

SELECTION OF CAMELINA MUTANTS RESISTANT TO ACETOLACTATE SYTHASE INHIBITOR
HERBICIDES

By

DUSTIN TIMOTHY WALSH

A thesis submitted in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE IN CROP SCIENCE

WASHINGTON STATE UNIVERSITY
Department of Crop and Soil Sciences

AUGUST 2010

To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of DUSTIN TIMOTHY WALSH find it satisfactory and recommend that it be accepted.

Scot H. Hulbert, Ph.D., Chair

Ian C. Burke, Ph.D.

William L. Pan, Ph.D.

ACKNOWLEDGMENTS

I would like to thank the Sun Grant Initiative for funding my degree program. A special thanks to Dr. Scot Hulbert for accepting me into the graduate program at WSU and for guiding me academically and in my research. Special appreciation is also extended to Dr. Ian Burke for the use of his lab equipment and for his advice and input on the study. I also thank Dr. Pan for his advice and guidance through my research.

Appreciation needs to be expressed to my family for their moral support. Finally, I thank my wife Erin, for her encouragement, patience, and understanding throughout this endeavor.

SELECTION OF CAMELINA MUTANTS RESISTANT TO ACETOLACTATE SYTHASE INHIBITOR HERBICIDES

Abstract

By Dustin Timothy Walsh, M.S.

Washington State University

August 2010

Chair: Scot H. Hulbert

Camelina (*Camelina satvia* L.) is a new alternative crop being developed as a low input, oilseed crop in the Inland Pacific Northwest. Adoption has been slow, in part because of limited weed control options and sensitivity to residual herbicide activity in soils. Other crops with resistance to these herbicides have been developed through mutation. Development of camelina with this resistance would allow great flexibility for the crop in rotation with other crops. M₂ seed from camelina mutagenized by exposure to 0.3% ethyl methane sulfonate (EMS) was screened for increased resistance with imazethapyr and sulfosulfuron. Five lines with resistance were identified and characterized. Four mutants identified in a screen for imazethapyr resistance (IM1, IM6, IM10, and IM18) appeared phenotypically identical and are likely controlled by the same semi-dominant gene. One mutant, designated SM4, identified in a screen for sulfosulfuron resistance was phenotypically different and was also controlled by a semi dominant gene. Plants treated with imazethapyr, sulfosulfuron, and flucarbazone were evaluated visually and for biomass production. All mutants appeared more resistant than the wild type visually, and SM4 appeared significantly more resistant than the IM mutants. The imazethapyr dose required to reduce plant growth 50% (GR₅₀) for Calena, IM1, and SM4 was 0.99mg ai/ha, 19.21mg ai/ha and 204.15mg ai/ha, respectively. GR₅₀ values for sulfosulfuron were 0.10 mg ai/ha, 0.46mg ai/ha and 15.56mg ai/ha, and for flucarbazone 1.5mg ai/ha, 4.67mg ai/ha and 10.57mg ai/ha. The herbicide concentration required to reduce enzyme activity by 50% (AR₅₀) was calculated by a crude enzyme extract containing ALS from Calena, IM1, and SM4. AR₅₀ concentrations of imazethapyr for Calena, IM1, and SM4 were 86.29uM, >400uM, and >400uM, respectively. AR₅₀ concentrations of

sulfosulfuron were 0.0061uM, 4.04uM, and 13.81uM and of flucarbazone were 0.62uM, 1.72uM, and 1.36uM respectively. Both IM1 and SM4 mutants were confirmed to have resistance to imazethapyr, sulfosulfuron, and flucarbazone. These mutant lines are available to industry and public breeders to create camelina varieties with increased resistance to these ALS herbicides.

Table of Contents

ACKNOWLEDGMENTS	iii
Abstract	iv
LIST OF TABLES	vii
LIST OF FIGURES	viii
Introduction	1
Camelina sativa	1
ALS herbicides	2
The ALS/AHAS enzyme.....	3
Development of Imidazolinone Resistant Crops.....	6
Conclusion.....	7
Literature Cited	9
Camelina Mutants Resistant to Acetolactate Synthase Inhibitor Herbicides	15
Abstract.....	15
Materials and Methods.....	17
Results and Discussion	22
Source of Materials	28
Literature Cited	29
Tables and Figures	32
Registration of Five Camelina (<i>Camelina sativa L.</i>) Genetic Stock Mutants with Resistance to Several ALS Herbicides	41
Abstract.....	41
Methods.....	43
Characteristics.....	45
References	49
Tables and Figures	51

LIST OF TABLES

Introduction

Table 1: Imidazolinone resistant crops released for production.	14
--	-----------

Camelina Mutants Resistant to Acetolactate Synthase Inhibitor Herbicides

Table 2: Segregation ratios for Wild type/Resistant F ₂ families.	32
---	-----------

Table 3: Whole plant and enzyme extraction dose response regression parameters	33
---	-----------

Registration of Two Camelina (*Camelina sativa* L.) Genetic Stock Mutants with Resistance to Several

ALS Herbicides

Table 4: Biomass of camelina mutants treated with ALS herbicides.	51
--	-----------

Table 5: Segregation ratios for wild type/resistant F ₂ families	52
--	-----------

LIST OF FIGURES

Camelina Mutants Resistant to Acetolactate Synthase Inhibitor Herbicides

Figure 1: Imazethapyr Whole Plant Dose Response, low rates.....	34
Figure 2: Imazethapyr Whole Plant Dose Response, high rates.....	35
Figure 3: Sulfosulfuron Whole Plant Dose Response.....	36
Figure 4: Flucarbazone Whole Plant Dose Response.....	37
Figure 5: Imazethapyr ALS Enzyme Dose Response.....	38
Figure 6: Sulfosulfuron ALS Enzyme Dose Response.....	39
Figure 7: Flucarbazone ALS Enzyme Dose Response.....	40

Introduction

Wheat (*Triticum aestivum* L.) is a major food crop grown in diverse production systems throughout the world depending on climate and soil conditions. Wheat is also a major crop in the U. S. and is the dominant crop in the dryland cropping systems of eastern Washington (Schillinger and Papendick 2008). Crop rotations in the region are designed around wheat production with a wheat – summer fallow rotation used in the driest regions (less than 38cm annual rainfall). In the intermediate zone (38cm to 50cm annual rainfall), a three – year rotation often is used. The most common rotation in these areas is a winter wheat - spring grain – summer fallow or chemical fallow. Occasionally a low input alternative crop is grown instead of the fallow year. In the high rainfall areas (greater than 50cm annual rainfall) the most common rotation is a winter wheat - spring grain – alternative crop. The spring grain can be either wheat or barley (*Hordeum spp.* L.). The greatest variation in three year rotations occurs with the use of an alternative crop (Schillinger and Papendick 2008). The alternative crop is often an oilseed crop, such as, spring canola (*Brassica spp.* L.), or a legume such as peas (*pisum sativum* L.), lentils (*Lens culinaris* Medikor) or chickpeas (*Cicer arietinum* L.). The alternative crop is utilized primarily for its benefit in the rotation, not for intrinsic economic value; however, the economic value does influence the alternative crop selected.

Camelina sativa

Heightened interest in biofuel crops for the Pacific Northwest (PNW) has increased research on camelina (*Camelina sativa* L.), a small seeded oil crop. Camelina can be a spring or fall planted crop. When seeded in soil with adequate moisture and temperature, seed will germinate and emerge within a few days. The plant initially forms a rosette above ground and then grows into an erect stalk with numerous leaves. Once elongated, flower buds and axial branches form from the apex. Camelina forms numerous small yellow flowers that develop into pear-shaped capsules containing 10 to 25 seeds (Zubur 1997).

Camelina oil is of interest for a variety of uses including food, fuel, and industrial applications including jet fuel. Oil yields for camelina are relatively high among oilseed crops, with oil content as high as 42 to 45% by weight (Vollman et al. 2007; Zubur 1997). Vollman et. al. (2007) found an average yield of 1850kg/ha of seed and 437.1g/kg oil yield in Australia, that resulted in 807kg/ha oil yield. The oil profile of camelina makes it a good candidate for both consumer and industrial applications. Camelina is similar to soybean (*Glycine Max* L.) and canola, which are already used for biodiesel production (Moser and Vaughn 2010). In the Northwest, camelina is primarily considered a non-food oil crop. It has been suggested that camelina will yield well while requiring less moisture and fertilizer than other alternative crops grown in the PNW (Putnam et al. 1993).

Overall, camelina has desirable traits as a rotation crop in the PNW; however there are a couple major barriers to growing camelina. One barrier that is similar to canola is the sensitivity to soil residual herbicides, specifically acetolactate synthase (ALS) inhibitors. Weed control is another issue with growing camelina as there are currently few registered herbicides for use on camelina (Ehrensing and Guy 2008). Residual herbicide concerns and limited weed control have limited farmers from planting camelina in the PNW.

ALS herbicides

Acetolactate synthase inhibitor herbicides are a popular group of enzyme targeted herbicides that are often used in PNW cropping systems. ALS inhibitor herbicides target acetolactate synthase, an important enzyme early in the synthesis of the branch-chain amino acids – valine, leucine, and isoleucine (Shaner et al. 1984). ALS is the target site for more than 50 commercial herbicides spanning five structurally distinct classes of chemicals, including sulfonylureas (SU; Chaleff and Mauvals 1984), imidazolinones (IMI; Shaner et al. 1984), pyrimidinylthiobenzoate (PTB; Stidham 1991), triazolopyrimidine (TP; Gerwick et al. 1990), and sulfonylaminocarbonyltriazolinones (SCT; Eliason et al.

