[b]	84	64	67	83	88
LSD (0.05)	1.1[b]	33	62	55	39	34
Number of genotypes/screen[c]	266 (—)	106 (4)	71 (8)	72 (6)	181 (3)	180 (0)

[a]Each result is the mean of two replicates.
[b]Plant damage score of 0–6 used in the first screen.
[c]Figures in parentheses indicate the number of lines identified in each screen with a plant necrosis score of 15% or less.

Table 2. Estimates of GR_{50} (the rate required to reduce dry weight 50%) values from logistic curves for plant dry weight (g plant^{-1}) of lentil genotypes treated with metribuzin herbicide in controlled-environment room experiments in Australia and Canada.

Genotype	GR_{50} (SE)	
	Australia	Canada
'PBA Flash'	99.6 (±23.5)	107.7 (±14.1)
SP1333	437.8 (±61.4)***	218.9 (±48.1)***
USSR-05-05-Rsel	388.1 (±49.4)***	—
METD	232.3 (±64.2)	—
M062	679.1 (±213.6)*	—
M009	4,775.0 (±1,182.5)**	2,734.2 (±1,104)**
M043	6,242.8 (±1,837.8)**	2,912.6 (±747.5)***
'CDC Maxim'	—	103.3 (±18.7)
'CDC Greenstar'	—	192.2 (±27)*

*P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 for significance compared with PBA Flash within each experiement using pairwise comparison (*SI* function in DRC).

Mutant genotypes M043 and M009 performed similarly and had statistically greater tolerance to metribuzin than all other genotypes, with GR_{50} values 62 and 48 times greater than that of PBA Flash (100 g ha^{-1}), respectively (Table 2). The other four putatively tolerant genotypes exhibited an intermediate level of tolerance to metribuzin, two to seven times greater than PBA Flash. In the Canadian experiment, in which comparisons with Canadian genotypes occurred, a four-parameter log-logistic model best described the effects of metribuzin on plant DW, and the models converged in DRC. The addition of very high rates (3,840, 5,761, and 8,641 g ha^{-1}) in this experiment allowed for improved parameter estimates to be obtained for the highly tolerant mutant types (Figure 1) compared with the Australian experiment. Consequently, a higher nonsignificant lack-of-fit test statistic was achieved (0.98). Plant DW of untreated PBA Flash was approximately four times higher in the Australian experiment compared with the Canadian experiment, most likely reflecting more favorable growth conditions due to higher light intensity and lower sand content in the soil medium used in the former. However, the GR_{50} of PBA Flash was similar in both experiments (approximately 100 g ai ha^{-1}), suggesting a high level of repeatability in herbicide phytotoxicity in this susceptible line across experiments. GR_{50} values of the mutant lines and SP1333 were lower in the Canadian experiment compared with the Australian experiment (Table 2), most likely due to the higher sand content in the Canadian potting media resulting in reduced binding of the herbicide to organic matter and increased availability (Savage 1976). Furthermore, the lower light intensities in the Canadian experiment may have led to reduced metribuzin metabolism, resulting in greater plant injury (Caldwell and O'Sullivan 1985). Despite some variation in the observed level of metribuzin tolerance across the dose responses in SP1333, M043, and M009, a high level of repeatability in the relative tolerance levels of these genotypes compared with PBA Flash occurred between experiments. M043 and M009 again exhibited the highest level of metribuzin tolerance, with GR_{50} values of 2,734 and 2,913 g ha^{-1}, respectively. These rates of metribuzin are approximately 10 times higher than the maximum postsowing PRE label rate for lentil in Australia, and over 13 times higher than the maximum label rate for POST application in lentil in Canada. SP1333 again showed an intermediate response, with a GR_{50} for plant DW two times higher than PBA Flash and similar to the Canadian cultivar CDC Greenstar (192 g ha^{-1}). The other Canadian genotype, CDC Maxim, performed similarly to PBA Flash.

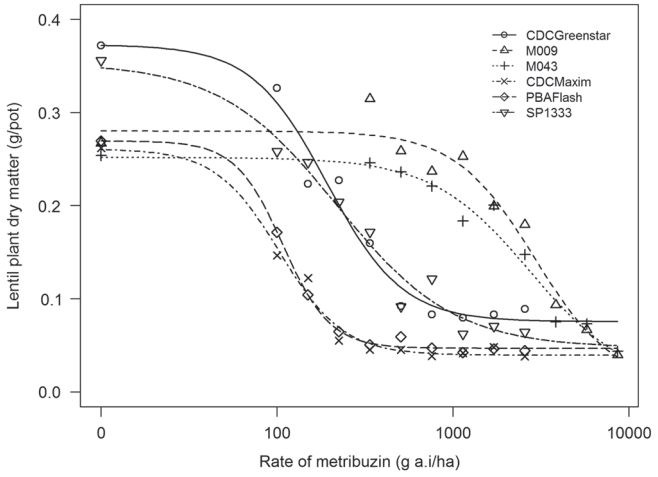

Figure 1. Dose–response curves of aboveground plant dry weight at 21 d after treatment with POST metribuzin herbicide of six lentil lines varying in herbicide tolerance under controlled-environment conditions in Canada.

Field Validation

A three-parameter log-logistic model best explained the relationship between lentil genotypes and metribuzin rate for plant DW under the two field environments of shade and no shade. The model converged in DRC, and a nonsignificant lack-of-fit test was achieved (0.54). Estimates of regression parameters and GR_{50} values for plant DW showed large differences in the response of genotypes to metribuzin rate under both environments (Table 3). Nonsignificant parameter estimates for GR_{50} were associated with high levels of standard error and occurred due to insufficient biomass reduction at the highest application rate leading to a poor fit of the model. In the unshaded treatment, 18 of the 24 genotypes had higher GR_{50} values than PBA Flash shaded, including PBA Flash unshaded. These ranged from the Canadian line MB1-3 to the metribuzin-sensitive Australian cultivar 'Nipper' (GRDC 2015), with GR 50 values 3.2 and 1.7 times greater than PBA Flash shaded, respectively. Of the other six genotypes, the known metribuzin-sensitive lines of VIR421 (Meier 2016) and 'PBA Herald XT' (GRDC 2015) had lower and equal GR_{50} values compared with PBA Flash shaded, respectively. GR_{50} values were not significant for 'PBA Jumbo,' SP1333, M043, and M009, as the lower limit could not be estimated at the range of rates evaluated. All genotypes with significant GR_{50} values under unshaded conditions had lower GR_{50} values when shaded, suggesting that metribuzin damage was accentuated in lentil under conditions of shade before herbicide application. A similar effect was observed in tomato by Pritchard and Warren (1980). Under shade, all genotypes, except for M009, M043, and SP1333, had significant parameter estimates and GR_{50} values within the range of rates evaluated. In shade, 15 genotypes had equal or lower GR_{50} values than PBA Flash shaded (Table 3). However, the Australian cultivar PBA Jumbo (1.8 times higher), the Canadian cultivars 'Plato' (1.7), 'Laird' (1.8), CDC Greenstar (1.8), and 'CDC KR-1' (2.0),

Table 3. Estimates of regression parameter *b* (slope ± SE) and GR_{50} (the rate required to reduce dry weight 50%) (± SE) for plant dry weight (g plant^{-1}) of lentil genotypes treated with metribuzin herbicide (0, 150, 300, 600, and 1,200 g ha^{-1}) under environments of induced shade (Shaded) and no shade (Unshaded) for 72 h before spray application at a field site in Saskatoon, SK, Canada, in 2014.

Genotype	Unshaded				Shaded			
	b	P-value (*b*)	GR_{50} ± SE	P-value (GR_{50})	*b*	P-value (*b*)	GR_{50} ± SE	P-value (GR_{50})
'PBA Flash'[a]	4.63 ± 2.2	≤ 0.05	1,071.4 ± 94.8**	≤ 0.0001	2.23 ± 0.6	≤ 0.001	514.4 ± 71.3	≤ 0.0001
VIR421	2.93 ± 1.3	≤ 0.05	583.1 ± 94.7	≤ 0.0001	1.55 ± 0.4	≤ 0.001	247.3 ± 54.7***	≤ 0.0001
M009[a]	15.83 ± 304.1	0.9585	1,489.7 ± 6,190.2	0.8099	2.04 ± NA	NA	11,535 ± NA	NA
M043[a]	6.2 ± 220.8	0.9776	8,241 ± 5.3e^{05}	0.9876	1.98 ± 17.4	0.9098	41,434 ± 1.8e^{06}	0.9821
SP1333	12.34 ± 68.7	0.8576	1,357.2 ± 933.4	0.1465	2.95 ± 2.3	0.204	1,623.2 ± 453.8*	≤ 0.001
3592-13[b]	2.31 ± 0.9	≤ 0.01	1,182.6 ± 220.2*	≤ 0.0001	2.3 ± 1.2	≤ 0.05	804 ± 150.7	≤ 0.0001
3674-15[b]	2.89 ± 1.4	≤ 0.05	1,181.8 ± 189.6**	≤ 0.0001	2.31 ± 0.8	≤ 0.01	611.1 ± 112.2	≤ 0.0001
'Blaze'[b]	2.79 ± 1.4	0.0522	1,263.3 ± 213.7**	≤ 0.0001	2.72 ± 0.9	≤ 0.01	718.7 ± 97.4	≤ 0.0001
'Eston'[b]	7.67 ± 41	0.8518	1,201.9 ± 66.6***	≤ 0.0001	4.03 ± 1.6	≤ 0.05	661.7 ± 65.5	≤ 0.0001
'CDC Greenstar'[b]	18.29 ± 329.7	0.9558	1,186.3 ± 247.3*	≤ 0.0001	2.52 ± 0.7	≤ 0.001	922.7 ± 95.3*	≤ 0.0001
'CDC KR-1'[b]	2.17 ± 0.7	≤ 0.01	1,266 ± 189**	≤ 0.0001	5.66 ± 2.9	≤ 0.05	1,014.7 ± 105.7**	≤ 0.0001
'Laird'[b]	3.75 ± 2.1	0.0802	1,280.5 ± 152**	≤ 0.0001	2.4 ± 0.7	≤ 0.01	949.6 ± 114.2*	≤ 0.0001
'CDC Maxim'[b]	3.32 ± 2.2	0.125	1,375.8 ± 216.4**	≤ 0.0001	3.48 ± 1	≤ 0.001	730.3 ± 95.7	≤ 0.0001
MB1-3[b]	1.61 ± 0.9	0.0653	1,641.6 ± 534.9*^	≤ 0.01	2.59 ± 1	≤ 0.05	683.9 ± 97.9	≤ 0.0001
MB1-4[b]	2.62 ± 2.5	0.2883	1,439.2 ± 364.5*	≤ 0.001	2.18 ± 0.8	≤ 0.01	680.1 ± 111.9	≤ 0.0001
'Milestone'[b]	3.06 ± 1.8	0.089	1,253.7 ± 176.3**	≤ 0.0001	2.67 ± 0.8	≤ 0.001	636.8 ± 87.2	≤ 0.0001
'Plato'[b]	2.12 ± 0.8	≤ 0.05	1,515.5 ± 300.3**	≤ 0.0001	3.04 ± 0.8	≤ 0.001	871.7 ± 83.2*	≤ 0.0001
'Boomer'[a]	1.84 ± 0.7	≤ 0.01	1,247.7 ± 180.9**	≤ 0.0001	1.84 ± 0.5	≤ 0.001	590.8 ± 93.9	≤ 0.0001
'Nipper'[a]	3.16 ± 1	≤ 0.01	883.2 ± 119.2*	≤ 0.0001	2.56 ± 0.8	≤ 0.01	562.1 ± 87.9	≤ 0.0001
'Nugget'[a]	2.33 ± 0.7	≤ 0.001	1,084 ± 162.1*	≤ 0.0001	2.73 ± 1	≤ 0.01	678.7 ± 92.6	≤ 0.0001
'PBA Ace'[a]	2.33 ± 0.7	≤ 0.001	1,077.2 ± 129.8**	≤ 0.0001	2.41 ± 0.7	≤ 0.001	626.1 ± 79.7	≤ 0.0001
'PBA Giant'[a]	2.6 ± 0.8	≤ 0.01	1,165.9 ± 115.2**	≤ 0.0001	4.67 ± 1.9	≤ 0.05	688.7 ± 57.9	≤ 0.0001
'PBA Herald XT'[a]	2.88 ± 1.2	≤ 0.05	868.8 ± 152.1	≤ 0.0001	2.01 ± 0.7	≤ 0.01	445.8 ± 80.4	≤ 0.0001
'PBA Jumbo'[a]	1.71 ± 1.7	0.3102	3,192.5 ± 3308.9	0.3351	2.8 ± 0.9	≤ 0.01	923.3 ± 108.9*	≤ 0.0001

*P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 for significance compared with PBA Flash shaded using pairwise comparison (*SI* function in DRC); *^P = 0.053.
[a]Australian lentil-breeding program origin.
[b]Canadian lentil-breeding program origin.

and the germplasm line SP1333 (3.2) all had higher GR_{50} values than PBA Flash shaded, indicating an improved level of metribuzin tolerance in these cultivars. This observed range in metribuzin tolerance among existing Australian and Canadian cultivars is relatively small and strongly influenced by environmental factors that increase metribuzin phytotoxicity. The increase in metribuzin tolerance of SP1333 over PBA Flash under shaded conditions was similar to that observed in the controlled-environment experiments. GR_{50} values could not be calculated for M009 and M043, as insufficient biomass reduction occurred at the rates used, even in the more damaging environment of induced shade. This result was not surprising, as GR_{50} values in the controlled-environment dose responses were at least two times greater than the highest field rate used, which in turn is five times greater than the label rate for metribuzin in lentil in Canada. The lack of response of the mutant lines to metribuzin under two field environments supports the controlled-environment results that showed they have a superior level of herbicide tolerance compared with all other genotypes evaluated. Further supporting the controlled-environment findings was the observation that no herbicide-damage symptoms (0% necrosis) occurred in M009 and M043 under the most damaging field treatment (shade plus metribuzin at 1,200 g ha^{-1}), unlike in all other genotypes (28% to 97% necrosis) (unpublished data). The DW GR_{50} values of PBA Flash were higher under field conditions in Canada than those achieved in both controlled-environment dose–response experiments. This apparently greater genotype tolerance may be explained by favorable variation in variables such as soil organic content (Savage 1976), herbicide incorporation into the soil due to inconsistent rainfall after application (Schroeder et al. 1985; Muehlbauer et al. 1995), positional evasion of herbicide by crop roots (Coble and Schrader, 1973), temperature (Caldwell and O'Sullivan 1985), and light intensity (Pritchard and Warren 1980) in the field when compared with controlled-environment pot experiments. However, the same relative trends in genotype tolerance between the mutant lines, SP1333 and the control lines PBA Flash, CDC Maxim, and CDC Greenstar persisted between the experiments and across conditions.

This study identified and validated a high level of metribuzin tolerance in two mutated genotypes and an intermediate level of tolerance in the germplasm line SP1333 compared with the

original mutant parent cultivar across controlled and field environments. The intermediate tolerance level in SP1333 is similar to that of the Canadian green lentil CDC Greenstar but superior to all Australian genotypes under field conditions and comparable to that found in a range of crops for which GR_{50} or equivalent measures have been used to determine tolerance improvements. These include a 2- to 3-fold improvement in tolerance between the susceptible and the most improved genotypes in winter wheat (Kleemann and Gill 2007), 3-fold improvement in soybean (Barrentine et al. 1982), 2-fold improvement in cowpea (Harrison 1988), 3-fold improvement in barley (Gawronski et al. 1987), and 2-fold improvement in narrowleaf lupin (Si et al. 2006). In the only previously reported case of mutagenesis used for discovering metribuzin tolerance, two separately induced mutants of narrowleaf lupin were identified with four and six times tolerance over the original wild type in controlled-environment conditions (Si et al. 2009). In another study, the authors showed a further 5-fold increase in tolerance was achieved in F_2 plants from a cross between these tolerant mutants in glasshouse studies (Si et al. 2011). The two highly tolerant mutant lines of lentil in this study exhibited greater than 25 times the tolerance of the parent cultivar and, importantly, no plant damage symptoms or biomass reductions at field rates approximately four times the label rate. At these levels, all 20 Australian and Canadian genotypes incurred high levels of plant damage. The estimated induced mutation rate in this study was 9.4×10^{-8} based on the one highly tolerant plant found in the M_2 generation compared with a lower estimated rate of 2.5×10^{-5} in the narrowleaf lupin study by Si et al. (2009).

Despite first being discovered in 1968, metribuzin remains a significant herbicide of choice in many farming systems in both the Southern and Northern Hemisphere, due in large part to its broad-spectrum grass and broadleaf weed control. However, more recently, it has been identified as having potential to control IMI-resistant broadleaf weeds such as wild mustard (*Sinapis arvensis* L.) in Canada (Beckie and Tardif 2012) and oriental mustard (*Sisymbrium orientale* L.), African mustard (*Brassica tournefortii* Gouan), and wild radish (*Raphanus raphanistrum* L.) in Australia (Boutsalis et al. 2016). It also has a lower risk for weed herbicide-resistance selection than the AHAS inhibitor group (Beckie and Tardif 2012), potentially delaying the onset of herbicide resistance in weed populations. The availability of lentil genotypes with high levels of metribuzin tolerance will provide breeding programs with an alternative or complementary herbicide tolerance trait to IMI and improve weed control options for growers. Further agronomic evaluation of this herbicide-tolerance trait in lentil is required, including grain yield assessments on soils varying in sand content to quantify its potential agronomic impact. An understanding of the heritability and the mechanisms of herbicide tolerance, along with identification of any associated fitness penalty or other deleterious mutations will be required to allow plant breeders to effectively incorporate this high level of tolerance. M043 and M009 were identified from the M_2 and M_3 field screens, respectively, and exhibited different morphological plant habits that may or may not be beneficial to plant breeders.

Mutagenesis and large-scale field selection proved to be an effective technique for generating a high level of metribuzin tolerance in lentil. While the in situ germplasm-screening approach was quicker and more cost-efficient, only a lower level of tolerance was identified. The mutagenesis approach generated a far greater level of metribuzin tolerance and had the benefits of the researcher being able to use an elite cultivar as the parent. Alternative approaches to generating herbicide-tolerant crops such as transformation techniques remain problematic in pulses, and it is unlikely that these approaches would ever lead to commercial cultivars in a minor crop like lentil, due to low return on investment and potential international trade issues (Devine 2005).

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Chapter 4.

Induced novel *psbA* mutation (Ala251 to Thr) in higher plants confers resistance to PSII inhibitor metribuzin in *Lens culinaris*.

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Overall percentage (%)	75%		
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.		
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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.

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Induced novel *psbA* mutation (Ala_{251} to Thr) in higher plants confers resistance to PSII inhibitor metribuzin in *Lens culinaris*

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Abstract

BACKGROUND: Weed competition is a major limitation to worldwide lentil (*Lens culinaris* Medik.) production in part due to limited effective safe herbicide options. Metribuzin is a photosystem II inhibiting herbicide that provides broad spectrum weed control, however it causes excessive injury in lentil. Dose response analysis of photosystem II inhibiting herbicides and DNA sequencing of the *psbA* chloroplast gene occurred to quantify the spectrum and mechanism of herbicide resistance in two ethyl-methanesulfonate (EMS) induced mutant lentils.

RESULTS: Compared to susceptible parent PBA Flash, the level of metribuzin resistance was 33-fold for mutant M043 and 10-fold for M009. No improvement in resistance occurred in either mutant to bromoxynil, diuron, bromacil and atrazine herbicides. Nucleotide sequencing of the *psbA* gene of both mutants identified a substitution at position 751 compared to PBA Flash. The resulting deduced amino acid sequence indicated an Ala_{251}Thr substitution as being most likely responsible for the high level of metribuzin resistance.

CONCLUSIONS: The Ala_{251}Thr substitution discovered in this study is unique in mutagenized higher plants and the first report of an induced *psbA* target site mutation in higher plants. This target site metribuzin resistance is likely to have a significant impact on lentil production in Australia and worldwide.

Keywords: cross-resistance; D1 protein; mechanism; substitution; target site; weed control

1 INTRODUCTION

Lentil (*Lens culinaris* Medik.) is a winter pulse crop in southern Australia. The area sown has increased from less than 20 000 ha in 1996 to over 220 000 ha in 2016[1] through a combination of improved genotypes, targeted effective agronomic research and relative high grain prices. Despite the availability of genotypes with improved resistance to imidazolinone (IMI: acetohydroxyacid synthase (AHAS) inhibitors), weed control remains a major limitation to lentil production. Photosystem II (PSII) inhibitor herbicides such as diuron (phenylureas), terbuthylazine (triazines) and metribuzin (triazinones) are used in Australian lentil production due to their broad spectrum weed control. In particular, they provide improved control of problematic weeds, such as milk thistle (*Sonchus oleraceus* L.), prickly lettuce (*Lactuca serriola* L.) (Davey C, https://grdc.com.au), IMI resistant oriental mustard (*Sisymbrium orientale* L.), African mustard (*Brassica tournefortii* Gouan) and wild radish (*Raphanus raphanistrum* L) (Boutsalis P, https://grdc.com.au) compared to the IMI herbicides.

PSII inhibiting herbicides disrupt photosynthesis by displacing the native plastoquinone at the Q_B binding site of the D1 protein in the PSII complex of the chloroplast.[2] The D1 protein is encoded by the chloroplast *psbA* gene, which has been sequenced in many higher plants including lentil[3] and found to be highly conserved among species.[4] Worldwide, over 70 weed species have evolved resistance to PSII inhibitor herbicides through alterations to this protein.[5] Research with algae and cyanobacteria has shown that specific amino acid substitutions between positions Phe_{211} and Leu_{275} in the QB binding niche of the D1 protein confer resistance to PSII inhibitor herbicides.[6] A summary of a number of studies with higher plants report eight substitutions in the D1 protein, Leu_{218}Val, Val_{219}Ile, Ala_{251}Val, Phe_{255}Ile, Phe_{255}Val, Ser_{264}Gly, Ser_{264}Thr and Asn_{266}Thr, as being associated with resistance to PSII inhibitors in various weed species.[7] The type and extent of herbicide resistance depends upon the individual substitution and the species involved, with no one substitution providing resistance to all PSII inhibitors.[6]

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The only reported case of a crop species possessing a target site resistance to a PSII herbicide inhibitor is in canola (*Brassica napus* L.). Triazine-resistant canola was developed through a back-crossing program with canola and a triazine-resistant biotype of birdsrape mustard (*B. rapa* L.) possessing a $Ser_{264}Gly$ amino acid substitution in the D1 protein.[8,9] Subsequent triazine-resistant canola cultivars exhibit high triazine resistance, low level triazinone resistance and similar levels of resistance to the phenylureas compared to susceptible canola.[10]

Two narrow-leafed lupin (*Lupinus angustifolius* L.) genotypes, with a 4- and 6-fold increase in metribuzin resistance over that of the susceptible parent genotype, were developed through chemical mutagenesis.[11] However, a further study concluded that the resistance was non-target site based and most likely due to P450 enzyme-mediated metabolism.[12]

Metribuzin is applied pre-emergent in Australian lentil production due to a low level of foliar tolerance in current cultivars. Metribuzin is also registered for pre or post-emergent application in lentil production in Canada but can cause crop phytotoxicity under certain conditions.[13] Further, the success of any pre-emergent strategy in either country is complicated by factors such as the relative rooting depths of the weed species, soil conditions and climatic factors influencing herbicide movement.[10] Improved resistance to metribuzin in lentil would allow safe post-emergent application and a subsequent increase in the effective period of weed control. To address these limitations, two lentil mutants with high levels of resistance to metribuzin, have recently been developed through EMS mutagenesis techniques and field selection.[14] Australian lentil breeding programs are incorporating this resistance into adapted cultivars with the view of developing metribuzin resistant genotypes. To date, the mechanism of resistance and the relative performance of the mutants to other PSII herbicides is unknown. This research aimed to identify the basis of metribuzin resistance in the two lentil mutants and to compare their respective resistance profiles to other commonly used PSII inhibitors.

2 MATERIAL AND METHODS

2.1 Plant materials

The EMS mutation induced metribuzin resistant lentil genotypes PBA Flash-EMS10-11SVHM043 (M043) and PBA Flash-EMS10-11SK-12PAHM009 (M009) and the susceptible parent cultivar PBA Flash were used in this research.[14] Seeds of all cultivars were produced near Riverton, South Australia during the winter growing season of 2013 and stored dry until required.

2.2 Dose-response experiments

Experiments were located in controlled environment growth room facilities at the Waite Research Precinct, Urrbrae, SA, Australia to investigate the resistance of the metribuzin resistant lentil mutants to the triazinone herbicide metribuzin (Mentor, 750 g a.i. kg^{-1}, Farm Oz Pty Ltd, St Leonards, NSW, Australia) and four other PSII inhibitor herbicides from different sub-families. These were atrazine (Farmozine, 600 g a.i. L^{-1}, Adama Australia, St Leonards, NSW, Australia) a triazine, diuron (Diuron, 900 g a.i. kg^{-1}, Nufarm Australia Ltd, Laverton North, Vic, Australia) a phenylurea, bromacil (Uragan, 800 g a.i. kg^{-1}, Adama Australia, St Leonards, NSW, Australia) a uracil, and bromoxynil (Bromacide 200, 200 g a.e. L^{-1}, Nufarm Australia Ltd, Laverton North, Vic, Australia) a nitrile.

Herbicide treatments were blocked by sub-family to avoid contamination, and randomized complete block designs were set-up within herbicides. Three replicates were used and the experiment (run) was repeated. Seeds of M009, M043 and PBA Flash were scarified by scoring the seed coat with a scalpel and four seeds of a single genotype were sown per pot (8 cm × 10 cm × 8 cm) filled with potting mix of 90% composted pine bark and 10% sand. All plants were grown under conditions of 14/10 h day/night day length, 20/10 °C day/night temperatures, light intensity of 1100 µmol m^2 s^{-1} and relative humidity maintained at 90%. All pots were watered after sowing (100 mL) and then twice weekly (50 mL) thereafter. Plants were thinned to two uniform seedlings per pot prior to herbicide application at the four- to five-node stage. Herbicide was applied using a laboratory track applicator with a twin nozzle (110° flat fan) moving boom situated 40 cm above the top of the plants, to generate an even measured spray distribution between the nozzles, and delivering 103 L ha^{-1} at 1 m s^{-1} and 250 kPa. Plants were watered immediately prior to herbicide treatment and then 24 h post treatment with water (50 mL). Twice weekly watering with 25% Hoagland's nutrient solution (50 mL) then commenced.

Metribuzin application rates were 0, 71.3, 142.5, 285, 570, 1140, 2280 g a.i. ha^{-1} in PBA Flash and 0, 285, 570, 1140, 2280, 4560, 9120 g a.i. ha^{-1} in the mutant genotypes. Application rates for all other herbicides were the same across genotypes and were bromacil at 0, 200, 400, 800, 1600, 3200, and 6400 g a.i. ha^{-1}; bromoxynil at 0, 12.5, 25, 50, 100, 200, and 400 g a.e. ha^{-1}; atrazine at 0, 150, 300, 600, 1200, 2400, and 4800 g a.i. ha^{-1}; and diuron at 0, 225, 450, 900, 1800, 3600, and 7200 g a.i. ha^{-1}. Herbicide application rates of 142.5 and 18 240 g a.i. ha^{-1} for metribuzin (mutants only); 6.3 g a.e. ha^{-1} for bromoxynil; 37.5 and 75 g a.i. ha^{-1} for atrazine; and 56.3 and 112.5 g a.i. ha^{-1} for diuron were included in the second run to improve the fit of curves. A non-ionic surfactant (0.1% (v/v) 100 g L^{-1} alcohol alkoxylate; BS1000; Crop Care, Murarrie, Queensland, Australia) and a combination vegetable oil and non-ionic surfactant (0.25% (v/v) 704 g L^{-1} ethyl and methyl esters of canola oil fatty acids and 196 g L^{-1} alcohol alkoxylate; Hasten; VicChem, Coolaroo, Victoria, Australia) were added to the diuron and atrazine treatments, respectively. Above ground biomass was collected at 21 days after treatment and samples were combined within experimental units and dried at 80 °C for 48 h in a laboratory oven.

2.3 Statistical analysis

Initial analysis of plant dry weight (DW) data from both experimental runs was conducted using ASREML in R (ASREML. Release 4.1. VSN International Ltd 2014). A significant interaction between run and treatment was identified and data were further analyzed within runs. Plant DW obtained from dose responses were analyzed with non-linear regression using the Dose Response Curve (DRC) package in the statistical analysis package R (R Development Core Team 2014). Estimates of growth reduction (GR_{50}), the effective dose of herbicide required to reduce the growth of the plants by 50%, were obtained from all the models and the selective index (SI) command of the DRC package was used to compare the relative differences of the GR_{50} values from susceptible parent PBA Flash.

