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Title: Molecular Basis of Target-Site Resistance to Acetolactate Synthase-Inhibiting Herbicides in Mayweed Chamomile (*Anthemis cotula* L.)

Abstract approved:

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Carol A. Mallory-Smith

Mayweed chamomile (*Anthemis cotula* L.) is an annual weed in the Asteraceae family that is commonly found in fields of the Pacific Northwest. Acetolactate synthase (ALS)-inhibiting herbicides are frequently used to control a broad spectrum of weed species including mayweed chamomile. Seeds of four biotypes of mayweed chamomile (KJ, KL1, KL2, and GW) suspected to be resistant to ALS-inhibiting herbicides were collected from different fields in Washington State, USA. Seeds from a susceptible (S) biotype of mayweed chamomile collected in Oregon were used as the control in all the experiments. Greenhouse studies were conducted to determine if the biotypes were resistant to four chemistry classes of ALS-inhibiting herbicides which were sulfonylureas (SU), imidazolinones (IMI), pyrimidinylthio-benzoate (PTB), and sulfonylamino-carbonyltriazolinone (SCT). A whole-plant dose-response assay confirmed cross-resistance to thifensulfuron+tribenuron (SU) and imazethapyr (IMI)

in resistant biotypes. There were high levels of resistance to thifensulfuron+tribenuron and moderate to low levels of resistance to imazethapyr. Propoxycarbazone (SCT) and cloransulam (TP) were applied at the recommended field rate. All resistant biotypes had moderate levels of resistance to propoxycarbazone while biotypes GW and KJ had moderate and low levels of resistance to and cloransulam, respectively. The resistance also was confirmed using an *in vitro* ALS assay. The herbicide concentrations that inhibited ALS activity by 50% (I_{50}) in the resistant biotypes were between 26- and 289-fold greater than in the S biotype for thifensulfuron+ tribenuron; 2- to 5-fold greater for imazethapyr; 3- to 18-fold greater for propoxycarbazone; and 4- to 18-fold greater for cloransulam. Once resistance was confirmed, the *ALS* gene was sequenced to determine if mutations occurred in the target-site. At least two *ALS* isoforms of *ALS* gene (*ALS1* and *ALS2*) were found in the mayweed chamomile biotypes. No mutations were observed in *ALS2*. The target-site mutations conferring the resistance to ALS-inhibiting herbicides were likely in *ALS1*. Sequence analysis of the *ALS1* gene identified four point mutations (Pro$_{197}$ to Leu, Gln, Thr or Ser) at position 197 in the resistant biotypes. Homozygous and heterozygous resistance and the existence of two different mutant *ALS* alleles were found in resistant biotypes. The ALS-resistant alleles and genotypes in resistant biotypes were diverse and related to the cross-resistance to ALS-inhibiting herbicides.

Molecular Basis of Target-Site Resistance to Acetolactate Synthase-Inhibiting
Herbicides in Mayweed Chamomile (*Anthemis cotula* L.)

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To my grandparent, Jantip Nonthathum, and my lovely friend, Bowling

Molecular Basis of Target-Site Resistance to Acetolactate Synthase-Inhibiting Herbicides in Mayweed Chamomile (*Anthemis cotula* L.)

INTRODUCTION

Acetolactate Synthase-inhibiting Herbicides

Acetolactate synthase (ALS)-inhibiting herbicides have been used since 1982. Chlorsulfuron, the first ALS-inhibiting herbicide, was introduced in 1982 for use in cereals. The ALS-inhibiting herbicides remain valuable for weed management in a variety of crop and non-crop situations because of their low use rates, low mammalian toxicity, and broad-spectrum weed control (Mazur and Falco 1989). There are five chemistry classes of ALS-inhibiting herbicides; imidazolinone (IMI), pyrimidinylthio-benzoate (PTB), sulfonylamino-carbonyltriazolinone (SCT), sulfonylurea (SU), and triazolopyrimidine (TP). The SU family has the most numerous active ingredients followed by IMI, TP, PTB, and SCT (Mallory-Smith and Retzinger 2003). The most widely used of the ALS-inhibiting herbicide chemical families are the SUs and the IMIs (Saari et al. 1994).

The mode of action of ALS-inhibiting herbicides is through binding of the herbicide with the ALS enzyme which is the first committed step of biosynthesis of the branched chain amino acids, valine, leucine, and isoleucine. Branched chain amino acids play an important role in protein synthesis required for plant growth (Monaco et al. 2002). Although SU and IMI herbicides do not directly bind to the ALS active site, they inhibit ALS activity by blocking a channel leading to the enzyme active site.

Ultimately, the herbicides decrease ALS affinity for its normal substrate (Duggleby et al. 2008; McCourt et al. 2006).

Herbicide Resistance

Herbicide resistance is defined by the Weed Science Society of America as "the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type" (WSSA 1998). The efficacy of the herbicide, the frequency of use, and the duration of phytotoxicity effect are the three primary components which contribute to selection pressure for herbicide resistance (Maxwell and Mortimer 1994). Evolved herbicide resistant weeds occur when gene frequencies within a weed population change as a result of selection pressure imparted by frequent use of one or more herbicide with the same mode of action (Christoffers 1999). The repeated use of a herbicide for as few as 3 to 5 yr to sensitive weed species can increase this selection pressure and the resistance of a particular weed species is generally detectable when the resistant plants comprise approximately 30% of the population (Monaco et al. 2002).

The first herbicide resistant weed species identified was common groundsel (*Senecio vulgaris* L.) with resistance to simazine (Ryan 1970). The International Survey of Herbicide Resistant Weeds currently reports 330 resistant weed biotypes, in 189 species (113 dicots and 76 monocots) which infest more than 300,000 fields in 60 countries (Heap 2009).

The mechanisms for resistance can be either nontarget site-based resistance or target site-based resistance. Nontarget-site resistance involves the exclusion of the

herbicide molecule from the target site. There are various potential mechanisms that could be responsible for nontarget site-based resistance including reduced uptake, reduced translocation, sequestration, and metabolism (Preston and Mallory-Smith 2001). Target-site resistance is a modification in the binding site to reduce or eliminate the ability of the herbicide to bind or interact (Preston and Mallory-Smith 2001). Target-site mutations have been identified in weeds resistant to different herbicide groups that inhibit PSII (Gronwald 1994), microtubule assembly (Yamamoto et al. 1998), and the enzymes ALS (Saari et al. 1994), acetyl-CoA carboxylase (ACCase) (Delye et al. 2005), and 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (Wakelin and Preston 2006).

Resistance to ALS-inhibiting Herbicides

Resistance to ALS-inhibiting herbicides evolved quickly (Tranel and Wright 2002). The first report of ALS resistance occurred in pricky lettuce (*Lactuca serriola* L.) only five years after the initial use of chlorsulfuron (Mallory-Smith et al. 1990). Currently, there are 101 weed species (70 dicots and 31 monocots) with evolved resistance to ALS-inhibiting herbicides (Heap 2009).

The nontarget-site resistance to ALS-inhibiting herbicides via herbicide metabolism has been reported less frequently than resistance caused by target-site mutations (Heap 2009; Tranel and Wright 2002). The metabolic detoxification of ALS-inhibiting herbicides has been documented in some weed species. For example, the metabolism of chlorsulfuron was increased by conjugation to glucose in rigid ryegrass (*Lolium rigidum* Gaudin) (Christopher et al. 1991) and the Cyt P450-

mediated metabolic resistance to penoxsulam was detected in late watergrass (*Echinochloa phyllogogon* (Stapf.) Koss.) (Yasuor et al. 2009).

The target-site resistance to ALS-inhibiting herbicides involves a decrease in the sensitivity of the ALS (Saari et al. 1994) caused by a substitution of one of five highly conserved regions (domain A, B, C, D and E) of *ALS* gene (Tranel and Wright 2002). The mutations of the *ALS* gene have been reported at amino acids Ala_{122} in domain C, Pro_{197} in domain A, Ala_{205} in domain D, Trp_{574} in domain B, and Ser_{653} in domain E (numbering based on ALS from *Arabidopsis thaliana* L.) (Boutsalis et al. 1999; Tranel and Wright 2002). Another mutation of the *ALS* gene was recently reported at amino acid Asp_{376} to Glu in SU-, IMI-, PTB- and TP-resistant smooth pigweed (*Amaranthus hybridus* L.) (Whaley et al. 2007).

ALS is a nuclear gene and the resistance *ALS* alleles are dominant or partially dominant over the susceptible alleles. Therefore, the resistance alleles can be transmitted by pollen and seed. (Tranel and Wright 2002).

Cross-resistance to ALS-inhibiting Herbicides

Cross-resistance occurs when a plant selected for resistance to specific chemical families also is resistant to other herbicides with the same mode of action (Monaco et al. 2002). Cross-resistance is evident among weed species resistant to ALS-inhibiting herbicides. Cross-resistance to ALS-inhibiting herbicides are due to the various amino acids substitutions and different mutations to this group of herbicides (Preston and Mallory-Smith 2001; Tranel and Wright 2002). In general, mutations at Pro_{197} confer resistance to the SU and TP herbicides; mutations at Ala_{122},

Ala_{205} and Ser_{653} confer resistance to IMI herbicides whereas a mutation at Trp_{574} confers resistance to IMI, SU, TP and PTB herbicides. Therefore, cross-resistance patterns can be classified into three groups: SU and TP resistance; IMI and PTB resistance; and SU, IMI, TP and PTB resistance (Tranel and Wright 2002). Cross-resistance among SU and IMI herbicides has been reported in many weed species (Tranel and Wright 2002; Tranel et al. 2009).