2004). Imidazolinone and sulfonylurea herbicides are effective at controlling a wide variety of both grass and broadleaf weeds at low application rates and are relatively non-toxic to mammals which make them generally safe herbicides to use. The use of acetolactate synthase inhibitor herbicides in the PNW has been a barrier to camelina adoption because of sensitivity to ALS herbicide residues in soils. Residual activity among the ALS inhibitor herbicides varies by each herbicide. For example, highly sensitive crops, such as canola, cannot be planted for 40 months after application of imazethapyr. Imazethapyr is primarily degraded by microbial activity. Microbial activity is dependent on temperature, moisture, and pH (Hanson and Thill 2001; Flint and Witt 1997). Herbicide degradation that is dependent on temperature and moisture is variable from year to year and has led to increased concerns about residual activity in the PNW. Dry summers, low pH soils, and long cold winters combine to leave a very small window for herbicide breakdown (Hanson and Thill 2001). The narrow window for degradation may increase levels of residual herbicide in the soil, resulting in increased damage to sensitive crops like camelina.

The ALS/AHAS enzyme

The enzyme acetolactate synthase (ALS, EC 2.2.1.6 [formerly EC 4.1.3.18] has two distinct metabolic roles in organisms which can lead to confusion. The enzyme is involved in the decarboxylation and condensation of pyruvate in the formation of branched- chain amino acids in most organisms; however, it is also involved in the catabolic process of butanediol fermentation in some microorganisms (Duggleby and Pang 2000). To address this confusion the name acetolactate synthase was suggested for use when referring to the enzyme involved in butanediol fermentation and acetohydroxyacid synthase (AHAS) when referring to the enzyme involved in branched- chain amino acid synthesis; however, the nomenclature has not been widely adopted (Gollop et al. 1989). In order to be consistent with the literature on herbicides and their classification, ALS will be used throughout this paper.

The branched-chain amino acids leucine, valine and isoleucine are formed by a common pathway (Duggleby and Pang 2000). ALS is the first enzyme in the common pathway for these amino acids and it utilizes the common precursor pyruvate for synthesis of 2-acetolactate or 2-aceto-2-hydroxybutyrate. In the synthesis of valine, two molecules of pyruvate are the initial substrate for ALS, ketol-acid reductoisomerase, dihydroxyacid dehydratase, and transaminase. The synthesis of isoleucine follows the same pathway, but one molecule of 2-ketobutyrate is substituted for one molecule of pyruvate. Leucine synthesis branches from valine synthesis by using 2-ketoisovalerate as the substrate for 2-isopropylmalate synthase. Synthesis continues through isopropylmalate isomerase, 3-isopropylmalate dehydrogenase and transaminase to result in leucine (Duggleby and Pang 2000). The amino acids valine, leucine and isoleucine are essential for growth and development in plants.

The ALS enzyme in plants has been identified as the target site for SU (Chaleff and Mauvals 1984) and IMI (Shaner et al. 1984) herbicides. The discovery that SU and IMI herbicides inhibit ALS has led to advances in the understanding of the enzyme. A number of plant ALS genes have been isolated and characterized, including Arabidopsis (*Arabidopsis thaliana* L.), tobacco (*Nicotiana tabacum* L.; Mazuret et al. 1987), oilseed rape (*Brassica napus* L.; Rutledge et al. 1991), corn (*Zea mays* L.; Fang et al. 1992), cotton (*Gossypium hirsutum* L.; Grula et al. 1995), cocklebur (*Xanthium* sp. L.; Bernasconi et al. 1995) and wild raddish (*Raphanus raphanistrum* L.; Tan and Medd 2002). Known plant species vary from one ALS locus in Arabidopsis to six ALS loci in cocklebur. At least one loci is expressed in all tissues throughout the plant, although the level of expression does vary by tissue in the plant (Oullett et al. 1992). The highest level of activity has been found in metabolically active meristematic tissues (Keeler et al. 1993; Schmitt 1990). In the case of *Brassica napus*, an allotetraploid, there are five ALS genes; some of this complexity is due to the two genomes (Rutledge et al. 1991). Camelina is in the same family as *Brassica napus*, and may be polyploid.

Inhibition of ALS by a herbicide causes accumulation of the phytotoxic substrate 2-ketobutyrate (Rhodes et al. 1987), amino acid content imbalance (Hofgen et al. 1995), inhibition of DNA synthesis (Stidham 1991), and a reduction of cell division and assimilate translocation (Kim and Vanden Born 1996). The physiological changes resulting from ALS inhibition result in a slow death of the plant beginning with the meristematic tissues and eventual necrosis of the entire plant (Duggleby and Pang 2000).

The enzyme is composed of two subunits with the active site being near the interface of these two subunits (Duggleby and Pang 2000). In competitive studies with pyruvate, sulfonylureas and imidazolinones have not been characterized as competitive with the substrate pyruvate (Durner et al. 1991; Shaneret al. 1984; Ahan el al. 1992; Chong and Duggleby 1997). Substrate binding competition studies with sulfonylurea and imidazolinone herbicides have demonstrated that their binding is mutually exclusive (Stidham and Shaner 1990; Durneret al. 1991; Landstein et al. 1993; Schlosset al. 1988). However, mutations in the ALS enzyme that confer resistance to one chemical class of herbicides often confer resistance to other classes. Additionally, there have been mutations that confer resistance to only one class of chemicals (Duggleby and Pang 2000). Substitutions at Ala 122 and Ser 653 confer resistance to IMI but not to SU, substitutions at Pro 197 confer resistance to SU but not IMI, and substitutions at Ala 205 and Trp 574 confer resistance to both SU and IMI (Tranel and Wright 2002). The different resistance patterns indicate that the binding sites for IMI and SU herbicides are different, but overlapping.

The separation of the inhibitor site from the active site of the enzyme helps explain the high number of mutants that are resistant to ALS inhibitors. The herbicide binding site can be altered without interfering with the active site of the enzyme, conferring resistance without disabling the enzyme. Reported resistance has been due to dominant or semi-dominant mutations in the catalytic

subunit gene of ALS (Duggleby and Pang 2000). Induced resistance is conferred by a single amino acid change from the wild-type enzyme, most commonly at Ala122, Pro197, Trp574, or Ser653 (Duggleby and Pang 2000). Yeast ALS has been most extensively characterized and most mutations found in other organisms correspond to one of ten mutations that confer resistance in yeast (Falco et al. 1989; Mazur and Falco 1989; Duggleby and Pang 2000). Levels of resistance are variable, ranging from 4 to 10,000 fold more resistant than wild type ALS, which results in variability among studies (Duggleby and Pang 2000). The number of mutations known and the characterization on ALS from multiple organisms provides a good background for the induction and characterization of herbicide resistant ALS in camelina.

Development of Imidazolinone Resistant Crops

Many crops have been developed and commercialized with imidazolinone resistance (Table 1). The first crop released was corn. Development began in 1982 with a selection of mutagenized callus tissue and was finally released in 1992 as IMI corn. Today it is sold under the name Clearfield® (BASF, North Carolina; Tan et al. 2005). Several other corn lines with imidazolinone resistance have been developed using ethyl methanesulfonate (EMS). The pollen of inbred corn lines was exposed to EMS and then imidazolinone resistant lines were selected (Tan et al. 2005).

Oilseed rape (*Brassica napus* L.) has also been developed as an imidazolinone resistant crop. *B. napus* is an allotetraploid believed to come from a cross of *B. campestris* L, and *B. oleracea* L., donors of genomes A and C, respectively (Rutledge et al. 1991). Five loci for the ALS gene have been reported, but only 2 of them are expressed and essential to plant growth and development. To develop imidazolinone resistant rape, microspores of oilseed rape were isolated and exposed to ethyl nitrosourea and developed into embryos and haploid plantlets which were doubled with colchicine

(Swanson et al. 1989). Plants were then screened with imazethapyr in soil. These resistant plants were developed and are now marketed as Clearfield® (BASF, N. Carolina) canola (Tan et al. 2005).

Lines of rice have also been developed with imidazolinone resistance by mutagenized seed. The rice was originally mutagenized with EMS and screened with imazethapyr (Croughan 1998). Varieties have been developed and are now marketed as Clearfield® (BASF, N. Carolina) rice.

Wheat has been mutagenized to obtain herbicide resistance, first with sodium azide, then later with EMS (Ponzial and Huci 2004). Wheat is hexaploid with three genomes: A, B, and D. Each genome is believed to have one ALS gene. The later EMS mutation yielded mutations in genomes A and B (Ponziak et al. 2004). One mutation is sufficient for resistance to ALS inhibitor herbicides in winter wheat, but two are required for sufficient resistance in spring wheat varieties (Tan et al. 2005).

Clearfield® sunflower has also been released, but it has a different history. Imidazolinone resistant sunflower was derived from wild populations found in Kansas and South Dakota (White et al. 2002). These traits have been bred into production sunflower lines. The inheritance of resistance in sunflowers is not clear and several models have been proposed. Miller and Khatib (2000) proposed that resistance is controlled by two genes with an additive effect. However, Bruniard (2001) proposed that the resistance is controlled by one semi-dominant gene and a second modifier gene because of lines with intermediate resistance that do not segregate.