2.4 *psbA* gene sequencing

A candidate gene approach was used to identify target site mutations in the *psbA* gene of the resistant mutants by polymerase chain reaction amplification and Sanger sequencing. DNA was extracted from leaf tissue of both mutants and the susceptible

parent line using a CTAB extraction procedure.[15] Three pairs of forward and reverse primers (sense: 5′-TAGAGAATTCGTGTGCTTGG-3′; antisense: 5′-AGCTGAATATGCAACAGCAA-3′, sense: 5′-TTTCTG GTGCCATTATTCCT-3′; antisense: 5′-AGGTTTCTTCCTCTTGACCA-3′, sense: 5′-GGTTCCCTATTCAGTGCTATG-3′; antisense: 5′-GTAATATC AACAAGGTTTATATTACTCC-3′) were designed based on the *psbA* gene sequence of lentil (GenBank KF186232.1) covering the complete 1.062 kb of the gene sequence.[5] Polymerase chain reaction occurred in a 25 μL reaction consisting of 10 × PCR buffer, 0.4 μL of each primer, 0.4 μL dNTPs, 1.6 μM MgCl2, 1 unit of Genscript Taq Polymerase and 50 ng of template DNA. The cycling program was an initial denaturation of 3 min at 95 °C, 40 cycles of 1 min at 94 °C, 1 min at 54 °C annealing, 1 min at 72 °C extension, 5 min at 72 °C final extension and 8 °C hold until samples removed from thermocycler. Polymerase chain reaction products were mixed with GenScript GelRed loading dye then run on a 1% agarose gel electrophoresis with Tris-acetate (TAE) buffer. Polymerase chain reaction product was gel-extracted using a QIAGEN® QIAquick® Gel extraction kit (QIAGEN Inc. Mississauga, ON, Canada). Sanger sequencing of eluted DNA was undertaken by Eurofins Genomics, Huntsville, Alabama, US. BioEdit software (Informer Technologies. Inc) was used to assemble and compare the gene sequence data of each genotype and *Arabidopsis thaliana* (GenBank Accession number X79898.1).

3 RESULTS

3.1 Dose-response experiments

Resistance to metribuzin was evident in both mutant lentil lines when whole plant DW GR_{50} values were compared to the susceptible parent genotype, PBA Flash (Table 1). No resistance was observed to any other herbicide, however, slight negative cross resistances to atrazine and bromacil, mostly in M009, were identified. Estimates of genotype resistance were generally lower in the second experimental run, but this depended upon herbicide applied and was mostly due to higher efficacy of some herbicides in this run (Table 1). However, the relative performance of the mutant lines compared to PBA Flash were consistent across runs (Fig. 1). The GR_{50} values for metribuzin in PBA Flash differed twofold between runs but both values were lower than the field label rate of 285 g ha^{-1}, indicating susceptibility to this herbicide as reported previously.[14] The GR_{50} values for metribuzin within each mutant genotype were similar between runs and both exhibited a very high level of resistance when compared to PBA Flash. M009 was 10.4 and 22.1 times greater than PBA Flash across runs one and two, respectively, compared with 32.6 and 55.6 for M043. These values are at least 7 (M009) and 20 (M043) times the maximum post-emergent field label rate for metribuzin in winter field crops in Australia and lentil in Canada. Resistance ratios between these genotypes were not significantly different despite higher values occurring with M043 in both runs (data not shown).

There was no difference in GR_{50} values for diuron and bromoxynil between the mutant lines and PBA Flash, with all genotypes exhibiting a low level of resistance to these herbicides. Dry matter of all genotypes was reduced by greater than 50% compared to the untreated control at the lowest application rate of diuron in run two and GR_{50} estimates were not significant, indicating a very low safety margin in lentil to this herbicide. All genotypes exhibited a higher relative level of resistance to bromacil when compared to diuron (Fig. 1). However, significant DW reductions occurred in all genotypes at rates between 800 and 1000 g ha^{-1}, which is below the range of registered rates for weed control in Australia (2800–5200 g ha^{-1}). Despite DW GR_{50} genotype estimates for bromacil differing twofold between runs, the resistance ratios between mutant genotypes and PBA Flash remained the same. Resistance ratios of M043 were similar to PBA Flash however M009 exhibited a negative cross-resistance of 0.6 times that of PBA Flash in both runs. Estimates of DW GR_{50} for atrazine were lower in run two when compared with run one as for diuron and bromoxynil. However, the relative performance of genotypes to this herbicide was similar to that of bromacil in that M009 had a negative cross-resistance (resistance ratios of 0.3 and 0.4 compared to PBA Flash), and M043 was similar to PBA Flash in run one but slightly lower in run two (0.6).

3.2 *psbA* gene sequencing

The entire 1062 kb of the chloroplast *psbA* gene of both the susceptible and resistant lentil genotypes was successfully sequenced and aligned with the *Arabidopsis thaliana* orthologue. This included the region that codes for the QB binding niche of the D1 protein, amino acid residues 211–275. The full coding sequence for the *psbA* gene and the translated amino acid sequence from M009, M043 and the susceptible parent PBA Flash were deposited into the GenBank database as MH681284, MH681285 and MH681286, respectively. Sequence analysis of all three genotypes identified a single nucleotide polymorphism (G to A) in mutants M009 and M043 at position 751 (Fig. 2). This nucleotide change conferred an alanine to threonine amino acid substitution at position 251 (Ala_{251}Thr) in both resistant genotypes.

4 DISCUSSION

The development of plant varieties with improved resistance to metribuzin has been the subject of research since reports of intraspecific variation in soybean in 1974.[16] Despite improvements in metribuzin resistance of up to six times that of the susceptible in crops such as winter wheat (*Triticum aestivum* L.),[17,18] soybean (*Glycine max* (L.) Merr.),[19] field pea (*Pisum sativum* L.),[20,21] barley (*Hordeum vulgare* L.)[22] and narrow-leafed lupin,[11] a target site-based resistance mechanism has not been reported. This contrasts with triazine resistance in canola where a Ser_{264}Gly chloroplast mutation is responsible for a high level of resistance to some PSII herbicides.[10] Sequence analysis of mutant *versus* wild type lentil in this study revealed a single point mutation resulting in an Ala_{251}Thr substitution in the chloroplast D1 protein as most likely responsible for the high level of metribuzin resistance in lentil mutants M009 and M043.

Metribuzin dose response experiments showed GR_{50} values were in excess of 11 and 34 times that of the susceptible genotype in M009 and M043, respectively. However, the difference between the two mutants was not significant and supported previous dose response findings, which found similar high levels of resistance (greater than 25 times) compared to PBA Flash in both mutants.[14] Very high metribuzin rates, in excess of 60 times label rates, were required to determine GR_{50} values of the mutants in these experiments. Variability in GR_{50} values across experimental runs occurred with some of the other herbicides. This may have been caused by small variations in plant growth within experimental units leading to differences in herbicide uptake or positional evasion of herbicide by crop roots,[23] particularly where low levels of tolerance existed. Despite some differences in absolute GR_{50} values across runs clear and consistent trends in relative genotype performance across all herbicides were observed. Both mutants failed

Table 1. Estimates of regression parameter *b* (slope ± SE) and GR_{50} (the rate required to reduce dry weight 50%) (± SE) for plant dry weight (g plant^{-1}) and resistance factors (RF) of two mutant lentil genotypes compared with susceptible PBA Flash treated with post-emergent PSII inhibitor herbicides from two experimental runs

		Run 1				Run 2			
Herbicide	Genotype	$b \pm$ SE	$GR_{50} \pm$ SE	P value (GR_{50})	RF	$b \pm$ SE	$GR_{50} \pm$ SE	P value (GR_{50})	RF
Metribuzin	M009	0.72 ± 0.12	2238.5 ± 531.9	≤ 0.001	10.8**	0.83 ± 0.13	2370.5 ± 530.3	≤ 0.001	22.1***
	M043	0.61 ± 0.14	7024.0 ± 1883.6	≤ 0.001	33.8**	0.79 ± 0.13	5964.6 ± 1314.2	≤ 0.001	55.6***
	PBA Flash	1.39 ± 0.17	208.1 ± 25.3	≤ 0.001		1.88 ± 0.36	107.2 ± 12.1	≤ 0.001	
Atrazine	M009	1.02 ± 0.21	38.3 ± 17.6	≤ 0.05	0.3***	1.38 ± 0.27	18.6 ± 4.6	≤ 0.001	0.4***
	M043	1.53 ± 0.27	122.2 ± 25.3	≤ 0.001	1.0	2.28 ± 0.45	30.5 ± 3.6	≤ 0.001	0.6***
	PBA Flash	1.45 ± 0.21	125.6 ± 26.0	≤ 0.001		0.94 ± 0.12	52.1 ± 9.7	≤ 0.001	
Bromacil	M009	1.22 ± 0.23	964.8 ± 188.7	≤ 0.001	0.6***	1.22 ± 0.18	2283.2 ± 442.7	≤ 0.001	0.6*
	M043	1.30 ± 0.26	1357.8 ± 242.5	≤ 0.001	0.9	1.22 ± 0.23	3226.6 ± 671.2	≤ 0.001	0.9
	PBA Flash	1.40 ± 0.26	1521.0 ± 255.3	≤ 0.001		1.86 ± 0.37	3614.9 ± 590.1	≤ 0.001	
Bromoxynil	M009	8.95 ± 17.42	20.2 ± 8.3	≤ 0.01	1.3	3.24 ± 0.76	13.5 ± 1.0	≤ 0.001	0.9
	M043	3.76 ± 0.96	20.5 ± 1.9	≤ 0.001	1.3	4.13 ± 0.80	15.1 ± 0.9	≤ 0.001	1.0
	PBA Flash	5.00 ± 1.02	15.9 ± 1.5	≤ 0.001		2.69 ± 0.45	14.5 ± 1.1	≤ 0.001	
Diuron	M009	1.85 ± 0.46	146.3 ± 33.8	≤ 0.001	0.9	0.60 ± 0.25	13.6 ± 13.5	0.32	0.6
	M043	1.09 ± 0.17	193.2 ± 52.2	≤ 0.001	1.1	0.58 ± 0.20	19.5 ± 13.5	0.16	0.9
	PBA Flash	1.10 ± 0.20	168.5 ± 53.5	≤ 0.01		0.43 ± 0.12	22.9 ± 15.6	0.15	

*P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 for significance compared with PBA Flash shaded using pairwise comparison (SI function in DRC).

to show an increase in resistance over PBA Flash to the other herbicides evaluated, indicating the Ala_{251}Thr mutation confers only metribuzin resistance in lentil. Increased susceptibility to bromacil and atrazine herbicides occurred in M009, however M043 generally performed similar to PBA Flash. M043 and M009 were identified from separate M_2 and M_3 field screens respectively and exhibit different morphological plant habits. Deleterious plant mutations are a likely outcome of the mutagenesis process and mutation events affecting P450, or similar enzymes involved in herbicide metabolism, could have occurred and led to the increased susceptibility identified in M009. Currently mutant M043 appears to be a superior genotype for use by plant breeders.

The Ala_{251}Thr substitution identified in this study is unique in higher plants except for the identification of mutations involving this substitution in *Chenopodium rubrum* cell cultures.[24] An Ala_{251}Val substitution was identified in a field population of *C. album* from Sweden where repeated use of the triazinone herbicides metamitron and metribuzin had occurred.[25] Similar to our findings, this *C. album* biotype exhibited a high level of resistance to metribuzin but a lack of cross-resistance to atrazine. A comparative study with the *C. album* biotype and two others possessing either a Ser_{264}Gly or Leu_{218}Val substitution found that the Ala_{251}Val biotype had the highest resistance to the triazinone herbicides but a lower resistance to the triazine herbicides than the Ser_{264}Gly biotype.[26] Metribuzin screening of *C. rubrum* cell cultures identified eight resistant cell lines all with double or triple mutations in the *psbA* gene, three of which involved the Ala_{251}Thr substitution.[24] Ratios of the response in resistant *versus* susceptible cell lines involving the Ala_{251}Thr substitution ranged from 6–794 times for metribuzin, 2–25 for bromacil, 4–14 times for atrazine and 3–13 for diuron, and were characterized by a stronger resistance to metribuzin. The relative higher resistance levels to the non-triazinone herbicides in these cell lines compared to the lentil mutants in the current study may be due to the presence of the additional chloroplast mutations induced in these cell lines. A singular Ala_{251}Val substitution along with Ala_{251} to one of Cys, Gly, Ile or Leu, all generated by chemical mutagenesis in *Chlamydomonas reinhardii* were found in general to be resistant to the triazinone herbicides, with the Val substitution conferring the highest resistance to metribuzin.[6] This is in contrast to the atrazine resistant *Chlamydomonas* mutant, Phe_{255}Tyr which was found to be very susceptible to the triazinone herbicides.[6] Interestingly, a singular Ala_{251}Thr substitution has not been reported in any study involving algae, bacteria or cell cultures unlike the Ala_{251}Val substitution.[27–29,6] Despite this, the findings with the Ala_{251}Thr in lentil in this study are consistent with previous findings that show substitutions at position 251 are linked to higher levels of metribuzin resistance than other PSII herbicides.

Induced mutation has been successfully used to develop high levels of target site resistance to AHAS herbicides in numerous crop species worldwide including maize (*Zea mays* L.), rice (*Oryza sativa* L.), wheat, canola, sunflower (*Helianthus annuus* L.), and lentil.[30,31] Target site resistance to AHAS inhibitor herbicides is due to a point mutation in the nuclear AHAS gene and generally not associated with any major fitness cost to the plant.[32] Chemical mutants such as EMS have been widely used to induce mutations in the chloroplast of plants mainly for the purpose of understanding photosynthetic processes.[33] EMS mutation was used in lentil to induce 'chlorophyll chimeras' in M1 plants with a number of mutants being successfully transmitted to the M2 generation.[34] However, there are no previous reports of a *psbA* target site gene mutation being induced by mutagenesis techniques in higher plants. Lupin genotypes with a sixfold increase in metribuzin resistance and a P450 enzyme mediated nuclear metabolism mechanism were developed from approximately 15 500 sodium azide mutagenized seeds at an estimated induced mutation rate of 2.5 by 10^{-5}.[11] Relatively lower levels of improved resistance to metribuzin were developed in lentil where gamma radiation was used to treat 1000 seeds but the mechanism of resistance was not reported.[35] In comparison, approximately 700 000 lentil seeds

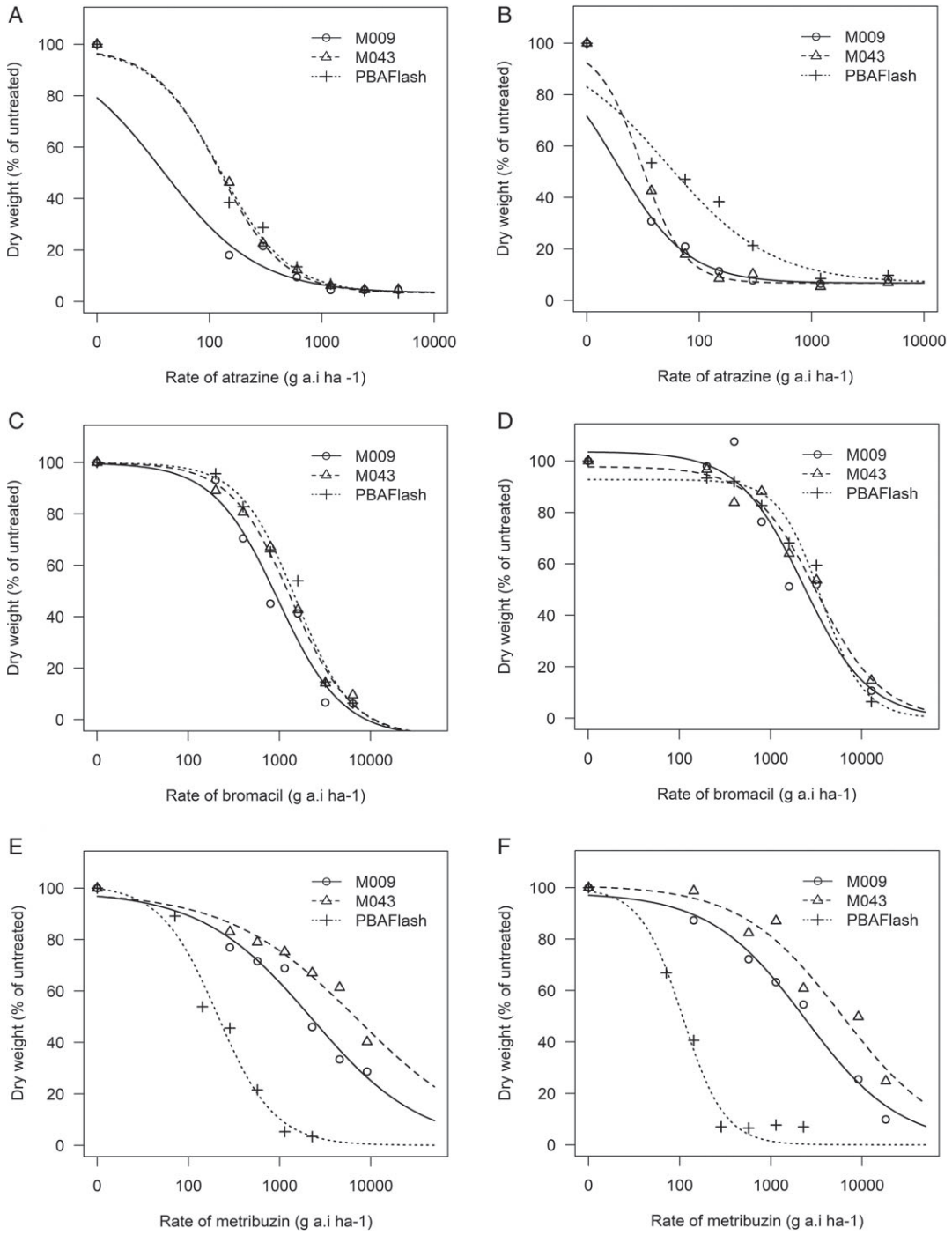

Figure 1. Plant dry weight of metribuzin resistant (M009 and M043) and susceptible (PBA Flash) genotypes 21 days after post-emergent treatment with multiple rates of atrazine (A, B), bromacil (C, D) and metribuzin (E, F) from two experimental runs.

were mutagenized with EMS at an estimated M_2 induced mutation rate of 9.4×10^{-8} to develop the mutants used in this study.[14] Diploid plant cells contain thousands of copies of their chloroplast genome but only two copies of their nuclear genome,[36] which is likely to be a major factor in the lower frequency of observed resistance mutations in the chloroplast *psbA* gene of the lentils used in this study compared with the nuclear metabolism mechanism found in lupin. Furthermore, it suggests that very large mutant populations are likely to be required to successfully identify chloroplast PSII inhibitor target site mutations in higher plants.

In contrast to the AHAS mutations, it is widely agreed that there is a significant fitness cost associated with the chloroplastic *psbA* gene mutation that encodes the $Ser_{264}Gly$ substitution in higher plants.[37] Alterations to the QB binding niche on the D1 protein reduce the binding of some PSII herbicides conferring resistance. However, it also results in a reduction in efficiency of electron transfer between plastoquinone QA and QB leading to a net lower photosynthetic rate and an associated reduction in plant productivity and yield.[38] There are no reports of a similar fitness cost associated with the $Ala_{251}Val$ mutation. However, analysis

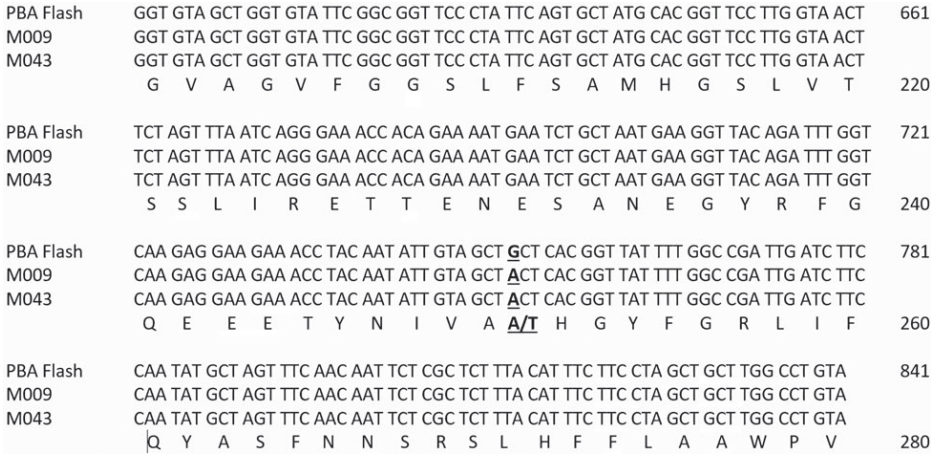

Figure 2. Nucleotide and deduced amino acid multiple sequence alignment of the QB binding niche (amino acid residues 211 to 275) of the D1 protein PSII *psbA* gene for metribuzin susceptible (PBA Flash) and metribuzin resistant (M009 & M043) lentil genotypes. Alignment is based on *Arabidopsis thaliana* (GenBank accession X79898.1). Bolded and underlined nucleotides and amino acids are associated with an alanine to threonine substitution at position 251 in the metribuzin resistant genotypes.

of *C. reinhardtii* mutants found that the substitutions $Ala_{251}Val$, $Gly_{256}Asp$, $Ser_{264}Gly/Ala$ greatly slowed electron transfer from QA to QB but substitutions at $Val_{219}Ile$, $Phe_{255}Tyr$ and $Leu_{275}Phe$ were found to have no effect on the rate of electron transfer.[39] Further research is clearly required with lentil mutants M009 and M043 to determine the presence and extent of any associated fitness penalty with the $Ala_{251}Thr$ substitution.

The high level of target site resistance to metribuzin confirmed in this study is likely to help with the development of lentil genotypes with improved broadleaf weed control options. A triazine-resistant canola industry based on the *psbA* target site mutation $Ser_{264}Gly$ has been successfully developed in Australia, despite the acknowledged fitness penalty and associated yield reduction. Further, triazine-resistant canola remains the major canola type grown in Australia some 20 years after its inception due to continued plant breeding efforts and their unique and advantageous weed control in this environment.[40] It is possible for metribuzin-resistant lentil to be as successful provided effective control of problem weeds can be achieved.

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Chapter 5.

Paternal leakage inheritance and a fitness cost are associated with the chloroplastic *psbA* gene controlled metribuzin tolerance in lentil (*Lens culinaris*).

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Paternal leakage inheritance and a fitness cost are associated with the chloroplastic *psbA* gene controlled metribuzin tolerance in lentil (*Lens culinaris*)

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Abstract

Reciprocal F_1, F_2 and F_3 populations of lentil (*Lens culinaris* Medik.) were developed by crossing between lines, with a chloroplastic *psbA* gene mutation conferring tolerance to the photosystem II inhibitor herbicide metribuzin, and sensitive parent PBA Flash, to understand the genetic control of the herbicide tolerance. Additionally, reciprocal BC_1F_2 populations were developed to identify any fitness penalty associated with the metribuzin tolerance. Phenotyping and genotyping results of the F_1, F_2, F_3 populations identified a predominantly maternal inheritance pattern, but with a level of paternal leakage. Paternal leakage occurred in approximately 20% of F_1 phenotypes, when including lines showing heteroplasmy (the existence of maternal and paternal chloroplasts within an individual). Field experiments with BC_1F_2 and BC_1F_3 lines confirmed this biparental inheritance pattern. Grain yield was reduced by 20 to 40% in metribuzin tolerant backcrossed lines compared with sensitive lines. Net assimilation rate at the onset of anthesis and plant dry weight at mid anthesis and maturity were also reduced in the tolerant lines suggesting reduced photosynthetic efficiency associated with the metribuzin tolerance results in lower dry weight and grain yield in the tolerant lines. The mode of inheritance and associated yield penalty of the tolerance trait will complicate its introgression in lentil breeding programs. However, the high level of tolerance and unique weed

control benefits of this trait suggest that this form of metribuzin tolerance in lentil, which is similar to triazine tolerant canola (*Brassica napus* L.), will be adopted.

Keywords: maternal, phenotyping, genotyping, NAR, heteroplasmy, PSII inhibitors

Introduction

Lentil (*Lens culinaris*) is an important pulse crop worldwide, providing an excellent food source high in protein and amino acids but relatively free of anti-nutritional factors (Bhatty 1988). World lentil production increased from 2.8 Mt in 1997 to more than 7.5 Mt in 2017 with over 55% from the developed countries of Canada, Australia and the United States (FAOSTAT 2019). Weed competition is a major limitation to lentil production with grain yield (GY) losses as high as 84% reported (Mohamed et al. 1997; McDonald et al. 2007). In countries where broadacre mechanised production of lentil occurs, the main means of weed control is by application of herbicides (Yennish et al. 2009). However, due to a lack of safe and effective options, variable levels of weed control and yield loss from crop phytotoxicity often occur (Brand et al. 2007).

Photosystem II (PSII) inhibitor herbicides such as the triazines, triazinones and uracils, are commonly used for broadleaf weed control in Australian lentil production due to their broad-spectrum weed activity. They are limited to a pre-emergent use in lentil due to a lack of selectivity between crop and weeds (White 2015). In addition, these herbicides are limited in use and effectiveness because of numerous application restrictions dependent on soil and weather conditions around the time of application. Lentil cultivars with improved tolerance to imidazolinone (IMI: acetohydroxyacid synthase [AHAS] inhibitors) herbicides are commercially available in Australia (Materne et al. 2011). However, PSII inhibitors are still widely used due to a broader range of broadleaf weeds controlled, and the increasing spread of IMI resistant weeds, caused by an over reliance of IMI herbicides (Boutsalis et al. 2016). Lentil lines with tolerance to the PSII inhibitor herbicide metribuzin have recently been developed (McMurray et al. 2019a). Metribuzin tolerance is attributed to an $Ala_{251}Thr$ substitution in the D1 protein encoded by the chloroplast *psbA* gene and is

being incorporated into Australian lentil breeding programs (McMurray et al. 2019b). However, the genetic factors controlling this tolerance have not been reported.

Although the development of other crop species with target site tolerance to metribuzin are not reported, there are numerous examples of weeds with evolved target site resistance, including *Poa annua* L. (Mengistu et al. 2000), *Kochia scoparia* L. (Mengistu et al. 2005), *Senecio vulgaris* L. (Park and Mallory-Smith 2006), *Capsella bursa-pastoris* L. (Perez-Jones et al. 2009) and *Chenopodium album* L. (Thiel and Varrelmann 2013). Furthermore, triazine tolerant (TT) canola (*Brassica napus* L.) with tolerance to the PSII inhibitor herbicide atrazine was developed through a back-crossing program with canola and a triazine-tolerant biotype of birdsrape mustard (*B. rapa* L.) (Beversdorf and Kott 1987). A $Ser_{264}Gly$ amino acid substitution in the chloroplast D1 protein of *B. rapa* was identified as being responsible for the triazine tolerance (Reith and Straus 1987) and is inherited maternally.

PSII inhibiting herbicides disrupt photosynthesis by displacing the native plastoquinone at the Q_B binding site of the D1 protein in the PSII complex of the chloroplast (Fuerst and Norman 1991). In reported weed species and TT canola, the $Ser_{264}Gly$ mutation removes a hydrogen bond that prevents triazine binding. It also reduces plastoquinone binding, thereby compromising photosynthesis and leading to a fitness cost in tolerant plants (Powles and Yu 2010). Less is known about fitness costs associated with other *psbA* mutations in higher plants. Analysis of mutants of *Chlamydomonas reinhardtii* found that the substitutions $Ala_{251}Val$, $Gly_{256}Asp$ and $Ser_{264}Gly/Ala$ greatly slowed electron transfer from Q_A to Q_B, but substitutions at $Val_{219}Ile$, $Phe_{255}Tyr$ and $Leu_{275}Phe$ had no effect on electron transfer rates (Rochaix and Erikson 1988). The $Ala_{251}Thr$ substitution attributed to the metribuzin tolerance in lentil is unique in higher plants except for its identification as part of double or triple mutations in *Chenopodium rubrum* cell cultures (Schwenger-Erger et al. 1993). The determination of the presence and extent of any fitness penalty associated with the $Ala_{251}Thr$ induced metribuzin tolerance is clearly required by lentil plant breeders incorporating this trait into their programs.

Therefore, the aims of this research in lentil were to: 1) identify the genetic controls of metribuzin tolerance, and 2) confirm the presence or absence of a fitness cost associated with the chloroplastic *psbA* gene conferring metribuzin tolerance.

Material and methods

Plant materials

The genetic controls of metribuzin tolerance and any associated fitness cost were investigated in two mutagenized lentil genotypes (PBA Flash-EMS10-11SK-12PAHM009 (M009)) and (PBA Flash-EMS10-11SVHM043 (M043) both with an $Ala_{251}Thr$ *psbA* mutation (McMurray et al. 2019b) in comparison to the parent line PBA Flash. Two separate streams of plant material were created. The first was developed at the University of Saskatchewan, Saskatoon, SK, Canada and the second at the Waite Research Precinct, Urrbrae, SA, Australia. The latter was created primarily to develop reciprocal BC_1F_2 derived lines for the fitness cost experiments, however, it was also used to confirm the findings from the experiments conducted in Canada.

The Canadian materials used seed sourced from a M_4 field multiplication near Riverton, SA, during winter, 2013. The two mutant lines were crossed with PBA Flash, including reciprocal crosses, to produce F_1 hybrid seed for phenotyping metribuzin response and for producing F_2 seed. All seeds were produced in a controlled environment room (CER) and grown in potting mix comprised of equal parts of Sunshine Mix #3 and #4 (Sun Grow, Seba Beach, AB, Canada).

In Australia, parental seeds were obtained from plants recovered from an M_4 metribuzin progeny screen (McMurray et al. 2019a) and all generations grown in a potting mix of 90% composted pine bark and 10% sand. Parental, F_1 and BC_1F_1 plants were grown in a CER both for phenotyping metribuzin response and producing F_2 seed. Ten random F_2 seeds from the two most productive F_1 plants within each cross, including reciprocals, were sown separately in an outdoor shade house facility for F_3 seed production. All F_2 plants were harvested and stored separately, and

all BC_1F_3 seed was obtained from bulk harvested plot-rows of field grown BC_1F_2 plants in the fitness cost experiment.