Resistance to one compound of a particular chemical family of ALS-inhibiting herbicide does not always confirm cross-resistance to all members of that chemical family (Tranel and Wright 2002). For example, in 1990 cross-resistance to ALS-inhibiting herbicides was reported in prickly lettuce which was resistant to eight other SU herbicides, imazapyr, imazethapyr, but not imazaquin (Mallory-Smith et al. 1990). Evolved resistance to bensulfuron in smallflower umbrella sedge (*Cyperus difformis* L.) did not result in broad cross-resistance to other SU herbicides (Merotto et al. 2009).

In addition to the cross-resistance between the SU and the IMI herbicides, there are reports of cross-resistance to other chemical families such as the cross-resistance in a smooth pigweed to the SU, IMI, PTB and TP (Whaley et al. 2007) and the cross-resistance in a smallflower umbrella sedge to SU, IMI, PTB, SCT, and TP (Merotto et al. 2009). The different patterns of cross-resistance among ALS-inhibiting herbicides indicate that different chemical structures have different modes of binding to ALS (Preston and Mallory-Smith 2001). Moreover, a partially overlapping binding

site on the ALS enzyme for the SU and IMI herbicides can support the occurrence of cross-resistance (McCourt et al. 2006).

Mayweed Chamomile

Mayweed chamomile (*Anthemis cotula* L.) is a winter annual weed in the Asteraceae family. Distinct characteristics include fern-like leaves, daisy-like flowers, and a bad odor (Gaines et al. 1972). Mayweed chamomile is a diploid (2n = 18) and primarily outcrossing species. However, self fertility has been documented in this species (Mitsuoka and Ehrendorfer 1972).

Mayweed chamomile is native to the Mediterranean region and was introduced to the United States. In the Pacific Northwest, mayweed chamomile is a troublesome weed in many crops including wheat (*Triticum aestivum* L.), pea (*Pisum sativum* L.) and lentil (*Lens culinaris* L.). Mayweed chamomile can emerge in late fall or early spring from moist soils and growth may be limited by the crop canopy in the early-season (Gealy et al. 1991). However, it has a rapid growth rate in late summer as crops senesce and may cause a serious harvest complication in pea fields (Stephens and Ogg 1989). Some ALS-inhibiting herbicides control mayweed chamomile. Chlorsulfuron+metsulfuron, trifensulfuron+tribenuron, tribenuron and chlorsulfuron have been reported to provide excellent control of mayweed chamomile in wheat. Imazethapyr applied alone or in combination with other herbicides can be used preplant incorporated or preemergence in chickpeas (*Cicer arietinum* L.), pea and lentil to control mayweed chamomile (Peachey et al. 2009). In 1997, mayweed chamomile was reported to have evolved resistance to ALS-inhibiting herbicides in

Idaho (Mallory-Smith, unpublished data). However, there are no published studies describing *ALS* resistance and the molecular basis of *ALS* resistance in mayweed chamomile.

Sunflower (*Helianthus annuus* L.), another species in the Asteraceae family, is reported to be resistant to SU herbicides, and the resistance mechanism is due to target site mutation of *ALS* gene at position Pro_{197} (Kolkman et al. 2004). In the *ALS* gene family of sunflower, there are three *ALS* isoforms of *ALS* gene (*AHAS1*, *AHAS2*, and *AHAS3*) which are expressed at different levels from different parts of the plant (Hawley 2006; Kolkman et al. 2004). *AHAS1* has the highest expression exhibited in leaf tissue and also is the most highly expressed gene in the family. Therefore, it is the most likely to be the target site for mutations conferring herbicide resistance (Hawley 2006). The *ALS* sequence from a related species like sunflower may be helpful as a reference for mayweed chamomile. The information about the *ALS* gene family in mayweed chamomile may provide more knowledge of the resistance mechanism.

The goal of this study was to gain a greater understanding of the resistance mechanism to ALS-inhibiting herbicides in mayweed chamomile. The specific objectives were to conduct greenhouse and laboratory experiments to determine if the mayweed chamomile biotypes (KJ, KL1, KL2, and GW) from Washington State, USA were resistant to four chemical families of ALS-inhibiting herbicides (SU, IMI, SCT, and TP) and determine the physiological and molecular basis of herbicide resistance.

MATERIALS AND METHODS

Plant Material

Seeds of mayweed chamomile biotypes KJ, KL1, KL2, and GW with suspected resistance to ALS-inhibiting herbicides were collected from different fields, over multiple years, and with varying histories of ALS-inhibiting herbicides (Table 1). Seeds from a susceptible (S) biotype of mayweed chamomile collected in 2008 at Hyslop Farm, Corvallis, Oregon were used as a control in all experiments. For the greenhouse studies, plants were grown at 25/20 C day/night temperature with a 14 h photoperiod and were watered as needed.

Table 1. Field history leading to the suspected evolution of resistance to ALS-inhibiting herbicides in mayweed chamomile biotypes from different fields in Washington State, USA.

Biotype	Crop	Year	ALS-inhibiting herbicides control history
KJ[a]	Winter wheat	2007	Thifensulfuron+ tribenuron
KL1	Peas	2003	Imazethapyr
	Winter wheat	2006	Thifensulfuron+ tribenuron
	Spring barley	2007	Thifensulfuron+ tribenuron
KL2	Winter wheat	2006	Chlorsulfuron+ metsulfuron
	Spring barley	2007	Thifensulfuron+ tribenuron
GW	Winter wheat	2006	Thifensulfuron
	Spring wheat	2007	Thifensulfuron+ tribenuron

[a] only one year history provided.

Whole-Plant Dose-Response Assay

Seeds were planted in 267-ml plastic pots containing commercial potting mix[1]. Plants at the four to six leaf stages were sprayed with thifensulfuron+tribenuron[2] at 0.002, 0.005, 0.016, 0.053 and 0.157 kg ai ha^{-1} and imazethapyr[3] at 0.005, 0.017, 0.053, 0.175 and 0.527 kg ai ha^{-1}. Only the recommended field rate of propoxycarbazone[4] (0.044 kg ai ha^{-1}) and cloransulam[5] (0.035 kg ai ha^{-1}) were used because of limited seed availability. The nonionic surfactant, R-11[6], at 0.25% by vol was added to the herbicide solutions. Shoot biomass was harvested two wk after herbicide treatment, dried at 60 C for 72 h, and weighed. Biomass data are reported as percentage of the untreated control. The experiment was performed in a completely randomized design. For each biotype, there were four plants of each replication and three replications of each herbicide treatment. The experiment was repeated.

***In vitro* ALS Activity Assay**

Mayweed chamomile plants were grown in the greenhouse as described previously. For the ALS extraction, 4 g of fresh tissue of each biotype were harvested at three wk after planting. Subsequent steps were derived from previous protocols (Hart et al. 1992; Ray 1984; Singh et al. 1988). Leaf tissue was frozen with liquid nitrogen and ground to a fine powder using a mortar and pestle. Ground tissue was homogenized with 10 ml of a reaction buffer of 50 mM potassium phosphate (pH 7.6) containing 200 mM sodium pyruvate, 1.25 mM $MgCl_2$, 1.25 mM thiamine pyrophosphate, and 2.5 µM flavin adenine dinucleotide. In addition to the reaction buffer, 1 g of polyvinylpolypyrrolidone (ratio 1:4 of fresh weight) was added to the

homogenate and mixed before filtering through 8 layers of cheesecloth. The
homogenate was centrifuged at 4 C and 12,500 rpm for 15 min. The supernatant
(protein extraction) was collected and the pellet was discarded.

Enzyme reactions were conducted in a microtiter plate and consisted of 50 μl
protein extraction and 50 μl herbicide solution (varying concentrations of herbicide as
shown in Table 2). The microtiter plate was incubated at 37 C for 90 min. Then, the
reaction was stopped by the addition of 25 μl 1.8 N H_2SO_4 and the product was
allowed to decarboxylate at 60 C for 15 min. The acetoin formed was determined by
incubating with 0.25% creatine and 2.5% α-naphtol in 175 μl 2.5 N NaOH (Westerfeld
1945). The solutions were heated for an additional 15 min at 60 C and the acetoin
content was measured at 535 nm absorption in a microtiter plate reader[7]. ALS activity
levels from leaf extractions were determined in the presence of four ALS-inhibiting
herbicides representing the major chemical families (Table 2). The experiment had
four replications.

Table 2. Different herbicide concentrations of four major chemical families of ALS-inhibiting herbicides used in the *in vitro* ALS activity assay.