Conclusion

Camelina varieties that have increased resistance to ALS herbicides would be beneficial in the PNW. A variety of camelina with increased resistance would enable more acres to be planted because residual herbicides would not affect the crop. A high level of resistance would provide a selective weed control option in the crop. An increase in camelina would increase the crop diversity in eastern Washington and boost Washington's contribution to biofuel production.

Literature Cited

- Ahan, T. W., D. W. Kim, and J. D. Chol. 1992. Inhibition of acetohydroxyacid synthase by sulfonylureas and imidazolinones. *Korean Biochem. J.* 25:636-641.
- Bernasconi, P., A. R. Woodworth, B. A. Rosen, M. V. Subramanian, and D. L. Siehl. 1995. A naturally occurring point mutation confers broad range tolerance to herbicides that target acetolactate synthase. *J. Biol. Chem.* 270:17381-17385.
- Bruniard, J. M.. 2001. Inheritance of imidazolinone resistance, characterization of cross-resistance pattern, and identification of molecular markers in sunflower (*Helianthus Annuus L.*) PhD Dissertation, North Dakota State Univ.
- Chaleff, R. S. and C. J. Mauvals. 1984. Acetolactate synthase is the site of action of two sulfonylurea herbicides in higher plants. *Science* 224:1443-1445.
- Chong, A. K. and R. G. Duggleby. 1997. Expression, purification and characterization of arabidopsis thaliana acetohydroxyacid synthase. *Biochem. J.* 327:161-169.
- Croughan, T. P., inventor; Board of Supervisors of Louisiana State University, assignee. 1998. Herbicide resistant rice. U.S. patent 5,773,704.
- Duggleby, R. G. and S. S. Pang. 2000. Acetohydroxyacid synthase. *J. of Biochem. Mol. Biol.* 33:1-36.
- Durner, J., V. Gailus, and P. Boger. 1991. New aspects on inhibition of plant acetolactate synthase by chlorsulfuron and imazaquin. *J. Plant Physiol.* 95:1144-1149.
- Ehrensing, D. T. and S. O. Guy. 2008. Camelina. Oregon State University Extension Bulletin: EM 8953-E.
- Eliason, R., J. J. Schoenau, A. M. Szmigielski, and W. M. Lavery. 2004. Phytotoxicity and persistence of flucabazone-sodium in soil. *Weed Sci.* 52:857-862.
- Falco, S. C., R. E. McDevitt, C. F. Chui, M. E. Hartnett, S. Knowlton, C. J. Mauvais, J. K. Smith, and B. J. Mazur. 1989. Engineering herbicide-resistant acetolactate synthase. *Dev. Ind. Microbiol.* 30:187-194.

- Fang, L. Y., P. R. Gross, C. H. Chen, and M. Lillis. 1992. Sequence of 2 acetohydroxyacid synthase genes from *Zea mays*. *Plant Mol. Biol.* 18:1185-1187.
- Flint, J. L. and W. W. Witt. 1997. Microbial Degredation of imazaquin and imazethapyr. *Weed Sci.* 45:585-591.
- Gerwick, B. C., M. V. Subramanian, and V. I. Loney-Gallant. 1990. Mechanism of action of the 1,2,4-triazolo[1,5-a]pyrimidines. *J. Pestic. Sci.* 29:357-364.
- Gollop, N., B. Damri, Z. Barak, and D. Chipman. 1989. Kinetics and mechanism of acetohydroxy acid synthase isozyme III from *Escherichia coli*. *J. Biochem.* 28:6310-6317.
- Gruha, J. W., R. L. Hudspeth, S. L. Hobbs, and D. M. Anderson. 1995. Organization, inheritance and expression of acetohydroxyacid synthase genes in the cotton allotetraploid *Gossypium hirsutum*. *Plant Mol. Biol.* 28:837-846.
- Hanson, B. D. and D. C. Thill. 2001. Effects of imazethapyr and pendimethalin on lentil (*Lens culinaris*), pea (*Pisum sativum*), and a subsequent winter wheat (*Triticum aestivum*) crop. *Weed Technol.* 15:190-194.
- Heap, I. 2010. The International Survey of Herbicide Resistant Weeds. Available at <http://www.weedscience.org>. Accessed January 27, 2010.
- Hofgen, R., B. Laber, I. Schuttler, A. K. KJlomas, W. Streber, and H. D. Pohlenz. 1995. Repression of acetolactate synthase activity through antisense inhibition. *J. Plant Physiol.* 107:469-477.
- Keeler, S. J., P. Sanders, J. K. Smith, and B. J. Mazur. 1993. Regulation of tobacco acetolactate synthase gene expression. *Plant Physiol.* 102:1009-1018.
- Kim, S. and W. H. Vanden Born. 1996. Chlorsulfuron decreases both assimilate export by source leaves and import by sink leaves in canola (*Brassica napus* L.) seedlings. *Pestic. Biochem.* 56:141-148.

- Landstein, D., S. Arad, Z. Barak, and D. M. Chipman. 1993. Relationships among the herbicides and functional sites of acetohydroxy acid synthase from *Chlorella emersonii*. *Planta* 191:1-6.
- Mazur, B. J., C. Chui, and J. K. Smith. 1987. Isolation and characterization of plant genes coding for acetolactate synthase, the target enzyme for two classes of herbicides. *J. Plant Physiol.* 85:1110-1117.
- Mazur, B. J. and S. C. Falco. 1989. The development of herbicide resistant crops. *Plant Mol. Biol.* 40:441-470.
- Miller, J. F. and K. Al-Khatib. 2000. Development of herbicide resistant germplasm in sunflower. *Proc. 15th Int. Sunflower Assoc. Conf. France.* 37-41
- Moser, B. R. and S. F. Vaughn. 2010. Evaluation of alkyl esters from *Camelina sativa* oil as biodiesel and as blend. *Bioresour. Tech.* 101:646-653.
- Oullett, T., R. G. Ritledge, and B. L. Miki. 1992. Members of the acetolactate synthase multigene family of *Brassica napus* have divergent patterns of expression. *Plant J.* 2:321-330.
- Ponziak, C. J., I. T. Birk, L. S. O'Donoghue, C. Menard, P. J. Huci, and B. K. Singh. 2004. Physiological and molecular characterization of mutation-derived imidazolinone resistance in spring wheat. *Crop Sci.* 44:1434-1443.
- Ponziak, C. J. and P. J. Huci. 2004. Genetic analysis of imidazolinone resistance in mutation-derived lines of common wheat. *Crop Sci.* 44:23-30.
- Putnam, D. H., J. T. Budin, L. A. Field, and W. M. Breene. 1993. Camelina: a promising low-input oilseed. Pages 314-322 in: J. Janick and J. E. Simon, eds. *New Crops*. New York: Wiley.
- Rhodes, D., A. L. Hogan, L. Deal, G. C. Jamieson, and P. Haworth. 1987. Amino acid metabolism of *Lemna minor* L. II responses to chlorsulfuron. *Plant Physiol.* 84:775-780.
- Rutledge, R. G., T. Quillet, J. Hattori, and B. L. Miki. 1991. Molecular characterization and genetic origin of *Brassica napus* acetohydroxyacid synthase multigene family. *Mol. Genet.* 229:31-40.

- Schillinger, W.F., and R.I. Papendick. 2008. Then and now: 125 years of dryland wheat farming in the Pacific Northwest. *Agron. J.* 100:S-166-S-182.
- Schloss, J. V., L. M. Ciskanik, and D. E. Van Dyk. 1988. Origin of the herbicide binding site of acetolactate synthase. *Nature* 331:360-362.
- Schmitt, G. K. Sign. B. K. 1990. Tissue Distribution of acetohydroxyacid synthase activity at various developmental stages of lima bean. *J. Pestic Sci.* 30:418-419.
- Shaner, D. L., P. C. Anderson, and M. A. Stidham. 1984. Imidazolinones: potent inhibitors of acetohydroxyacid synthase. *Plant Physiol.* 76:545-546.
- Stidham, M. A. 1991. Herbicidal inhibitors of branched chain amino acid biosynthesis. *Weed Sci.* 39, 428-434.
- Stidham, M. A. 1991. Herbicides that inhibit acetohydroxyacid synthase. *Weed Sci.* 39:428-434.
- Stidham, M. A. and D. L. Shaner. 1990. Imidazolinone inhibition of acetohydroxyacid synthase in vitro and vivo. *Pestic. Sci.* 29:335-340.
- Swanson, E. B., M. J. Hargesel, M. J. Arnoldo, M. Sippell, and R. S. V. Wong. 1989. Microspore mutagenesis and selection: canola plants with field resistance to imidazolinone. *Thor. Appl. Genet.* 78:525-530.
- Tan, S., R. R. Evans, M. L. Dahmer, B. K. Singh, and D. L. Shaner. 2005. Imidazolinone-tolerant crops: history, current status, and future. *Pest Manag. Sci.* 61:246-257.
- Tan, M. K. and R. W. Medd. 2002. Characterization of the acetolactate synthase (ALS) gene of *Raphanus raphanistrum* L. and the molecular assay of mutation associated with herbicide resistance. *Plant Sci.* 163:195-205.
- Tranel, P. J. and T. R. Wright. 2002. Resistance of weeds to ALS-inhibiting herbicides: what have we learned?. *Weed Sci.* 50:100-712.