Canadian phenotyping of F_1 and F_2 plants

All plants were grown in pots (8 cm by 10 cm by 8 cm) filled with a custom soil mix of 60% Sunshine Mix #3 and 40% coarse sand in a CER under conditions of 14/10 h day/night, 21/15 °C day/night temperatures, light intensity of 540 μmol m^{-2} s^{-1} and relative humidity maintained at 40%. All seed coats were scarified with a scalpel and four seeds of each parent, F_1 or F_2 line were sown per pot.

The F_1 experiment was arranged as a square array consisting of seven columns (one pot per column) by seven rows. Within the array three pots containing a total of 12 F_1 seeds each of M043 x PBA Flash, reciprocals (PBA Flash x M043), M009 x PBA Flash and reciprocals (PBA Flash x M009) were randomly arranged along with nine or ten pots of each parent.

The F_2 experiment was arranged as two rectangular arrays of pots, one array for all crosses involving M009 and the other for all crosses involving M043. The arrays consisted of seven columns as for the F_1 experiment, by 25 rows (M009) and 24 rows (M043). Each pot within each row was sown to seed from the same individual F_1 plant (4 seeds per pot, 28 seeds in total), except for four rows which were sown equally to each parent. In the M009 array, 11 individual M009 x PBA Flash and 10 individual PBA Flash x M009 F_1 hybrid plants were represented. The two rows of each parent were randomly arranged within the array. The M043 array was sown as per the first and contained F_2 seed from 10 M043 x PBA Flash F_1 plants and 10 reciprocals along with two rows of each parent. Pots received 100 ml of water after sowing and were then watered twice weekly with 100 ml of 25% Hoagland's nutrient solution. Metribuzin (Sencor®, 750 g a.i. kg^{-1}, Bayer Crop Science, Calgary, AB, Canada) was applied using a laboratory track applicator with a single even flat spray nozzle 8001 EVS (Lechler, St Charles, IL, USA) delivering 108 L ha^{-1} at 240 kPa at a rate of 750 g a.i. ha^{-1}, at 12 days after sowing (DAS). Plants were watered immediately prior to herbicide treatment, then 24 h post-treatment with water (50 ml), and thereafter twice weekly with 100 ml of 25% Hoagland's nutrient solution. All plants were assessed individually for growth stage (node number) immediately

prior to spraying, which occurred when the majority of plants were at the five-node above ground stage of development. Seeds that had failed to emerge and seedlings with unopened cotyledons at the time of spraying were excluded from analysis. At 14 days after treatment (DAT) plants were assessed for herbicide injury. Plant injury was scored as the percentage of necrotic plant tissue.

Australian phenotyping of F_1, F_2 and F_3 plants

The Australian phenotyping experiment followed the Canadian experiment, but with the addition of F_3 seed and the following modifications. Pots were filled with potting mix of 90% composted pine bark and 10% sand. Daylength in the CER was as for Canada, but other conditions were 20/10 °C day/night temperatures, light intensity of 1,100 μmol m^2 s^{-1} and relative humidity maintained at 90%. Each rectangular array of pots consisted of eight columns by variable numbers of rows per F_1, F_2 and F_3 groupings.

The F_1 array consisted of three rows containing a total of 10 F_1 seeds (three pots) from M043 x PBA Flash and 17 reciprocals (five pots), and 16 seeds (four pots) from M009 x PBA Flash and 24 (six pots) reciprocals, randomly arranged with each parent. The F_2 arrays were grouped by the two mutant parents and each array consisted of 36 rows. Each parent array contained F_2 seed from six separate F_1 plants, six separate reciprocal F_1 plants and each parent. Seed of each F_2 and reciprocal F_2 line was sown into three consecutive rows containing 22 pots (88 seeds) along with two pots, each containing two seeds of each relevant parent (eight seeds). The F_3 arrays were grouped by the two mutant parents and each parent array consisted of eight rows. Within each array two consecutive rows contained seed from each F_3 family or reciprocal F_3 family, consisting of three pots (12 seeds) from each of five separate F_2 plants derived from the same F_1 (totaling 15 pots) plus one pot containing two seeds of each relevant parent. Metribuzin (Mentor, 750 g a.i. kg^{-1}, Farm Oz Pty Ltd, St Leonards, NSW, Australia) was applied to all plants 13 DAS using a laboratory track applicator with a twin nozzle (110° flat fan) moving boom situated 40 cm above the top of the plants, to generate an even measured spray distribution between the nozzles, and delivering 103 L ha^{-1} at 1 m s^{-1} and 250 kPa.

Genotyping

DNA was extracted from leaf tissue of all plants in the Canadian phenotyping experiment and from selected plants in the Australia phenotyping experiment. In Canada, a 2 mm^2 piece of plant tissue was harvested with tweezers from the second youngest emerged leaf. Each leaf tissue sample was placed in an individual well of a PCR micro-plate with 40 µl of 0.25M NaOH prior to shaking for 30 sec followed by heating at 95 °C for 2 min. Samples were cooled at room temperature prior to the addition of 60 µl of 0.5M Tris-HCl (pH 8.0), then shaken for 30 sec, heated at 95 °C for 2 min and stored in a refrigerator. DNA was diluted ten-fold in sterile water and 2.0 µl of the resulting dilution used in a 10 µl KASP reaction. The assay was completed using the forward primer allele 1 (sensitive) 5' – GAAGGTGACCAAGTTCATGCTAGGAAGAAACCTACAATATTGTAGCTG – 3', forward primer allele 2 (tolerant) 5' – GAAGGTCGGAGTCAACGGATTGAGGAAGAAACC TACAATATTGTAGCTA – 3' and reverse primer 5' – GCGAGAATTGTTGAAACTAGCATAT TGGAA – 3' designed using LGC PrimerPicker software. The reaction was run on a StepOnePlus™ Real-Time PCR system with the following program: step 1, 21 °C for 2 min (florescence read); step 2, 95 °C for 15 min (hot start); step 3, 94 °C for 20 sec (denaturing); step 4, 65 °C for 1 min (annealing and extension), repeat step 3-4, 10x reducing the annealing and extension temperature 0.8 degrees each cycle; step 5, 94 °C for 20 sec (denaturing); step 6, 57 °C for 1 min (annealing and extension), repeat step 5-6, 48x taking a fluorescence reading at 21 °C for 2 min after cycles 32, 36, 40, 44 and 48. Multiple end-point fluorescence readings were taken towards the end of the PCR in order to identify the optimal time-point for segregation of genotypes.

A similar method was used in Australia with the following modifications. Fresh leaf tissue was harvested for genetic analysis from 87 parental, all F_1 plants, 368 selected F_2 and 237 selected F_3 lines with the latter two providing a representation of all F_2 and F_3 crosses and reciprocals. Lyophilized leaf tissue was ground in 1.1 mL tubes with stainless steel ball bearings in a MM-300 Retsch-mill grinder for 1 min at 30 strokes s^{-1}. Genomic DNA was extracted using a modified CTAB protocol (Doyle and Doyle 1987). Primers used in the reaction were as for the Canadian experiment

but designed with Kraken™ workflow management software. Reaction was run using the LGC Genomics SNPline™ system with the following program: step 1, 94 °C for 15 min; step 2, 94 °C for 20 sec; step 3, 61 °C (-0.6 °C per cycle) for 60 sec, repeat step 2-3, 10x; step 4, 94 °C for 20 sec; step 5, 55 °C for 60 sec, repeat step 4-5, 26x; step 6, fluorescence read at 37 °C; step 7, 94 °C for 20 sec; step 8, 55 °C for 60 sec; step 9, fluorescence read at 37 °C, repeat step 7-9, 3x. Fluorescence readings were taken using a BMG PHERAstar spectrophotometer and SNP calls were made using Kraken™ software.

BC_1F_2 and BC_1F_3 fitness cost experiments

The BC_1F_2 and bulked BC_1F_3 populations were grown at field sites of Riverton and Turretfield, SA, over two successive years to ascertain if a fitness cost was associated with the chloroplastic *psbA* gene mutation that encodes the $Ala_{251}Thr$ substitution in lentil mutants M009 and M043. The sites represented favourable lentil growing areas in southern Australia with mean annual rainfall totals of 528 and 464 mm at Riverton and Turretfield, respectively. The soil type at Riverton was a calcareous clay loam over light clay with a pH (H_20) ranging from 6.7 on the surface to 8.9 at a depth of 60 cm, and at Turretfield, an alkaline, calcareous clay loam with a pH (H_20) ranging from 7.5 to 8.2. Annual rainfall totals were 439 and 361 mm in 2015 and 825 and 633 mm in 2016 at Riverton and Turretfield, respectively.

In 2015 three pairs of BC_1F_2s from the same F_1 parents, but backcrossed to a different PBA Flash plant, derived from PBA Flash x (M009 x PBA Flash) and PBA Flash x (M043 x PBA Flash) and their reciprocals (Table 5.1), and all parents were sown in 1m paired row plots in a randomised complete block design with three replicates. All lines were included once within each block except for PBA Flash (four times, representing each different PBA Flash plant used in the backcross) and the mutant parents (twice). Seed rows were formed by banding basal fertilizer at a depth of 8 cm and rate of 7 kg N ha^{-1}, 15 kg P $ha^{-1}$and 1.8 kg Zn ha^{-1} using a small plot cone seeder with six narrow tynes on 0.225 m row spacing. An unsown row separated every paired row plot. All seed coats were scarified pre-sowing and 40 seeds were sown at 2.5 cm spacing into two adjacent 1m rows at a depth

of 5 cm on July 8 and 9 at Turretfield and Riverton, respectively. These sowing dates were approximately 4 weeks later than the optimal sowing time in these environments due to delayed seed availability from indoor seed multiplication. General insecticide and fungicide application were as best agronomic practice for lentil. Both sites were hand weeded to remove weed competition and the Turretfield site received two supplementary irrigation events of 56 mm in spring. Net assimilation rate (NAR) in g of whole plant dry weight (DW) per square meter of leaf surface area (LA) per day (d) was calculated as per Williams (1946):

$$\mathrm{NAR} = \frac{[\,(\mathrm{DW2}-\mathrm{DW1})\,(\log_e \mathrm{LA2} - \log_e \mathrm{LA1})\,]}{[\,(\mathrm{LA2}-\mathrm{LA1})\,(t2-t1)]} \quad (1)$$

Three plants of uniform size were sampled from each paired row-plot initially in mid-September ($t1$) and then 16 (Turretfield) and 17 (Riverton) days later ($t2$) with the second sampling occurring at the onset of anthesis in PBA Flash. Plant samples from each plot were bulked in paper bags and stored in a cool dark room for processing. Random leaflets from all parts of each plant were removed with tweezers and placed on white paper (210 mm by 297 mm) on a flatbed scanner for leaf area measurement. Remaining leaflets were removed from plants and placed in a second envelope and all remaining plant parts placed in a third envelope. All envelopes were oven-dried at 80 °C for 48 h prior to weighing. Total leaf surface area for each sample was estimated from the total plant leaf weight using the weight of the known measured leaf area. At maturity all paired row plots were hand harvested separately into bags, dried and threshed for DW and GY determination. All grain samples were bagged separately and kept for use as seed in the 2016 BC_1F_3 experiments.

Methods used in the BC_1F_3 field experiments in 2016 were as for the BC_1F_2 experiments with some modifications. The experimental design consisted of four replicates at each site and all seed was sourced from the 2015 experiments. Seed was sown at a density of 120 seeds m^{-2} in 1.35 m by 5 m plots. Experiments were sown with a small plot cone seeder into retained straw residue at a depth of 5 cm at Riverton (June 3) and Turretfield (June 15). Basal fertilizer was banded below the seed and a trailing steel roller provided a level and uniform surface for herbicide application and machine

harvesting. Each plot was divided into two sections and at the five node growth stage metribuzin (Stacato, 750 g a.i. kg^{-1}, Sipcam Australia, Geelong, Vic, Australia) at 1,000 g a.i. ha^{-1} (more than 3x the maximum label rate for clay soil types in Australia) was applied to the first 1.5 m by 6 row section of each plot using a hand-held sprayer at 107 L ha^{-1} using four Air Mix 110-015 low-pressure nozzles on 0.5 m spacing at an operating pressure of 240 kPa. Plant survival was estimated by counting all plants in the 4 internal rows by 1 m within the sprayed section prior to spraying, and then counting surviving plants at 21 days after spray application from the same section. Plant DW was estimated from the unsprayed area at the anthesis stage in PBA Flash. Cuts of 0.5 m by the four internal rows were taken at two locations in each plot, and the two sub-samples were combined and oven-dried at 80 °C for 48 h. GY was estimated by harvesting the remaining unsprayed area with a small plot harvester at maturity.

Data analyses

Data from both the Canadian and Australian genotyping experiments were compared to their relevant phenotyping data to determine the reaction of all parents and progeny plants to metribuzin herbicide.

All data from the fitness cost field studies were analysed using linear mixed models. The fixed component of the model included site, variety and site by variety fixed effects and block was random. Additional fixed or random effects were added to model potential sources of spatial variation (Gilmour et al. 1997). A separable autoregressive process of order 1 in the row and columns was used to model spatial correlation between plot errors. All data were analysed using the ASReml-R software (Butler et al. 2009) in the R environment (R core team, 2018). The empirical logistic transformation (McCullagh and Nelder 1989) was used to improve the normality of the residuals of the percentage survival data:

$$z = \log\left[\frac{\left(p+\frac{1}{2n}\right)}{\left(1-p+\frac{1}{2n}\right)}\right] \quad (2)$$

where p is the percentage of live plants and n is the total number of plants prior to herbicide application. The line BC1F2-2015-29 was removed from the analysis due to a severely retarded plant

growth habit compared to all other lines, most likely due to a deleterious mutation not associated with the herbicide trait. To understand the factors associated with the differences in NAR, DW, GY and plant survival, the fixed component of the model was decomposed into a number of contrasts based on the parents, crossing structure and assumed maternal inheritance nature of the herbicide tolerant trait (Table 5.1). To quantify the level of fitness cost associated with the herbicide tolerance trait, a second classification of the 23 back-crossed lines was constructed based on the mutant parent used in the cross and the obtained plant survival data for each line from the 2016 experiments. Additional contrasts were set up using a Wald test to determine differences in NAR, GY and DW between tolerant and sensitive line groupings.

Results

Response of parents to metribuzin

Lentil plant response to metribuzin was classified into three categories based on the level of whole plant necrosis in both the Canadian and Australian genetic studies. Plants were considered tolerant if there was no evidence of necrosis or very minor necrotic speckling on individual leaflets and less than 6% necrosis on a whole plant basis; intermediate when necrotic regions were readily observed and often resulted in leaflet or leaf death and accounted for between 6 and 50% of total plant leaf area; and sensitive when leaf and stem necrotic regions or death was greater than 50% of total plant area and was generally associated with whole plant death. Of the 281 PBA Flash control plants phenotyped across both experiments, 280 were classified as sensitive with 276 of these showing severe levels of whole plant necrosis (greater than 90% leaf and stem area necrosis) regardless of the origin of the plant (Tables 5.2 and 5.3). Conversely, all of the 128 M009 and 142 M043 mutant parent plants were classified as tolerant with no necrotic regions observed on leaflets or stems of any plant.

Phenotype response of F_1, F_2 and F_3 lines to metribuzin

Plant necrosis levels of individual F_1 and F_1 reciprocals from crosses between both mutant parents and PBA Flash varied from 0 to 100% in both studies (Tables 5.2 and 5.3). Ratios of tolerant, intermediate and sensitive F_1 plants from M009 x PBA Flash and reciprocal were 5:1:0 and 0:1:9, respectively in the Canadian study (Table 5.2) compared with 15:0:1 and 1:1:4 for the same F_1 cross and reciprocal, respectively, in the Australian study (Table 5.3). Similar ratios were observed in the F_1 progeny of crosses and reciprocals of M043 x PBA Flash where ratios of tolerant, intermediate and sensitive plants were 3:1:0 and 1:1:8 in the Canadian experiment and 7:1:0 and 1:1:5 in the Australian studies, respectively. The results suggested that metribuzin tolerance in both lentil mutants is predominantly maternally inherited with occasional paternal transmission (paternal leakage, McCauley 2013) resulting in either paternal or more commonly biparental inheritance in some individuals.

Individual F_2 and reciprocal F_2 lines were treated separately due to paternal leakage observed in the F_1 phenotypes. Results of the F_2 segregation patterns are presented in Tables 5.2 and 5.3 and support a maternal inheritance with a level of paternal leakage across both experiments and both mutant genotypes. In the Canadian study 50% of F_2 lines from M009 x PBA Flash, 40% of the reciprocals, 50% of M043 x PBA Flash and 70% of the reciprocals exhibited uniparental maternal inheritance. Only two lines (18%) from the M009 x PBA Flash hybrid and one line (10%) from the M043 x PBA Flash reciprocal showed uniparental paternal inheritance. The remaining lines of all crosses exhibited a mixed phenotypic response with the level of paternal leakage detected ranging from 4 to 85% based on the percentage of F_2 progeny exhibiting the paternal phenotype. The Australian study supported these results with 33% of F_2 lines from M009 x PBA Flash and reciprocals along with 50% of M043 x PBA Flash and 67% of the reciprocals exhibiting uniparental maternal inheritance. Only 1 line (17%) from M009 x PBA Flash had uniparental paternal inheritance. The remaining 12 lines from all crosses of both mutants showing a mixed phenotype with the level of paternal leakage ranging from 1 to 97%.

Results of the Australian F_2 phenotyping of the M009 x PBA Flash cross showed that one of the two F_1 plants used for F_3 seed production had a mixed tolerance response with 97% of the F_2 progeny (F2C5F1-1) showing a paternal phenotype. The other F_1 line showed uniparental paternal inheritance in the F_2 phenotyping (F2C5F1-2). One reciprocal line (F2C5RF1-3) showed maternal inheritance and the other had a mixed response in the F_2 screening with 30% of the F_2 progeny (F2C5RF1-2) exhibiting a paternal phenotype. Phenotyping of the F_3 progeny from the same F_1 plants supported the F_2 phenotyping results, although all F_3 plants from F_2 lines with paternal leakage detected were classified as sensitive, despite only 70% being identified as sensitive in the F_2.

In the M043 x PBA Flash cross F_2 phenotyping, one line (F2C4F1-1) derived from the two F_1 plants used for F_3 phenotyping exhibited uniparental maternal inheritance and the other (F2C4F1-2) had a mixed tolerance response with 82% of the F_2 progeny (F2C5F1-1) showing a paternal phenotype. Both reciprocal lines (F2C4RF1-1 and F2C4RF1-2) showed maternal inheritance in the F_2 screen. Phenotyping of the F_3 progeny from these plants also supported the F_2 phenotyping results although, again all F_3 plants from the F_2 line with paternal leakage were classified as sensitive.

Genotype response of F_1, F_2 and F_3 lines to metribuzin

The KASP marker effectively separated mutant parents and PBA Flash into discrete clusters in the Canadian experiment (Table 5.2, Fig. 5.1a). A similar result occurred in the Australian experiment (Table 5.3) although two of 24 M009 mutant parents were genotyped as sensitive, a result which disagreed with the phenotyping results and is unexplained.

Using the parental clustering of fluorescence data, individual plants of the F_1, F_2 and F_3 generations were classed tolerant, as for M043 or M009, or sensitive like PBA Flash. Lines with a fluorescence reading located between the two parental clusters were classified as ambiguous, and likely possess some level of both tolerant and sensitive chloroplasts (Fig. 5.1b,c). The genotyping data was compared with the phenotyping data for all individual lines in the Canadian experiment and selected respective lines in the Australian experiment. Generally, genotyping results of the F_1 hybrids were in support of their respective phenotyping data however, there were a low number of

discrepancies that in the majority of instances were when an intermediate phenotype was observed. Correlations between F_1 genotype and phenotype data were $R^2 = 0.85$ in the Canadian experiment and $R^2 = 0.88$ in the Australian experiment.

Stronger correlations between genotype and phenotype data occurred for F_2 lines ($R^2 = 0.99$ (Canada) and $R^2 = 0.97$ (Australia)). Again, a lack of a complete correlation between all genotypes and phenotypes occurred and was almost entirely due to lines with a mixed phenotype response to metribuzin and/or ambiguous genotype results. Discrepancies between genotyping and phenotyping could be due to variable numbers of tolerant and sensitive chloroplasts, or chloroplast genomes in some lines potentially weakening both the fluorescence signal and the tolerance to metribuzin. Genotyping of F_3 plants accurately predicted the herbicide response except for a small number of instances where an intermediate phenotype was matched with a sensitive genotype classification. In all lines a maternal genotype was observed regardless of whether the F_3 line had originated from a F_2 line exhibiting a level of paternal inheritance or not.

BC_1F_2 and BC_1F_3 fitness cost experiments

The analyses performed identified significant genotype by site interactions for GY, 2015DW and plant survival, but not for NAR or 2016DW (data not shown). The Wald test using the decomposition of site by variety fixed effects into the various contrasts described in Table 5.1, showed that the higher level interactions, incorporating the crossing structure, parent type and assumed maternal inheritance nature of the tolerance were significant for all variates except for Parents:Sex:Mutant:ReciprocalRep:Dup for NAR and that they did not interact with site except for Parents:Sex:Mutant:ReciprocalRep for plant survival (Table 5.4). These results suggest that differences occurred between some backcrossed lines and reciprocals regardless of how close genetically they were related to each other, and these differences were similar across sites. Therefore, data is presented at the individual genotype level and across sites in Fig. 5.2. Furthermore, the back-transformed means of plant survival were highly correlated between sites and are also presented across sites.

Mean site GY was 3.6 (2015) and 2.1 (2016) t ha^{-1} at Riverton compared with 2.8 (2015) and 4.0 (2016) t ha^{-1} at Turretfield. The 2015 results reflected the more favourable growing conditions at Riverton compared with Turretfield, with the latter requiring two irrigation events to secure yield. Rainfall and growing conditions were very favourable in 2016. However, the Riverton site was located on a relatively poorly structured heavy soil type which when combined with above average rainfall in 2016, led to waterlogging and reduced yields compared to Turretfield.

The two mutant parents had similar GY in both years, but were 44 and 35% lower than that of PBA Flash at Riverton and Turretfield, respectively, in 2015 and 46% lower at both sites in 2016 (Fig. 5.2a,b). The contrast term sex was highly significant for GY and DW. In 2015 GY was 3.53 t ha^{-1} when the mutant line was used as a male in the backcross compared with 2.91 t ha^{-1} when used as the female (contrast estimate (CE) = 0.62, SE = ±0.06). In 2016 there was an interaction between site and sex for GY. Grain yields at Riverton were 2.35 t ha^{-1} when the mutant was used as male in the backcross compared with 1.92 t ha^{-1} when used as the female and even though the direction of yield difference was the same as for Turretfield, 4.37 t ha^{-1} (male) compared to 3.75 t ha^{-1} (female) (CE = 0.19, SE = ±0.1), the difference between the two was larger. The GY reduction associated with using the mutant as a female compared to as a male in the backcross ranged from 14 to 18%, suggesting a fitness cost is associated with the herbicide tolerance gene.

The relative performance of individual BC_1F_2 lines in 2015 were generally similar to their corresponding BC_1F_3 yields in 2016 (R^2 = 0.6, P < 0.001) (Fig. 5.2a,b). The higher order contrast interactions of Parents:Sex:Mutant:ReciprocalRep and Parents:Sex:Mutant:ReciprocalRep:Dup were significant for GY and DW in both the BC_1F_2 (2015) and the BC_1F_3 (2016) experiments. This suggests that the difference in using the mutant lines as a female or male is not the same across the different PBA Flash parents and that there could be differences between the duplicate pairs of the same cross. For example, the genetically similar paired lines of BC1F2-2015-20 and -32 were significantly different in GY in both 2015 and 2016, however, the paired lines from the same cross, BC1F2-2015-11 and -23 had similar yield in both years. Furthermore, the highest and lowest yielding

lines in both years came from different combinations of the sex, mutant, reciprocalrep and dup contrasts suggesting that not all lines within a specific cross were exhibiting the same level of GY reduction or fitness cost (Fig. 5.2a,b). DW at maturity in 2015 and at the onset of anthesis in PBA Flash in 2016 (data not presented) showed a similar pattern of relative line performance as the respective GY with linear relationships of $R^2 = 0.98$, $P < 0.001$ (2015) and $R^2 = 0.77$, $P < 0.001$ (2016).

The parent PF term for NAR was close to significant ($P = 0.07$), but the parent PM term was not significant. This suggested that the two mutant parents had similar NAR values, but averaged slightly lower than PBA Flash. The NAR of PBA Flash, M009 and M043 was 4.41, 4.11 and 3.74 g DW m^{-2} LA d^{-1} respectively (CE = 0.48, SE = ± 0.25). As for GY and DW the parent sex term was significant for NAR ($P < 0.05$). The mean NAR of all lines using the mutant parent as a female in the backcross was lower than that of lines where the mutant was used as a male, 4.01 compared to 4.36 g DW m^{-2} LA d^{-1} (CE = 0.35, SE = ± 0.15), respectively. The Parent: sex term interacted with mutant and reciprocalrep ($P < 0.01$) for NAR suggesting that the differences in NAR from using the mutant parents as female varied depending upon mutant parent and PBA Flash back cross parent (reciprocalrep).

A very high level of necrosis resulting in complete plant death was observed in the plot area of PBA Flash treated with metribuzin in the BC_1F_3 experiments at both sites. Plant survival percentage of PBA Flash was 0% compared with 99 and 93% in M043 and M009, respectively. Percentage survival of the BC_1F_3 lines varied from 0 to 100% and as found for GY and DW results varied across the dup contrast (Fig. 5.2c). Given that a very low plant mortality rate occurred in the mutant parent plots, possibly due to non-herbicide related plant death such as wind damage or pest predation, a plant survival rating of > 90% in the BC_1F_3 lines was considered as tolerant. Conversely a plant survival rate of < 10% was considered sensitive, while an intermediate classification was given for plant survival rates ≥ 10% and ≤ 90%. Sensitive and tolerant genotypes were present in each group derived from the crosses PBAFlash//M043/PBAFlash, M043/PBAFlash//PBAFlash and M009/PBAFlash//PBAFlash (Fig. 5.2c). In the group of lines derived from

PBAFlash//PBAFlash/M009 three of the five lines showed an intermediate response for plant survival to metribuzin and the other two a sensitive reaction. These findings provide further evidence that a strict uniparental maternal inheritance of the tolerance trait is not occurring in lentil. GY decreased linearly with plant survival in both years, R^2 = 0.64, P <0.001 in 2015 and R^2 = 0.83, P <0.001 in 2016. A relatively weak linear decrease in the NAR of all lines occurred with plant survival (R^2 = 0.38, P <0.001). However, eight of the 10 lines with the highest NAR values were all classed as sensitive and the other two as intermediate for plant survival to metribuzin (Fig. 5.2c,d). This is further evidence to support the hypothesis that a fitness cost is associated with the metribuzin tolerance trait in lentil.

Fitness cost assessment

The extent of the apparent fitness cost associated with the metribuzin tolerance gene in lentil was quantified as follows. The mean NAR, DW and GY for backcrossed lines rated as tolerant, according to the BC_1F_3 plant survival data, were compared with the mean of all lines rated as sensitive within each mutant group, regardless of if the mutant was used as a female or not in the backcross (Table 5.5). Lines rated as intermediate in tolerance were omitted from this comparison as they were likely to possess variable levels of associated fitness costs. The interaction of site and genotype for NAR was not significant. The NAR for the tolerant M009 line group was reduced by 16% compared to the sensitive M009 line group, with a similar 13% reduction for the same comparison occurring in the M043 lines. The site by genotype interaction was significant for GY and DW at maturity in 2015, but not for DW at anthesis in 2016. In all comparisons the tolerant groups had significantly lower contrast estimates than the sensitive groups, however, the amount of the reduction varied from 18 to 41% across sites and measurements (Table 5.5). GY and DW reductions between tolerant and sensitive M043 derived groups were approximately 25% at both sites in 2015, but varied from approximately 40% at Turretfield to approximately 25% at Riverton for the M009 derived group. In 2016, DW reductions at anthesis between tolerant and sensitive groups were similar across sites, averaging 18% in M009 derived groups and 28% in the M043 derived groups. However, GY

reductions between the two groups in 2016 varied across sites. For both groups, the lower yielding Riverton site had greater reductions, approximately 10% more than those recorded at Turretfield.

Discussion

The metribuzin tolerant lentil populations used in separate genetic experiments in this research were created, phenotyped and genotyped in different countries but using highly comparable methods. Phenotyping results from the screening of F_1 and F_2 progeny with metribuzin in both experiments indicated that the chloroplast *psbA* controlled metribuzin tolerance in lentil had a maternal inheritance pattern with a level of paternal leakage. Genotype data from both studies along with field collected phenotype data from independently created BC_1F_3 lines further confirmed this inheritance pattern.

The transmission of organelle genes, such as those found in the chloroplast, are largely thought to follow non-Mendelian genetics in plants. They have vegetative segregation, uniparental inheritance, intra-cellular segregation and reduced recombination in comparison with nuclear genes (Birky 2001). Although uniparental maternal inheritance of chloroplast genes is the usual form of transmission in angiosperms (Corriveau and Coleman 1988; Zhang et al. 2003), biparental plastid inheritance has been reported. Cytological evidence of plastid DNA in the generative and/or sperm cells of pollen was found in 43 of 235 angiosperm species investigated, however, it was not identified in lentil (Corriveau and Coleman 1988). Restriction fragment length polymorphism analysis has confirmed biparental chloroplast inheritance in *Medicago sativa* L. (Lee et al. 1988; Schuman and Hancock 1989; Masoud et al. 1990) and *Nicotiana tabacum* L. (Horlow et al. 1990). In more recent studies, where relatively larger number of progenies were assessed, low-level frequencies (< 5%) of paternal chloroplast inheritance has been reported in sunflower (*Helianthus verticillatus* Small.) (Ellis et al. 2008) and canola (Schneider et al. 2015). Despite a negative response for lentil in the Corriveau and Coleman (1988) cytological study, RFLP analysis of progeny from interspecific crosses of lentil identified paternal chloroplasts in 1 of 10 F_1 progeny (Rajora and Mahon 1995). This latter finding suggests biparental or at least a form of paternal leakage could occur in lentil.