Herbicide treatment	Concentration
	μM
Chlorsulfuron (SU)	10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^2
Imazethapyr (IMI)	10^{-1}, 1, 3, 10, 30, 10^2, 3×10^2, 10^3
Propoxycarbazone (SCT)	10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^2
Cloransulam (TP)	10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-1}, 1, 10, 10^2

Partial *ALS* Gene Sequencing

To determine the molecular basis for resistance, mayweed chamomile plants of each biotype were grown as previously described. A 70 mg leaf tissue sample from each plant was collected and placed in a 1.5-ml microcentrifuge tube at -80 C. Leaf tissue was ground in liquid nitrogen in the microcentrifuge tube. DNA was extracted using a DNeasy Plant Mini Kit[8]. The plants were then treated at the four- to six- leaf stage with thifensulfuron+tribenuron at 0.032 kg ai ha^{-1} plus the nonionic surfactant, R-11, at 0.25% by vol to confirm resistance.

DNA extraction for the partial *ALS* gene (1,750 base pairs) was performed on four plants from all mayweed chamomile biotypes plus two additional biotypes A and B which had already been confirmed to be resistant to SU herbicides (Perez-Jones et al. 2004). DNA extraction for the *ALS1* gene (293 base pairs) was performed on 96 plants consisting of 16 plants of each biotype S, KJ, KL1, KL2, and GW, and eight plants each of biotype A and B.

Overlapping primer pairs were designed in three fragments to amplify the partial *ALS* gene (1,750 base pairs) which included the region of domains A, B, C, D, and E. Three sets of degenerate primer pairs were designed based on conserved sequences in three isoforms of *ALS* gene of sunflower (Kolkman et al. 2004). After sequencing the amplified fragments, specific primer pairs (*ALS1*) were designed to amplify 293 base pairs including domain A and D (Table 3).

The fragments of partial *ALS* gene for each biotype were amplified in three separate polymerase chain reactions (PCR) in a C1000 thermal cycler[9]. The PCR

reaction consisted of 30-50 ng of template DNA, 0.2 μM of each forward and reverse primer, 0.2 mM of each deoxynucleotide, 1x PCR buffer, and 1 unit of Tag DNA polymerase[10] in a total volume of 15 μl. The amplification protocol consisted of a 4 min incubation at 95 C, follow by 35 cycles of a denaturation step at 94 C for 30 s, an annealing step at 50 C for 30 s, an extension step at 74 C for 1 min, and a final extension cycle at 72 C for 10 min. Amplification products were resolved on a 2% agarose gel containing 0.01% ethidium bromide.

The amplified cDNA fragments were cloned using a TOPO® TA cloning kit[11]. *Escherichia coli* strains TOP10 were transformed with plasmid vector harboring the PCR product and plated on Luria-Bertani broth (LB) plus agar supplemented with 0.1% kanamycin. Plates were incubated overnight at 37 C. Positive colonies were selected. The PCR reactions were performed using the M13F and M13R primers. The PCR reaction mixture contained template DNA from a positive colony, 0.2 μM of each forward and reverse primer, 0.2 mM of each deoxynucleotide, 1x PCR buffer, and 1 unit of Tag DNA polymerase in a total volume of 50 μl. The reaction consisted of one denaturation step of 4 min at 95 C, 35 cycles of 45 s at 94 C, 45 s at 50 C, and 2 min at 72 C, and followed by a final extension step of 10 min at 72 C. PCR products were purified using a QIAquick® PCR purification kit[12] and sequenced using an automatic ABI PRISM® 3770 genetic analyzer and a BigDye® terminator v. 3.1 cycle sequencing kit[13]. To prevent PCR errors, four clones per PCR product were sequenced in both directions and aligned using the CLC Sequence Viewer v. 6.1[14].

Gene specific primers were designed for *ALS1* to amplify a fragment of about 293 base pairs including the regions of domains A and D (Table 3). The PCR reaction mixture consisted of 30-50 ng of template DNA, 0.2 μM of each forward and reverse primer, 0.2 mM of each deoxynucleotide, 1x PCR buffer, and 1 unit of Tag DNA polymerase in a total volume of 25 μl. After a denaturation step of 3 min at 94 C, a program of 35 cycles was used, consisting of 30 s at 94 C, 30 s at 63 C, and 45 s at 72 C, and followed by a final extension step of 10 min at 72 C. The amplified DNA fragment was purified using a QIAquick® PCR purification kit. The purified DNA was sent immediately for sequencing to an automatic ABI PRISM® 3770 genetic analyzer and a BigDye® terminator v. 3.1 cycle sequencing kit. The sequencing chromatograms were visualized and edited using Finch TV v. 1.4.0[15]. The DNA sequences were aligned and compared using the CLC Sequence Viewer v. 6.1.

Table 3. Oligonucleotide primers used for polymerase chain reaction (PCR) and sequencing for partial *ALS* gene.

Region	Target region	Primers	Sequence 5' - 3'	Amplicon size obtained (bp)	Annealing temp. (C)
I	Domain C, A, and D	Forward 1	TSGAAGCCTTAGAACGKGAAGGTG	913	50
		Reverse 1	GTGTGGCTGYTTATTCTTCCC		
II	Domain B	Forward 2	GGGATGCATGGSACGGTTTAT	848	50
		Reverse 2	CCSGGTGTATCCAACATYTTCTG		
III	Domain E	Forward 3	ATAACAAACCGAGGCAGTGG	571	50
		Reverse 3	CACAAACTGCTTAGGCGTTACA		
ALS1	Domain A and D	Forward 4	TGCACGCGCTTCAGGTAAACC	293	63
		Reverse 4	TCAAAACCGGACCAGGCCTA		

Statistical Analysis

The shoot biomass data for each herbicide treatment were analyzed using ANOVA and means were separated by a least significant difference (LSD) test using PROC GLM in SAS v. 9.2[16]. Dose-response curves were obtained by a nonlinear regression using a log-logistic equation (Equation 1) (Seefeldt et al. 1995; Streibig et al. 1993).

$$y = C + \frac{D-C}{1+(x/GR_{50})^b} \quad [1]$$

Where y represents shoot dry weight (percentage of untreated control) at herbicide rate x, C is the mean response at the highest herbicide rate (lower limit), D is the mean response when the herbicide rate is zero (upper limit), b is the slope of the line at GR_{50}, and GR_{50} is the herbicide rate required for 50% growth reduction. A homogeneity test by a one way analysis of variance (ANOVA) using the program R v. 2.8.0[17] was conducted of shoot biomass for the two dose-response experiments.

For the *in vitro* ALS activity assay, dose-response curves were obtained using the log-logistic equation as described previously. I_{50} values, which are the herbicide concentration that inhibited ALS activity by 50%, were obtained using a mathematical model (log-logistic curve).

Shoot biomass data of mayweed chamomile biotypes as affected by different rates of thifensulfuron+tribenuron and imazethpyr in the whole-plant dose-response assay were used to conduct the nonlinear regression analysis of the dose-response curve using the program R v. 2.8.0 (R Development Core Team 2008) with the *drc* (dose-response curves) extension package (Ritz and Streibig 2005). Programmed

commands for dose-response analysis in the *drc* package enabled R to graph the distribution of data and the regression lines (Knezevic et al. 2007). In order to create dose-response curves using all mayweed chamomile biotypes, the value for the control of each population was adjusted to fit the parameters of a model for separate control measurements[18]. The new model predicted a single common upper limit (at 100% of untreated control) for all curves. Dose-response curves with the adjusted model were also constructed using *in vitro* ALS activity data of mayweed chamomile biotypes as influenced by chlorsulfuron, imazethapyr, propoxycarbazone and cloransulam.

RESULTS

Whole-Plant Dose-Response Assay

Dose-response assays conducted on whole plants treated with thifensulfuron+tribenuron (SU) and imazethapyr (IMI) confirmed the resistance to these herbicides in four suspected resistant biotypes (KJ, KL1, KL2, and GW). All suspected resistant biotypes had high level of resistance to thifensulfuron+tribenuron (Figure 1; Table 4). The GR_{50} obtained from a mathematical model (log-logistic curve) for biotypes KJ ($GR_{50}= 0.18\pm0.053$ kg ai ha^{-1}) and GW ($GR_{50}= 0.14\pm0.038$ kg ai ha^{-1}) were estimated to be more than 100-fold greater than the GR_{50} for the S bioype ($GR_{50}= 0.001\pm0.0004$ kg ai ha^{-1}) while the GR_{50} for biotype KL2 ($GR_{50}= 0.08\pm0.016$ kg ai ha^{-1}) was approximately 60-fold greater than the GR_{50} for S biotype. The KL1 biotype had more than a 100-fold level of resistance to thifensulfuron+tribenuron; however, the GR_{50} could not be predicted because of the non-significant estimation ($p > 0.05$) by the log-logistic model. Only the suspected resistant biotypes survived the application of thifensulfuron+tribenuron at levels greater than the use rate of 0.016 kg ai ha^{-1}.

A differential response to imazethapyr was found among the four suspected resistant biotypes (Figure 2; Table 5). The biotype GW had a low level of resistance to imazethapyr. The value of GR_{50} for biotype GW ($GR_{50}= 0.04\pm0.008$ kg ai ha^{-1}) was slightly higher compare to the S biotype ($GR_{50}= 0.01\pm0.003$ kg ai ha^{-1}). Biotypes KJ, KL1 and KL2 had high levels of resistance to imazethapyr. The GR_{50} for the resistant

biotypes KJ (GR_{50} = 0.41±0.079 kg ai ha^{-1}), KL1 (GR_{50} = 0.18±0.056 kg ai ha^{-1}) and KL2 (GR_{50} = 0.18±0.037 kg ai ha^{-1}) were 33-, 14- and 15-fold greater than S biotype, respectively.