Vollman, J., T. Moritz, C. Kargl, S. Baumgartner, and H. Wagentristl. 2007. Agronomic evaluation of camelina genotypes selected for seed quality characteristics. *Industrial Crops and Products* 26: 270-277.

White, A. D., M. D. K. Owen, R. G. Hartzler, and J. Cardina. 2002. Common Sunflower resistance to acetolactase synthase-inhibiting herbicides. *Weed Sci.* 50:432-437.

WSU Variety Testing, Washington State University Extension, WSU. 2009. Uniform Cereal Variety Testing. *Crop and Soil Sciences*, Pullman. <http://variety.wsu.edu/2009/index.htm>

Zubur, J.. 1997. Oil-seed crop: *Camelina sativa*. *Industrial Crops and Products* 6:113-119.

Table 1: Imidazolinone resistant crops released for production.

Crop	Mutant	Codon	Amino Acid Change	Mutation Selection Method
Corn	Several	Several	Several	Callus and Seed Mutagenesis
Rice	93AS3510	654	Gly to Gla	Seed Mutagenesis
Rice	Several	653	Ser to Asp	Seed Mutagenesis
Wheat	Several	653	Ser to Asp	Seed Mutagenesis
Wheat	TealIMI 11A	653	Ser to Asp	Seed Mutagenesis
Oilseed Rape	PM1	653	Ser to Asp	Microspore Mutagenesis
Oilseed Rape	PM2	574	Trp to Leu	Microspore Mutagenesis
Sunflower	Two	205	Ala to Val	Natural Selection

Camelina Mutants Resistant to Acetolactate Synthase Inhibitor Herbicides

Dustin T Walsh, Ian C. Burke, and Scot H. Hulbert¹

Abstract

Camelina (*Camelina satvia* L.) is a small acreage crop grown as a low input oil seed crop in the inland Pacific Northwest. Adoption has been slow, in part because of limited weed control options and sensitivity to residual herbicide activity in soils. M₂ seed from camelina mutagenized by exposure to 0.3% ethyl methane sulfonate (EMS) was screened for increased resistance to imazethapyr and sulfosulfuron. Five lines with resistance were identified and characterized. Four mutants identified in a screen for imazethapyr resistance (IM1, IM6, IM10, and IM18) appeared phenotypically identical and controlled by the same semi-dominant gene. One mutant identified in a screen for sulfosulfuron resistance was phenotypically different but also appears to be controlled by a single codominant gene. The imazethapyr dose required to reduce plant growth 50% (GR₅₀) for Calena, IM1, and SM4 was 0.99mg ai/ha, 19.21mg ai/ha and 204.15mg ai/ha, respectively. GR₅₀ values for sulfosulfuron were 0.10 mg ai/ha, 0.46mg ai/ha and 15.56mg ai/ha, and for flucarbazone 1.5mg ai/ha, 4.67mg ai/ha and 10.57mg ai/ha. The herbicide concentration required to reduce enzyme activity by 50% (AR₅₀) was calculated by a crude enzyme extract, containing ALS from Calena, IM1, and SM4. AR₅₀ concentrations of imazethapyr for Calena, IM1, and SM4 were 86.29uM, >400uM, and >400uM, respectively. AR₅₀ concentrations of sulfosulfuron were 0.0061uM, 4.04uM, and 13.81uM and of flucarbazone were 0.62uM, 1.72uM, and 1.36uM respectively. Both IM1 and SM4 mutants were confirmed to have increased resistance to imazethapyr, sulfosulfuron, and flucarbazone.

Nomenclature: *Camelina sativa* L., flucarbazone, imazethapyr, sulfosulfuron.

Keywords: Dose response, seed mutagenesis, herbicide resistance.

¹ Graduate Student, Assistant Professor, and Professor respectively, Department of Crop and Soil Sciences, Johnson Hall 201, Washington State University, Pullman, WA 99164, Corresponding author's email: dtwalsh@wsu.edu

Camelina has been identified as a potential oilseed and rotation crop for wheat production in the Pacific Northwest. As with other crops in the mustard family, camelina is highly sensitive to acetolactate synthase (ALS, EC 2.2.1.6 [formerly EC 4.1.3.18]) inhibitor herbicides. In addition, camelina currently has few herbicides registered for use. These factors have limited the adoption of camelina as an oilseed crop in the Pacific Northwest. Canola has similar sensitivity and has overcome these problems through the release of ALS herbicide resistant lines.

Acetolactate synthase, also known as acetoxyacid synthase, is an essential enzyme in the synthesis of essential branched-chain amino acids valine, leucine, and isoleucine in plants (Duggleby and Pang 2000). ALS is the target site for more than 50 commercial herbicides spanning five structurally distinct classes of chemicals (Heap 2010). The ALS inhibiting herbicide classes include sulfonylureas (SU; Chaleff and Mauvals 1984), imidazolinones (IMI; Shaner et al. 1984), pyrimidinylthiobenzoate (PTB; Stidham 1991), triazolopyrimidine (TP; Gerwick et al. 1990), and sulfonylaminocarbonyltriazolinones (SCT; Eliason et al. 2004). Inhibition of ALS results in a slow death of the plant with symptoms first appearing in the meristematic tissues (Duggleby and Pang 2000). Resistant plants have been produced by mutagenesis in maize (*Zea mays* L.) (Newhouse et al. 1991), *Arabidopsis thaliana* L. (Haughn and Somerville 1986), sugar beet (*Beta vulgaris* L.; Wright and Penner 1998; Hart et al. 1992), canola (*Brassica napus* L.; Swanson et al. 1989), soybean (*Glycine max* L.; Sabastian et al. 1989), tobacco (*Nicotiana tabacum* L.; Chaleff and Ray 1984), cotton (*Gossypium hirsutum* L.; Rajasekaran et al. 1996) rice (*Oryza sativa* L.; Croughan 1998) and wheat (*Triticum aestivum* L.; Ponzial and Huci 2004). Most selected resistance is due to altered forms of the ALS enzyme that are less sensitive to inhibition by one or more ALS-inhibiting herbicides.

There are five amino acid substitutions in the ALS protein which are most commonly associated with ALS herbicide resistance in plants. The various substitutions can result in resistance to one or multiple chemical families. The mutation resulting in substitution of Ser653 results in resistance to

imidazolinone herbicides, but not cross resistance to other ALS inhibitors (Lee et al. 1999). Substitutions of Pro197 for Thr, Arg, Leu, Gln, Ser, or Ala induce resistance to sulfonylureas, but not imidazolinones, but substitutions for His, Leu, or Ile result in resistance to sulfosulfuron and imidazolinone herbicides (Yu et al. 2003; Thill 1997; Tranel and Wright 2002). Substitution of Ala122 confers resistance to imidazolinone herbicides and low levels of resistance to sulfonylureas (Bernasconi et al. 1995; Tranel and Wright 2002). Ala205 substitutions result in moderate resistance to all classes of ALS herbicides (Bernasconi et al. 1995). Substitutions of Trp574 or Asp376 cause high levels of resistance to imidazolinone, sulfonylurea and triazolopyrimidine herbicides (Tranel and Wright 2002; Whaley et al. 2007). To explain the variety of resistance characteristics, Ott et al. (1996) proposed a hypothetical model of the ALS enzyme where the amino acids coalesce to form a herbicide binding site at the entrance of the enzyme substrate (Ponziak et al. 2004; Tan et al. 2005).

The objectives of this research were to select and characterize camelina mutant plants with increased resistance to ALS inhibitor herbicides. Plants with increased resistance would be very useful in increasing the amount of camelina planted. The risk of camelina crop damage from residual herbicides has limited planting of camlina, but would be eliminated by planting a camelina variety with resistance to the residual herbicide. Development of ALS inhibitor resistant camelina would aid in increased production of this oil crop.

Materials and Methods

Mutation and Selection of Camelina

Camelina seed was mutagenized in several experiments. In experiment 1, seven batches of 10g of camelina (cv. Cheyenne) was treated with 0%, 0.05%, 0.1%, 0.15%, 0.2%, 0.25%, and 0.3% EMS in a 0.1M phosphate buffer, pH 7.5. All seven batches were planted on April 14, 2007 by broadcasting and raking in a 7.62m by 29.26m field plot. In experiments 2, 3, and 4 batches 110g of camelina seed was treated with 1L of 0.3% EMS in a 0.1M phosphate buffer, pH 7.5. Experiment 2 had four batches of

Calena and three batches of Cheyenne, experiment 3 had 6 batches of Cheyenne, and experiment 4 had three batches of Calena. All mutagenized seed was broadcast and raked in a 57.91m by 15.54m plot during the last week of April, 2008. All plots were in a Palouse silt loam soil at the WSU Cook Agronomy Farm. Plots were harvested in 15 separate bulks on September 8, 2008. 350g subsamples of M₂ seed from each of the 15 bulks was planted in 4.57 m by 10.05 m plots by harvest bulk on September 10, 2008.

M₂ plots planted September 12, 2008 were treated with a 52.5g ai/ha imazethapyr with 0.25% nonionic surfactant¹ on September 26, 2008. Resistant selections were designated IM 1-27 (Imazethapyr Mutant) and were transplanted into pots and moved to the greenhouse before the first hard frost.

On February 3, 2009, another subsample of M₂ seed was planted in 15 3.86m by 15.54m plots and sprayed with 17.5g ai/ha sulfosulfuron and 0.25% non-ionic surfactant. Resistant selections from these plots designated SM 1-8 (Sulfosulfuron Mutant) and were allowed to mature in the field.