The detection and quantification of chloroplast paternal leakage in lentil was not an aim of this research, nor was it expected, and specific organelle studies are required to confirm the extent to which it occurs and its significance in lentil. Based on the F_1 herbicide phenotyping results, we detected paternal chloroplast leakage in 14 and 23% of progeny from M009 crosses, and in 23 and 24% of progeny from M043 crosses, when including lines showing potential heteroplasmy (the existence of maternal and paternal chloroplasts within an individual (Birky 2001)). It should be noted that the whole plant phenotyping assay used in this study may not have accurately detected low levels of paternal chloroplast leakage, as evidenced by a heteroplasmic genotype but a uniparental phenotype in the same F_1, and therefore the actual frequency of paternal leakage inheritance in lentil could be higher than found in this study. The suggested paternal leakage transmission detection rates in this research however, are far higher than those identified in canola and sunflower and also higher than the 1 in 10 ratio identified previously in lentil, although an accurate estimate of the frequency of paternal leakage was not possible in the latter study as a very low number of F_1 plants were assessed (Rajora and Mahon 1995). However, given that the initial evidence for uniparental maternal chloroplast inheritance was generally based on studies with small progeny numbers (Ellis et al. 2008; Schneider et al. 2015), the finding by Rajora and Mahon (1995) may indicate that paternal leakage occurs more regularly in lentil than in other angiosperms, where much higher numbers were required for paternal plastid identification. Very high paternal plastid transmission rates were reported in *Medicago sativa* where only 5 of 212 progeny showed no evidence of paternal plastid fragments (Masoud et al. 1990). This finding provides some evidence that the relative high frequencies we observed in lentil could occur in angiosperms.

The relatively high level of chloroplast paternal leakage identified in lentil in this study potentially complicates the incorporation of the metribuzin herbicide tolerance trait into breeding programs. The major discrepancies between genotype and phenotype data occurred in the F_1 generation and were predominately linked to plants showing a heteroplasmic phenotype. This relative high frequency of heteroplasmy, particularly when only low levels of paternal leakage of sensitive

chloroplasts occurs, may reduce the value of a rapid marker platform such as KASP in the introgression of this trait by plant breeders. Assay tissue samples are typically taken from one plant part and may misrepresent the level of chloroplast DNA at the whole plant level in F_1 plants displaying biparental inheritance of chloroplasts, necessitating a different sampling or marker approach (Weihe et al. 2009). The accuracy of the KASP marker when compared with the phenotyping data did improve across generations (F_1 to F_2 to F_3) with almost 100% correlation in the F_3 generation regardless of maternal or paternal inheritance. This finding is consistent with the understanding that the distribution of organelles and their DNA to daughter cells during mitosis is a random process and resolution of chloroplast heteroplasmy typically will occur in the following rounds of cell division with sorting-out of plastids completed before flower formation (Greiner et al. 2014). This provides a reasonable explanation for why F_2 and F_3 families derived from F_1 plants showing evidence of paternal leakage might be observed to have a different herbicide response to that of their F_1 parent. Furthermore, Frey (1998), with reference to *Senecio vulgaris*, which is polymorphic for a chloroplast mutation that confers resistance to triazine herbicides, suggests that a polymorphic plant that survives treatment with triazine herbicides will most likely have eliminated sensitive chloroplasts and will be insensitive to further treatment. In this context, breeding programs incorporating the metribuzin herbicide trait in lentil are advised to spray selected early generation plants with the herbicide to eliminate any lines with a low level of paternally inherited sensitive chloroplasts.

Plant tolerance to some PSII inhibitors occurs due to alterations at the Q_B binding site in the D1 protein which reduce the binding affinity of the herbicide (Fuerst and Norman 1991). This alteration can also result in reduced efficiency of electron transfer between plastoquinone Q_A and Q_B leading to a lower net photosynthetic rate and reduction in plant productivity (Devine and Shukla, 2000). A fitness cost is associated with the $Ser_{264}Gly$ substitution in a number of weed species and triazine tolerant canola, but less is known about fitness costs of other *psbA* mutations (Powles and Yu 2010). Despite the presence of backcrossed lentil lines with varying levels of paternal leakage

inheritance in our initial analysis, results confirmed that a fitness cost is associated with the $Ala_{251}Thr$ substitution conferring metribuzin tolerance. In the analysis comparing only tolerant and sensitive reciprocal BC_1F_2 and BC_1F_3 lentil lines, GY was reduced in the tolerant progeny group by 20 to 40% and 26 to 37% for M009 and M043 derived lines, respectively. The results are similar to those found in TT canola, the only commercial crop with a chloroplast *psbA* mutation for tolerance to a PSII inhibitor herbicide. Studies across environments and canola genotypes with near-isogenic or reciprocal hybrids found yield penalties of 20 to 30% were associated with the TT trait (Beversdorf et al. 1988; Robertson et al. 2002). The latter study was conducted across 21 environments in Australia with mean site GY of 0.2 to 3.4 t ha^{-1}. An average GY reduction of 25% was suggested as being associated with the tolerance, and a GY loss of up to 39% was reported in an irrigated study. Our study was limited to two favourable lentil producing environments with average site GY in the top half of this range (2.1 to 4.0 t/ha). Combinations of light and temperature are known to amplify, neutralize or even reverse the negative effect of the $Ser_{264}Gly$ mutation on photosynthesis and plant growth (Vila-Aiub et al. 2009). A similar response with the $Ala_{251}Thr$ mutation could explain the variation in reduction of DW and GY in lentil across environments. Further evaluation of the metribuzin tolerance trait across more environments is required to ascertain if the fitness cost identified in this study changes under low yield conditions.

The NAR can provide a measure of the photosynthetic efficiency of plants (Watson 1952; Vernon and Allison 1963). Given that net reduction in photosynthetic rate is a result of alterations to the Q_B binding site, it was expected that tolerant lentil backcrossed lines would have lower NAR values. The NAR values measured in PBA Flash were 4.41 g DW m^{-2} LA d^{-1} and generally comparable with that measured in cereals (Watson 1952; Cannell 1967) and *M. sativa* (Tan and Tan 1981). The NAR of mutant parent M043 was 15% lower than PBA Flash, however, mutant parent M009 had levels similar to both PBA Flash and M043. The NAR of the tolerant group of BC_1F_2 lines of M009 and M043 had similar values to their mutant parents and were reduced by 16 and 13% respectively, when compared with their respective sensitive groups. The reductions in NAR at the

onset of flowering in 2015 translated into DW reductions at maturity of 27 to 41% and 25 to 26% in the M009 and M043 tolerant groups respectively. These DW reductions were similar to those identified for GY at maturity in 2015 and suggest that reduced DW production is accounting for the GY reduction in the metribuzin tolerant lines, as found for triazine tolerant canola (Robertson et al. 2002). Based on a single measure at one time point in the season, the level of reduction in the NAR of the tolerant lines when compared with the sensitive lines was generally less than half of those achieved for DW and GY. The measure of NAR assumes that the increase in leaf area is proportional to the rate of dry matter increase and this can be affected by mutual shading of leaves in the canopy. Given that the sensitive lines and PBA Flash produced greater levels of DW than the tolerant lines it is likely that they may have had increased levels of canopy shading, leading to an under estimation of NAR in comparison with tolerant lines and mutant parents. Despite this, it was clear that NAR was reduced in lines rated as tolerant to metribuzin in this study.

In summary, this study showed that the metribuzin tolerance trait in lentil has a predominately maternal inheritance pattern, but with occasional paternal leakage and an associated fitness penalty. The fitness penalty resulted in a 20 to 40% reduction in GY due to reduced DW production most likely attributable to a decrease in photosynthetic rate. This yield reduction appears similar to that found in triazine tolerant canola across a wide yield range in Australia. Despite a significant yield penalty, triazine tolerant canola is the major canola type grown in Australia due to its unique and advantageous weed control spectrum, and improved economics of production compared to other canola options (Zhang et al. 2016; Duke 2005). Given that lentil is a significantly less competitive crop than canola and has fewer herbicide options available, a similar uptake of this technology may occur, providing plant breeders can effectively incorporate the tolerance into agronomically accepted plant types.

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Table 5.1 Lentil parents and BC_1F_2 lines and corresponding factor levels from the decomposition of the site x variety treatment structure

Parent/line	Pedigree	Factor level[a]						
		P	PF	PM	SX	M	RR	D
PBAFlash		Y	2	1	3	3	Y	1
M009		Y	3	2	3	3	Y	1
M043		Y	3	3	3	3	Y	1
BC_1F_2-2015-2	(M009/PBAFlash)/PBAFlash-1	N	1	1	F	M009	1	1
BC_1F_2-2015-26	(M009/PBAFlash)/PBAFlash-1	N	1	1	F	M009	1	2
BC_1F_2-2015-27	(M009/PBAFlash)/PBAFlash-4	N	1	1	F	M009	2	1
BC_1F_2-2015-3	(M009/PBAFlash)/PBAFlash-4	N	1	1	F	M009	2	2
BC_1F_2-2015-30	(M009/PBAFlash)/PBAFlash-5	N	1	1	F	M009	3	1
BC_1F_2-2015-6	(M009/PBAFlash)/PBAFlash-5	N	1	1	F	M009	3	2
BC_1F_2-2015-1	PBAFlash-1/(M009/PBAFlash)	N	1	1	M	M009	1	1
BC_1F_2-2015-25	PBAFlash-1/(M009/PBAFlash)	N	1	1	M	M009	1	2
BC_1F_2-2015-28	PBAFlash-4/(M009/PBAFlash)	N	1	1	M	M009	2	1
BC_1F_2-2015-4	PBAFlash-4/(M009/PBAFlash)	N	1	1	M	M009	2	2
BC_1F_2-2015-29	PBAFlash-5/(M009/PBAFlash)	N	1	1	M	M009	3	1
BC_1F_2-2015-5	PBAFlash-5/(M009/PBAFlash)	N	1	1	M	M009	3	2
BC_1F_2-2015-20	(M043/PBAFlash)/PBAFlash-7	N	1	1	F	M043	4	1
BC_1F_2-2015-32	(M043/PBAFlash)/PBAFlash-7	N	1	1	F	M043	4	2
BC_1F_2-2015-33	(M043/PBAFlash)/PBAFlash-10	N	1	1	F	M043	5	1
BC_1F_2-2015-9	(M043/PBAFlash)/PBAFlash-10	N	1	1	F	M043	5	2
BC_1F_2-2015-11	(M043/PBAFlash)/PBAFlash-12	N	1	1	F	M043	6	1
BC_1F_2-2015-23	(M043/PBAFlash)/PBAFlash-12	N	1	1	F	M043	6	2
BC_1F_2-2015-19	PBAFlash-7/(M043/PBAFlash)	N	1	1	M	M043	4	1
BC_1F_2-2015-31	PBAFlash-7/(M043/PBAFlash)	N	1	1	M	M043	4	2
BC_1F_2-2015-10	PBAFlash-10/(M043/PBAFlash)	N	1	1	M	M043	5	1
BC_1F_2-2015-34	PBAFlash-10/(M043/PBAFlash)	N	1	1	M	M043	5	2
BC_1F_2-2015-12	PBAFlash-12/(M043/PBAFlash)	N	1	1	M	M043	6	1
BC_1F_2-2015-24	PBAFlash-12/(M043/PBAFlash)	N	1	1	M	M043	6	2

[a] P (Parents), parents or lines; PF (Parent PBAFlash), PBAFlash or mutants;
PM (Parent mutant), M009 or M043; SX (Sex), mutant parent maternal or paternal;
M (Mutant), mutant line derived from M009 or M043;
RR (ReciprocalRep), one of six PBAFlash plants used in backcross; D (Duplicate), line from same BC_1F_1

Table 5.2 Phenotypic response and associated genotype of lentil parents, F_1 and F_2 generations to 750 g a.i. ha^{-1} of metribuzin applied at the five node growth stage under controlled environment conditions, Canada

Generation	Female	Male	Line	Observed number of plants					
				Phenotype (% Necrosis)			Genotype (KASP Assay *PsbA* $Ala_{251}Thr$)		
				0-5	6-50	51-100	T[a]	S[b]	A[c]
Parent			PBAFlash-P1	0	0	36	0	3	0
			PBAFlash-P2	0	0	29	0	4	0
			M009	28	0	0	23	0	0
			M043	38	0	0	21	0	0
F_1	M009	PBAFlash-P2		10	2	0	9	3	0
	PBAFlash-P2	M009		0	1	9	1	6	1
	M043	PBAFlash-P1		9	3	0	9	2	1
	PBAFlash-P1	M043		1	1	8	1	8	1
Parent			PBAFlash-1	0	0	57	0	8	0
			PBAFlash-2	0	0	54	0	8	0
			M043	54	0	0	8	0	0
			M009	51	0	0	8	0	0
F_2	M009-1	PBAFlash-P2	M001a-4	27	0	0	26	0	0
			M001a-6	27	0	0	27	0	0
			M001a-9	27	0	0	27	0	0
			M001a-11	27	0	0	27	0	0
			M001a-12	0	0	26	0	26	0
			M001a-20	8	1	17	7	19	0
			M001a-31	8	0	0	8	0	0
			M001a-23	13	0	4	12	5	0
			M001a-24	0	0	28	0	28	0
			M001a-25	6	0	19	6	19	0
			M001a-28	27	0	0	27	0	0
	PBAFlash-P2	M009-1	M001b-4	1	0	25	1	25	0
			M001b-5	4	0	20	4	21	0
			M001b-6	4	0	18	5	17	0
			M001b-22	1	0	24	1	24	0
			M001b-23	2	0	24	2	24	0
			M001b-24	0	0	27	0	28	0
			M001b-25	0	0	25	0	28	0
			M001b-26	0	1	24	0	25	0
			M001b-27	0	0	28	0	28	0
			M001b-30	0	0	27	0	27	0

Table 5.2 (**Cont'd.)** Phenotypic response and associated genotype of lentil parents, F_1 and F_2 generations to 750 g a.i. ha^{-1} of metribuzin applied at the five node growth stage under controlled environment conditions, Canada

Generation	Female	Male	Line	Observed number of plants					
				Phenotype (% Necrosis)			Genotype (KASP Assay *PsbA* $Ala_{251}Thr$)		
				0-5	6-50	51-100	T[a]	S[b]	A[c]
F_2	M043-1	PBAFlash-P1	M002a-3	25	0	2	25	2	0
			M002a-4	28	0	0	28	0	0
			M002a-6	28	0	0	28	0	0
			M002a-7	28	0	0	25	0	3
			M002a-8	25	0	3	23	3	2
			M002a-12	28	0	0	27	0	1
			M002a-24	14	1	13	13	15	0
			M002a-25	28	0	0	27	0	1
			M002a-26	4	0	22	4	22	0
			M002a-28	20	0	7	19	8	0
	PBAFlash-P1	M043-1	M002b-5	24	0	0	23	0	1
			M002b-20	0	0	28	0	25	3
			M002b-22	0	0	28	0	25	3
			M002b-23	0	0	27	0	26	1
			M002b-25	0	0	28	0	24	4
			M002b-26	15	0	12	13	13	1
			M002b-27	0	0	28	1	26	1
			M002b-29	1	0	27	2	25	1
			M002b-30	0	0	28	0	28	0
			M002b-31	0	0	28	0	28	0

[a] Tolerant, as clustering and florescence data of line agreed with mutant parent

[b] Sensitive, as clustering and florescence data of line agreed with PBA Flash parent

[c] Ambiguous, as clustering and florescence data of line located between the two parental clusters

Table 5.3 Phenotypic response and selected associated genotype of lentil parents, F_1, F_2 and F_3 generations to 750 g a.i. ha^{-1} of metribuzin applied at the 5 node growth stage under controlled environment conditions, Australia

Generation	Female	Male	Line	Observed number of plants					
				Phenotype (% Necrosis)			Genotype (KASP Assay *PsbA* $Ala_{251}Thr$)		
				0-5	6-50	51-100	T[a]	S[b]	A[c]
Parent			PBAFlash-P4	0	0	49	0	15	5
			PBAFlash-P2	1	0	55	1	19	0
			M009	49	0	0	22	2	0
			M043	50	0	0	20	0	0
F_1	M009	PBAFlash-P2		15	0	1	13	1	2
	PBAFlash-P2	M009		4	4	16	1	21	2
	M043	PBAFlash-P4		7	1	0	6	1	1
	PBAFlash-P4	M043		2	2	9	0	8	4
F_2	M009	PBAFlash-P2	F2C5F1-1	2	1	72	3	9	1
			F2C5F1-2	0	0	67	0	10	0
			F2C5F1-3	85	3	0	11	0	0
			F2C5BCF1-2	84	0	0	10	0	0
			F2C5BCF1-3	85	0	0	10	0	0
			F2C5BCF1-6	58	5	24	4	4	2
	PBAFlash-P2	M009	F2C5RF1-1	62	5	21	60	11	4
			F2C5RF1-2	18	5	54	20	13	0
			F2C5RF1-3	0	0	88	0	10	0
			F2C5RF1-4	0	2	82	0	10	0
			F2C5RF1-5	0	0	87	0	10	0
			F2C5RF1-6	4	0	83	4	10	0
	M043	PBAFlash-P4	F2C4F1-1	84	0	0	10	0	0
			F2C4F1-2	14	3	58	5	4	0
			F2C4F1-5	35	2	50	5	5	0
			F2C4BCF1-8	53	4	24	5	3	2
			F2C4BCF1-9	82	0	0	10	0	0
			F2C4BCF1-11	88	0	0	10	0	0
	PBAFlash-P4	M043	F2C4RF1-1	0	1	75	0	3	7
			F2C4RF1-2	0	0	66	0	9	0
			F2C4RF1-3	32	0	56	28	10	2
			F2C4RF1-4	0	0	86	0	10	0
			F2C4RF1-5	0	0	88	0	10	0
			F2C4RF1-6	0	0	88	0	10	0
F_3	M009	PBAFlash-P2	F3C5F1-1-1	0	0	6	0	12	0
			F3C5F1-1-2	0	0	11	0	12	0
			F3C5F1-1-3	0	1	6	0	12	0
			F3C5F1-1-6	0	1	7	0	11	0
			F3C5F1-1-8	0	1	10	0	12	0
			F3C5F1-2-1	0	0	6	0	2	0
			F3C5F1-2-2	0	0	5	0	5	0
			F3C5F1-2-5	0	0	3	0	5	0
			F3C5F1-2-7	0	0	0	0	12	0
			F3C5F1-2-8	0	0	9	0	1	0

Table 5.3 (Cont'd) Phenotypic response and selected associated genotype of lentil parents, F_1, F_2 and F_3 generations to 750 g a.i. ha^{-1} of metribuzin applied at the 5 node growth stage under controlled environment conditions, Australia

Generation	Female	Male	Line	Observed number of plants					
				Phenotype (% Necrosis)			Genotype (KASP Assay *PsbA* $Ala_{251}Thr$)		
				0-5	6-50	51-100	T[a]	S[b]	A[c]
F_3	PBAFlash-P2	M009	F3C5RF1-2-1	0	0	4	0	4	0
			F3C5RF1-2-3	0	0	10	0	1	0
			F3C5RF1-2-4	0	0	10	0	2	0
			F3C5RF1-2-6	0	0	7	0	2	0
			F3C5RF1-2-7	0	0	9	0	1	0
			F3C5RF1-3-1	0	0	12			
			F3C5RF1-3-2	0	0	12			
			F3C5RF1-3-3	0	0	12			
			F3C5RF1-3-4	0	0	12			
			F3C5RF1-3-5	0	0	4	0	6	0
	M043	PBAFlash-P4	F3C4F1-1-2	3	0	0	4	0	0
			F3C4F1-1-4	3	0	0	10	0	0
			F3C4F1-1-5	11	0	0	12	0	0
			F3C4F1-1-6	5	0	0	12	0	0
			F3C4F1-1-10	11	1	0	12	0	0
			F3C4F1-2-1	0	0	8	0	12	0
			F3C4F1-2-2	0	0	8	0	12	0
			F3C4F1-2-5	0	0	12	0	11	0
			F3C4F1-2-7	0	0	9	0	12	0
			F3C4F1-2-9	0	0	10	0	12	0
	PBAFlash-P4	M043	F3C4RF1-1-2	0	0	12			
			F3C4RF1-1-3	0	0	5	0	7	0
			F3C4RF1-1-4	0	0	10	0	2	0
			F3C4RF1-1-7	0	0	10	0	2	0
			F3C4RF1-1-9	0	0	11			
			F3C4RF1-2-1A	0	0	11	0	1	0
			F3C4RF1-2-1B	0	0	7	0	4	0
			F3C4RF1-2-2	0	0	8	0	3	0
			F3C4RF1-2-3	0	0	6	0	6	0
			F3C4RF1-2-4	0	0	11	0	1	0

[a] Tolerant, as clustering and florescence data of line agreed with mutant parent

[b] Sensitive, as clustering and florescence data of line agreed with PBA Flash parent

[c] Ambiguous, as clustering and florescence data of line located between the two parental clusters

Table 5.4 Significance levels of terms from the decomposition of the site x variety treatment structure to determine the factors associated with differences in NAR (g DW m^{-2} LA d^{-1}), DW (t ha^{-1}), GY (t ha^{-1}) and plant survival (%) of lentil parents and BC_1F_2 (2015) and BC_1F_3 (2016) lines at two sites in SA

	2015			2016		
Terms [a]	NAR	DW (maturity)	GY	DW (anthesis)	GY	Plant survival
S	.	***	***	***	***	***
P	ns	***	***	ns	.	***
P:PF	.	***	***	***	***	***
P:PM	ns	ns	ns	ns	ns	***
P:SX	*	***	***	***	***	***
P:M	ns	ns	ns	**	***	***
P:M:RR	***	***	***	.	ns	***
P:SX:M	ns	**	*	ns	ns	***
P:SX:M:RR	**	***	***	***	***	***
P:SX:M:RR:D	ns	***	***	***	***	***
S:P	ns	ns	ns	ns	ns	ns
S:P:PF	ns	***	***	ns	ns	*
S:P:PM	ns	ns	ns	ns	ns	.
S:P:SX	ns	ns	ns	ns	**	ns
S:P:M	ns	ns	ns	ns	**	ns
S:P:M:RR	ns	ns	ns	.	ns	*
S:P:SX:M	ns	***	**	ns	ns	ns
S:P:SX:M:RR	ns	ns	ns	ns	ns	**
S:P:SX:M:RR:D	ns	ns	ns	ns	ns	ns

[a] S (Site); P (Parents), parents or lines; PF (Parent PBAFlash), PBAFlash or mutants; PM (Parent mutant), M009 or M043; SX (Sex), mutant parent maternal or paternal; M (Mutant), mutant line M009 or M043; RR (ReciprocalRep), one of six PBAFlash plants used in backcross; D (Duplicate), duplicate line from same BC_1F_1

*** $P<0.001$; ** $P<0.01$; * $P<0.05$; $P<0.1$; ns, not significant

Table 5.5 Mean and contrast estimates of NAR (g DW m^{-2} LA d^{-1}), DW (t ha^{-1}) and GY (t ha^{-1}) for lentil mutant (M009 or M043) x PBA Flash BC_1F_2 (2015) and BC_1F_3 (2016) lines grouped by tolerance (T) or sensitivity (S) to metribuzin and the percentage reduction due to metribuzin tolerance at two field sites of Riverton and Turretfield, SA in 2015 and 2016

Variate	Site	M009					M043				
		Line group mean[b]		Contrast estimate	*P*-value (Contrast	Reduction due to T	Line group mean[b]		Contrast estimate	*P*-value (Contrast	Reduction due to T (%)
		T	S	(T:S)±SE	estimate)	(%)	T	S	(T:S)±SE	estimate)	
NAR	NS[a]	3.76	4.47	-0.71±0.313	0.023	16	3.90	4.49	-0.594±0.225	0.008	13
DW (2015)	Riverton	5.75	7.83	-2.09±0.364	≤0.0001	27	5.82	7.76	-1.95±0.275	≤0.0001	25
	Turretfield	3.56	6.01	-2.46±0.232	≤0.0001	41	4.23	5.73	-1.51±0.159	≤0.0001	26
DW (2016)	NS[a]	3.01	3.66	-0.654±0.215	0.0024	18	2.70	3.77	-1.07±0.154	≤0.0001	28
GY (2015)	Riverton	3.05	4.06	-1.0±20.4	≤0.0001	25	2.99	4.15	-1.16±0.154	≤0.0001	28
	Turretfield	2.03	3.38	-1.33±0.146	≤0.0001	40	2.39	3.25	-0.86±0.101	≤0.0001	26
GY (2016)	Riverton	1.68	2.46	-0.77±0.16	≤0.0001	32	1.64	2.61	-0.97±0.12	≤0.0001	37
	Turretfield	3.36	4.18	-0.82±0.13	≤0.0001	20	3.49	4.81	-1.33±0.087	≤0.0001	28

[a] NS, site interaction not significant

[b] M009 (T, tolerant): BC_1F_2/F_3-2015/16-2, -3, -30; M009 (S, sensitive): BC_1F_2/F_3-2015/16-1, -6, -25, -27; M043 (T): BC_1F_2/F_3-2015/16-10, -11, -23, -32, -33; M043 (S): BC_1F_2/F_3-2015/16-9, -12, -24, -31, -34

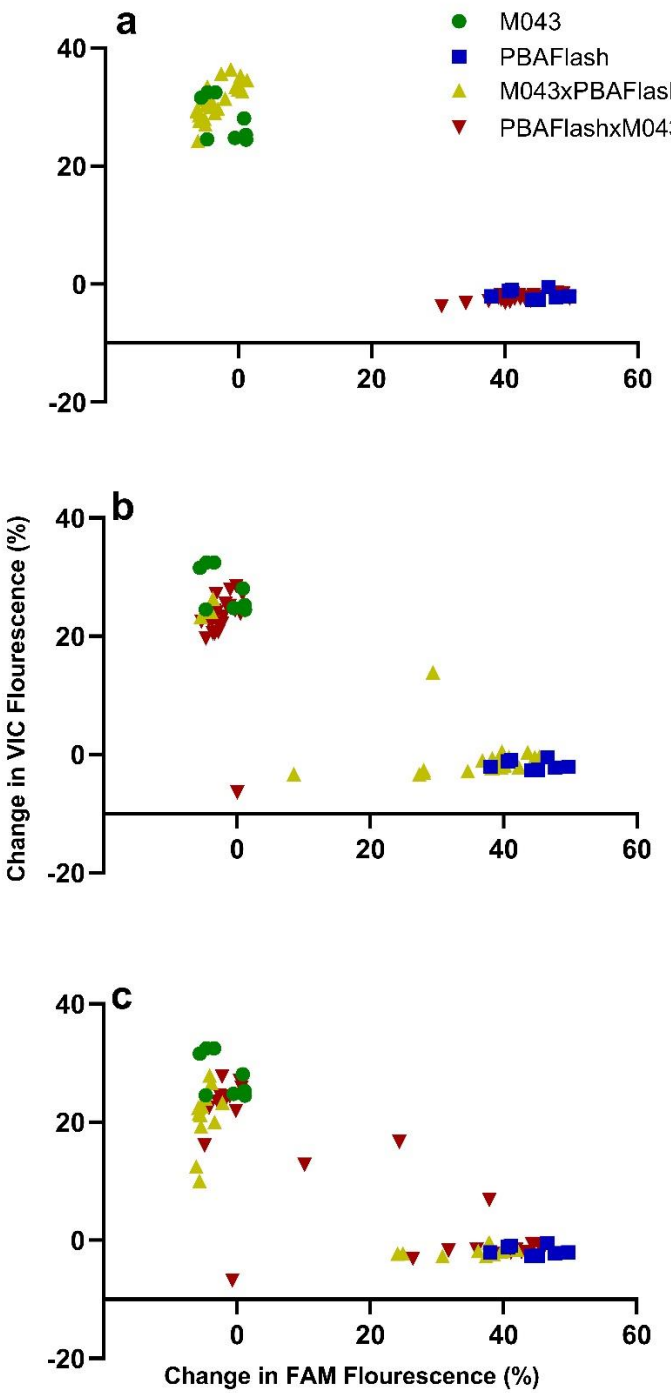

Fig. 5.1 Allelic discrimination plot of KASP SNP genotyping of populations from F_2 M043xPBAFlash (M002a-4 (a), M002a-26 (b), M002a-24 (c)) and reciprocal PBAFlash/M043 (M002b-31 (a), M002b-5 (b), M002b-26 (c)) and parents M043 and PBA Flash from the Canadian genetic study. The three populations within the F_2 and F_2 reciprocal represent an example of no apparent paternal leakage (a), majority apparent paternal leakage (b) and partial apparent paternal leakage (c) according to phenotyping results (Table 5.2).