At the recommended field rate of propoxycarbazone (0.044 kg ai ha^{-1}), shoot biomass of all resistant biotypes was significantly different from S biotype (LSD = 23% of untreated control) and were 3-fold greater than those of S biotype (Figure 3). While at the recommended field rate of cloransulam (0.035 kg ai ha^{-1}), shoot biomass of biotypes KJ and GW was significantly different from of the S biotype (LSD = 9% of untreated control) and was approximately 2- and 3-fold higher than those of S biotype, respectively (Figure 4).

In vitro ALS Activity Assay

The *in vitro* ALS assay confirmed that resistance to ALS-inhibiting herbicides in all the resistant mayweed chamomile biotypes was associated with an altered target site. I_{50} values in the resistant biotypes were between 26- and 289-fold greater than in the S biotype for chlorsulfuron; 2- to 5-fold greater for imazethapyr; 3- to 18-fold greater for propoxycarbazone except for biotype KJ which was susceptible to propoxycarbazone; and 4- to 18-fold greater for cloransulam (Figure 5; Table 6).

Partial *ALS* Gene Sequencing

To identify the molecular basis for resistance, a 1,750-bp *ALS* coding region of mayweed chamomile including the five conserved domains (A, B, C, D, and E) from all biotypes were sequenced separately in three regions (I, II, and III) (Table 3).

According to the alignment of each fragment, at least two different isoforms of *ALS* gene (*ALS1* and *ALS2*) were detected in clones of individual mayweed chamomile plants (Figure 6). Single target-site mutations were found only in region I, specifically in domain A, of *ALS1*. No mutations were observed in *ALS2*. Sequence analysis of the *ALS1* gene resulted in four point mutations at position 197 in the resistant biotypes: Pro_{197} to Leu in KJ, KL1, KL2, GW and B; Pro_{197} to Gln in KJ, KL1and KL2; Pro_{197} to Thr in KL1, KL2 and GW; and Pro_{197} to Ser in KL2, GW, A and B (Figure 7; Table 7).

Either 8 or 16 plants were analyzed from each biotype. The analysis of the DNA sequence for S biotype revealed a Pro (CCA) at position 197 in all plants examined whereas the DNA sequence for resistant biotypes had various substitutions at Pro_{197}. Within resistant biotypes, two plants of biotype KL1 and one plant of biotype KL2 had a Pro at position 197 which is the same amino acid sequence as the wild type. The herbicide susceptibility of those three plants was confirmed by the herbicide treatment. These results indicated that there was segregation for resistance in both biotypes (Table 7).

The number of mutations and frequencies were different among the biotypes. The biotypes A, B, GW, and KJ had higher number of homozygous resistant plants comparing to the biotypes KL1 and KL2 (Table 7). The substitution from Pro_{197} to Gln was not observed in homozygous resistance whereas other three substitutions were found in homozygous resistance at least one biotype. Biotypes KJ, KL1, and GW were found in two different mutant *ALS* alleles.

The mutation from Pro_{197} to Ser was found more often in biotypes A, B, and GW compared to other biotypes (Table 7). Biotype A was 100% homozygous for Pro_{197} to Ser substitution. Biotype B was 88% homozygous for mutation Pro_{197} to Ser and 12% heterozygous for Pro_{197} to Leu. Biotype GW was 56% homozygous and 19% heterozygous for Pro_{197} to Ser. Biotype GW also had one resistant allele coding for Leu and another resistant allele coding for Ser (13%). The resistant biotype KJ was found to have one resistant allele coding for Leu and another resistant allele coding for Gln (25%). Most resistant individuals were homozygous (38%) and heterozygous (31%) for the substitution from Pro_{197} to Leu. Biotypes KL1 and KL2 had less than 13% homozygous resistant individuals. Biotype KL1 had three mutations at position 197 (Leu, Gln, and Thr). Biotype KL1 also had one resistant allele coding for Leu and another resistant allele coding for Thr (6%) and one mutant allele coding for Leu and another mutant allele coding for Gln (6%). Biotype KL2 had four different mutations from Pro_{197} to Leu, Gln, Thr or Ser and only 6% homozygous for mutation from Pro_{197} to Leu.
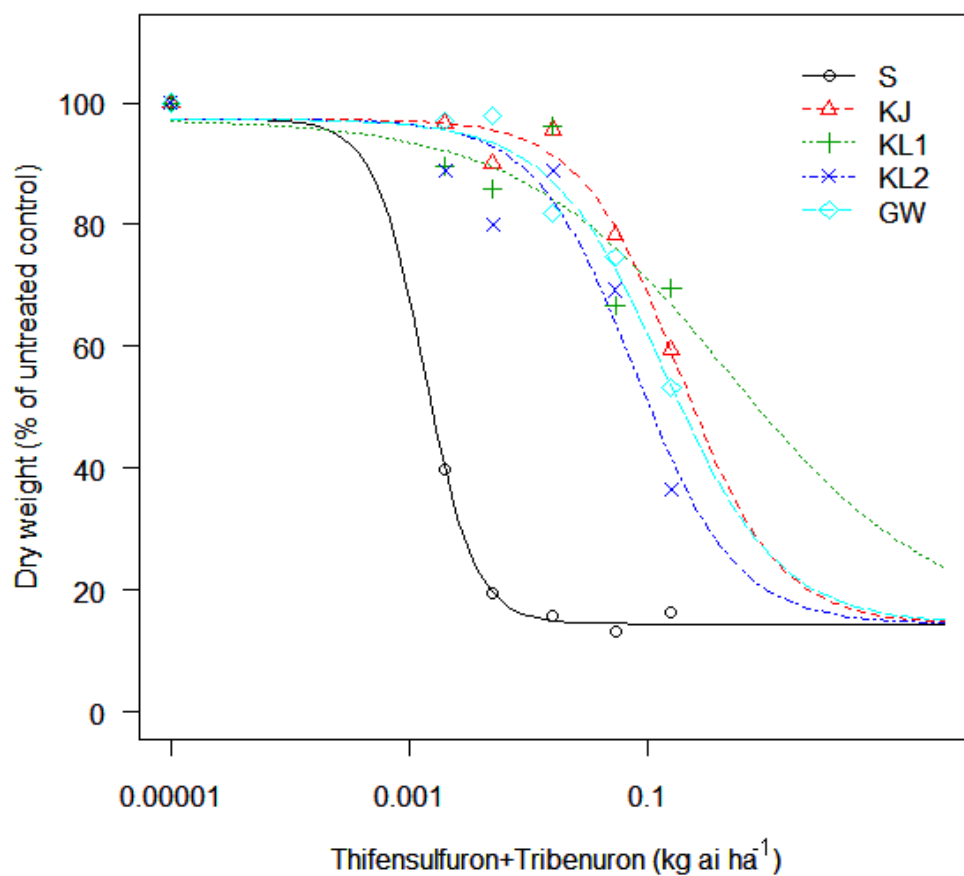
Figure 1. Shoot biomass of mayweed chamomile biotypes in response to different rates of thifensulfuron+tribenuron.

Table 4. The herbicide rate required for 50% growth reduction (GR_{50}) of mayweed chamomile biotypes in response to different rates of thifensulfuron+tribenuron.

Biotype	GR_{50} (kg ai ha^{-1}±SE) [a]	R/S [b]
S	0.001±0.0004 *[c]	
KJ	0.18±0.053 **	137
KL1	0.46±0.389 [NP]	-
KL2	0.08±0.016 **	59
GW	0.14±0.038 **	103