Confirmation of resistant selections

All greenhouse experiments used either 500 cm³ square pots or flats of 59 cm³ cells filled with sunshine mix #1 (Sun Grow Horticulture Inc., Bellevue). Seeds were planted 0.25 cm deep and covered with soil. Plants were grown under a 32/25 C (± 3 C) day/night temperature regime with a 16-h photoperiod. Natural light was supplemented with overhead sodium vapor lighting at 980 μmol/m²/s. All pots were watered daily to field capacity. When assaying for resistance, seedlings were treated with herbicides using a moving-nozzle cabinet sprayer equipped with a flat-fan nozzle tip calibrated to deliver 168 L ha⁻¹ of spray solution at 206 kPa in a single pass over the foliage. Plants treated with herbicides were visually evaluated 21 days after treatment (DAT) for discoloration, stunting and biomass. For biomass evaluations, plants were harvested at soil level and above ground fresh weight was measured; plants were then dried and weighed for dry weight.

To confirm selections for resistance, M₃ seed from IM1-IM27 and several susceptible check varieties were planted in 59 cm³ square cells, with six seedlings per cell. The experiment was a randomized complete block design with four replications. The seedlings were treated with a moving cabinet sprayer at the two- to three- leaf stage with a 52.5g ai/ha imazethapyr and 0.25% non-ionic surfactant¹.

M₃ seed from resistant selections SM1-SM8 was also planted and treated with a moving cabinet sprayer at the two- to three- leaf stage with 17.5g ai/ha sulfosulfuron per hectare in solution with 0.25% non-ionic surfactant¹.

Segregation of Resistance

Crosses were made in the greenhouse between all M₃ plants with resistant and susceptible plants from the variety Calena by growing them in gallon pots, emasculating the female flower (Calena) and pollinating with a flower from the resistant mutant. F₁ plants were grown and the seed harvested to produce F₂ families. F₂ plants were grown in 500cm³ square pots and thinned to six plants per pot just prior to treatment. For segregation assays, seedlings were treated at the two- to three-leaf stage with a 52.5g ai/ha imazethapyr per hectare rate and 0.25% non-ionic surfactant¹. When evaluated, plants were categorized into three categories; 1) resistance similar to the resistant parent 2) dead or severely discolored with no signs of growth, similar to the susceptible parent and 3) intermediate between the two parents, green and growing slowly. The experiment was performed twice. Chi square goodness of fit tests were used to determine if segregation fit expected Mendelian ratios.

After the plants were evaluated, the least injured plant from each pot was selected to advance to the next generation. Several of these plants did not produce seed, but those that did were advanced to make F₄ families. A sample of the progeny was checked for segregation of resistance.

Whole Plant Dose Response

To evaluate the level of resistance in mutants and susceptible camelina lines in response to treatment with ALS inhibitor herbicides, seedlings of the susceptible variety Calena, and resistant mutant lines IM1 and SM4 were treated with three ALS inhibitor herbicides (imazethapyr, sulfosulfuron, and flucarbazone) from different chemical families (IMI, SU, and TCP respectively). The experiment was a randomized complete block design arranged in a split-plot treatment arrangement with herbicide rate as the whole plot and camelina line as the subplot. Each herbicide was a separate experiment and each experiment was conducted twice.

Seeds were planted, six per 500 cm³ square pot, and were treated at the two- to three- leaf stage. Rates of 0, 0.79, 1.57, 3.15, 6.30, 12.60, 25.20, and 50.39 mg ai/ha were applied for Imazethapyr. Sulfosulfuron rates were 0, 0.83, 1.66, 3.32, 6.64, 13.27, 26.54, and 53.08 mg ai/ha. Rates for flucarbazone were 0, 0.46, 0.92, 1.84, 3.68, 7.37, 14.74, and 29.47 mg ai/ha. Visual stunting and discoloration was evaluated at 7, 14, and 21 DAT. Three plants were randomly selected from each pot, and fresh weights were taken of above ground tissues 21 DAT. The samples were then dried at 50C for 4d, and weighed again for dry weights.

An additional dose response experiment with SM4 and Calena was conducted with higher rates of imazethapyr as sufficient reduction of biomass was not observed in the first dose response experiment to determine GR₅₀. Rates of imazethapyr used were 0, 6, 12, 25, 50, 101, 202, 302, 403, 504, 605, and 806 mg ai/ha. Otherwise, the experiment was conducted as described for the previous experiment.

Plant fresh biomass data was modeled using the equation:

$$y = a (1 + (x/x_0)^b)^{-1}$$

Where, y is the plant biomass, x is herbicide rate, a is the lower asymptote, x_0 is the herbicide rate that caused 50% reduction in plant biomass (GR₅₀) and b is the slope of the line at x_0 . R/S ratios were calculated by dividing the resistant line GR₅₀ by the susceptible line GR₅₀ (Seefeldt et al. 1995).

Enzyme Assay for ALS Activity

A crude extract from young stem and leaf tissue from camelina plants was derived in order to measure acetolactate synthase activity *in vitro* (Singh et al. 1988; Ponziak et al. 2004). Young stem and leaf tissue was cut and ground with a mortar and pestle in a 100mM potassium phosphate, 4mM thiamine pyrophosphate, 200mM pyruvate, 20mM magnesium chloride, 20uM flavin adenine dinucleotide buffer. Five grams of plant tissue was ground with 10mL buffer for 5 minutes, and then filtered through cheese cloth. Filtered homogenate was then centrifuged at 3700rpm for 30 min. The resulting supernatant was then used in the assay.

The assay was performed in a 100-uL volume in 96 well microtiter plates. The herbicides sulfosulfuron, and flucarbazone were diluted to final concentrations of 1.56, 3.125, 6.25, 12.5, 25, 50, 100, and 200 uM. Imazethapyr was diluted to final concentrations of 3.125, 6.25, 12.5, 25, 50, 100, 200, and 400 uM. Other test solutions in the plates included distilled water (control) and distilled water plus 5% sulfuric acid (no enzyme activity). To bring the wells to 100uL, 50uL of crude extract supernatant was added to wells containing 50uL test solution. Plates were incubated at 37 C for 2 hours. Then 25uL of 5% sulfuric acid was added to each well and the plates were incubated at 60 C for 15 min. A color change solution was prepared containing 0.25 and 2.5% (w/v) of creatine and naphthol, respectively, in 2N NaOH and 175uL was added to each well.

Acetolactate synthase activity was calculated from the amount of acetoin produced. Total acetoin was measured by colorimetric analysis at 532nm using a plate reader (Westerfeld 1945). The ALS activity data was expressed as a percentage of the mean of the water controls minus the absorbance of the no enzyme activity treatments.

Percent acetoin produced data was modeled using the equation:

$$y = a (1 + (x/x_0)^b)^{-1}$$

Where, y is the ALS activity, x is the herbicide concentration, a is the lower asymptote, and x_0 is the herbicide concentration that caused 50% reduction in ALS activity, (AR_{50}) and b is the slope of the line at x_0 . R/S ratios were calculated by dividing the resistant line AR_{50} by the susceptible line AR_{50} (Seefeldt et al. 1995).

Results and Discussion

Camelina seed (cv. Calena and Cheyenne) was planted after EMS treatment in May of 2008. When M_2 bulk populations in field plots were treated with imazethapyr, 27 were selected as potential mutants due to lack of symptoms. Of these 27, four, designated IM1, IM6, IM10, and IM18, were confirmed to have heritable resistance in greenhouse tests of the M_3 progeny. M_2 field plots treated in 2009 with sulfosulfuron resulted in eight potential mutants with sulfosulfuron resistance. Of these eight, one, designated SM4, was confirmed to have heritable resistance in M_3 progeny tests.

Segregation of Resistance Genes

When confirming the M_2 mutants for resistance, a total of 48 M_3 progeny were treated from each parental M_2 . For each line all plants were either susceptible or resistant, indicating only homozygotes were selected in the field. To identify the inheritance pattern of the resistance, crosses between resistant mutants and susceptible wild type plants were made. The F_1 - seed from these crosses were planted and grown to produce F_2 progeny. The progeny segregation for IM1/wild type and SM4/wild type F_2 families fit the model for a single dominant gene (Table 1). There was a range in apparent levels of resistance, with the most resistant plants appearing similar to the resistant parents, others similar to the susceptible parent or intermediate in resistance. The range of resistance is consistent with a model where resistance is controlled by a single codominant gene with the homozygous resistant types being more resistant than the heterozygotes. When the seedlings were classified into resistant, intermediate and susceptible classes a ratio of 149:450:210 was observed for IM1 families and a ratio of 173:452:196 was observed for SM4 families. These ratios deviated

significantly from the expected 1:2:1 ratio ($\chi^2 = 19.44$, $P < 0.001$; $\chi^2 = 9.679$, $p < 0.01$) due to an excess of intermediate types and a deficiency of resistant types. When the resistant and intermediate classes were combined, a 599:210 ratio was observed for IM1 families and a 625:196 ratio was observed for SM4 families which fits a 3:1 ratio ($\chi^2 = 0.396$, $p > 0.05$, and $\chi^2 = 0.556$, $p > 0.05$). It therefore seemed likely that the resistant and intermediate classes were not being accurately classified. To examine this, seed was collected from 32 F_2 plants that were classified as resistant. When 90-100 seedlings were sprayed three of these 32 F_3 families segregated for resistance while the other 29 families appeared homozygous resistant.