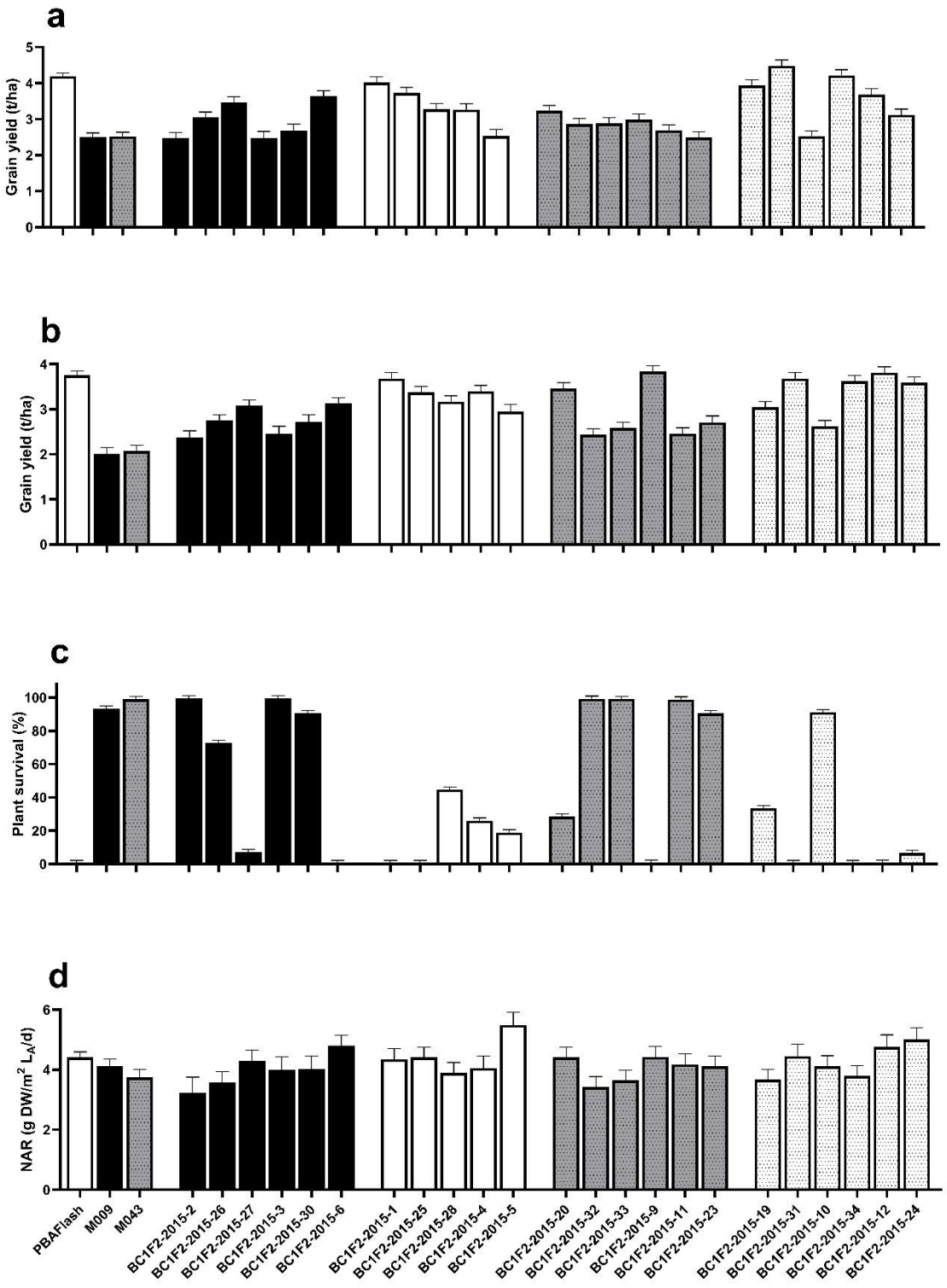

Fig. 5.2 Mean response of BC_1F_2 (a and d) and BC_1F_3 (b and c) lines and reciprocals derived from M009/PBAFlash//PBAFlash (lines -2 to -6, reciprocals -1 to -5) and M043/PBAFlash//PBAFlash (lines -20 to -23, reciprocals -19 to -24) compared to their parents for (a) GY 2015, (b) GY 2016, (c) plant survival following post-emergent metribuzin application (1,000 g a.i.ha^{-1}) 2016, (d) net assimilation rate 2015 at field sites Riverton and Turretfield, SA.

Chapter 6.

Field performance of metribuzin tolerant lentil (*Lens culinaris*).

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Contribution to the Paper	Designed, conducted all research experiments, assisted with data analysis, interpreted all data, and drafted and constructed the manuscript and was corresponding author.		
Overall percentage (%)	75%		
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.		
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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.

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Field performance of metribuzin tolerant lentil (*Lens culinaris*)

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Abstract

Lentil (*Lens culinaris*) is the major pulse crop grown in southern Australia; however, production is limited by weed competition. Two lentil genotypes (M009 and M043) with target site mutation providing tolerance to the photosystem II inhibitor herbicide metribuzin have been developed. Field experiments over two years at contrasting locations compared plant growth and grain yield (GY) response of M009 and M043 to the susceptible parent PBA Flash and an additional line, SP1333 with improved metribuzin tolerance, to post-emergent metribuzin application. Interactions between location_year (site), genotype and rate were highly significant for all variables measured. Logistic regression analysis showed clear differences in the response of M009 and M043 compared with both PBA Flash and SP1333 at all sites. Plant injury (% necrosis) increased, and plant density and GY decreased by 95 to 100% in PBA Flash to metribuzin applied at 840 g a.i. ha^{-1} across all sites; however, no or weak relationships existed between rate and these variables for M009 and M043. This suggested an agronomically useful level of metribuzin tolerance exists in M009 and M043. Plant dry weight (DW) of M009 and M043 decreased with increasing metribuzin rate at some sites and indicated a level of herbicide sensitivity at high rates on light textured soil types. Plant DW and GY was reduced by between 26 and 61% in the untreated control plots of M009 and M043 compared to

PBA Flash suggesting a fitness cost is associated with the tolerance. Agronomically, M043 was superior to M009.

Additional keywords: photosystem II inhibitor, post-emergent, mutation. plant injury, fitness cost

Introduction

Lentil is the major pulse crop grown in southern Australia with area expanding from less than 500 ha in 1993 to over 350,000 ha in 2017 (Brouwer 2002; ABARES 2018). Lentil is grown mainly as a cash crop, but has a number of rotational benefits (Long 2002). Weed competition is a major limitation to lentil production due to inherent low levels of crop competitiveness (Knott and Halila 1988; Muehlbauer *et al*. 1995; Hanson and Thill 2001). Grain yield (GY) reductions of up to 84% from weed competition have been reported in lentil (Mohamed *et al*. 1997; McDonald *et al*. 2007). Weed control in broad acre mechanised cropping systems, such as those used in Australia, is primarily achieved through the application of herbicides (Yennish *et al*. 2009). However, due to a lack of safe and effective herbicide options in lentil, variable levels of yield loss from crop phytotoxicity and weed control often occur (Brand *et al*. 2007).

The recent availability of lentil cultivars with tolerance to imidazolinone (IMI: acetohydroxyacid synthase [AHAS] inhibitors) herbicides has increased broadleaf weed control options in Australian lentil production (Materne *et al*. 2011). However, the overreliance of IMI herbicides in lentil and other rotation crops such as barley, wheat and canola has led to the development of multiple IMI resistant broadleaf weed species, including oriental mustard (*Sisymbrium orientale* L.), African mustard (*Brassica tournefortii* Gouan), and wild radish (*Raphanus raphanistrum* L.) (Boutsalis *et al*. 2016). Additionally, the IMI herbicides fail to effectively control a number of problematic weeds in lentil including milk thistle (*Sonchus oleraceus* L.), prickly lettuce (*Lactuca serriola* L.) and bifora (*Bifora testiculata* L. Spreng.) (Davey 2014). The

limited availability of effective herbicide options for broadleaf weed control in lentil has necessitated alternative herbicide control measures to be developed.

Recently, lentil genotypes M009 and M043, with tolerance to the photosystem II inhibitor herbicide metribuzin, have been developed through mutation and field selection (McMurray *et al*. 2019a). Metribuzin is an aminotriazinone or asymmetrical triazine (Hatzios and Penner 1988), controlling a range of grass and broadleaf weeds. Metribuzin has a relative lower risk of herbicide resistance development than the AHAS inhibitors, and is a registered herbicide in lentil in a number of countries. It is only registered for pre-emergent use in lentil in Australia due to severe crop damage from post-emergent application (White 2015). Metribuzin is widely used in Australia, but less on light textured soils that are low in organic matter due to a high risk of herbicide leaching and subsequent crop damage from post-application rainfall events (Sharom and Stephenson 1976; Peter and Weber 1985; Allen and Walker 1987; Gill and Bowran 1990; Kim and Feagley 1998). To avoid this risk, recommended application rates vary from 135 to 285 g a.i. ha^{-1} depending upon soil texture (White 2015). Reduced herbicide rates are used to lower the risk of crop damage in lentil, but this can lead to inadequate weed control (Gosheh and El-Shatnawi 2003).

Metribuzin tolerance in lentil genotypes M009 and M043 is due to an $Ala_{251}Thr$ substitution in the D1 protein encoded by the chloroplast *psbA* gene (McMurray *et al*. 2019b). Dose response studies have shown a high level of tolerance to post-emergent metribuzin applications for both mutants compared to parent PBA Flash under both controlled conditions and a preliminary field trial in Canada. The tolerance is currently being incorporated into Australian lentil breeding programs with the aim of developing adapted metribuzin tolerant cultivars (McMurray *et al*. 2019b). To date there has been no agronomic assessment of the tolerant lines in the field in Australia. Further, the effect of post-emergent application on GY of the tolerant mutants compared to the parent line is unknown.

The aim of this research was to assess the field performance of the metribuzin tolerant lentil genotypes and PBA Flash to an agronomically useful rate of post-emergent metribuzin. A germplasm

line, SP1333, with two to four times the tolerance to metribuzin as PBA Flash in growth room studies (McMurray *et al*. 2019a), was also included as it represented the highest level of metribuzin tolerance available from the natural variation in lentil.

Material and methods

Field experiments were located at Pinery and Paskeville, South Australia, in 2015 and 2016. These locations represent a light and medium textured soil type respectively, in the key lentil growing regions of South Australia. Details of the locations and management of the experiments are presented in Table 6.1.

A common seed source of each of the two tolerant lentil genotypes (PBA Flash-EMS10-11SVHM043 (M043) and PBA Flash-EMS10-11SK-12PAHM009 (M009)), parent genotype PBA Flash and germplasm line SP1333 was used in all experiments. PBA Flash is a widely grown cultivar in Australia due to its broad adaptation, earlier maturity, improved salinity and boron tolerance and greater harvestability than other commercial cultivars. It has a level of metribuzin tolerance that is representative of most Australian cultivars (McMurray *et al*. 2019a). SP1333 is a green lentil line from Argentina and was identified in metribuzin herbicide germplasm screens with a two to four-fold increase in tolerance compared to PBA Flash. Seed of all genotypes was adjusted for germination percentage and weight, and treated with thiram plus thiabendazole (P-Pickel T®, 360 g l^{-1} + 200 g l^{-1}, Crop Care, Pinkenba, Qld, Australia) seed dressing at 200 ml kg^{-1}. The trials were randomised complete block designs with four replicates, sown at a density of 120 seeds m^{-2} in plots measuring 1.35 m by 10 m using a small plot cone seeder with six narrow tynes on 0.225 m row spacing. All plots were direct drilled into standing cereal residue at a depth of 50 to 60 mm with basal fertilizer banded below the seed (Table 6.1). A steel roller trailed the seeder and provided a level and uniform surface for herbicide application. General insecticide and fungicide application in all experiments followed local agronomic practice for lentil. Selective grass herbicides were applied during the

vegetative growth phase to control annual ryegrass (*Lolium rigidum* Gaudin) and volunteer cereals (Table 6.1). Hand weeding was employed where needed to remove weed competition.

Metribuzin (Stacato, 750 g kg^{-1}, Sipcam Australia, Geelong, Vic, Australia) at rates of 0, 210, 420 and 840 g a.i. ha^{-1} was applied at the fifth node growth stage in PBA Flash with a hand-held sprayer at 107 L ha^{-1} using four Air Mix 110-015 low-pressure nozzles on 0.5 m spacing at an operating pressure of 240 kPa. Recommended pre-emergent application rates of metribuzin for lentil in Australia are 135 (sand), 210 (loam) and 285 g ha^{-1} on clay (White 2015). The 210 g ha^{-1} application rate, representing the midpoint of this range, was used as the lowest rate, as there was no recommendation for post-emergent use and neither site represented a true sand or clay soil type. An additional rate of 630 g ha^{-1} was included in 2016. Soil samples for soil moisture estimates were taken at the time of herbicide application by sampling soil across the sites in a W pattern at depths of 0 to 2 cm, 2 to 10 cm and 10 to 30 cm. All samples were weighed upon collection, oven dried at 80°C for 72 hours then re-weighed. Plant injury scores were recorded on a plot basis at 21 days after herbicide treatment (DAT) by estimating the percentage of necrotic tissue in the plot. Plant density was assessed 42 DAT by counting the number of alive plants in 1 m sections of the four internal rows at two locations in each plot. Plant dry weight (DW) was estimated 21 DAT and approximately 1 week after plants in the nil metribuzin treatment of PBA Flash had commenced flowering (50% of plants within the plot with at least one flower). Cuts of 0.5 m of the four internal rows were taken at two locations in each plot, the two sub-samples combined, and oven-dried at 80°C for 48 hours. Grain yield was estimated by harvesting the lentils with a small plot harvester at maturity.

Data analysis

All measured variables were initially analysed using linear mixed models, and multi-environment trial (MET) analysis was conducted across sites (location_year) in the R environment (R Core Team, 2014) using the ASReml-R software (ASReml. Release 4.1. VSN International Ltd 2014) (Butler *et al*. 2009). Raw data for plant injury underwent square root transformations to meet model assumptions. Additional site specific extraneous fixed and random terms were included in the

analysis as required. The residual errors for each site were modelled using spatial methods. The method of residual maximum likelihood (REML) was used for variance parameter estimation. The relationship between the measured variables and herbicide rate for each genotype was examined using non-linear regression analysis in GraphPad PRISM Version 8.00. Four-parameter log-logistic models were applied to all variables using either:

$$Y=100/[1+10\text{^}((\log ED_{50}-X)*b)] \quad (1)$$

$$Y=a/[1+10\text{^}((\log ED_{50}-X)*b)] \quad (2)$$

where Y is the dependent variable (% necrosis (1), DW (2), plant density (2), GY (2)) at metribuzin rate X, a is the maximum value for the dependent variable (the asymptote), b indicates the slope of the relationship and ED_{50}, the effective dose of metribuzin required to reduce the dependent variable by 50%.

Results

Analysis of the three-way interaction between site, genotype and metribuzin rate was highly significant ($P<0.001$) for plant injury, DW, plant density and GY and largely due to the variable response of PBA Flash and SP1333 to metribuzin rate across sites. Logistic dose response curves were fitted to all variables to explain the response of lentil genotypes to metribuzin rate at each location within each year. Estimates of regression parameters b (slope) and ED_{50} are presented in Table 6.2 and back transformed data is shown in Fig. 6.1 to 6.5. Regression analysis of all variables showed clear differences between the response of M009 and M043 to metribuzin when compared with PBA Flash and SP1333 (Table 6.2). PBA Flash and SP1333 generally had higher measures of plant damage at Pinery than Paskeville, and in 2016 compared to 2015.

PBA Flash and SP1333 showed a strong positive relationship between metribuzin rate and % plant necrosis compared with low levels of response in the mutant genotypes (Fig. 6.1). Metribuzin applied at the highest rate (840 g ha^{-1}) resulted in necrosis levels of greater than 85% in PBA Flash and SP1333, but only up to 15% in M009 and M043, across all sites. Estimated ED_{50} values of PBA

Flash ranged from 445 (Paskeville_15) to 151 g ha^{-1} of metribuzin (Pinery_16). In comparison, ED_{50} values of SP1333 ranged from 545 (Paskeville_15) to 391 g ha^{-1} (Pinery_16), and apart from similar levels at the former, were two to four times higher than those of PBA Flash. At all sites the ED_{50} values of M009 and M043 were greater than the maximum rate applied and suggested that a high level of foliar metribuzin field tolerance exists in these genotypes across seasons and soil types.

In the absence of metribuzin application, the mean DW at 21DAT was 38 and 36% lower in M009 and M043 respectively, compared to PBA Flash (175kg ha^{-1}) across sites. Genotype SP1333 had the highest levels of DW 21 DAT in the untreated control plots (211 kg ha^{-1}). Generally, all genotypes had higher DW 21 DAT values at Paskeville than Pinery, and higher DW 21 DAT in 2015 than 2016 (Fig. 6.2). Mean plant DW anthesis of M009 and M043 in the untreated control metribuzin treatment were similar and approximately 45% lower than PBA Flash (5059 kg ha^{-1}) across sites. The DW of genotype SP1333 was 25% lower than PBA Flash in the untreated control plots at this growth stage, despite having similar values at 21DAT. Similarly, to DW 21 DAT, DW anthesis values of the untreated genotypes were higher at Paskeville than Pinery in both years (Fig. 6.3).

The ED_{50} values for DW 21 DAT of PBA Flash and SP1333 varied from 116 to 4 and 368 to 219 g ha^{-1} of metribuzin, respectively, across sites (Table 6.2). Plant DW 21 DAT of the tolerant genotypes was less responsive to metribuzin rate (Fig. 6.2). At 210 g ha^{-1} of metribuzin reductions in DW 21 DAT, when compared to the untreated control, varied from 15 to 30% in M009 and 0 to 18% in M043, and were less than for PBA Flash (62 to 80%) and SP1333 (35 to 52%). In comparison, reductions at the 840 g ha^{-1} of metribuzin, when compared with the untreated control, were approximately 40% in both tolerant genotypes, with the exception being M043 in 2016 where a lower reduction of 20% (Paskeville) and no reduction (Pinery) occurred. In contrast, PBA Flash and SP1333 incurred reductions in DW of greater than 70% at all sites for the same rate comparison. As found for plant injury, ED_{50} estimates of DW 21 DAT for the tolerant genotypes were greater than the maximum rate applied, or unable to be calculated due to a lack of relationship between the dependent and independent variables. Low regression coefficient values at both locations in 2016 was further

indication of a poor relationship between metribuzin rate and DW 21DAT for the tolerant genotypes (Table 6.2).

Estimates of DW ED_{50} values of PBA Flash at anthesis ranged from 238 to 95 g ha^{-1} and were higher than those measured at 21 DAT at all sites suggesting a level of plant recovery had occurred. The DW ED_{50} values of SP1333 at anthesis were generally similar to their respective values at 21 DAT for each site and ranged from 1.5 to 2.5 times higher than those of PBA Flash. Genotypes M009 and M043 were clearly more tolerant of metribuzin than PBA Flash and SP1333 at anthesis (Fig. 6.3). Estimates of DW ED_{50} values were either higher than the maximum rate applied or unable to be calculated for these tolerant genotypes. However, they were close to the highest application rate at the light textured site of Pinery in 2015. A decrease in anthesis DW of approximately 45% occurred at the 840 g ha^{-1} rate when compared with the untreated control at this site, suggesting a level of susceptibility in both genotypes at very high rates on lighter soil types. There was no relationship between metribuzin rate and DW at anthesis in M043 at Paskeville in 2015. However, DW of M009 was reduced by 21% at 210 g ha^{-1} but only 33% at 840 g ha^{-1} compared to the untreated control. The foliar disease botrytis grey mould (casual agents *Botrytis cinerea* & *B. fabae*) was observed in M009 and SP1333 at this site prior to control with a routine foliar fungicide application in early spring. However, the disease was not observed in M043 or PBA Flash and may have compromised the performance of M009 and SP1333 to metribuzin at this site.

There were clear genotype differences in the relationships between plant density and metribuzin rate across all sites (Fig. 6.4). Non-significant estimates of *b* slope for genotypes M009 and M043 indicated that they were highly tolerant (Table 6.2). Plant density decreased strongly with metribuzin rate for PBA Flash and SP1333 at all sites. Plant density values for these two genotypes were generally less than 10 plants m^{-2} at 840 g ha^{-1} of metribuzin, with SP1333 at Paskeville_2015 being the exception (44 plants m^{-2}).

Mean GY of PBA Flash without metribuzin application was 1.7 and 1.9 t ha^{-1} at Paskeville and Pinery respectively, in 2015, and 3.7 t/ha at both sites in 2016. In the absence of metribuzin,

grain yields of all three genotypes with improved tolerance were significantly less than PBA Flash ranging from 35 to 55% lower in SP1333, 32 to 61% lower in M009 and 26 to 49% lower in M043. The relationships between GY and metribuzin rate for all genotypes followed similar patterns to that of plant injury and plant density (Fig. 6.5). PBA Flash incurred strong and similar reductions in GY to increasing rates of metribuzin at all sites. The GR_{50} estimates of PBA Flash ranged from 405 (Paskeville_15) to 251 g ha^{-1} (Pinery_15) and were generally less than half of the values obtained for SP1333. There was no relationship between GY and metribuzin rate for SP1333 at Paskeville_15 indicating a level of recovery had occurred from the initial plant damage observed at this site. At the highest metribuzin application rate, GY of PBA Flash was reduced by at least 95%, when compared with the untreated control, at all sites. There was no significant relationship between GY and metribuzin rate for genotypes M009 and M043 at all sites. This result supports those of the other variables and suggests both genotypes have higher levels of metribuzin tolerance than PBA Flash and SP1333 under field conditions. However, at both Pinery sites the GY of the tolerant genotypes after treatment with 840 g ha^{-1} of metribuzin was reduced by approximately 20% compared to the untreated control. This suggests a level of susceptibility to high rates of metribuzin on lighter textured soil and agrees with the DW findings at this location.

Discussion

The development of lentil genotypes with a *psbA* target site mutation conferring high levels of metribuzin tolerance provides plant breeders with an alternative herbicide tolerance trait to IMI, for improving weed control options. However, these genotypes had not been evaluated under field conditions in Australia. Evaluation over two years at contrasting field locations showed unlike PBA Flash and SP1333, M009 and M043 exhibited an agronomically useful level of tolerance to post-emergent applications of metribuzin under Australian conditions.

Metribuzin is only recommended for pre-emergent use in lentil at rates of 135 to 285 g ha^{-1} in Australia (White 2015). Minimal plant injury was observed across all environments in the mutant

genotypes when metribuzin was applied post-emergent at 210 g ha^{-1} (0 to 7% necrosis in M009 and 0 to 4% in M043). In contrast, plant necrosis levels were as high as 81% in PBA Flash and 12% in SP1333 for the same treatments. The highest level of plant necrosis observed in the mutant genotypes was at Pinery_15 to 840 g ha^{-1} (15.6% in M009 and 11% in M043). This rate is equivalent to three times the maximum pre-emergent label rate for lentil on a clay soil type, and resulted in necrosis levels of 100% in PBA Flash and 97% in SP1333. These findings show that the mutant genotypes have an agronomically useful level of metribuzin tolerance compared to Australian cultivars. Furthermore, there was no significant relationship between metribuzin rate and the variables GY and plant density in the tolerant genotypes. However, GY reductions of 10 to 20% when compared to the nil metribuzin treatment occurred in both genotypes at the highest rate at Pinery, and indicated a level of crop sensitivity to high rates on light textured soil types.

Despite the observed improvement in tolerance to metribuzin, DW of M009 and M043 was reduced by increasing rates of metribuzin at some sites. The average ED_{50} values of DW 21 DAT for M009 and M043, at the three sites where DW was reduced by metribuzin rate, were 1,243 and 1,479 g ha^{-1} of metribuzin respectively, and outside the range of rates applied. These values were lower than those reported for M009 and M043 in multiple controlled environment dose response experiments (2,239 to 4,775 for M009 and 2,913 to 7,024 g ha^{-1} for M043; McMurray *et al*. 2019a,b). However, the field result may have underestimated the average ED_{50} value of both genotypes as it failed to include estimates from Pinery_16 due to a lack of response between DW and metribuzin rate at this site. Average ED_{50} values of DW 21 DAT for PBA Flash and SP1333, from these same three sites, were 65 and 332 g ha^{-1}, respectively. In the case of PBA Flash these values were also lower than reported in the controlled environment studies (100 to 208 for PBA Flash and 219 to 438 g ha^{-1} for SP1333; McMurray *et al*. 2019a,b). Average anthesis DW ED_{50} estimates for M009 and M043 were similar or slightly lower than those achieved at 21 DAT and suggests plants had not recovered from the initial plant damage by this growth stage. This finding could explain the low levels of GY loss which occurred at high metribuzin rates at Pinery in the tolerant genotypes.

However, as it was not possible to estimate the ED_{50} for grain yield at any site in either tolerant genotype, a level of late plant recovery is likely to have occurred at all sites as found in barley by Kleemann and Gill (2007).

Despite the lower genotype DW ED_{50} estimates under field conditions, the relative level of improvement in tolerance between the tolerant genotypes and PBA Flash was comparable with those reported in growth room studies. Genotypes M009, M043 and SP1333 were improved 19, 23 and 5-fold, respectively over PBA Flash in the field, and 11 to 22, 27 to 48, and 2 to 4-fold, respectively in growth room studies. No other reports of crops with a target site tolerance to metribuzin are available to make comparisons. However, smaller improvements in metribuzin tolerance of up to six times that of the intolerant genotype have been reported in field experiments with wheat (*Triticum aestivum* L.) (Kleemann and Gill 2007), soybean (Barrentine *et al*. 1982), field pea (*Pisum sativum* L.) (Al-Khatib *et al*. 1997), and narrow-leafed lupin (*Lupinus angustifolius* L.) (Si *et al*. 2009).

Plant DW and GY of the mutant genotypes were lower than that of PBA Flash in the untreated control treatments across all environments. Anthesis DW was 39 to 52% lower and GY 26 to 61% lower, and these comparable results suggest that the source of the reduced GY is likely to be reduced plant biomass accumulation. Chloroplastic *psbA* gene mutations such as the $Ser_{264}Gly$ in triazine tolerant (TT) canola reduce plastoquinone binding, compromising photosynthesis and leading to a fitness cost in the tolerant plant (Powles and Yu 2010). Research with near-isogenic and reciprocal canola hybrids identified yield penalties of 20 to 30% associated with TT canola (Beversdorf *et al*. 1988, Robertson *et al*. 2002). A fitness penalty was identified in research with reciprocal lentil lines of M009 and M043 backcrossed to PBA Flash producing a GY reduction of 20 to 40% (McMurray *et al*. unpublished data). The latter study suggested a fitness cost similar to that in TT canola exists in the metribuzin tolerant lentil genotypes, partially explaining the lower yields of the tolerant genotypes in this study. Generally, the two mutant genotypes performed similarly to each other in terms of their response to metribuzin across environments. However, M009 did incur greater reductions than M043 for DW (21 DAT and anthesis), plant density and GY at some sites, although

these reductions were not always at the same site. Agronomically, M009 was more susceptible to the foliar disease botrytis grey mould than M043 and more susceptible to bromacil and atrazine herbicides in controlled environment experiments (McMurray *et al*. 2019b). M043 appears to be the superior line for use by plant breeders incorporating the metribuzin tolerance trait into adapted lines.

Despite agronomically superior levels of field metribuzin tolerance identified in M009 and M043 compared to PBA Flash, questions remain around their relatively poor agronomic performance compared to susceptible cultivars. Intercrossing M043 with a range of adapted elite lentil lines is required to improve the agronomic type. A concerted breeding effort with targeted lentil parents addressing the low biomass and yield constraints could potentially reduce the productivity gap, as reported for TT canola in Australia (Zhang *et al*. 2016). Photosystem II inhibitors are an integral part of lentil production in Australia despite current levels of genotype susceptibility. This reliance is likely to remain into the future due to the increasing frequency of occurrence of IMI resistant weeds (Boutsalis *et al*. 2016). The importance of weed management in lentil crops, and the continued success of the TT canola industry in Australia, support the potential for developing metribuzin tolerant lentil cultivars.