[a] GR_{50} values were calculated from regression curves presented in Figure 1.
[b] resistance ratio = GR_{50} of resistant biotype x GR_{50} of S biotype^{-1}.
[c] *, **, and [NP] represent $p \leq 0.05$, $p \leq 0.001$ and not predicted, respectively.
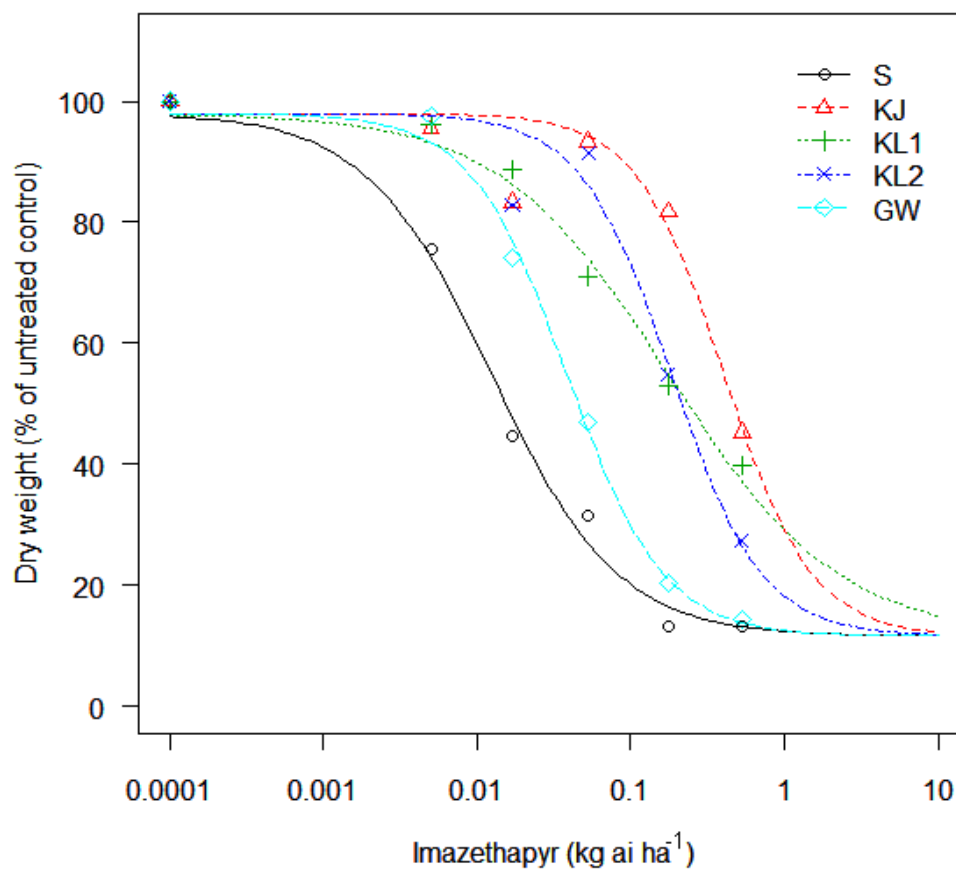
Figure 2. Shoot biomass of mayweed chamomile biotypes in response to different rates of imazethapyr.

Table 5. The herbicide rate required for 50% growth reduction (GR_{50}) of mayweed chamomile biotypes in response to different rates of imazethapyr.

Biotype	GR_{50} (kg ai ha^{-1}±SE)[a]	R/S[b]
S	0.01±0.003 **[c]	
KJ	0.41±0.079 **	33
KL1	0.18±0.056 *	14
KL2	0.18±0.037 **	15
GW	0.04±0.008 **	3

[a] GR_{50} values were calculated from regression curves presented in Figure 2.
[b] resistance ratio = GR_{50} of resistant biotype x GR_{50} of S biotype^{-1}.
[c] * and ** represent $p \leq 0.05$ and $p \leq 0.001$, respectively.
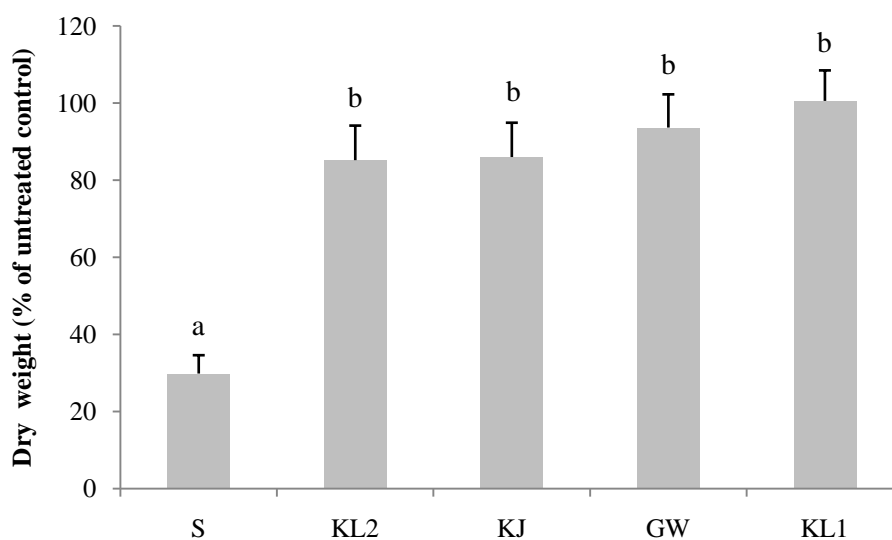
Figure 3. Shoot biomass and SE (T bar) of mayweed chamomile biotypes in response to recommended field rate of propoxycarbazone (0.044 kg ai ha^{-1}). Dry weight values with the same letter are not significantly different (p ≤ 0.05).
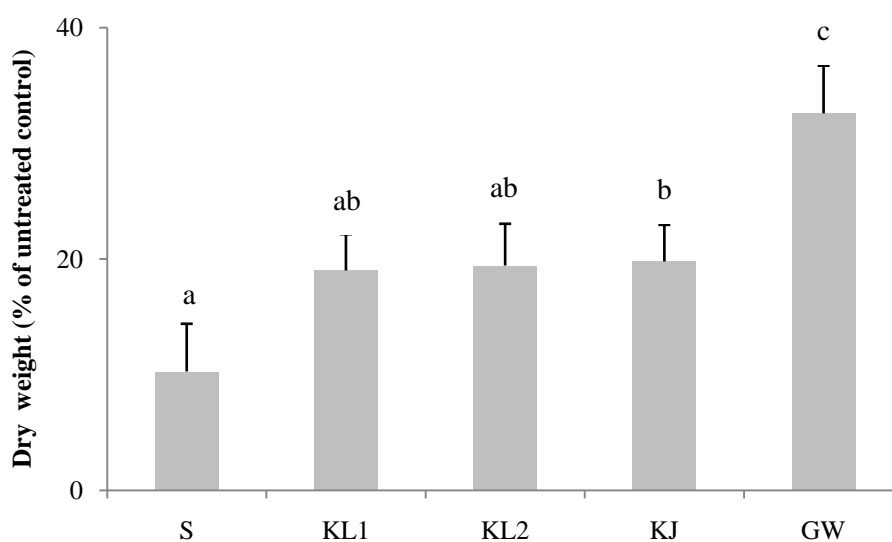
Figure 4. Shoot biomass and SE (T bar) of mayweed chamomile biotypes in response to recommended field rate of cloransulam (0.035 kg ai ha^{-1}). Dry weight values with the same letter are not significantly different (p ≤ 0.05).
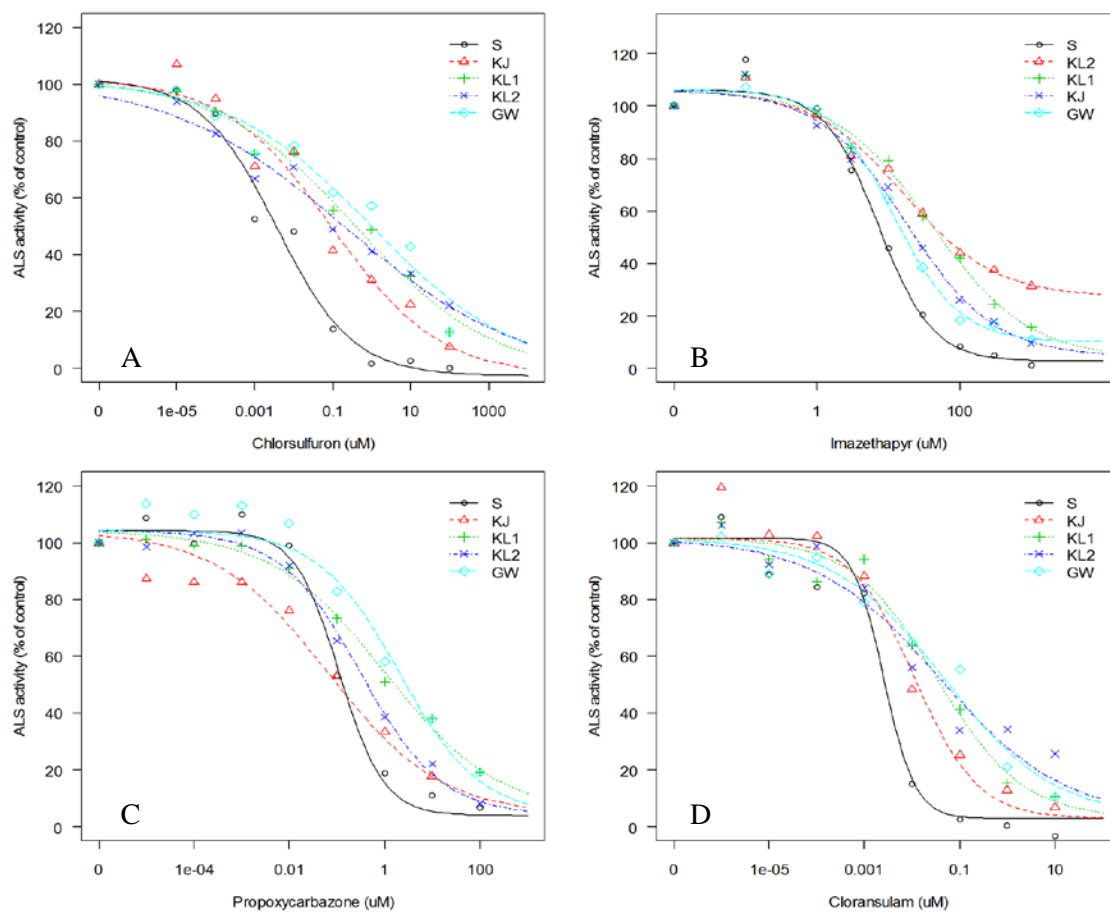
Figure 5. *In vitro* ALS activity of mayweed chamomile biotypes in response to A) chlorsulfuron, B) imazethapyr, C) propoxycarbazone and D) cloransulam. Note that each herbicide was used at different concentrations.