When F_2 seedlings from Cheyenne crossed to IM6 and IM18 were classified into resistant, intermediate and susceptible classes, 117:279:155 and 145:354:147 ratios were observed for Cheyenne/IM6 and Cheyenne/IM18 respectively. The ratio for Cheyenne/IM6 fit the expected 1:2:1 ratio ($\chi^2 = 5.33$, $P > 0.05$) as did Cheyenne/IM18 ($\chi^2 = 5.96$, $P > 0.05$). When the resistant and intermediate classes were combined, the data also fit a 3:1 ratio at 0.05 level of significance (Table 1). The segregation data indicates that the resistance for these mutants also fit the model for a single codominant gene, similar to IM1 derived seedlings. However, the homozygous resistant seedlings may have been more accurately distinguished from the heterozygotes for these F_2 populations.

Overall the results indicate that resistance is co-dominant for all mutants, but the homozygous resistant class can be difficult to distinguish from the heterozygotes with intermediate resistance. It might be possible to find herbicide rates or screening conditions where the homozygotes and heterozygotes could be more accurately distinguished, but no attempts were made to do this.

Allelism tests with different mutants

All four mutants selected with imazethapyr appeared to have a similar level of resistance in that they were stunted by the herbicide but are not killed like the susceptible wild type. If the mutations were in different loci, they may have an additive effect when crossed, resulting in a higher level of

resistance. To determine if there would be an additive effect crosses among all four mutants were made and the F₁ seed was grown to produce F₂ families. A sample of the F₂ seed was then planted in flats and seedlings were treated at the two- to three-leaf stage with 52.5g ai/ha imazethapyr and 0.25% non-ionic surfactant¹. The plants were evaluated 21 DAT to determine if the families were segregating for resistance. All four parents expressed resistance similarly, so separating true crosses from self pollinated contaminants was difficult, but in other segregation experiments crosses have been true approximately 90% of the time, therefore most families were assumed to be true crosses.

Of the 20 crosses among IM1, IM6, IM10, and IM18 that were tested in the F₂ for segregation, no susceptible plants were found. If the mutations that confer resistance were in different loci one out of 16 plants would have been expected to be susceptible. Small or discolored plants were removed prior to herbicide application because they may have been interpreted as susceptible plants if they had died. One hundred and forty five F₂ plants from two F₁ plants between IM1 and IM6, 155 F₂ plants from two F₁ plants between IM1 and IM10, and 314 F₂ plants from four F₁ plants between IM1 and IM18 were screened with imazethapyr for segregation. No susceptible plants were found. In addition to a lack of susceptible progeny, no plants were observed that had levels of resistance above that of the parents. All were stunted to approximately the same extent as the parents. A chi square test confirmed that the results are statistically different from a 15:1 ratio indicating that the mutations are in the same locus.

Crosses between IM1 and SM4 were also made and F₂ progeny were tested for segregation. Of 247 plants none were found to be susceptible which is significantly different than a 15:1 ratio. Indicating that the mutations are also at the same loci.

Whole Plant Response to Various Doses of Imazethapyr, Sulfosulfuron, and Flucarbazone

IM1 was selected to represent all four IM mutants in dose response experiments because the four mutants were not noticeably different in previous herbicide tests and segregation experiments. The dose response series of experiments was performed to quantify the relative resistance of the mutants to

each other and to the susceptible variety Calena. In the imazethapyr dose response experiment, seedlings derived from both IM1 and SM4 had increased resistance compared to the susceptible variety Calena. Visually, SM4 appeared healthier for all imazethapyr and sulfosulfuron treatments, with mild discoloration only in the higher rates of sulfosulfuron. The morphology of SM4 was notably different when treated with imazethapyr than the nontreated plants. When treated, the second and third leaves appeared more rounded and thicker than when nontreated. This trend was not apparent when treated with sulfosulfuron. SM4 derived plants appeared to gain biomass and initiated growth at secondary growing points, but did not elongate at the primary growing point like nontreated plants, resulting in short bushy plants at the time of harvest. IM1 derived plants had greater stunting and discoloration than SM4 derived plants, but not as severe as Calena when treated with imazethapyr. When treated with sulfosulfuron, IM1 derived plants appeared similar to the susceptible line in their degree of stunting and discoloration. Neither IM1 nor SM4 derived seedlings were noticeably different in stunting and discoloration from susceptible lines when treated with flucarbazone. The susceptible Calena appeared less stunted and discolored when treated with flucarbazone than the other herbicides, indicating flucarbazone may have less of an effect on camelina.

Based on biomass and GR_{50} , IM1 was 19 fold more resistant to imazethapyr than the susceptible line (Figure 1), while SM4 was >500 fold more resistant (Figure 2, Table 2). Both IM1 and SM4 also had increased resistance to sulfosulfuron; 4.6 and 155 fold respectively (Figure 3, Table 2). The increased resistance of IM1 to sulfosulfuron could be detected by the biomass data, although it had not been visually obvious. IM1 and SM4 do have increased resistance of 3.11 and 7.05 fold to flucarbazone, respectively (Figure 4, Table 2). Over all three herbicide treatments SM4 had greater resistance than IM1, although both mutant lines had greater resistance than the susceptible Calena.

Sensitivity of Camelina ALS Enzyme to Imidazolinone, Sulfosulfuron, and Flucarbazone

The whole plant dose response confirmed the two mutants had increased resistance to one or more herbicides. ALS herbicide resistance in mutagenized populations is most commonly due to changes in the target site of the ALS enzyme. The *in vitro* assay determines if resistance seen in the whole plant is a result of the ALS enzyme being insensitive to the herbicide.

When treated with imazethapyr the AR₅₀ for the susceptible line was 86.29 uM. Concentrations used for the two resistant lines were not high enough to reduce the ALS activity by 50%, therefore the AR₅₀ is greater than 400uM (Figure 5; Table 2). This insensitivity of the mutant enzyme reinforces the response seen in the whole plant for both IM1 and SM4.

When extracted ALS enzyme was tested with sulfosulfuron the results also mirrored the results for the whole plant dose response experiments. The AR₅₀ was 0.04 uM for IM1, only 6.6 fold more resistant as compared to the susceptible wild type. The AR₅₀ for SM4 was 13.8 uM, more than 2000 fold more resistant than the susceptible wild type (Figure 6; Table 2). The greater resistance at the enzyme level to sulfosulfuron agrees with the greater resistance observed in the whole plant dose response experiments with for the SM4 derived line.

IM1 and SM4 both was more resistant to flucarbazone, 2.8 fold and 2.2 fold for IM1 and SM4, respectively, than the wild type (Figure 7; Table 2). The increase in resistance to flucarbazone was less than for the other herbicides, and was also less at the enzyme level than at the whole plant level. Both IM1 and SM4 were about two times more resistant at the enzyme level, and more resistant at the whole plant level than the wild type camelina.

The increased resistance to imazethapyr and sulfosulfuron observed at the whole plant level for both mutants is likely from an insensitive form of the enzyme, as demonstrated by the *in vitro* enzyme dose response. The different levels indicate that the mutations are either different substitutions or in different, unequally expressed loci. IM1 and SM4 are resistant to imazethapyr and sulfosulfuron. Both mutants are resistant to flucarbazone, but the level of resistance observed in IM1 and SM4 is less than

that observed for the other two herbicides. In the *in vitro* enzyme dose response the ALS activity was not reduced to zero, even for the wild type, whereas the wild type was killed in the whole plant dose response experiments. The less than complete inhibition may occur in both experiments. ALS-inhibiting herbicides typically concentrate in the meristematic regions of growing plants. At the whole plant level there is enough enzyme inhibition in the meristematic regions to result in the death of the plant.

Although these mutants would be stunted by field applications of these three herbicides, they could be used to develop camelina varieties that would not sustain damage when planted into soil with treated with imazethapyr or sulfosulfuron. Imazethapyr is commonly used for broadleaf weed control in lentils and peas in the Pacific Northwest. Sulfosulfuron and flucarbazone are used for selective grass control in wheat. Conventional camelina varieties are susceptible to damage from low rates of all three of these chemicals. If varieties were available that could be planted without concern for injury from these chemicals more growers would be able to include camelina as a crop in the Pacific Northwest. Herbicide resistance in camelina could increase rotational flexibility and potential biofuel production for this region their rotations. By advancing these mutants through field screening they may be developed into released varieties.

Source of Materials

¹ Induce. Helena Chemical Company., 225 Schilling Boulevard. Suite 300, Collierville, TN38017.

² Potting media, LC1 Sunshine Mix. Sun Gro Horticulture Distribution Inc., 15831 N. E. 8th St. Suite 100, Bellevue, WA 98008.