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Table 6.1. Details of site and trial management of the lentil post-emergent metribuzin spray application experiments conducted at two field locations in South Australia in 2015 and 2016

		Paskeville		Pinery	
		2015	2016	2015	2016
Soil type[A]		Hypercalcic Calcaresol		Lithocalcic Calcaresol	
Soil texture		Clay loam/light clay		Light sandy loam/sandy clay loam	
pH (CaCl2)	0-10cm	6.1	7.7	7.8	7.8
	10-60cm	7.8	7.9	8.1	8.2
Organic carbon	0-10cm	2.22	2.54	1.77	1.31
	10-60cm	0.82	-	0.49	0.38
Soil moisture (%)	0-2cm	16.0	21.0	16.2	17.0
	2-10cm	16.5	16.8	15.3	12.7
	10-30cm	18.2	16.6	15.9	16.7
Rainfall (mm)	Annual	291.6	612.6	352.0	562.5
	Apr.-Oct.	211.6	431.2	236.2	389.4
Sow date		26 May	31 May	21 May	20 May
Basal fertiliser		7kg N ha^{-1}, 15 kg P ha^{-1}, 1.8 kg Zn ha^{-1}			
Herbicides					
Pre-sowing	Trifluralin (480 g L^{-1}) 1.2 L ha^{-1}				
Post-sowing	Quizalofop-P-ethyl[B] (200 g L^{-1}) 0.15 L ha^{-1}	June 29 July 28 Aug 16		June 26	
	Clethodim[B] (240 g L^{-1}) 0.5 L ha^{-1}	June 29 July 28 Aug 16	July 18	June 26	July 1
	Butroxydim[C] (250 g kg^{-1}) 160 g ha-1		Aug 8		
	Haloxyfop[B] (520 g L^{-1}) 0.075 L ha^{-1}		July 18		July 1
Treatments	Metribuzin (750 g kg^{-1})	June 29	July 1	June 25	June 19

[A] Isbell, 2016

[B] Combination vegetable oil and non-ionic surfactant (1% (v/v) 704 g L-1 ethyl and methyl esters of canola oil fatty acids and 196 g L-1 alcohol alkoxylate; Hasten; VicChem, Coolaroo, Vic, Australia) added

[C] Petroleum oil (1% (v/v) 471 g L^{-1} paraffin oil; Supercharge®; Crop Care, Murarrie, Qld, Australia) added

Table 6.2 Estimates of regression parameter *b* (slope ±SE) and ED_{50} (the effective dose of metribuzin required to reduce the dependent variable by 50%) (±SE) for plant injury (% necrosis), plant dry weight 21 days after treatment and anthesis (kg ha^{-1}), plant density (plants m^{-2}) and grain yield (t ha^{-1}) of lentil genotypes treated with metribuzin herbicide (0, 210, 420, 630, 840 g ai ha^{-1}) at two field locations in South Australia in 2015 and 2016. Back transformed ED_{50} estimates in parentheses

Genotype	Paskeville 2015			Pinery 2015			Paskeville 2016			Pinery 2016		
	b	R^2	Log ED_{50}	*b*	R^2	Log ED_{50}	*b*	R^2	Log ED_{50}	*b*	R^2	Log ED_{50}
							Plant injury[A]					
PBAFlash	4.37±1.46	0.99	2.65±0.026 (445)	5.24±0.04	1.00	2.36±0.000 (227)	5.41±0.65	1.00	2.30±0.004 (200)	4.42±0.25	1.00	2.18±0.008 (151)
SP1333	4.66±0.06	1.00	2.74±0.002 (545)	5.19±0.28	1.00	2.61±0.003 (402)	4.24±0.37	1.00	2.59±0.009 (392)	4.63±1.06	0.99	2.59±0.022 (391)
M009	2.56±0.01	1.00	3.27±0.001 (1846)	2.53±0.19	1.00	3.21±0.023 (1636)	0.66±0.63	0.56	5.13±2.25	0.17±0.06	0.92	8.35±1.99
M043	4.19±0.43	1.00	3.24±0.033 (1737)	2.60±0.18	1.00	3.28±0.025 (1903)	9.44±5.29	0.96	3.15±1.25 (1402)	0.53±0.84	0.92	4.95±0.85
							Dry weight 21 days after treatment[B]					
PBAFlash	-0.81±0.04	1.00	2.07±0.026 (116)	-0.51±0.15	1.00	1.87±0.24 (74)	-0.31±0.24	0.95	0.58±2.47 (3.8)	-0.52±0.38	0.93	1.17±1.13 (14.7)
SP1333	-1.20±0.11	1.00	2.57±0.11 (368)	-0.85±0.12	1.00	2.42±0.047 (263)	-0.61±0.15	0.94	2.22±0.13 (364)	-1.00±0.18	0.94	2.34±0.071 (219)
M009	-0.91±0.41	0.95	2.97±0.17 (937)	-0.86±0.04	1.00	3.20±0.023 (1569)	-0.82±0.29	0.71	3.09±0.14 (1223)	-0.11±0.58	0.36	4.00±13.01
M043	-1.19±0.73	0.91	3.17±0.12 (1484)	-0.94±0.01	1.00	3.04±0.004 (1102)	-1.40±0.91	0.40	3.27±0.25 (1851)	-1.18±2.39	0.06	3.63±1.46
							Dry weight anthesis[B]					
PBAFlash	-1.72±0.76	0.98	2.38±0.11 (238)	-3.83±0.013	1.00	2.30±0.000 (198)	-1.93±0.19	1.00	2.04±0.046 (111)	-2.89±0.28	1.00	1.98±0.034 (95)
SP1333	-2.35±0.09	1.00	2.67±0.008 (463)	-2.98±0.21	1.00	2.45±0.013 (280)	-2.24±0.24	1.00	2.45±0.028 (279)	-2.66±0.30	1.00	2.28±0.019 (189)
M009		ns[C]		-1.81±0.016	1.00	2.95±0.002 (891)	-1.36±0.34	0.97	3.31±0.074 (1346)	-0.69±0.58	0.82	3.14±0.38 (1367)
M043		ns		-2.90±0.76	0.99	2.96±0.26 (914)	-1.28±0.73	0.88	3.27±0.23 (1865)	-2.06±0.96	0.92	3.01±0.078 (1018)
							Plant density[B]					
PBAFlash	-1.88±0.36	0.92	2.47±0.048 (295)	-4.73±0.75	0.97	2.42±0.023 (265)	-3.66±0.85	0.94	2.36±0.029 (231)	-2.63±0.79	0.93	2.25±0.052 (178)
SP1333	-1.33±0.28	0.86	2.79±0.058 (691)	-4.42±0.95	0.96	2.66±0.018 (455)	-3.75±0.16	1.00	2.54±0.007 (345)	-4.36±0.79	0.94	2.66±0.021 (460)
M009	-1.28±1.11	0.32	3.42±0.439 (2592)	-3.10±11.79	0.08	3.32±1.45 (2070)		ns			ns	
M043		ns			ns		-2.71±6.30	0.06	3.30±0.89 (2016)		ns	
							Grain yield[B]					
PBAFlash	-2.70±0.51	0.94	2.61±0.035 (405)	-3.44±0.30	0.99	2.40±0.13 (251)	-4.03±0.41	0.98	2.53±0.017 (341)	-3.17±0.33	0.98	2.41±0.041 (259)
SP1333		ns		-3.80±0.76	0.95	2.66±0.023 (462)	-6.90±1.25	0.91	2.86±0.012 (729)	-6.42±1.42	0.93	2.76±0.018 (577)
M009		ns		-0.72±0.52	0.40	3.83±0.71		ns		-0.39±0.55	0.24	4.84±2.80
M043	-2.89±7.86	0.13	3.24±0.81 (1743)	-2.23±0.18	0.53	3.18±0.18 (1516)		ns			ns	

[A] Equation fitted: Y=100/(1+10^((LogED_{50}-X)**b*)) where *b* is the slope; [B] Equation fitted: Y=*a*/(1+10^((LogED_{50}-X)**b*)) where *a* is the upper limit and *b* the slope; [C] ns = fit not significant

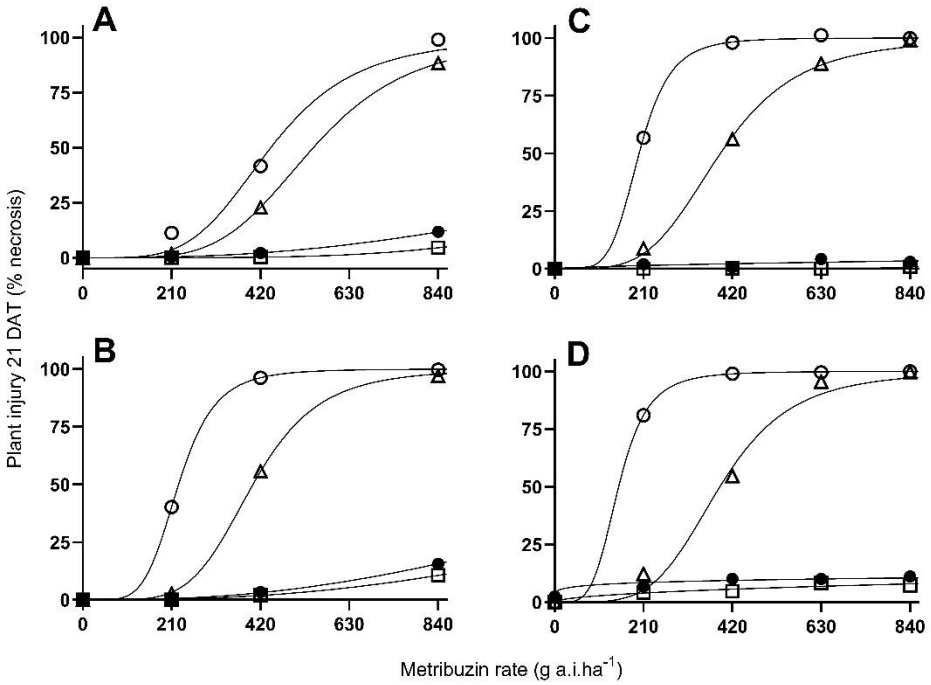

Fig. 6.1. The effect of post-emergent metribuzin application on plant injury of four lentil genotypes; PBA Flash (○) SP1333 (△) M009 (●) M043 (□), at (A) Paskeville_2015, (B) Pinery_2015, (C) Paskeville_2016, and (D) Pinery_2016.

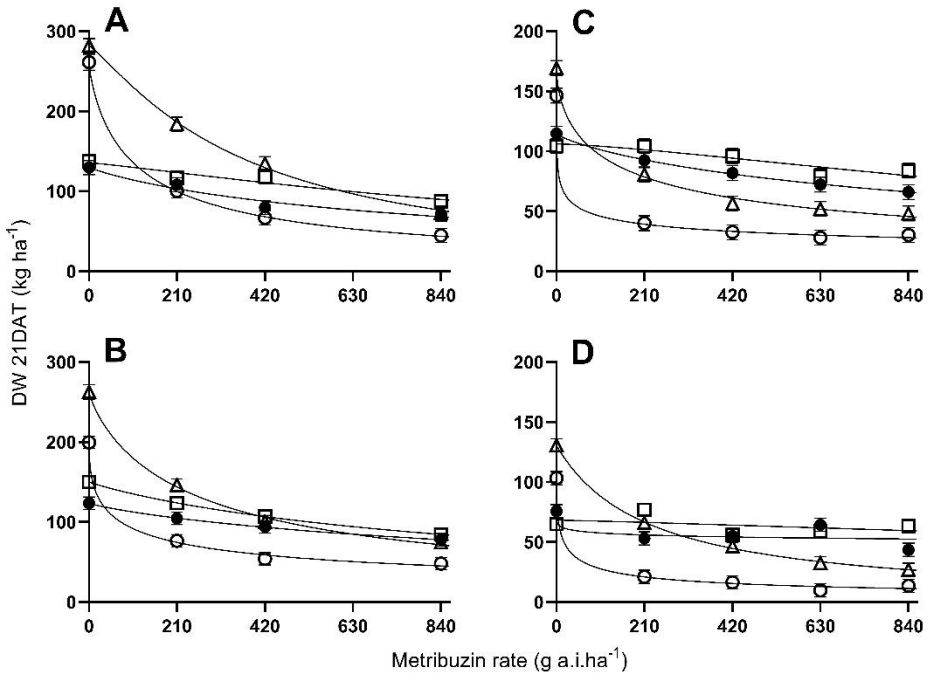

Fig. 6.2. The effect of post-emergent metribuzin application on plant dry weight (DW) 21 days after treatment (DAT) of four lentil genotypes; PBA Flash (○) SP1333 (△) M009 (●) M043 (□), at (A) Paskeville_2015, (B) Pinery_2015, (C) Paskeville_2016, and (D) Pinery_2016.

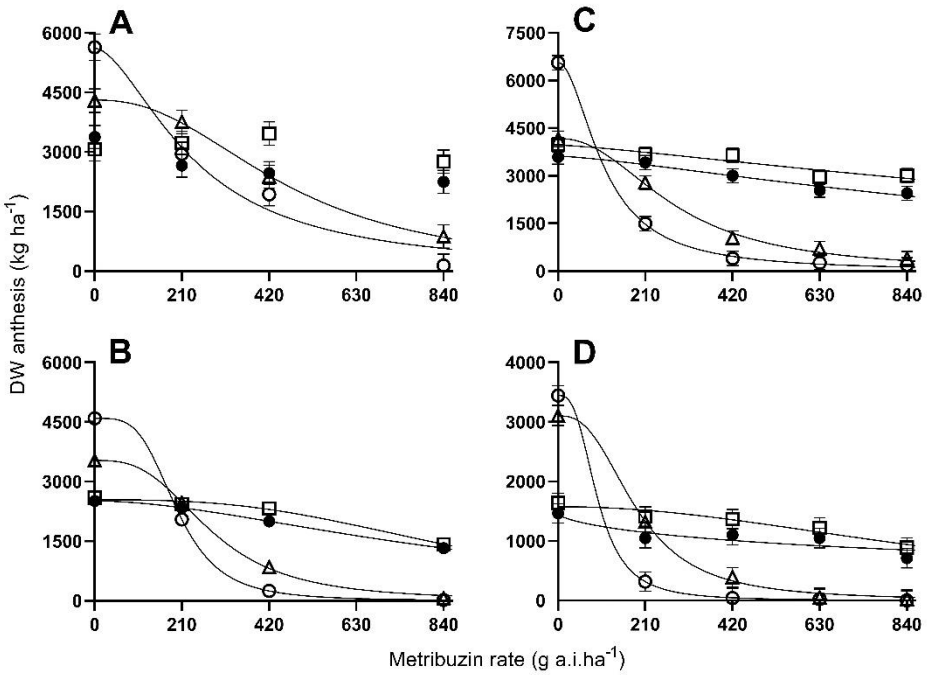

Fig. 6.3. The effect of post-emergent metribuzin application on plant dry weight (DW) anthesis of four lentil genotypes; PBA Flash (○) SP1333 (△) M009 (●) M043 (□), at (A) Paskeville_2015, (B) Pinery_2015, (C) Paskeville_2016, and (D) Pinery_2016.

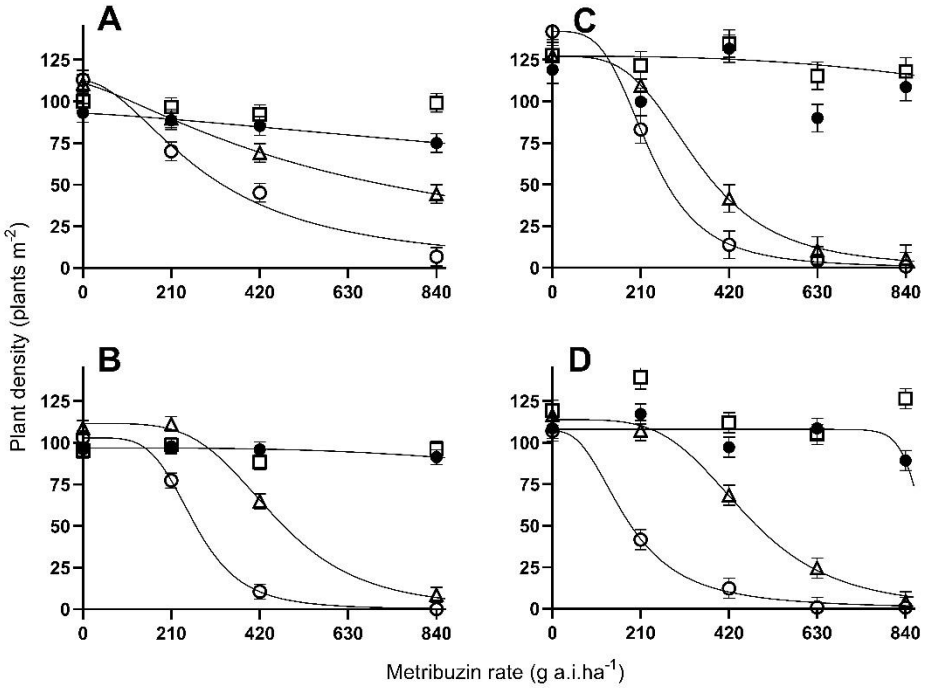

Fig. 6.4. The effect of post-emergent metribuzin application on plant density of four lentil genotypes; PBA Flash (○) SP1333 (△) M009 (●) M043 (□), at (A) Paskeville_2015, (B) Pinery_2015, (C) Paskeville_2016, and (D) Pinery_2016.

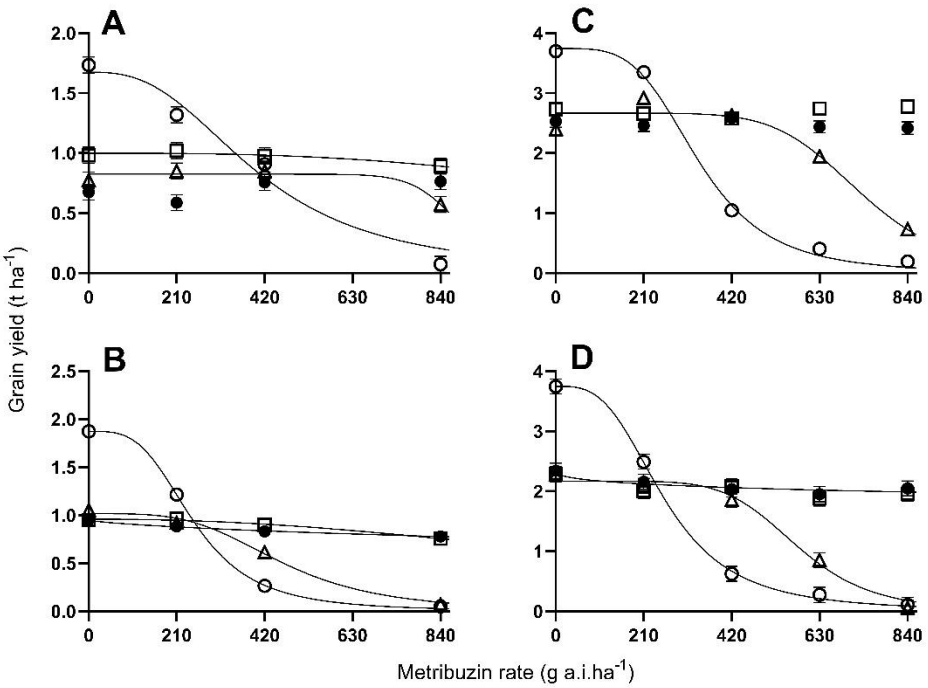

Fig. 6.5. The effect of post-emergent metribuzin application on grain yield of four lentil genotypes; PBA Flash (○) SP1333 (△) M009 (●) M043 (□), at (A) Paskeville_2015, (B) Pinery_2015, (C) Paskeville_2016, and (D) Pinery_2016.

Chapter 7.

General Discussion

GENERAL DISCUSSION

Weeds and their cost-effective control are a significant limitation to lentil (*Lens culinaris* Medik.) production worldwide. In general, changing only one management practice has little effect on weed dynamics, but integrating management methods allows the utilization of techniques that individually are considered ineffective (Young *et al*. 2000). The escalating frequency of weeds resistant to imidazolinone (IMI: acetohydroxyacid synthase [AHAS] inhibitors) herbicides in Australia and North America following the release of multiple IMI tolerant crops, including lentil, lends support to this statement (Beckie and Tardif 2012; Beckie *et al*. 2013, Boutsalis *et al*. 2016). However, the rapid uptake and production dominance of IMI tolerant lentil cultivars in these countries suggests that the continued production of this crop, in broad acre mechanised systems at least, will continue to rely on chemical weed control. The use of alternative chemical weed control technologies to IMI, such as metribuzin, in combination with the implementation of effective cultural and mechanical weed control tactics, is suggested as a sustainable strategy to maximise weed suppression and reduce the reliance on any single one tactic in lentil (Redlick *et al*. 2017).

In this study, the novel development and characterisation of genotypes with an induced *psbA* ($Ala_{251}Thr$) target site mutation responsible for high level tolerance to the photosystem II (PSII) inhibitor herbicide, metribuzin is reported. This high level of tolerance provides lentil breeding programs with an alternative or complimentary herbicide group to IMI and will enable a novel post-emergent metribuzin application to occur in Australia. Additionally, the discovery and agronomic validation of a non-target site intermediate level of metribuzin tolerance via the germplasm line SP1333 provides an alternative source of metribuzin tolerance that will aid in reducing current crop phytotoxicity risks associated with the existing pre-emergence metribuzin usage pattern.

Metribuzin was first reported in 1968 however, it is still routinely used in Australian lentil production despite the inherent risk of crop phytotoxicity and the recent availability of IMI tolerant cultivars (Friesen and Wall 1986; Muehlbauer *et al*. 1995; Yasin *et al*. 1995; Gosheh and El-Shatnawi

2003; Elkoca *et al*. 2005; Materne *et al*. 2011). The increasing occurrence of IMI resistant weeds and the need to use an alternative mode of action to the AHAS inhibitors are the likely present-day drivers of this usage pattern. Metribuzin is registered for post-emergent use in Canada, but many application restrictions exist due to crop safety concerns (Fedoruk *et al*. 2011; Redlick *et al*. 2017; SMA 2019). Research aimed at improving lentil cultivar tolerance to metribuzin in a similar manner to this study is occurring in Canada (Meier 2016) and highlights the importance placed on this herbicide in ongoing lentil production worldwide.

The level of tolerance of commercial lentil cultivars to metribuzin is insufficient for safe post-emergent application under Australian conditions, with grain yield losses of up to 67% when label approved pre-emergent rates were applied (see Chapter 2). The field evaluation trial in Canada comparing Australian and Canadian cultivars under conditions of high and low metribuzin damage provided the first understanding that cultivars from both countries had similar levels of field tolerance to metribuzin (see Chapter 3). This suggests that reasons for the recommendation of a post-emergent metribuzin application in lentil in Canada, but not Australia, are due to differences in soil types and environmental conditions, and not because of inherent genotypic differences in tolerance of cultivars between the two countries.

The improved knowledge gained on weather and soil conditions conducive to metribuzin phytotoxicity from post-emergent application in lentil presented in Chapter 2 of this study, contribute to the broader understanding of the behavior of metribuzin in the environment. It is well known that plant phytotoxicity to metribuzin is increased under soil conditions of low moisture, high pH, low organic matter and high sand content (Ladlie *et al*. 1976; Savage 1976; Sharom and Stephenson 1976; Peter and Weber 1985; Gill and Bowran 1990). However, prior to this study there was little reported on the influence of weather conditions on metribuzin induced plant damage under field conditions in Australia. Weather characteristics of reduced light intensity, increased relative humidity and changes in ambient temperature were associated with increased post-emergent metribuzin phytotoxicity in lentil in this study and are likely to be transferrable to lupin and field pea, where this application

timing for metribuzin occurs routinely. Conversely, these conditions are likely to increase herbicide uptake and plant phytotoxicity and could be used advantageously to manage difficult to control weeds. This practice would only be successful where a high level of selectivity between the crop and target weed species exists, as could be the case for the two herbicide tolerant lentils developed in this study.

The knowledge of soil and weather conditions favouring damage to lentil from metribuzin application can be used by plant breeders interested in developing field screens and nurseries for selecting lines with improved tolerance to this herbicide. This will be particularly useful when incorporating the intermediate level of tolerance from SP1333 into superior germplasm. Only relatively low levels of differentiation in herbicide tolerance exist between SP1333 and sensitive lines (see Chapters 3 and 6) and uniform low levels of plant phytotoxicity will be required to accurately identify genotypes with improved tolerance. The findings in Chapter 2 suggest that a light textured sandy soil type, high in pH and low in organic matter content would be suited for a screening nursery. Seed should be sown at a depth of less than 5 cm and early in the growing season when soil moisture levels are low. Metribuzin would ideally be applied prior to rainfall events when humidity is high and light intensity is relatively low. In contrast, the high level of target site tolerance in the tolerant genotypes allows them to be easily differentiated from sensitive lines through the use of high herbicide rates or via the use of specific molecular markers (see Chapters 3,4 and 6).

The complexity of multiple soil and weather conditions contributing to the level of plant phytotoxicity from metribuzin made it difficult to isolate any one factor responsible for crop damage in lentil across environments in southern Australia. However, heavy rainfall within 10 days of herbicide application, particularly on light textured soils or where soil moisture was low, was strongly linked to plant damage. The rainfall amount linked to plant damage depended upon factors, such as soil type, soil moisture, rainfall intensity and metribuzin rate, but were not quantified in this study. Further research quantifying the amount of rainfall that leads to crop phytotoxicity post-metribuzin

application, taking into account herbicide rate, soil type and soil moisture level, would be of considerable benefit to growers and farm advisors.

The development of a high level of metribuzin tolerance in lentil and subsequent confirmation that it is due to an $Ala_{251}Thr$ target site mutation encoded by the chloroplastic *psbA* gene is unique in higher plants and, a significant breakthrough in herbicide tolerant crop research (See Chapters 3 and 4). Despite concerted research efforts in over 10 crop species worldwide, utilising a range of methods including germplasm screening and mutagenesis, target site tolerance to a PSII inhibitor herbicide has not been reported (see Chapter 1 and 3). The exception is TT canola; however, this was developed through the introgression of atrazine tolerance from a related resistant weed species into domesticated canola (Beversdorf and Kott 1987). Previous attempts to develop metribuzin tolerance using mutagenesis generated non-target site tolerance and resulted in improvements of up to six times greater than the sensitive control (Si *et al*. 2009; Pan *et al*. 2012; Sharma *et al*. 2017). This is considerably lower than the levels identified in the tolerant genotypes in this research (11 to 48 times in M009 and 22 to 62 times in M043), but similar to the non-target site level identified in SP1333 (two to four times). Reasons for both the identification of higher levels of tolerance and a target site tolerance mechanism being identified in this study and not in the others are detailed in Chapter 4, but are primarily thought to be due to the initial size of the mutated population screened.

A unique aspect of the selection method used to identify target site tolerant genotypes in this study was the use of very large field screens. Mutant M_2 and M_3 field screens of approximately 14 ha each were conducted using commercial farm equipment. Not only were very large amounts of seed required for this process, but, given only one tolerant plant in each screen was identified, a significant amount of experimental skill, attention to detail, persistence and good fortune. Operational and logistic challenges encountered, included: identification of a 14 ha site with sufficient soil uniformity to allow a single differentiating rate of metribuzin; protecting individual plants from pest attack; logistics issues with using large scale farm machinery including variability in seeding depth, evenness of residue cover, seed and herbicide spray overlaps, machinery wheel tracks and timeliness

of operations; and finally the ability to effectively and timely 'scout' a large field site. Despite these challenges, the use of EMS mutagenesis, large population sizes and subsequent large-scale field selection proved an effective approach for identifying a chloroplastic target site mutation endowing a high level of metribuzin tolerance in lentil. Additionally, this approach allows the researcher to use a desired cultivar as the parent and to select plants under a relevant production system. Given the success of this study, this approach could be used to discover tolerance to other herbicides in lentil and potentially other crops, particularly minor crops where transgenic approaches are unlikely to be cost-effective or will face international trade issues (Devine 2005).

The intermediate level of metribuzin tolerance discovered in the Argentinian sourced accession SP1333, was identified through the *in situ* hydroponic sand screening of 750 *Lens* accessions from the Australian Grains Genebank and PBA (see Chapter 3 and Appendices 1 to 6). Accessions were randomly selected to represent diverse sources of origin as no obvious region or plant characteristic for targeting herbicide tolerance was known. Further improvements in metribuzin tolerance greater than the level identified in SP1333 could be possible through screening a wider range of *Lens* accessions, potentially with a focus on material from Argentina. The *in situ* screening approach was quicker and less resource intensive than the field mutagenesis process, however, a lower level of tolerance was identified and standard errors within screens were high leading to the identification of false positives. Further refinement of the screening technique used in this study or the development of alternative screening methods would be warranted if a larger number of lines is to be screened. This would be particularly important where only a relatively low level of tolerance improvement compared to the control line is expected.

Two years of field evaluation trials confirmed that genotypes M009 and M043 were tolerant to agronomically useful levels of metribuzin applied post-emergent under Australian conditions (see Chapter 6). This finding suggests that the metribuzin tolerance trait has potential to significantly improve weed control options in lentil in Australia and North America. It is also likely to have application in numerous lentil producing countries worldwide. From an agronomic perspective, the

ability to apply metribuzin post-emergent in lentil will facilitate higher application rates and improved flexibility in timing of application leading to more effective weed control when compared to the existing pre-emergent technology (Mao 2018).

Opportunities for alternative and improved weed control with maximum label rates of post-emergent metribuzin, include: 1) IMI (Group B) resistant broadleaf weeds such as oriental mustard (*Sisymbrium orientale* L.), African mustard (*Brassica tournefortii* Gouan), and wild radish (*Raphanus raphanistrum* L.) (Boutsalis *et al*. 2016) and volunteer IMI tolerant canola 2) current difficult to control weeds in lentil including sowthistle (*Sonchus oleraceus* L.), prickly lettuce (*Lactuca serriola* L.) and bifora (*Bifora testiculata* L. Spreng.) (Davey 2014; Preston 2002), and 3) the emerging problem of the acetyl coA carboxylase inhibitor, cyclohexanediones (Dim) resistant annual rye grass (*Lolium rigidum* Gaud.) (Mao 2018). Further improvement in weed control could be achieved through the development of dual herbicide (metribuzin + IMI) tolerant lentil cultivars, which would allow for combination PSII and AHAS inhibitor herbicides to be used, as has occurred recently in canola with TT and IMI (DPIRD 2018). The current recommendation for metribuzin in lentil is post-sowing pre-emergence application (White 2015). In autumn-sown lentils in southern Australia, this application period can be as little as five or six days, which often leads to application occurring under sub-optimal conditions. A post-emergent application will allow a 3 to 4 week herbicide application window and increase the ability to avoid the sub-optimal weather conditions detailed in Chapter 2.