Table 6. The 50% inhibition value (I_{50}) obtained for ALS enzyme extracted from mayweed chamomile biotypes incubated with various concentrations of chlorsulfuron, imazethapyr, propoxycarbazone and cloransulam.

| Biotype	I_{50}[a]					R/S[b]
	uM		95% CI[c]			
Chlorsulfuron (SU)						
S	0.004	**[d]	0.003	-	0.005	
KJ	0.096	*	0.056	-	0.135	26
KL1	0.408	*	0.213	-	0.604	112
KL2	0.207	NP	0.087	-	0.326	> 50
GW	1.056	*	0.532	-	1.581	289
Imazethapyr (IMI)						
S	7.308	**	6.513	-	8.103	
KJ	17.872	**	13.967	-	21.777	2
KL1	39.701	*	26.502	-	52.899	5
KL2	16.195	**	11.375	-	21.016	2
GW	12.022	**	10.327	-	13.716	2
Propoxycarbazone (SCT)						
S	0.112	**	0.086	-	0.137	
KJ	0.065	*	0.040	-	0.091	-
KL1	1.038	*	0.632	-	1.444	9
KL2	0.314	*	0.213	-	0.415	3
GW	2.039	*	1.370	-	2.707	18
Cloransulam (TP)						
S	0.003	**	0.002	-	0.003	
KJ	0.011	**	0.008	-	0.014	4
KL1	0.033	**	0.023	-	0.043	13
KL2	0.038	*	0.023	-	0.054	15
GW	0.047	*	0.030	-	0.063	18

[a] I_{50} values were calculated from regression curves presented in Figure 5.
[b] resistance ratio = I_{50} of resistant biotype x I_{50} of S biotype^{-1}.
[c] 95% confidence intervals.
[d] *, **, and [NP] represent $p \leq 0.05$, $p \leq 0.001$ and not predicted, respectively.

```
        191                                       200
        Ala      Ile    Thr    Gly    Gln    Val   Pro*    Arg    Arg    Met
ALS1-A  GCT     ATT    ACG    GGC    CAA    GTG  ┌TCA┐  CGT    AGG    ATG
ALS1-KL1 GCT    ATT    ACG    GGC    CAA    GTG  │CTA│  CGT    AGG    ATG
ALS1-S  GCT     ATT    ACG    GGC    CAA    GTG  │CCA│  CGT    AGG    ATG
ALS2-A  GCG     ATT    ACT    GGT    CAG    GTG  │CCC│  CGT    AGG    ATG
ALS2-KL1 GCG    ATT    ACT    GGT    CAA    GTG  │CCC│  CGT    AGG    ATG
ALS2-S  GCG     ATT    ACT    GGT    CAG    GTG  └CCC┘  CGT    AGG    ATG
```

Figure 6. Partial nucleotide and deduced amino acid sequence alignment of two isoforms of *ALS* gene (*ALS1* and ALS2) from susceptible biotype (S), and two resistant biotypes (A and KL1). *ALS1* and *ALS2* nucleotides were aligned from each clone of the same individual plant. Single nucleotide polymorphisms (SNPs) are highlighted and the mutations conferring herbicide resistant at codon 197 are underlined.

```
601 GCT ATT ACG GGC CAA GTG CCA CGT AGG ATG ATT S

    ... ... ... ... ... ... .T. ... ... ... ...  KJ,KL1,KL2,GW,B

191  A   I   T   G   Q   V   P/L  R   R   M   I


601 GCT ATT ACG GGC CAA GTG CCA CGT AGG ATG ATT S

    ... ... ... ... ... ... .A. ... ... ... ...  KJ,KL1,KL2

191  A   I   T   G   Q   V   P/Q  R   R   M   I


601 GCT ATT ACG GGC CAA GTG CCA CGT AGG ATG ATT S

    ... ... ... ... ... ... A.. ... ... ... ...  KL1,KL2,GW

191  A   I   T   G   Q   V   P/T  R   R   M   I


601 GCT ATT ACG GGC CAA GTG CCA CGT AGG ATG ATT S

    ... ... ... ... ... ... T.. ... ... ... ...  KL2,GW,A,B

191  A   I   T   G   Q   V   P/S  R   R   M   I
```

Figure 7. Partial nucleotide and deduced amino acid sequence alignment of the *ALS* gene from S, KJ, KL1, KL2, GW, A and B biotypes. Shaded amino acids indicate Pro (CCA) to Leu (CTA), Pro (CAA) to Gln (CAA), Pro (CCA) to Thr (ACA), and Pro (CCA) to Ser (TCA) at position 197.

Table 7. The variable amino acids of the *ALS1* gene at the codon 197 and the phenotype after thifensulfuron+tribenuron treatment (0.032 kg ai ha^{-1}) from 96 plants of mayweed chamomile biotypes (S, KJ, KL1, KL2, GW, A and B).

Biotype	Number of plants analysed	Genotype at position 197	Number of plants where a specific genotype was detected	Phenotype[a]
S	16	Pro	16	S
KJ	16	Leu	6	R
		Pro/Leu	5	R
		Pro/Gln	2	R
		Leu/Gln	3	R
KL1	16	Pro	2	S
		Leu	2	R
		Pro/Gln	6	R
		Pro/Thr	3	R
		Pro/Leu	1	R
		Leu/Gln	1	R
		Leu/Thr	1	R
KL2	16	Pro	1	S
		Leu	1	R
		Pro/Leu	6	R
		Pro/Gln	1	R
		Pro/Thr	2	R
		Pro/Ser	3	R
		N/A[b]	2	R
GW	16	Ser	9	R
		Thr	1	R
		Pro/Ser	3	R
		Pro/Thr	1	R
		Leu/Ser	2	R
A	8	Ser	8	R
B	8	Ser	7	R
		Pro/Leu	1	R

[a] the phenotype was recorded at two weeks after application of herbicide.
[b] not available.

DISCUSSION

Both whole-plant dose-response and *in vitro* ALS assays were successful in confirming SU resistance in the mayweed chamomile biotypes. This is probably due to the fact that the mayweed chamomile biotypes from the different fields were selected with SU herbicides (Table 1). Biotype KL1, which was collected in a field with a history of IMI herbicides, was found to have a high level of resistance to IMI herbicides in both whole-plant dose-response and enzyme activity assays.

According to the whole-plant dose-response assay, the resistant biotypes KJ, KL1, and KL2 had high levels of resistance to thifensulfuron+tribenuron (approximately more than 60-fold greater than S biotype) and imazethapyr (approximately more than 10-fold greater than S biotype) while biotype GW also had a high level of resistance to thifensulfuron+tribenuron (approximately more than 100-fold greater than S biotype) but low level of resistance to imazethapyr (approximately 3-fold greater than S biotype). These levels of ALS-inhibiting herbicide resistance are similar to rigid ryegrass in Australia where the resistant populations had 50% to 100% of the individuals resistant to sulfometuron (SU) while 2% to 10% were resistant to imazethapyr (IMI) (Yu et al. 2008). The resistant mayweed chamomile biotypes were confirmed to have moderate levels of resistance to propoxycarbazone (3-fold greater than S biotype) while biotypes GW and KJ had moderate (3-fold greater than S biotype) and low (2-fold greater than S biotype) levels of resistance to cloransulam, respectively.

For the *in vitro* ALS assay, the resistant mayweed chamomile biotypes had high levels of resistance to chlorsulfuron (26- to 289-fold greater than S biotype), moderate levels of resistance to propoxycarbazone (3- to 18-fold greater than S biotype regardless of the result from biotype KJ) and cloransulam (4- to 18-fold greater than S biotype), and low levels of resistance to imazethapyr (2- to 5-fold greater than S biotype). For biotype KJ, it was not possible to determine the resistant ratio to propoxycarbazone since ALS activity data did not fit to log-logistic model. However, the whole-plant dose-response assay and sequencing data confirmed that biotype KJ was resistant to propoxycarbazone by target-site mutations. Therefore, this failure may be from the unstable and quick degradation of extracted enzyme.

The resistant ratios from the *in vitro* ALS assay were not consistent with those from the whole-plant dose-response assay. The whole-plant dose-response assay provides a more realistic level of herbicide resistance because the plant growth stage, time and rate of application are comparable with a field situation (Perez-Jones 2007). However, the *in vitro* ALS assay is complex but less time-consuming compared to the whole-plant dose-response assay.

Cross-resistance was confirmed in the resistant mayweed chamomile biotypes using whole-plant dose-response and enzyme activity assays. The cross-resistance in each biotype was indicated to be at least three chemistry classes of ALS-inhibiting herbicides. Biotypes KJ and GW had cross-resistance to the SU, IMI, SCT, and TP classes of ALS-inhibiting herbicides while biotypes KL1, and KL2 had cross-resistance to the SU, IMI, and SCT classes.