Literature Cited

- Bernasconi, P., A. R. Woodworth, B. A. Rosen, M. V. Subramanian, and D. L. Siehl. 1995. A naturally occurring point mutation confers broad range tolerance to herbicides that target acetolactate synthase. *J. Biol. Chem.* 270:17381-17385.
- Chaleff, R. S. and C. J. Mauvals. 1984. Acetolactate synthase is the site of action of two sulfonylurea herbicides in higher plants. *Science* 224:1443-1445.
- Chaleff, R. S. and T. B. Ray. 1984. Herbicide resistant mutants from tobacco culture. *Science* 223:1148-1151.
- Croughan, T. P., inventor; Board of Supervisors of Louisiana State University, assignee. 1998. Herbicide resistant rice. U.S. patent 5,773,704.
- Duggleby, R. G. and S. S. Pang. 2000. Acetohydroxyacid synthase. *J. of Biochem. Mol. Biol.* 33:1-36.
- Eliason, R., J. J. Schoenau, A. M. Szmigielski, and W. M. Lavery. 2004. Phytotoxicity and persistence of flucabazone-sodium in soil. *Weed Sci.* 52:857-862.
- Gerwick, B. C., M. V. Subramanian, and V. I. Loney-Gallant. 1990. Mechanism of action of the 1,2,4-triazolo[1,5-a]pyrimidines. *J. Pestic. Sci.* 29:357-364.
- Hart, S. E., J. W. Saunders, and D. Penner. 1992. Chlorsulfuron resistant sugar beet: cross-resistance and physiological basis of resistance. *Weed Sci.* 40:378-383.
- Haughn, G.W. and C. R. Somerville. 1986. Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. *Mol. Gen. Genet.* 204:430-434.
- Heap, I. 2010. The International Survey of Herbicide Resistant Weeds. Available at <http://www.weedscience.org>. Accessed January 27, 2010).
- Lee, Y., A. K. Chang, and R. G. Duggleby. 1999. Effect of mutagenesis synthase at serine 653 of *Arabidopsis thaliana* acetohydroxyacid synthase on the sensitivity to imidazolinone and sulfonylurea herbicides. *FEBS letters* 452:341-345.

- Newhouse, K. E., B. K. Singh, D.L. Shaner and M. A. Stidham. 1991. Mutations in corn (*Zea mays* L.) conferring resistance to imidazolinones. *Theor. Appl. Genet.* 83:65-70.
- Ott, K. H., G. W. Kwagh, G. W. Stockton, V. Sidorov, and G. Kakefude. 1996. Rational molecular design and genetic engineering of herbicide resistant crops by structure modeling and site-directed mutagenesis of acetohydroxyacid synthase. *J. Mol. Biol.* 263:359-368.
- Ponziak, C. J., I. T. Birk, L. S. O'Donoghue, C. Menard, P. J. Huci, and B. K. Singh. 2004. Physiological and molecular characterization of mutation-derived imidazolinone resistance in spring wheat. *Crop Sci.* 44:1434-1443.
- Ponziak, C. J. and P. J. Huci. 2004. Genetic analysis of imidazolinone resistance in mutation-derived lines of common wheat. *Crop Sci.* 44:23-30.
- Rajasekaran, K., J. W. Gula, and D. M. Anderson. 1996. Selection and characterization of mutant cotton (*Gossypium hirsutum* L.) cell lines resistant to sulfonylurea and imidazolinone herbicides. *Plant Sci.* 199:115-124.
- Sabastian, S.A., G. M. Fader, D. R. Ulrich, D. R. Forney, and R. S. Chaleff. 1989. Semidominant soybean mutation for resistance to sulfonylurea herbicides. *Crop Sci.* 29:1403-1408.
- Seefeldt, S. S., J. E. Jensen, and E. P. Fuerst. 1995. Log-logistic analysis of herbicide dose-response relationships. *Weed Tech.* 9:218-227.
- Shaner, D. L., P. C. Anderson, and M. A. Stidham. 1984. Imidazolinones: potent inhibitors of acetohydroxyacid synthase. *Plant Physiol.* 76:545-546.
- Singh, B. K., M. A. Stidham, and D. L. Shaner. 1988. Assay of acetohydroxyacid synthase. *Analytical Biochem.* 171:173-179.
- Stidham, M. A. 1991. Herbicides that inhibit acetohydroxyacid synthase. *Weed Sci.* 39:428-434.

- Swanson, E. B., M. J. Harrgesel, M. J. Arnoldo, M. Sippell, and R. S. V. Wong. 1989. Microspore mutagenesis and selection: canola plants with field resistance to imadazolinone. *Theor. Appl. Genet.* 78:525-530.
- Tan, S., R. R. Evans, M. L. Dahmer, B. K. Singh, and D. L. Shaner. 2005. Imidazolinone-tolerant crops: history, current status, and future. *Pest Manag. Sci.* 61:246-257.
- Thill, D. C. 1997. Sulfonylurea herbicide resistance in plants. US Patent RE35661.
- Tranel, P. J. and T. R. Wright. 2002. Resistance of weeds to ALS-inhibiting herbicides: what have we learned? *Weed Sci.* 50:100-712.
- 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 herebicides. *Weed Sci.* 55:83-90.
- Wright, T. R. and D. Penner. 1998. Cell celection and inheritance of imidazolinone resistance in sugar beet (*Beta vulgaris*). *Theor. Appl. Genet.* 96:612-620.
- Yu, Q., X. Q. Zhang, A. Hashem, M. J. Walsh, and S. B. Powles. 2003. ALS gener prolene (197) mutations confer ALS herbicide resistance in eight seperate wild radish (*Raphanus raphanistrum*). *Weed Sci.* 51:831-838.

Tables and Figures

Table 2: Segregation of resistance to imazethapyr in F₂ families derived from resistant by susceptible crosses.

F ₂	Number of Plants				Chi-sq 1:2:1	Chi-sq 3:1
	Total	Resistant	Intermediate	Susceptible		
Calena/IM 1	809	149	450	210	19.4351*	0.39596
Cheyenne/IM 6	551	117	279	155	5.3303	2.8802
Cheyenne/IM 18	646	145	354	147	5.9628	1.7358
Calena/SM4	821	173	452	196	9.6796*	0.5558

* indicate Chi-square values that deviate significantly (P<.01) from expected ratios.

Table 3: Whole plant and enzyme extraction dose response regression parameters (and standard errors) for two mutant lines and a susceptible line treated with three different ALS inhibitor herbicides.

Camelina line	Regression parameters (and standard errors)					
	Whole plant dose response			Enzyme extract dose response		
	GR ₍₅₀₎	R ²	R/S	AR ₍₅₀₎	R ²	R/S
Imazethapyr						
Calena –S	.99(.02)	0.99	-	86.29(26.7)	0.95	-
IM 1	19.21 (3.69)	0.88	19.40	>400	-	-
Calena – S	.35 (.21)*	.99*		-	-	-
SM4	204.15 (37.87)*	.88*	583.28*	>400	-	-
Sulfosulfuron						
Calena – S	0.10 (.16)	0.98	-	0.0061(0.1)	0.88	-
IM 1	0.46 (.05)	0.99	4.6	0.04(0.15)	0.97	6.56
SM4	15.56 (8.8)	0.53	155.6	13.81(6.04)	0.96	2263.93
Flucarbazone						
Calena – S	1.5 (.13)	0.99	-	0.62(0.20)	0.99	-
IM 1	4.67 (1.34)	0.87	3.11	1.72(1.88)	0.92	2.77
SM4	10.57 (5.01)	0.62	7.05	1.36(0.42)	0.98	2.19

*Imazethapyr whole plant dose response was performed at two rate ranges, those lines with * are from the higher rate experiment.

Figure 1: Reduction in plant biomass as a result of different rates of imazethapyr, low rates.