Despite large improvements in the metribuzin tolerance of genotypes M009 and M043 confirmed in the field in this study, further understanding of their performance under diverse field conditions is required. Small reductions of plant dry weight (DW) occurred as metribuzin application rate increased on these genotypes. Grain yield reductions were also observed at the highest rate (3x maximum label pre-emergence rate) in some environments (see Chapter 6). However, there was little or no relationship between visual plant injury symptoms or reductions in plant density and metribuzin rate. Reductions in DW were also observed in the controlled environment dose response experiments

(Chapters 3 and 4), although generally at higher application rates. Reasons for variation in the metribuzin application rate required for similar DW reductions between different experiments are discussed in detail in Chapters 3, 4 and 6, including potential interactions with disease in the field experiments. Another potential interaction in the field is due to antagonism between metribuzin and grass herbicides. A phytotoxic interaction was reported between metribuzin and the grass herbicide tridiphane in soybean (*Glycine max* (L.) Merr.) and tomato (*Lycopersicon esculentum* Mill.), with the order and timing of herbicide applications important in determining the extent of injury (Gaul *et al*. 1995). It is highly likely that grass selective herbicides would be applied with or at a similar timing to post-emergent metribuzin in Australia, as occurred in the field study in Chapter 6. Further agronomic research, including understanding any interactions with grass herbicides, is required to define safe and effective application rates of post-emergent metribuzin in lentil. This research should occur in adapted metribuzin tolerant lentil genotypes and not in the original mutant selections, as in this study, to reduce issues associated with deleterious mutations present in M043 and M009.

Little is known about the characteristics of the $Ala_{251}Thr$ mutation as the only previous report is from three *Chenopodium rubrum* cell cultures all with double or triple mutations in the D1 protein (Schwenger-Erger *et al*. 1993). In contrast, a detailed understanding exists on how the $Ser_{264}Gly$ mutation confers high level tolerance to atrazine in TT canola and over 60 weed species globally (Powles and Yu 2010). The $Ser_{264}Gly$ mutation is responsible for tolerant biotypes having a tolerance factor to the herbicide atrazine of 1,000 at the binding site and 100 at the whole plant level when compared with the sensitive biotypes (Devine and Shukla 2000). Whole plant dose response studies in this research suggested tolerant factors ranging from 11 to 48 in genotype M009 and 22 to 62 in M043 (see Chapters 3 and 4). In both cases these range of tolerance factors are less than the 100 suggested for TT canola and could explain why a level of DW reduction was observed from metribuzin applications at higher rates. Detailed binding site studies using the tolerant lentil genotypes are required to understand how the $Ala_{251}Thr$ mutation confers metribuzin tolerance and to what level.

The two tolerant genotypes differed in cross-tolerance to atrazine with M009 showing a negative response, but this was not evident in M043 (Chapter 4). Negative cross-tolerance has been reported previously in species tolerant to PSII inhibitors, including bentazon sensitivity in TT biotypes of *B. napus* (Van Oorschot and Van Leeuwen 1988). A susceptibility to atrazine in any metribuzin tolerant lentil cultivar would be of concern to Australian lentil growers. Triazine herbicides, such as simazine or terbuthalzine, are often used in conjunction with metribuzin due to contrasting differences in solubility and leaching index characteristics providing more durable weed control. Additional cross-tolerance experiments not reported in this study found that germplasm line SP1333 not only had an improved level of tolerance to metribuzin compared to PBA Flash, but also to other PSII inhibitors including diuron and atrazine. A crossing program between SP1333 and M009 and selection of progeny under simazine pressure could potentially be a solution to the triazine sensitivity in M009. However, throughout this study M043 was repeatedly observed to be agronomically superior to M009 and is recommended for use by plant breeders (see Chapters 3, 4, 5 and 6).

It is widely agreed that a fitness cost is associated with the $Ser_{264}Gly$ mutation that confers target site tolerance to atrazine in TT canola and numerous weed species; however, little is known about other PSII inhibitor mutations (Powles and Yu, 2010). Results of field experiments investigating fitness cost with BC_1F_2 and BC_1F_3 populations and their reciprocals, created from the tolerant genotypes and PBA Flash, were consistent with this accepted view. Reductions in DW and GY of 20 to 40% were associated with the metribuzin tolerance in lentil in field trials (see Chapter 5). These reductions were similar, although perhaps slightly higher, than those identified in studies with TT canola (Beversdorf *et al*. 1988; Robertson *et al*. 2002).

Despite the presence of a fitness cost and associated yield penalty in TT canola, it remains the dominant canola type grown in Australia some 25 years after its inception (Zhang *et al*. 2016). However, after initially being grown in Canada, TT canola was abandoned in favour of alternative herbicide systems in the 1990s (Devine 2005). Reasons given for the dominance of TT canola in

Australia include a combination of robust cost effective weed control, equal or better gross margins than alternative herbicide systems, good fit in the farming system, similar yield to alternative canola types (progress in closing yield gap) and the ability to exploit specific weed problems (Duke 2005; Zhang *et al*. 2016). Apart from similar yields, the above reasons are all pertinent to metribuzin use in lentil production in southern Australia. Given the high sensitivity of lentil to weed competition, a yield penalty for improved weed control is more likely to be accepted in lentil than in a more competitive crop like canola. Reasons were not given for the reduction in yield gap between TT and the alternative canola options, however, the authors suggested further investigation is warranted (Zhang *et al*. 2016).

An earlier study in TT canola showed variation occurred for yield in TT canola hybrids and led the authors to suggest that yields of some TT hybrids were better in some genetic backgrounds compared to others (Grant and Beversdorf 1985). A similar study is warranted in lentil using a diverse range of parents varying in plant characteristics such as early vigour, height, branching habit, biomass level, growth rate, phenology and leaf morphology with the aim of identifying parents that produce higher yielding progeny. A reduction in the yield gap between metribuzin tolerant and other lentil types will not only be beneficial to profitability, but essential in maximising the uptake of this technology and its associated weed control benefits to lentil production.

The confirmations that target site metribuzin tolerance in lentil is predominately due to maternal inheritance but with occasional paternal leakage was a significant outcome of this study (see chapter 5). Traditionally it has been widely accepted in angiosperms, that the chloroplast genome is maternally inherited (Birky 2001). However, a number of species have occasional paternal transmission leading to paternal or biparental inheritance (McCauley *et al*. 2007). Furthermore, RFLP analysis of progeny from interspecific crosses of lentil identified paternal chloroplasts in one of 10 F_1 progeny (Rajora and Mahon 1995). Given the above, it is not completely unexpected that paternal chloroplast leakage occurs in lentil. However, the finding that it occurred in approximately 20% of F_1 progeny is both higher than the previous lentil study and the level found in crops such as canola

and sunflower (Ellis *et al*. 2008; Schneider *et al*. 2015). It should be noted that the whole plant phenotyping method used on the F_1 progeny in Chapter 5 was not designed to detect paternal leakage rates and may not have accurately detected all biparental chloroplast inheritance, particularly when occurring at low levels. It is therefore probable that the actual paternal leakage rate of chloroplasts in lentil could be higher than the 20% suggested in Chapter 5.

The understanding that biparental chloroplast inheritance is occurring in lentil is critical for lentil breeding programs as it complicates the introgression of the metribuzin tolerance into adapted cultivars. In particular, rapid marker platforms like KASP, will be unreliable in detecting low levels of biparental leakage (see Chapter 5). Plant breeders will need to routinely incorporate the phenotyping methods detailed in Chapters 3, 4 and 5 to not only identify heteroplasmic plants, but also for resolving low levels of chloroplast heteroplasmy by eliminating sensitive chloroplasts during early generation development (Greiner *et al*. 2014; Frey 1998).

The confirmation that biparental inheritance and paternal transmission of chloroplast genes occurs in lentil provides further evidence that biparental inheritance via paternal leakage can occur in angiosperms. It also now provides the opportunity for further investigation into recombination and segregation of chloroplast DNA, plastid development and structural requirements for photosynthesis in this crop. A chloroplast inherited metribuzin tolerance trait is likely to provide a more useable trait for these areas of research than chlorophyll chimeras, which were used in lentil by Miller *at al*. (1984). A potential consequence of the discovery of biparental transmission of chloroplast DNA in lentil could be in the area of transgenic research. Chloroplast genomes are often proposed as the site for insertion of engineered genes in angiosperms because of a lack of transmission through pollen reducing the chance of gene escape, but this is not true for lentil. Finally, this new understanding in lentil may provide increased insights into the evolutionary biology of population structures and phylogeography of this crop (Lambertini 2016).

In summary, lentil genotypes with high and intermediate levels of metribuzin tolerance when compared with Australian and Canadian cultivars have been developed and characterised. Field

validation in southern Australia found that the target site metribuzin tolerance in M009 and M043 provided an agronomically useful level of tolerance to post-emergent application of this herbicide. Germplasm line SP1333 showed a level of tolerance between the tolerant genotypes and the commercial cultivar PBA Flash. All three lentil genotypes with improved metribuzin tolerance are in use as parents in Australian breeding programs; however, M043 is the genotype of choice. The confirmation of a maternal inheritance pattern with occasional paternal leakage and an associated fitness cost linked to biomass and yield reductions potentially complicates the introgression of the target site tolerance into adapted cultivars. However, the detailed knowledge of the genetic controls of inheritance and associated fitness cost of the target site along with screening methodologies and molecular markers provided by this study will aid plant breeders to incorporate the tolerance into agronomically accepted plant types rapidly and effectively. Intercrossing M043 with a wide range of adapted elite lentil lines to identify higher yielding progeny is required to reduce the productivity gap, as has occurred in TT canola. Further agronomic research to define application rates for safe and effective metribuzin use in adapted metribuzin tolerant genotypes is also required. The potential of developing a metribuzin tolerant lentil industry in Australia, similar to that which has occurred in TT canola, now exists. Additionally, the development of dual herbicide tolerant lentil cultivars through combining the metribuzin and IMI target site tolerances is now possible.

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(Literature Review and General Discussion)

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Appendices

Appendix 1 Additional information on lentil genotype, source, Australian Grains Genebank (AGG) accession number (ATC), country of origin and plant damage response to metribuzin (0 to 6) in hydroponic sand screen pot assay 1 conducted in Chapter 3

Genotype	Source	ATC #	Origin	Necrosis (0 to 6)
69	AGG	70154	Algeria	3.5
77	AGG	70157	Algeria	4.5
1225	AGG	70415	Chile	5
36076	AGG	70530	Ethiopia	5
36137	AGG	70534	Ethiopia	4.5
36139	AGG	70535	Ethiopia	5
9920321	AGG	70876	Bangladesh	4.5
9920343	AGG	70874	Bangladesh	5
9920357	AGG	70867	Bangladesh	5
070689-0701	AGG	73240	Turkey	4.5
070785-0102	AGG	73004	Turkey	4
090689-0101	AGG	73246	Turkey	5
090785-0201	AGG	73011	Turkey	4.5
11-3-135	AGG	70459	Libya	4.5
200785-0202	AGG	73048	Turkey	4
210785-0101	AGG	73053	Turkey	4.5
210785-0501	AGG	73055	Turkey	5
220785-0301	AGG	73060	Turkey	5
240785-1102	AGG	73064	Turkey	4.5
74TA 548	AGG	70493	Syria	4
74TA 577	AGG	70494	Mexico	4
74TA-138	AGG	70491	Morocco	5
74TA-72	AGG	70490	Iraq	4.5
75B	AGG	70248	Afghanistan	4.5
76TA 66012	AGG	70497	Jordan	4
96-047L*99R099	PBA		Australia	5.25
99-088L*02H037	PBA		Australia	4.339
ABAWI# 2	AGG	73263	Peru	5
Adas	AGG	71102	Iran	4.5
ANICIA	AGG	73746	France	5
Aproszemü Lenese	AGG	73653	Hungary	5
ARMENIAN 88	AGG	73434	Armenia	5
B 969	AGG	73743	Georgia	6
B92-143	AGG	73381	Bulgaria	5
B92-169	AGG	73382	Bulgaria	4.5
B92-175	AGG	73383	Russian Federation	3.5
BELOCERKOVSKAJA 24	AGG	73727	Ukraine	4.5
BERATI	AGG	73838	United States	4.5
BGRC 025686	AGG	70554	Tunisia	4.5
BKK&WJK-1	AGG	73427	Spain	5
Boomer	PBA		Australia	4.5
BOYACA 2	AGG	70238	Colombia	4.5
BREWER	PBA		North America	4.5
CALLISTO	AGG	70069	Unknown	5
CASTELLANA	AGG	73216	Spain	5
CASTELLUCCIO LENTIL	AGG	73416	Italy	5
CDC BLAZE	PBA		Canada	5
CDC MATADOR	PBA		Canada	4.5
CDC ROBIN	PBA		Canada	5
CDC ROSETOWN	PBA		Canada	5
CDC ROULEAU	PBA		Canada	4.5

Genotype (cont'd.)	Source	ATC #	Origin	Necrosis (0 to 6)
CNPH 84-125	AGG	73200	Brazil	6
COBBER	AGG	70743	Australia	4.5
CPI 72029	AGG	70467	Iran	5
CRIMSON	AGG	73830	United States	3
CYPRIOT LOCAL	AGG	70245	Cyprus	4.5
CYPRUSI	AGG	71144	Cyprus	4.5
DE-17	AGG	73453	Ecuador	5
DIGGER	AGG	70742	Australia	5
DUPUY	AGG	72943	France	4.5
E92-1	AGG	73364	Egypt	4.5
EL 2	AGG	70249	Ethiopia	4.5
ESTON	PBA		Canada	5
FRENCH GREEN	AGG	70794	France	6
Gestreifte Linse	AGG	73646	Germany	4.5
GIZA 9	AGG	71129	Egypt	4
HALEROVA COCKA	AGG	73713	Czechoslovakia	4.5
HROTOVICKA VELKOZRNNA	AGG	73679	Czech Republic	5
ICE BEAN	AGG	73457	China	4
ILL 106	AGG	70977	Guatemala	5
ILL 112	AGG	70985	Turkey	3.5
ILL 113	AGG	70986	Turkey	4
ILL 120	AGG	70993	Turkey	5
ILL 123	AGG	70996	Turkey	4
ILL 128	AGG	71000	Turkey	4
ILL 131	AGG	71003	Turkey	4.5
ILL 1337	AGG	70200	Iran	5
ILL 134	AGG	71006	Turkey	5
ILL 136	AGG	71008	Turkey	3.5
ILL 139	AGG	71012	Turkey	5
ILL 141	AGG	71014	Turkey	5
ILL 147	AGG	71021	Turkey	5
ILL 155	AGG	70022	Turkey	6
ILL 171	AGG	71046	Turkey	4
ILL 180	AGG	71056	Turkey	4.5
ILL 1808	AGG	70259	Afghanistan	5
ILL 1819	AGG	70265	Afghanistan	4.5
ILL 182	AGG	71058	Turkey	4
ILL 1823	AGG	70268	Afghanistan	4.5
ILL 1861	AGG	70270	Sudan	5
ILL 1921	AGG	71149	United Kingdom	5
ILL 210	AGG	70492	Costa Rica	2
ILL 214	AGG	71093	Afghanistan	3.5
ILL 2148	AGG	70285	Jordan	5
ILL 215	AGG	70025	Afghanistan	5
ILL 216	AGG	71095	Afghanistan	5
ILL 2194	AGG	70294	Pakistan	4
ILL 2198	AGG	70296	Pakistan	5
ILL 233	AGG	71112	Iran	4.5
ILL 236	AGG	71115	Egypt	4.5
ILL 238	AGG	71117	Ukraine	5
ILL 2425	AGG	70302	Colombia	4.5
ILL 2439	AGG	70771	Ethiopia	5
ILL 248	AGG	71128	Pakistan	4.5
ILL 279	AGG	71164	Greece	5
ILL 307	AGG	71192	Greece	2.5
ILL 309	AGG	71194	Greece	4.5
ILL 3395	AGG	74532	Turkmenistan	5
ILL 4367	AGG	70376	Syria	4.5
ILL 4469	AGG	70386	Syria	4
ILL 4535	AGG	70401	Syria	5
ILL 4909	AGG	74537	Russian Federation	3.5

Genotype (cont'd.)	Source	ATC #	Origin	Necrosis (0 to 6)
ILL 5067	AGG	70472	Jordan	5
ILL 512	AGG	70951	Bulgaria	4.5
ILL 6110	AGG	70550	Argentina	4.5
ILL 6111	AGG	70551	Argentina	4.5
ILL 6648	AGG	70574	Syria	4.5
ILL 69	AGG	73856	Cyprus	4
ILL 8215	AGG	74656	Uzbekistan	4
ILL 8250	AGG	74432	Uzbekistan	4
ILL 8252	AGG	74434	Uzbekistan	5
ILL 8288	AGG	74439	China	4
ILL 8289	AGG	74700	China	4
ILL 8323	AGG	74720	Tajikistan	4
ILL 837	AGG	70153	Lebanon	5
ILL 8401	AGG	74462	Russian Federation	4.5
ILL 8430	AGG	74779	Azerbaijan	4.5
ILL 8522	AGG	74828	Azerbaijan	4
ILL 921	AGG	70164	Tunisia	5
ILL 9798	AGG	74868	Tajikistan	4
INVINCIBLE	AGG	70785	Unknown	4.5
ITALIJANKO SOCIVO	AGG	73644	Unknown	2.5
JANA	AGG	73333	Bulgaria	4
LAB10*B1998-99-00-10KHM001	PBA		Unknown	4
LAB10*B1998-99-00-10KHM002	PBA		Unknown	4.5
LAB10*B1998-99-00-10KHM003	PBA		Unknown	4.5
LAB10*B1998-99-00-10KHM004	PBA		Unknown	5
LAB10*B1998-99-00-10KHM005	PBA		Unknown	5
LAB10*B1998-99-00-10KHM006	PBA		Unknown	5
LAB10*B1998-99-00-10KHM007	PBA		Unknown	5
LAB10*B1998-99-00-10KHM008	PBA		Unknown	4
LAB10*B1998-99-00-10KHM009	PBA		Unknown	5
LAB10*B1998-99-00-10KHM010	PBA		Unknown	5
LAB10*B1998-99-00-10KHM011	PBA		Unknown	4.5
LAB10*B1998-99-00-10KHM012	PBA		Unknown	4.5
LAB10*B1998-99-00-10KHM013	PBA		Unknown	4.5
LAB10*B1998-99-00-10KHM014	PBA		Unknown	4.5
LAB10*B1998-99-00-10KHM015	PBA		Unknown	4.5
LAB10*B1998-99-00-10KHM016	PBA		Unknown	4.5
LAB10*B1998-99-00-10KHM017	PBA		Unknown	5
LAB10*B1998-99-00-10KHM018	PBA		Unknown	5
LAB10*B1998-99-00-10KHM019	PBA		Unknown	5
LAB10*B1998-99-00-10KHM020	PBA		Unknown	4.5
LAB10*B1998-99-00-10KHM021	PBA		Unknown	5
LAB10*B1998-99-00-10KHM022	PBA		Unknown	5
LAB10*B1998-99-00-10KHM023	PBA		Unknown	4.5
LAB10*B1998-99-00-10KHM024	PBA		Unknown	4.5
LAB10*B1998-99-00-10KHM025	PBA		Unknown	5
LAB10*B1998-99-00-10KHM026	PBA		Unknown	4.5
LAB10*B1998-99-00-10KHM027	PBA		Unknown	0
LAB10*B1998-99-00-10KHM028	PBA		Unknown	4.5
LAB10*B1998-99-00-10KHM029	PBA		Unknown	3
LAB10*B1998-99-00-10KHM030	PBA		Unknown	4.5
LAB10*B1998-99-00-10KHM031	PBA		Unknown	4.5
LAB10*B1998-99-00-10KHM032	PBA		Unknown	3.5
LAB10*B1998-99-00-10KHM033	PBA		Unknown	5
LAB10*B1998-99-00-10KHM034	PBA		Unknown	4

Genotype (cont'd.)	Source	ATC #	Origin	Necrosis (0 to 6)
LAB10*B1998-99-00-10KHM035	PBA		Unknown	5
LAB10*B1998-99-00-10KHM036	PBA		Unknown	5
LAB10*B1998-99-00-10KHM037	PBA		Unknown	5
LAB10*B1998-99-00-10KHM038	PBA		Unknown	5
LAB10*B1998-99-00-10KHM039	PBA		Unknown	5
LAB10*B1998-99-00-10KHM040	PBA		Unknown	5
LAB10*B1998-99-00-10KHM041	PBA		Unknown	5
LAB10*B1998-99-00-10KHM042	PBA		Unknown	4.5
LAB10*B1998-99-00-10KHM043	PBA		Unknown	5
LAB10*B1998-99-00-10KHM044	PBA		Unknown	5
LAB10*B1998-99-00-10KHM045	PBA		Unknown	5
LAB10*B1998-99-00-10KHM046	PBA		Unknown	4.5
LAB10*B1998-99-00-10KHM047	PBA		Unknown	5
LAB10*B1998-99-00-10KHM048	PBA		Unknown	6
LAB10*B1998-99-00-10KHM049	PBA		Unknown	5
LAB10*B1998-99-00-10KHM050	PBA		Unknown	5
LAB10*B1998-99-00-10KHM051	PBA		Unknown	5
LAB10*B1998-99-00-10KHM052	PBA		Unknown	3.5
LAB10*B1998-99-00-10KHM053	PBA		Unknown	6
LAB10*B1998-99-00-10KHM055	PBA		Unknown	5
LAB10*B1998-99-00-10KHM056	PBA		Unknown	6
LAB10*B1998-99-00-10KHM057	PBA		Unknown	6
LAB10*B1998-99-00-10KHM058	PBA		Unknown	5
LAB10*B1998-99-00-10KHM059	PBA		Unknown	4.5
LAB10*B1998-99-00-10KHM060	PBA		Unknown	4.5
LAB10*B1998-99-00-10KHM062	PBA		Unknown	4
LAIRD	AGG	70789	Canada	5
LARGE BLONDE PROCODE	AGG	71147	Hungary	3.5
LC00600854E	PBA		North America	5
LC01600743E	PBA		North America	5
LC01601724T	PBA		North America	5
LC01601751T	PBA		North America	4
LC01601752T	PBA		North America	6
LENKA	AGG	73721	Czech Republic	4.5
LENS 157	AGG	73693	Libya	4.5
LENS 163	AGG	73699	Iran	4
LENS 196	AGG	73723	Iraq	4.5
LENS 198	AGG	73724	Iraq	5
LENS 400	AGG	70449	Yemen	5
LENS 58\\75	AGG	70461	Greece	5
LENS 62	AGG	73608	Greece	5
LENS 72	AGG	73618	Greece	3.5
LENTEJA VERDINA	AGG	73215	Spain	4.5
LENTEJAS SELECCIONADAS	AGG	71154	Argentina	4.5
Linea-30	AGG	70571	Argentina	5
M89-15	AGG	73452	Morocco	5
M93-1	AGG	73419	Mexico	4.5
MALAZGIRT89	PBA			4.5
MARIETTE	AGG	72945	France	3.5
MARKET SAMPLE	AGG	70939	India	3.5
MASOUR LENTILS	AGG	73196	India	4.5
MASSAR	AGG	73109	Pakistan	5
MASURO	AGG	73369	Nepal	4.5
MASURO(DHAEL)	AGG	73372	Nepal	5
MOUNTAIN LENTIL #1	AGG	73417	Italy	4.5
NASLADA	AGG	73373	Bulgaria	5
Nipper	PBA		Australia	5.5
PAK 40835	AGG	70565	Pakistan	4
PALLAGI KEK	AGG	71142	Hungary	4.5
PAN 6	AGG	70333	Nepal	4.5
PARDINA	AGG	73826	Spain	4

Genotype (cont'd.)	Source	ATC #	Origin	Necrosis (0 to 6)
PBA Blitz	PBA		Australia	5
PBA Flash	AGG	75320	Australia	4.5
Petite Rouge d'Egypte	AGG	73678	Unknown	6
PETROVSKAJA JUBILEJNAJA	AGG	73741	Russian Federation	4
PETROVSKAJA YUBILEJNAJA	AGG	72929	Former Soviet Union	4
PI 297759	AGG	70104	Algeria	5
PI 312179	AGG	70120	Mexico	5
PI 319366	AGG	70121	Mexico	5
PI 339282	AGG	70128	Turkey	6
PI 374116	AGG	70276	Morocco	4.5
PI 472385	AGG	72673	India	5
PI 472571	AGG	72858	Iran	4.5
PI 472581	AGG	72868	Iran	4.5
PI 472591	AGG	72878	Iran	4.5
PI 472607	AGG	72894	Iran	5
PI 509316	AGG	70624	Turkey	5
PI 509320	AGG	70628	Turkey	3.5
PI 509333	AGG	70641	Turkey	5
PI 509390	AGG	73024	Turkey	5
PI 509391	AGG	70699	Turkey	4.5
PI 509406	AGG	70714	Turkey	5
PI 606573	AGG	73461	Iraq	4
PI 606593	AGG	73362	China	5
ROSE	AGG	70786	United States	4.5
RUSKA TALIROVA	AGG	73718	Unknown	3.5
SP 1386	AGG	70436	Chile	5
SPANISH BROWN	AGG	73836	Spain	4.5
Späths Hellerlinse	AGG	73668	Germany	6
STELA	AGG	73833	Bulgaria	5
STEPNAJA 244	AGG	71589	Ukraine	4.5
SULTON MERCIMEK	AGG	73035	Turkey	4
TADZIKSKAJA 95	AGG	73738	Tajikistan	4
TALIN 6	AGG	73433	Armenia	4
TALLINSKAJA 6	AGG	72927	Former Soviet Union	4.5
TG. FRUMOS	AGG	73672	Romania	5
Valticka Halerova	AGG	73714	Czech Republic	5
VERDINA	AGG	73217	Spain	6
Weihenstephaner Linse	AGG	70462	Germany	2.5
Assay mean				4.6
LSD (0.05)				1.1

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Appendix 2 Additional information on lentil genotype, source, Australian Grains Genebank (AGG) accession number (ATC), country of origin and plant damage response to metribuzin (% necrosis) in hydroponic sand screen pot assay 2 conducted in Chapter 3

Genotype	Source	ATC #	Origin	Necrosis (%)
69	AGG	70154	Algeria	8
1264	AGG	71388	Chile	95
130785	AGG	73421	Turkey	100
96-047L*99R099	PBA		Australia	100
99-088L*02H037	PBA		Australia	72
AKCA MERCIMEGI	AGG	71513	Turkey	85
B 1156	AGG	73748	Georgia	100
B 986	AGG	73744	Italy	100
B92-183	AGG	73386	Czech Republic	100
B92-195	AGG	73387	Bulgaria	100
B92-213	AGG	73393	Russian Federation	50
BALADI	AGG	71458	Syria	100
Boomer	PBA		Australia	100
Borinskaja	AGG	74455	Russian Federation	100
CIPAL 709	AGG	75313	Australia	100
CRIMSON	AGG	73830	United States	98
Dneprovskaja 3	AGG	74560	Ukraine	74
GIZA	AGG	71223	Egypt	100
GRADECKA	AGG	71624	Yugoslavia	100
HEBRON	AGG	72406	Brazil	100
HOFFMAN # 19	AGG	[A]		100
HOFFMAN # 6	AGG	[B]		*
HOFFMAN # 93	AGG	[D]		*
HYBRIDE INRA	AGG	73702	France	36
IG 140929	AGG	[c]		*
IL-19	AGG	71643	India	95
ILL 1712	AGG	75301	Ethiopia	68
ILL 1941	AGG	71618	Morocco	75
ILL 210	AGG	70492	Costa Rica	100
ILL 307	AGG	71192	Greece	80
ILL 322	AGG	71207	Greece	98
ILL 345	AGG	71231	Mexico	100
ILL 362	AGG	71248	Chile	59
ILL 363	AGG	71249	Chile	4
ILL 456	AGG	71420	Chile	16
ILL 466	AGG	71433	Chile	90
ILL 485	AGG	71456	Lebanon	100
ILL 513	AGG	71484	Palestine	100
ILL 515	AGG	71486	Azerbaijan	89
ILL 577	AGG	71549	Turkey	99
ILL 7577	AGG	74613	Afghanistan	95
ILL 8256	AGG	74436	Uzbekistan	100
ILL 8257	AGG	74437	Turkmenistan	100
ILL 8286	AGG	74438	China	100
ILL 8394	AGG	74459	Tajikistan	100
ILL 8407	AGG	74464	Uruguay	100
ILL 8411	AGG	74466	Libya	100
ILL 8432	AGG	74781	Azerbaijan	100
ILL 8457	AGG	74470	Libya	100
ILL 8594	AGG	74508	Ecuador	100
ILWL 130	AGG	[A]		*
ILWL 14	AGG	[B]		*
Indianhead	AGG	70787	Canada	99