Based on enzyme activity assays, the resistance to ALS-inhibiting herbicides was associated with an altered target-site. Sequencing of the partial mayweed chamomile *ALS* was indicated that at least two *ALS* isoforms of *ALS* gene (*ALS1* and *ALS2*) were detected in mayweed chamomile. The target-site mutations conferring the resistance to ALS-inhibiting herbicides in all resistant mayweed chamomile biotypes were likely in *ALS1*. Similar findings in Asteraceae species has been reported in sunflower which has at least three isoforms of the *ALS* and only one *ALS* isoform of the resistant biotypes resulted in amino acid substitutions from Pro_{197} to Leu and Ala_{205} to Val (Kolkman et al. 2004).

The amino acid substitutions among the resistant mayweed chamomile biotypes were found in *ALS1* at position 197 from Pro to Leu, Gln, Thr, and Ser. (Figure 7). Substitution at Pro_{197} is normally found in target-site resistance to ALS-inhibiting herbicides. Pro_{197} is conserved amino acids in domain A, which is one of five highly conserved regions within the *ALS* gene (Tranel and Wright 2002). There are 23 different plant species carry the mutations referred to Pro_{197} and 8 different amino acid substitutions have been reported to confirm the substitutions from Pro_{197} to His, Thr, Arg, Leu, Gln, Ser, Ala, and Ile (Tranel et al. 2009). Substitutions at Pro_{197} commonly resulted in strong resistance to SU herbicides and generally in lesser degree to IMI herbicides (Saari et al. 1994).

However, the amino acid substitutions at Pro_{197} were different in each resistant mayweed chamomile biotype. All four mutations were found in one biotype (KL2) (Figure 7). Within a single resistant mayweed chamomile biotype, multiple mutations

and a combination of homozygous and heterozygous resistance to ALS-inhibiting herbicides were detected (Table 7). Biotypes KL1 and KL2 were found high levels of heterozygous resistance where one allele at position 197 was proline and another was a resistant allele. The existence of two different mutant *ALS* alleles at position 197 was found in biotypes KJ, KL1, and GW. Similar findings have been reported in resistant rigid ryegrass to sulfometuron (Yu et al. 2008) and resistant corn poppy (*Papaver rhoeas* L.) to tribenuron (Kaloumenos et al. 2009). The segregation of resistace to thifensulfuron+tribenuron was detected in biotypes KL1 and KL2.

Even though the resistant mayweed chamomile biotypes had the same target-site mutation at Pro_{197}, the levels of resistance to certain herbicides differed. The various levels of resistance in different biotypes may occur because of the different substitutions found in each biotype. Biotypes GW, A, and B which had the predominant mutation of Pro_{197} to Ser (Table 7) had high and moderate levels of resistant to SU and TP herbicides, respectively, but low levels of resistance to IMI herbicides (data for biotypes A and B not shown.). Similar results were reported in SU resistant prostrate pigweed (*Amaranthus blitoides* S. Wats.) which had the same target-site mutation but had no resistance to IMI (Sibony and Rubin 2003). The Pro_{197} substitutions to Leu, Gln, Thr, or Ser of biotypes KJ, KL1, and KL2 resulted in resistance to SU, IMI, and SCT herbicides. In kochia, the substitutions of Pro_{197} by Thr, Arg, Leu, Gln, Ala, and Ser resulted in the cross-resistance between SU and IMI herbicides (Guttieri et al. 1995).

In summary, mayweed chamomile had more than one isoform of *ALS* gene and the mutations were located in *ALS1*. This was probably due to the higher expression of *ALS1* compared to *ALS2*. There were four mutations at Pro_{197} that conferred the resistance to ALS-inhibiting herbicides. However, these mutations did not have negative effects on ALS activity. This segregation of resistance was expected in a species such as mayweed chamomile because it is an outcrossing species. The ALS-resistant alleles and genotypes in resistant biotypes were diverse and related to the cross-resistance to ALS-inhibiting herbicides.

CONCLUSION

Mayweed chamomile is a troublesome weed in many cropping systems throughout the Pacific Northwest. In 1997, mayweed chamomile was reported to have evolved resistance to ALS-inhibiting herbicides (SU and IMI) in Idaho. However, the mechanism of resistance for this biotype was either unknown or had not been reported (Heap 2009). This study is the first report of resistance and details the mechanisms of resistance of suspected resistant mayweed chamomile biotypes from Washington State and Idaho.

The suspected resistant mayweed chamomile biotypes were confirmed to be resistant to ALS-inihbiting herbicides. All resistant mayweed chamomile biotypes had high levels of resistance to SU and moderate to low levels of resistance to imazethapyr and propoxycarbazone. The low levels of resistance to TP were found in biotypes KJ and GW.

Mayweed chamomile possessed at least two isoforms of the *ALS* gene (*ALS1* and *ALS2*). No mutations were found in *ALS2*. The target-site mutations conferring the resistance to ALS-inhibiting herbicides in all resistant mayweed chamomile biotypes were likely in *ALS1*. Probably, *ALS1* was highly expressed compared to *ALS2*. The resistance and cross-resistance in the resistant mayweed chamomile biotypes were due to *ALS* target-site mutations at position 197. Among resistant biotypes, four amino acid substitutions were identified from Pro_{197} to Leu, Gln, Thr, and Ser. Within a single resistant biotype, multiple mutations were detected. The resistant alleles were found to be either homozygous or heterozygous. Some resistant individuals of

biotypes KJ, KL1, and GW were found containing two different 197 resistant alleles. The segregation of the resistant alleles was found in biotypes KL1 and KL2.

Even though the resistant mayweed chamomile biotypes had the same target-site mutations at Pro_{197}, the levels of resistance to certain herbicides differed. The various levels of resistance in different biotypes may cause by the different amino acid substitutions among biotypes, the multiple *ALS* mutations within biotype, and the segregation of the resistant alleles.

Cross-resistance patterns in the resistant mayweed chamomile biotypes depended on the type of mutation. The substitutions to Leu, Gln, Thr or Ser were found in biotypes KJ, KL1, and KL2 resulting in high levels of resistance to both SU and IMI herbicides. Biotypes GW, A, and B, which had substitutions predominantly to Ser had high levels of resistance to SU but low levels of resistance to IMI. The cross-resistance to at least three chemistry groups of ALS-inhibiting herbicides with numerous mutations caused more difficulty for weed management practices. The occurrence of cross-resistance in mayweed chamomile can reduce weed management options and increase control cost.

To the best of our knowledge, this was the first study on *ALS* mutations in mayweed chamomile in which many biotypes were evaluated. The study demonstrated a diverse genotypic variation associated with herbicide resistance within a single biotype. The results from this study improve our understanding of both the dynamic nature and the genetic diversity of herbicide resistance in outcrossing weed species.

BIBLIOGRAPHY

Boutsalis, P., J. Karotam, and S. B. Powles. 1999. Molecular basis of resistance to acetolactate synthase-inhibiting herbicides in *Sisymbrium orientale* and *Brassica tournefortii*. Pestic. Sci. 55:507-516.

Christoffers, M. J. 1999. Genetic aspects of herbicide-resistant weed management. Weed Tech. 13:647-652.

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

Delye, C., X. Q. Zhang, S. Michel, A. Matejicek, and S. B. Powles. 2005. Molecular bases for sensitivity to acetyl-coenzyme a carboxylase inhibitors in black-grass. Plant Physiol. 137:794-806.

Duggleby, R. G., J. A. McCourt, and L. W. Guddat. 2008. Structure and mechanism of inhibition of plant acetohydroxyacid synthase. Plant Physiol. Biochem. 46:309-324.

Gaines, X. M, D. G. Swan, and H. C. Keller. 1972. Weeds of Eastern Washington and Adjacent Areas. WA: Camp-Na-Bor-Lee Association.

Gealy, D. R., S. A. Squier, and A. G. Ogg. 1991. Photosynthetic productivity of mayweed chamomile (*Anthemis cotula*). Weed Sci. 39:18-26.

Gronwald, J. W. 1994. Resisance to photosystem II inhibiting herbicides. Pages 27-60 in S. B. Powles and J. A. M. Holtum, eds. Herbicide Resistance in Plants, Biology and Biochemistry. Boca Raton, FL: Lewis Publishers.

Guttieri, M. J., C. V. Eberlein, and D. C. Thill. 1995. Diverse mutations in the acetolactate synthase gene confer chlorsulfuron resistance in kochia (*Kochia scoparia*) biotypes. Weed Sci. 43:175-178.

Hart, S. E., J. W. Saunder, and D. Penner. 1992. Chlorsulfuron-resistant sugarbeet: cross resistance and physiological basis of resistance. Weed Sci. 40:378-383.

Hawley, R. M. 2006. The acetohydroxyacid synthase gene family its role in herbicide resistant sunflowers. M.S. thesis. Corvallis, OR: Oregon State University. 61 p.

Heap, I. M. 2009. The international survey of herbicide resistant weeds. http://www.weedscience.com. Accessed: June 15, 2009.

Kaloumenos, N. S., C. A. Dordas, G. C. Diamantidis, and I. G. Eleftherohorinos. 2009. Multiple Pro197 substitutions in the acetolactate synthase of corn poppy (*Papaver rhoeas*) confer resistance to tribenuron. Weed Sci. 57:362-368.