Genotype (cont'd.)	Source	ATC #	Origin	Necrosis (%)
ITALIJANKO SOCIVO	AGG	73644	Unknown	95
K-726	AGG	71680	Pakistan	100
KISLIK YESIL 21	AGG	72389	Turkey	95
KROKHMAL #6	AGG	73412	Ukraine	85
KURD	AGG	71460	Syria	14
LAB10*B1998-99-00-10KHM027	PBA		Unknown	35
LAB10*B1998-99-00-10KHM029	PBA		Unknown	38
LAB10*B1998-99-00-10KHM070	PBA		Unknown	10
LAIRD LENTIL	AGG	72407	Canada	42
LENS 155	AGG	73691	Afghanistan	71
LENS 81	AGG	73626	Greece	99
LENTEJA	AGG	71467	Mexico	100
LEREN	AGG	73472	Spain	93
LOKALNA SREDNOEDRA	AGG	71596	Macedonia	100
LP 54-2987	AGG	71374	Chile	84
Luna 09	AGG	74575	Ukraine	63
Nipper	PBA		Australia	100
NORTHERN RED	AGG	71461	Syria	95
OBRAZTZOV CHIFLIK 7	AGG	73353	Bulgaria	20
PAK 20	AGG	73425	Pakistan	100
PBA Blitz	PBA		Australia	76
PBA Flash	AGG	75320	Australia	83
PBAFlash-EMS10-11SVHM015	Mutant Flash		Australia	*
PBAFlash-EMS10-11SVHM063	Mutant Flash		Australia	*
PI 429836	AGG	71692	Iran	20
PI 472172	AGG	72462	India	100
PI 472297	AGG	72585	India	100
PI 472313	AGG	72601	India	100
PI 472360	AGG	72648	India	100
PI 472365	AGG	72653	India	100
PI 509333	AGG	70641	Turkey	100
PI 606564	AGG	73448	Nepal	100
PI 606593	AGG	73362	China	100
POPULACAO IBIRUBA	AGG	72402	Brazil	100
RED LENTIL	AGG	73269	China	100
RISOVAYA	AGG	71588	Armenia	100
RPIP 33-071-10420A	AGG	71500	Iran	100
RPIP 33-071-10713	AGG	71953	Iran	98
RPIP 33-071-10722	AGG	71962	Iran	100
RPIP 33-071-11030	AGG	72221	Iran	97
RPIP 33-071-11112	AGG	72282	Iran	61
RPIP 33-079-10999	AGG	72324	Jordan	58
RPIP 33-085-10602	AGG	72336	Lebanon	80
RPIP 33-153-11133	AGG	72366	Turkey	76
SITNA	AGG	71601	Yugoslavia	100
Späths Hellerlinse	AGG	73668	Germany	53
STEPPE 244	AGG	71566	Ukraine	100
STONKA-1	AGG	73375	Bulgaria	95
SULTANI	AGG	71518	Turkey	46
TIPO CASTELLUCCIO PICCOLE	AGG	71228	Italy	100
USSR-05-05	AGG	73266	Tajikistan	37
VULGARIS	AGG	71219	Turkey	100
Weihenstephaner Linse	AGG	70462	Germany	95
Assay mean				84
LSD (0.05)				33

[A] *L. culinaris subsp. odemensis,* [B] *L. culinaris subsp. orientalis,* [C] *L. ervoides,* [D] *L. nigricans,* * Failed to germinate
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Appendix 3 Additional information on lentil genotype, source, Australian Grains Genebank (AGG) accession number (ATC), country of origin and plant damage response to metribuzin (% necrosis) in hydroponic sand screen pot assay 3 conducted in Chapter 3

Genotype	Source	ATC #	Origin	Necrosis (%)
19	AGG	71640	Jordan	19
69	AGG	70154	Algeria	50
3020	AGG	71424	Chile	98
4023	AGG	71438	Chile	77
100785-0401	AGG	73015	Turkey	15
180785-0701	AGG	73043	Turkey	4
290685-0602	AGG	72950	Turkey	1
96-047L*99R099	PBA		Australia	95
99-088L*02H037	PBA		Australia	56
A-1-1-1	AGG	72779	India	94
ABAWI# 1	AGG	73262	Peru	49
Boomer	PBA		Australia	0
CARZINHO	AGG	72403	Brazil	97
CNPH 84-123	AGG	73199	Brazil	88
IL-30	AGG	71644	India	98
ILL 1953	AGG	71628	Iran	32
ILL 293	AGG	71178	Greece	14
ILL 306	AGG	71191	Greece	96
ILL 312	AGG	71197	Greece	45
ILL 320	AGG	71205	Hungary	8
ILL 323	AGG	71208	Yugoslavia	50
ILL 339	AGG	71225	Spain	0
ILL 361	AGG	71247	Chile	75
ILL 362	AGG	71248	Chile	29
ILL 363	AGG	71249	Chile	46
ILL 425	AGG	71369	Chile	17
ILL 456	AGG	71420	Chile	50
ILL 500	AGG	71471	Mexico	93
ILL 580	AGG	71552	Turkey	15
ILL 968	AGG	71719	Iran	98
Indianhead	AGG	70787	Canada	100
ITALIJANKO SOCIVO	AGG	73644	Unknown	98
K-2124	AGG	71577	Russian Federation	100
K-479	AGG	71664	Uzbekistan	93
KURD	AGG	71460	Syria	74
LAB10*B1998-99-00-10KHM070	PBA		Unknown	49
LAIRD LENTIL	AGG	72407	Canada	27
LOKALNA SITNA	AGG	71595	Yugoslavia	68
Luna 09	AGG	74575	Ukraine	76
Miser	AGG	71597	Ethiopia	91
Nipper	PBA		Australia	30
OBRAZTZOV CHIFLIK 7	AGG	73353	Bulgaria	53
PBA Blitz	PBA		Australia	46
PBA Flash	AGG	75320	Australia	26
PENZENSKAYA 14	AGG	71584	Former Soviet Union	28
PI 451763	AGG	72393	United States	100
PI 458503	AGG	72397	Mexico	100
PI 472165	AGG	72455	India	98
PI 472280	AGG	72568	India	100
PI 472317	AGG	72605	India	98
PI 472359	AGG	72647	India	96
PI 472368	AGG	72656	India	100
PI 472370	AGG	72658	India	97

Genotype (cont'd.)	Source	ATC #	Origin	Necrosis (%)
PI 472611	AGG	72898	Iran	90
PI 513271	AGG	73088	Pakistan	99
PLASNICKA	AGG	71603	Yugoslavia	95
Precoz	PBA		Argentina	7
RPIP 33-039-11177	AGG	72379	Cyprus	81
RPIP 33-071-10448	AGG	71759	Iran	96
RPIP 33-071-10483	AGG	71780	Iran	99
RPIP 33-071-10691	AGG	71934	Iran	75
RPIP 33-071-10725	AGG	71964	Iran	76
RPIP 33-071-10924	AGG	72141	Iran	100
RPIP 33-071-11037	AGG	72228	Iran	35
SCHWARZE LINSE	AGG	71491	Mexico	86
TALINSKAYA 6	AGG	71591	Armenia	3
TUB85-083-01	AGG	73228	Turkey	78
TUB86-16-07	AGG	73229	Turkey	49
USSR-05-05	AGG	73266	Tajikistan	45
VULGARIS	AGG	71219	Turkey	99
YASSI	AGG	71538	Turkey	98
Assay mean				64
LSD (0.05)				62

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Appendix 4 Additional information on lentil genotype, source, Australian Grains Genebank (AGG) accession number (ATC), country of origin and plant damage response to metribuzin (% necrosis) in hydroponic sand screen pot assay 4 conducted in Chapter 3

Genotype	Source	ATC #	Origin	Necrosis (%)
19	AGG	71640	Jordan	98
69	AGG	70154	Algeria	6
36008	AGG	70525	Ethiopia	100
36033	AGG	70527	Ethiopia	100
09920313	AGG	70870	Bangladesh	100
09920356	AGG	70866	Bangladesh	100
180785-0701	AGG	73043	Turkey	34
290685-0602	AGG	72950	Turkey	76
78S26014	AGG	70502	Jordan	45
96-047L*99R099	PBA		Australia	100
99-088L*02H037	PBA		Australia	81
ADSABOGEBA-CAIROSUPERMARKET	AGG	70741	Egypt	100
Boomer	PBA		Australia	77
ILL114	AGG	70987	Turkey	100
ILL116	AGG	70989	Turkey	95
ILL126	AGG	70999	Turkey	100
ILL142	AGG	71015	Turkey	100
ILL145	AGG	71019	Turkey	100
ILL154	AGG	71028	Turkey	99
ILL161	AGG	71035	Turkey	99
ILL163	AGG	71037	Turkey	68
ILL169	AGG	71044	Turkey	90
ILL170	AGG	71045	Turkey	80
ILL183	AGG	71059	Turkey	50
ILL188	AGG	71064	Turkey	100
ILL1907	AGG	71011	Turkey	100
ILL1917	AGG	71081	Ethiopia	36
ILL1953	AGG	71628	Iran	95
ILL198	AGG	71075	Turkey	90
ILL217	AGG	71096	Afghanistan	43
ILL220	AGG	71099	India	83
ILL224	AGG	71103	Belgium	78
ILL225	AGG	71104	Yemen	100
ILL239	AGG	71118	Former Soviet Union	41
ILL244	AGG	71123	Pakistan	51
ILL271	AGG	71156	Greece	36
ILL291	AGG	71176	Algeria	9
ILL292	AGG	71177	Algeria	24
ILL293	AGG	71178	Greece	13
ILL312	AGG	71197	Greece	60
ILL320	AGG	71205	Hungary	29
ILL323	AGG	71208	Yugoslavia	4
ILL339	AGG	71225	Spain	30
ILL362	AGG	71248	Chile	68
ILL363	AGG	71249	Chile	2
ILL425	AGG	71369	Chile	100
ILL456	AGG	71420	Chile	46
ILL5438	AGG	70486	Tunisia	45
ILL580	AGG	71552	Turkey	63
ILL6393	AGG	70566	Pakistan	97
Indianhead	AGG	70787	Canada	100
LAIRDLENTIL	AGG	72407	Canada	64
LENS390	AGG	70446	Yemen	100

Genotype (cont'd.)	Source	ATC #	Origin	Necrosis (%)
LENTOJA	AGG	71465	Guatemala	100
MORAVSKA	AGG	71145	Czech Republic	75
OBRAZTZOV CHIFLIK 7	AGG	73353	Bulgaria	41
PAK40827	AGG	70562	Pakistan	78
PallagiSarga	AGG	71143	Hungary	50
PBA Flash - big (Willamulka 2011)	PBA		Australia	91
PBAFlash	AGG	75320	Australia	70
PENZENSKAYA 14	AGG	71584	Former Soviet Union	38
PI509361	AGG	70669	Turkey	95
PI509383	AGG	70691	Turkey	43
PI509386	AGG	70694	Turkey	53
PI509413	AGG	70721	Turkey	45
PI509419	AGG	70727	Turkey	73
PI509421	AGG	70729	Turkey	39
PI509430	AGG	70738	Turkey	100
Precoz	PBA		Argentina	60
SP1333	AGG	70435	Argentina	8
SP77	AGG	70425	Chile	25
TALINSKAYA 6	AGG	71591	Armenia	79
Assay mean				67
LSD (0.05)				55

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Appendix 5 Additional information on lentil genotype, source, Australian Grains Genebank (AGG) accession number (ATC), country of origin and plant damage response to metribuzin (% necrosis) in hydroponic sand screen pot assay 5 conducted in Chapter 3

Genotype	Source	ATC #	Origin	Necrosis (%)
1	AGG	71449	Lebanon	10
69	AGG	70154	Algeria	44
70	AGG	70155	Algeria	82
73	AGG	70156	Algeria	95
1010	AGG	70405	Chile	46
3003	AGG	70416	Chile	101
36041	AGG	70528	Ethiopia	90
6/9	AGG	71406	Chile	85
9920300	AGG	70869	Bangladesh	100
9920325	AGG	70856	Bangladesh	99
11-3-103	AGG	70458	Morocco	70
11B	AGG	70246	Afghanistan	95
180785-0701	AGG	73043	Turkey	49
188-67	AGG	71835		103
26512-68	AGG	70138	Turkey	55
290685-0602	AGG	72950	Turkey	81
31670-70	AGG	70140	Turkey	99
31974-70	AGG	70142	Turkey	60
32214-70	AGG	70143	Turkey	59
32725-71	AGG	70144	Turkey	83
96-047L*99R099	PBA		Australia	86
99-088L*02H037	PBA		Australia	84
ADASS	AGG	71627		92
ARI 00243	AGG	70536	Cyprus	93
ARI 00336	AGG	70538	Cyprus	78
BGRC 025689	AGG	70557	Tunisia	101
Boomer	PBA		Australia	99
BOYACA 1	AGG	70237	Colombia	83
CUNDINA MARCO 7	AGG	70242	Colombia	86
Daghestanica	AGG	70009	Russian Federation	96
EL 39	AGG	70250	Ethiopia	67
F144	AGG	70150	Egypt	98
ILL 1090	AGG	70173	Iran	84
ILL 1138	AGG	70178	Egypt	76
ILL 1146	AGG	70185	Egypt	87
ILL 1175	AGG	70192	Lebanon	101
ILL 1452	AGG	70215	Iran	93
ILL 1509	AGG	70219	Iran	95
ILL 1511	AGG	70220	Lebanon	96
ILL 157	AGG	71031	Syria	73
ILL 159	AGG	71033		73
ILL 160	AGG	71034	Turkey	76
ILL 174	AGG	71049	Turkey	43
ILL 176	AGG	71051	Turkey	102
ILL 1764	AGG	70252	Afghanistan	100
ILL 1784	AGG	70254	Afghanistan	85
ILL 1802	AGG	70257	Afghanistan	102
ILL 1813	AGG	70261	Afghanistan	101
ILL 1822	AGG	70267	Afghanistan	87
ILL 1824	AGG	70269	Afghanistan	95
ILL 187	AGG	71063	Turkey	101
ILL 1915	AGG	71080	Egypt	93
ILL 1927	AGG	71318	Chile	64

Genotype (cont'd.)	Source	ATC #	Origin	Necrosis (%)
ILL 204	AGG	71083	Ethiopia	100
ILL 213A	AGG	70024	Afghanistan	99
ILL 218	AGG	71097	Afghanistan	94
ILL 219	AGG	70005	India	79
ILL 2214	AGG	70297	Lebanon	101
ILL 230	AGG	71109	Pakistan	96
ILL 2449	AGG	70304	Afghanistan	99
ILL 2452	AGG	70307	Afghanistan	80
ILL 247A	AGG	70031	Ethiopia	73
ILL 254	AGG	71135	Greece	102
ILL 278	AGG	71163	Greece	57
ILL 315	AGG	71200	France	82
ILL 320B	AGG	70033	Hungary	79
ILL 344	AGG	70006	Italy	95
ILL 349	AGG	70007	Mexico	96
ILL 358	AGG	71244		97
ILL 393	AGG	71297	Chile	95
ILL 403	AGG	71314	Chile	63
ILL 405	AGG	71316	Chile	82
ILL 422	AGG	70946	Chile	83
ILL 436	AGG	71384	Chile	56
ILL 4368	AGG	70377	Cyprus	100
ILL 4383	AGG	70380	Turkey	100
ILL 4463	AGG	70385	Syria	103
ILL 447	AGG	71399	Chile	85
ILL 4486	AGG	70387	Syria	100
ILL 4493	AGG	70390	Syria	100
ILL 453	AGG	71412	Chile	28
ILL 4542	AGG	70402	Syria	91
ILL 472	AGG	71443	Syria	99
ILL 476	AGG	71447	Syria	63
ILL 493	AGG	71464	Syria	9
ILL 514	AGG	71485	Turkey	97
ILL 53	AGG	70082	Iraq	32
ILL 560	AGG	71532	Turkey	73
ILL 6080	AGG	70544	Pakistan	89
ILL 6091	AGG	70546	Pakistan	96
ILL 623B	AGG	70037	Macedonia	84
ILL 625	AGG	70038	Macedonia	103
ILL 638	AGG	70137	Lebanon	99
ILL 754	AGG	70146	Iran	94
ILL 757	AGG	70147	Cyprus	76
ILL 920	AGG	70163	Tunisia	100
ILL1953	AGG	71628	Iran	101
ILL271	AGG	71156	Greece	64
ILL291	AGG	71176	Algeria	94
ILL292	AGG	71177	Algeria	49
ILL293	AGG	71178	Greece	96
ILL320	AGG	71205	Hungary	66
ILL323	AGG	71208	Yugoslavia	94
ILL339	AGG	71225	Spain	84
ILL362	AGG	71248	Chile	66
ILL363	AGG	71249	Chile	98
ILL425	AGG	71369	Chile	77
ILL456	AGG	71420	Chile	62
ILL5438	AGG	70486	Tunisia	98
ILL580	AGG	71552	Turkey	83
ILL6393	AGG	70566	Pakistan	46
Indianhead	AGG	70787	Canada	97
K-221	AGG	71657	Russian Federation	97
K-780	AGG	71684	Pakistan	100

Genotype (cont'd.)	Source	ATC #	Origin	Necrosis (%)
KENITRA PETITE VERTE NO.	AGG	71201	Greece	98
Kirmizi	AGG	70126	Turkey	98
L 1200	AGG	70282	Ethiopia	98
LAIRDLENTIL	AGG	72407	Canada	90
LENS 159\\75	AGG	70464	Iran	97
LENS 398	AGG	70448	Yemen	44
LENS 428	AGG	70450	Yemen	96
LG 128	AGG	70345	India	100
LP 54-1642	AGG	71325	Chile	67
LP 54-1960	AGG	71336	Chile	99
LP 54-1990	AGG	71337	Chile	100
LP 54-2969	AGG	71373	Chile	99
LP 54-608	AGG	71300	Chile	99
Nipper	PBA		Australia	92
PAK 40635	AGG	70560	Pakistan	94
PAN 16	AGG	70340	Nepal	100
PAN 8	AGG	70334	Nepal	96
PAN 9	AGG	70335	Nepal	100
PBA Blitz	PBA		Australia	97
PBA Flash	AGG	75320	Australia	85
PENZENSKAYA14	AGG	71584	Former Soviet Union	93
PI 211732	AGG	70092	Afghanistan	80
PI 251248	AGG	70099	Egypt	82
PI 297285	AGG	70103	Argentina	75
PI 298631	AGG	70107	Peru	57
PI 300563	AGG	70117	Lebanon	96
PI 302398	AGG	70119	Jordan	102
PI 319367	AGG	70122	Mexico	67
PI 339284	AGG	70129	Turkey	64
PI 339305	AGG	70134	Turkey	83
PI 374119	AGG	70277	Morocco	92
PI 509323	AGG	70631	Turkey	101
PI 509362	AGG	70670	Turkey	83
PI 509378	AGG	70686	Turkey	77
PI 509388	AGG	70696	Turkey	85
PI 509389	AGG	70697	Turkey	80
PI 509392	AGG	70700	Turkey	97
PI 509415	AGG	70723	Turkey	62
PI 509422	AGG	70730	Turkey	78
PI509383	AGG	70691	Turkey	62
PI509413	AGG	70721	Turkey	77
PI509421	AGG	70729	Turkey	53
PL 59-1225	AGG	71437	Chile	97
Precoz	PBA		Argentina	41
RED CHIEF	AGG	70002	United States	93
RPIP 33-071-10146	AGG	71708	Iran	99
RPIP 33-071-10417	AGG	71499	Iran	77
RPIP 33-071-10419	AGG	71733	Iran	103
RPIP 33-071-10482	AGG	71779	Iran	84
RPIP 33-071-10498	AGG	71794	Iran	98
RPIP 33-071-10502	AGG	71798	Iran	62
RPIP 33-071-10511	AGG	71807	Iran	74
RPIP 33-071-10515	AGG	71811	Iran	44
RPIP 33-071-10538	AGG	71834	Iran	99
RPIP 33-071-10577	AGG	71855	Iran	57
RPIP 33-071-10586	AGG	71864	Iran	76
RPIP 33-071-10601	AGG	71867	Iran	100
RPIP 33-071-10624	AGG	71881	Iran	53
RPIP 33-071-10638	AGG	71888	Iran	88
RPIP 33-071-10643	AGG	71891	Iran	98
RPIP 33-071-10655	AGG	71901	Iran	100

Genotype (cont'd.)	Source	ATC #	Origin	Necrosis (%)
RPIP 33-071-10903	AGG	71505	Iran	96
SLOVENIAN KRAYODA	AGG	71492		97
SP1333	AGG	70435	Argentina	6
SP77	AGG	70425	Chile	91
TALINSKAYA 6	AGG	71591	Armenia	88
Yerli	AGG	70136	Turkey	97
Assay mean				83
LSD (0.05)				39

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Appendix 6 Additional information on lentil genotype, source, Australian Grains Genebank (AGG) accession number (ATC), country of origin and plant damage response to metribuzin (% necrosis) in hydroponic sand screen pot assay 6 conducted in Chapter 3

Genotype	Source	ATC #	Origin	Necrosis (%)
1	AGG	71449	Lebanon	96
69	AGG	70154	Algeria	88
1010	AGG	70405	Chile	92
40781	AGG	73174	Pakistan	98
010785-0403	AGG	72964	Turkey	84
040689-0201	AGG	73435	Turkey	95
040689-0301	AGG	73235	Turkey	80
060689-0301	AGG	73260	Turkey	60
100785-0102	AGG	73012	Turkey	95
160689-0102	AGG	73438	Turkey	45
180785-0701	AGG	73043	Turkey	97
300685-0603	AGG	72955	Turkey	94
31974-70	AGG	70142	Turkey	96
96-047L*99R099	PBA		Australia	91
99-088L*02H037	PBA		Australia	80
Boomer	PBA		Australia	92
CNPH 84-021	AGG	73197	Brazil	95
E-2	AGG	72785		95
ESTON	AGG via PBA	70053	Canada	95
ILL 44	AGG	73854	Syria	93
ILL 6408	AGG	74430	Bulgaria	92
ILL 8209	AGG	74654		82
ILL 8236	AGG	74669		95
ILL 8332	AGG	74442		68
ILL 8333	AGG	74725		96
ILL 8512	AGG	74493	India	96
ILL 8561	AGG	74506	Morocco	94
ILL0051	AGG via PBA	70080	Iraq	100
ILL0052	AGG via PBA	70081	Iraq	98
ILL0098	AGG via PBA	70085	Morocco	95
ILL0166	AGG via PBA	70086	Turkey	94
ILL0183	AGG via PBA	70087	Turkey	93
ILL0209	AGG via PBA	70088	Afghanistan	98
ILL0210	AGG via PBA	70089	Costa Rica	88
ILL0212	AGG via PBA	70090	Afghanistan	100
ILL0213	AGG via PBA	70091	Afghanistan	97
ILL0215(ATFCC DUPLICATE2)	AGG via PBA	70093	Afghanistan	91
ILL0217	AGG via PBA	70094	Afghanistan	96
ILL0218	AGG via PBA	70095	Afghanistan	54
ILL0222	AGG via PBA	70026	Pakistan	99
ILL0226	AGG via PBA	70096	Pakistan	100
ILL0227	AGG via PBA	70097	Pakistan	98
ILL0228A	AGG via PBA	70027	Pakistan	93
ILL0229	AGG via PBA	70028	Pakistan	55
ILL0230	AGG via PBA	70029	Pakistan	100
ILL0230(ATFCC DUPLICATE1)	AGG via PBA	70030	Pakistan	93
ILL0230(ATFCC DUPLICATE2)	AGG via PBA	70098	Pakistan	93
ILL0246	AGG via PBA	70100	Pakistan	95
ILL0266	AGG via PBA	70101	Pakistan	98
ILL0268	AGG via PBA	70102	Argentina	72
ILL0316	AGG via PBA	70105	Greece	66

Genotype (cont'd.)	Source	ATC #	Origin	Necrosis (%)
ILL0344(ATFCC DUPLICATE1)	AGG via PBA	70035	Italy	88
ILL0379	AGG via PBA	70114	Chile	73
ILL0385	AGG via PBA	70115	Chile	95
ILL0427	AGG via PBA	70116	Chile	55
ILL0473	AGG via PBA	70036	Syria	51
ILL0484	AGG via PBA	70118	Lebanon	96
ILL0497	AGG via PBA	70008	Mexico	86
ILL0514	AGG via PBA	70123	Turkey	83
ILL0536	AGG via PBA	70125	Turkey	78
ILL0549	AGG via PBA	70127	Turkey	96
ILL0557	AGG via PBA	70130	Turkey	100
ILL0559	AGG via PBA	70131	Turkey	99
ILL0564	AGG via PBA	70132	Turkey	96
ILL0568	AGG via PBA	70133	Turkey	81
ILL0581	AGG via PBA	70135	Turkey	96
ILL0657	AGG via PBA	70139	Turkey	71
ILL0674	AGG via PBA	70141	Turkey	73
ILL0712	AGG via PBA	70145	Morocco	100
ILL0779	AGG via PBA	70148	Syria	91
ILL0817	AGG via PBA	70149	Egypt	91
ILL0821	AGG via PBA	70151	Egypt	94
ILL0822	AGG via PBA	70152	Egypt	93
ILL0838	AGG via PBA	70044	Lebanon	96
ILL0859	AGG via PBA	70158	Algeria	65
ILL0885	AGG via PBA	70159	Lebanon	93
ILL0901	AGG via PBA	70161	Iran	90
ILL0910	AGG via PBA	70162	Iran	92
ILL0920A	AGG via PBA	70045	Tunisia	91
ILL0937	AGG via PBA	70165	Afghanistan	98
ILL0941	AGG via PBA	70166	Pakistan	92
ILL0943	AGG via PBA	70167	Pakistan	96
ILL0955	AGG via PBA	70168	Cyprus	98
ILL1008	AGG via PBA	70169	Iran	93
ILL1078	AGG via PBA	70170	Iran	83
ILL1079	AGG via PBA	70171	Iran	79
ILL1085	AGG via PBA	70172	Iran	76
ILL1122	AGG via PBA	70174	Iran	63
ILL1124	AGG via PBA	70175	Iran	96
ILL1137	AGG via PBA	70177	Egypt	93
ILL1139	AGG via PBA	70179	Lebanon	96
ILL1140	AGG via PBA	70180	Egypt	98
ILL1141	AGG via PBA	70181	Egypt	99
ILL1142	AGG via PBA	70182	Egypt	97
ILL271	AGG	71156	Greece	100
ILL292	AGG	71177	Algeria	45
ILL320	AGG	71205	Hungary	61
ILL6393	AGG	70566	Pakistan	94
Indianhead	AGG	70787	Canada	100
LENS 33	AGG	73583	Greece	94
LENS 39	AGG	73588	Greece	96
LENS 45	AGG	73593	Greece	95
LENS 6	AGG	73558	Greece	96
LENS 69	AGG	73615	Greece	89
LENS 85	AGG	73630	Greece	91
LENS 88	AGG	73633	Greece	98
LP 54-1642	AGG	71325	Chile	58
MARDOM	AGG	73428		53
MASOOR	AGG	73142	Pakistan	92
MASOOR DL-6	AGG	73219	Pakistan	99
MASSOR	AGG	73168	Pakistan	95
MOUNTAIN LENTIL#2	AGG	73418	Italy	93

Genotype (cont'd.)	Source	ATC #	Origin	Necrosis (%)
Nipper	PBA		Australia	94
PALOUSE	AGG	73827	North America	98
PI 298631	AGG	70107	Peru	87
PI 435960	AGG	72385	Iran	96
PI 472119	AGG	72409	India	96
PI 472122	AGG	72412	India	95
PI 472136	AGG	72426	India	90
PI 472145	AGG	72435	India	96
PI 472175	AGG	72465	India	99
PI 472182	AGG	72472	India	96
PI 472265	AGG	72553	India	100
PI 472273	AGG	72561	India	83
PI 472276	AGG	72564	India	99
PI 472320	AGG	72608	India	97
PI 472334	AGG	72622	India	96
PI 472363	AGG	72924	India	92
PI 472378	AGG	72666	India	100
PI 472379	AGG	72667	India	100
PI 472395	AGG	72683	India	90
PI 472612	AGG	72899	Iran	68
PI 472614	AGG	72901	Iran	92
PI 472625	AGG	72912	Iran	98
PI 472635	AGG	72922	Iran	98
PI 472923	AGG	72938		86
PI 513278	AGG	73095	Pakistan	98
PI165019	AGG	70021	Turkey	78
PI509421	AGG	70729	Turkey	68
RPIP 12-071-07018	AGG	72396	United Sta	61
RPIP 33-071-10515	AGG	71811	Iran	89
RPIP 33-071-10577	AGG	71855	Iran	56
RPIP 33-071-10607	AGG	72339	Lebanon	85
RPIP 33-071-10624	AGG	71881	Iran	75
RPIP 33-071-10671	AGG	71915	Iran	91
RPIP 33-071-10678	AGG	71921	Iran	73
RPIP 33-071-10681	AGG	71924	Iran	93
RPIP 33-071-10687	AGG	71930	Iran	91
RPIP 33-071-10719	AGG	71959	Iran	98
RPIP 33-071-10737	AGG	71975	Iran	97
RPIP 33-071-10754	AGG	71990	Iran	95
RPIP 33-071-10769	AGG	72005	Iran	97
RPIP 33-071-10775	AGG	72009	Iran	96
RPIP 33-071-10795	AGG	72026	Iran	89
RPIP 33-071-10811	AGG	72041	Iran	98
RPIP 33-071-10816	AGG	72045	Iran	99
RPIP 33-071-10828	AGG	72056	Iran	98
RPIP 33-071-10836	AGG	72064	Iran	62
RPIP 33-071-10849	AGG	72074	Iran	94
RPIP 33-071-10867	AGG	72089	Iran	94
RPIP 33-071-10871	AGG	72092	Iran	95
RPIP 33-071-10940	AGG	72156	Iran	71
RPIP 33-071-10944	AGG	72160	Iran	90
RPIP 33-071-10952	AGG	72168	Iran	94
RPIP 33-071-10959	AGG	72172	Iran	98
RPIP 33-071-10965	AGG	72177	Iran	90
RPIP 33-071-10970	AGG	72182	Iran	93
RPIP 33-071-10982	AGG	72352	Turkey	95
RPIP 33-071-10994	AGG	72190	Iran	100
RPIP 33-071-10997	AGG	72193	Iran	78
RPIP 33-071-11007	AGG	72199	Iran	83
RPIP 33-071-11012	AGG	72204	Iran	93
RPIP 33-071-11026	AGG	72217	Iran	56

Genotype (cont'd.)	Source	ATC #	Origin	Necrosis (%)
RPIP 33-071-11046	AGG	72234	Iran	90
RPIP 33-071-11065	AGG	72251	Iran	98
RPIP 33-071-11069	AGG	72254	Iran	99
RPIP 33-071-11149	AGG	72303	Iran	92
Slovenska Krajova Levocska	AGG	73663		86
SLOVENSKA MODRA	AGG	73680		96
SP1333	AGG	70435	Argentina	17
Assay mean				88
LSD (0.05)				34

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