Knezevic, S. Z., J. C. Streibig, and C. Ritz. 2007. Utilizing R software package for dose-response studies: the concept and data analysis. Weed Tech. 21:840–848.

Kolkman, J. M., M. B. Slabaugh, J. M. Bruniard, S. Berry, B. S. Bushman, C. Olungu, N. Maes, G. Abratti, A. Zambelli, J. F. Miller, A. Leon, and S. J. Knapp. 2004. Acetohydroxyacid synthase mutations conferring resistance to imidazolinone or sulfonylurea herbicides in sunflower. Theor. Appl. Genet. 109:1147-1159.

Mallory-Smith C. A. and E. J. Retzinger. 2003. Revised classification of herbicides by site of action for weed resistance management strategies. Weed Tech. 17:605-619.

Mallory-Smith C. A., D. C. Thill, and M. J. Dial. 1990. Identification of sulfonylurea herbicide-resistant prickly lettuce (*Lactuca serriola)*. Weed Tech. 4:163-168.

Maxwell, B. D. and A. M. Mortimer. 1994. Selection for herbicide resistance. Pages 1-25 in S.B. Powles and J.A.M. Holtum, eds. Herbicide Resistance in Plants, Biology and Biochemistry. Boca Raton, FL: Lewis Publishers.

Mazur, B. J. and S. C. Falco. 1989. The development of herbicide resistant crops. Annu. Rev. Plant Physiol. Mol. Biol. 40:441-470.

McCourt, J. A., S. S. Pang, J. King-Scott, L.W. guddat, and R. G. Dubbleby. 2006. Herbicide-binding sites revealed in the structure of plant acetohydroxyacid synthase. PNAS 103:569-573.

Merotto, A., M. Jasieniuk, M. D. Osuna, F. Vidotto, A. Ferrero, and A. J. Fischer. 2009. Cross-resistance to herbicides of five ALS-inhibiting groups and sequencing of the *ALS* gene in *Cyperus difformis* L. J. Agric. Food Chem. 57:1389-1398.

Mitsuoka, S. and F. Ehrendorfer. 1972. Cytogenetics and evolution of *Matricaria* and related genera (Asteraceae-Anthemideae). Plant Syst. Evol. 120:155-200.

Monaco, T. J., S. C. Weller, and F. M. Ashton. 2002. Weed Science Principle and Practice. NY: John Wiley & Sons, Inc.

Peachey E., D. Ball, R. Parker, J. Yenish, D. Morishita, and P. Hutchinson, eds. 2009. Pacific Northwest Weed Management Handbook. Corvallis, OR: Oregon State University. 543 p.

Perez-Jones, A. 2007. Glyphosate-resistance in Italian ryegrass (*Lolium multiflorum*): evaluation and investigation of the mechanisms of resistance. Ph.D dissertation. Corvallis, OR: Oregon State University. 75 p.

Perez-Jones, A., K. W. Park and C. A. Mallory-Smith. 2004. *Anthemis cotula* resistance to ALS inhibitors. Pages 44-45 in Proceedings of the 57th National Poster Presentation. Colorado Springs, CO: The Western Society of Weed Sience.

Preston, C. and C. A. Mallory-Smith. 2001. Biochemical mechanisms, inheritance, and molecular genetics of herbicides resistance in weeds. Pages 23-60 in S. Powles and D. Shaner, eds. Herbicide Resistance and World Grains. Boca Raton, FL: CRC Press.

R Development Core Team. 2008. R: A language and environment for statistical computing. Vienna, Austria: R foundation for statistical computing.

Ray, T. B. 1984. Site of action of chlorsulfuron-inhibition of valine and isoleucine biosynthesis in plants. Plant Physiol. 75: 827-831.

Ritz, C. and J. C. Streibig. 2005. Bioassay analysis using R. J. Statistic Software 12:1-22.

Ryan, G. F. 1970. Resistance to common groundsel to siamazine and atrazine. Weed Sci. 18:614-616.

Saari, L. L., J. C. Cotterman, and D. C. Thill. 1994. Resistance to acetolactate synthase inhibiting herbicides. Pages 83-139 in S. B. Powles and J. A. M. Holtum, eds. Herbicide Resistance in Plants, Biology and Biochemistry. Boca Raton, FL: Lewis Publishers.

Sibony, M. and B. Rubin. 2003. Molecular basis for multiple resistance to acetolactate synthase-inhibiting herbicides and atrazine in *Amaranthus blitoides* (prostrate pigweed). Planta 216:1022-1027.

Singh, B. K., M. A. Stidham, and D. L. Shaner. 1988. Assay of acetohydroxyacid synthase. Analytical Biochem. 171:173-179.

Seefeldt, S. S., J. E. Jensen, and E. P. Fuerst. 1995. Log-logistic analysis of herbicide dose-response relationships. Weed Tech. 9:218-227.

Stephens, R. H. and A. G. Ogg, Jr. 1989. Growth analysis over time of mayweed chamomile (*Anthemis cotula*) interference with peas (*Pisum sativum*). Pages 150 in Proceedings of the 42nd National Poster Presentation. Honolulu, HA: The Western Society of Weed Sience.

Streibig, J. C., M. Rudemo, and J. E. Jensen. 1993. Dose-response curves and statistical models. Pages 29-53 in J. C. Streibig and P. Kudsk, eds. Herbicide Bioassay. Boca Raton, FL: CRC Press.

Tranel, P. J. and T. R. Wright. 2002. Resistance of weeds to ALS-inhibiting herbicides: what have we learned? Weed Sci. 50:700-712.

Tranel, P. J., T. R. Wright, and I. M. Heap. 2009. ALS mutations from herbicide-resistant weeds. http://www.weedscience.com. Accessed: June 15, 2009.

Wakelin, A. M. and C. Preston. 2006. A target-site mutation is present in a glyphosate-resistant *Lolium rigidum* population. Weed Research 46:432-440.

Westerfeld, W. W. 1945. A colorimetric determination of blood acetoin. J. Biol. Chem. 161:495-502.

Whaley, C. M., H. P. Wilson, and J. H. Westwood. 2007. A new mutation in plant *ALS* confers resistance to five classes of ALS-inhibiting herbicides. Weed Sci. 55:83-90.

[WSSA] Weed Science Society of America. 1998. "Herbicide Resistance" and "Herbicide Tolerance" Defined. Weed Tech. 12:789.

Yamamoto, E., L. Zeng, and W. V. Baird. 1998. A tubulin missense mutations correlate with antimicrotubule drug resistance in *Eleusine indica*. Plant Cell 10:297-308.

Yasuor, H., M. D. Osuna, A. Ortiz, N. E. Saldain, J. W. Echert, and A. J. Fischer. 2009. Mechanism of resistance to penoxsulam in late watergrass (*Echinochloa phyllogogon* (Stapf.) Koss.). J.Agric. Food Chem. 57:3653–3660.

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

APPENDIX

Sources of Materials

[1] Potting mix, Sunshine Mix#1, Sun Grow Horticulture Inc., 15831 N.E. 8th Street, Suite 100 Bellevue, WA 98008.

[2] HARMONY® EXTRA XP Herbicide label, DuPont Crop Protection, P.O. Box 80705, CRP 705/L1S11, Wilmington, DE 19880.

[3] Pursuit® Herbicide label, BASF Corporation, Polyurethanes RBU, 138Meats Avenue, Orange, CA 92865.

[4] Olympus® Herbicide label, Bayer CropScience, Crop Protection Division, 2 T.W. Alexander Drive RTP, NC 27709.

[5] FirstRate® Herbicide label, Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN 46268.

[6] R-11® Spreader-activator nonionic surfactant label, Wilbur-Ellis Company, 345 California Street, 27th Floor, San Francisco, CA 94104.

[7] VersaMax™ microplate reader, Molecular Devices, 1311 Orleans Drive, Sunnyvale, CA 94089.

[8] DNeasy Plant Mini Kit, Qiagen Inc., 27220 Turnberry Lane, Suite 200, Valencia, CA 91355.

[9] The C1000 thermal cycler, Bio-Rad Laboratories Inc., 1000 Alfred Nobel Drive Hercules, CA 94547.

[10] Tag DNA Polymerase, Qiagen Inc., 27220 Turnberry Lane, Suite 200, Valencia, CA 91355.

[11] TOPO® TA cloning kit, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, CA 92008.

[12] QIAquick® PCR purification kit, Qiagen Inc., 27220 Turnberry Lane, Suite 200, Valencia, CA 91355.

[13] ABI PRISM® 3770 genetic analyzer and a BigDye® terminator v. 3.1 cycle sequencing kit, Applied Biosystems Inc., 850 Lincoln Centre DriveFoster City, CA 94404.

[14] CLC Sequence Viewer v. 6.1, CLC bio, 245 First Street, Suite 1826, Cambridge, MA 02142.

[15] Finch TV v. 1.4.0, Geospiza, Inc., 100 West Harrison, North Tower, Suite #330, Seattle, WA 98119.

[16] SAS v. 9.2, SAS Institute Inc., 100 SAS Campus Drive, Cary, NC 27513.

[17] R v. 2.8.0, The R Project for Statistical Computing, http://www.r-project.org/.

[18] Model for separate control measurements, Analysis of bioassay using R, http://www.bioassay.dk/.