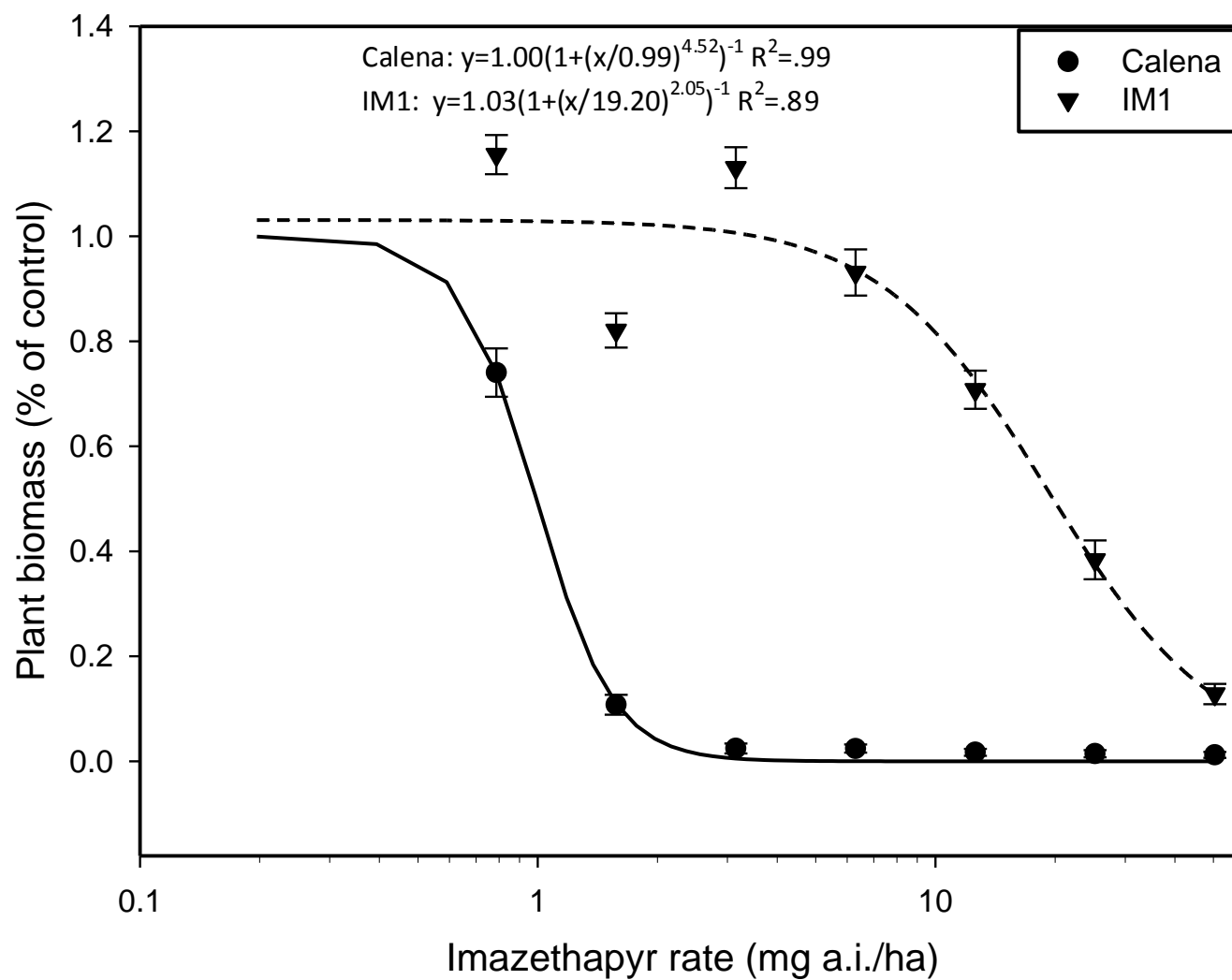


Figure 2: Reduction in plant biomass as a result of different rates of imazethapyr, high rates.

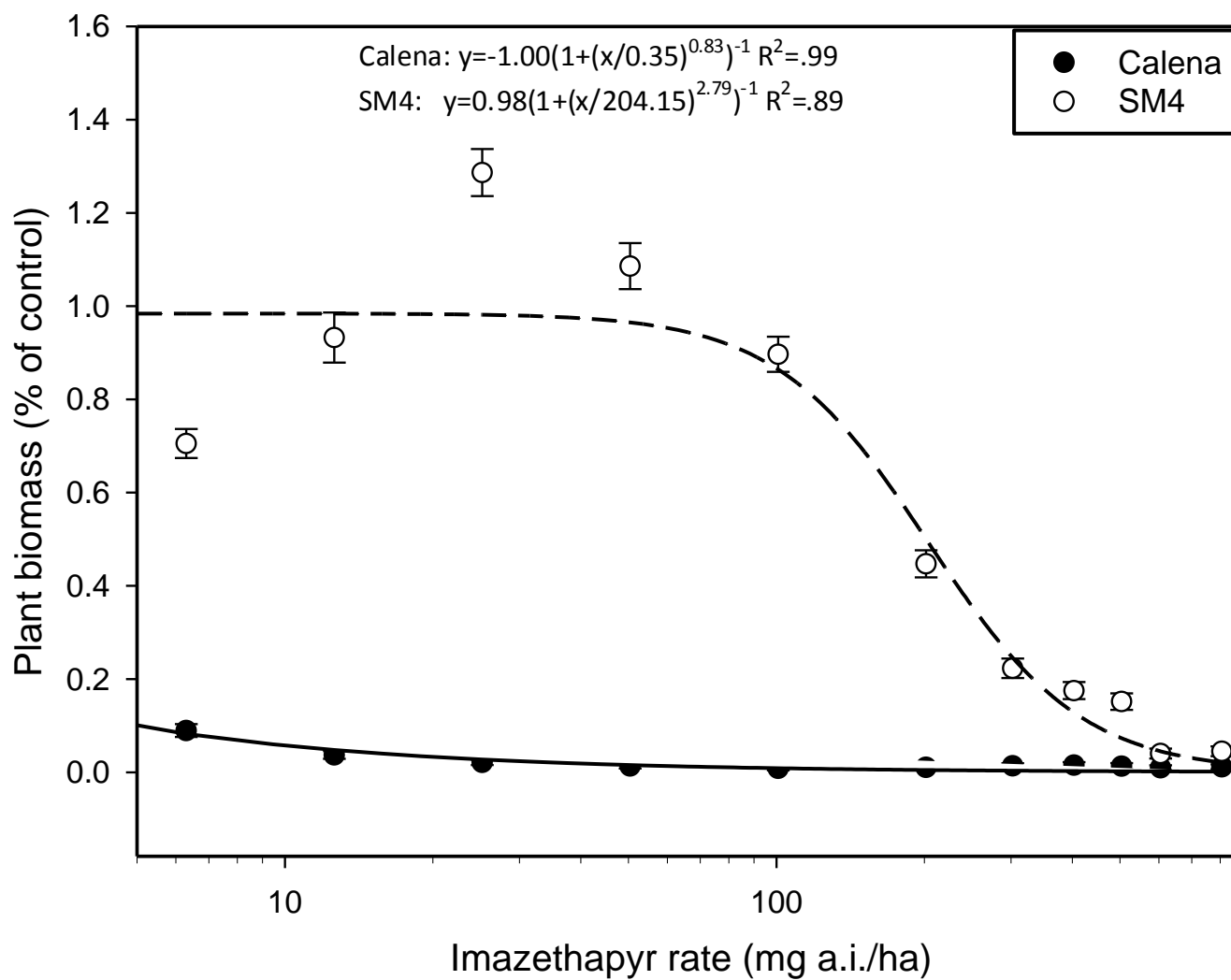


Figure 3: Reduction in plant biomass as a result of different rates of sulfosulfuron

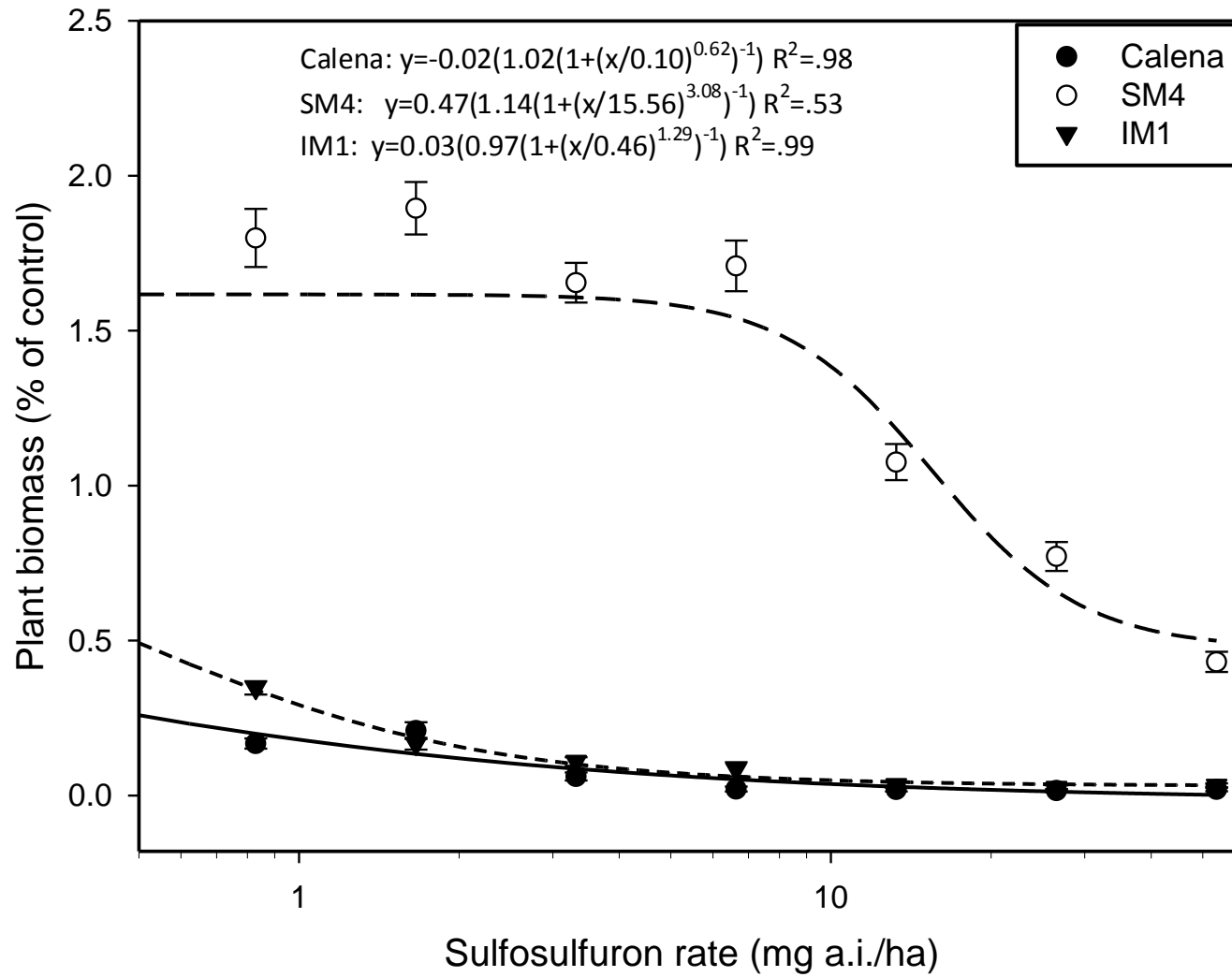


Figure 4: Reduction in plant biomass as a result of different rates of flucarbazone.

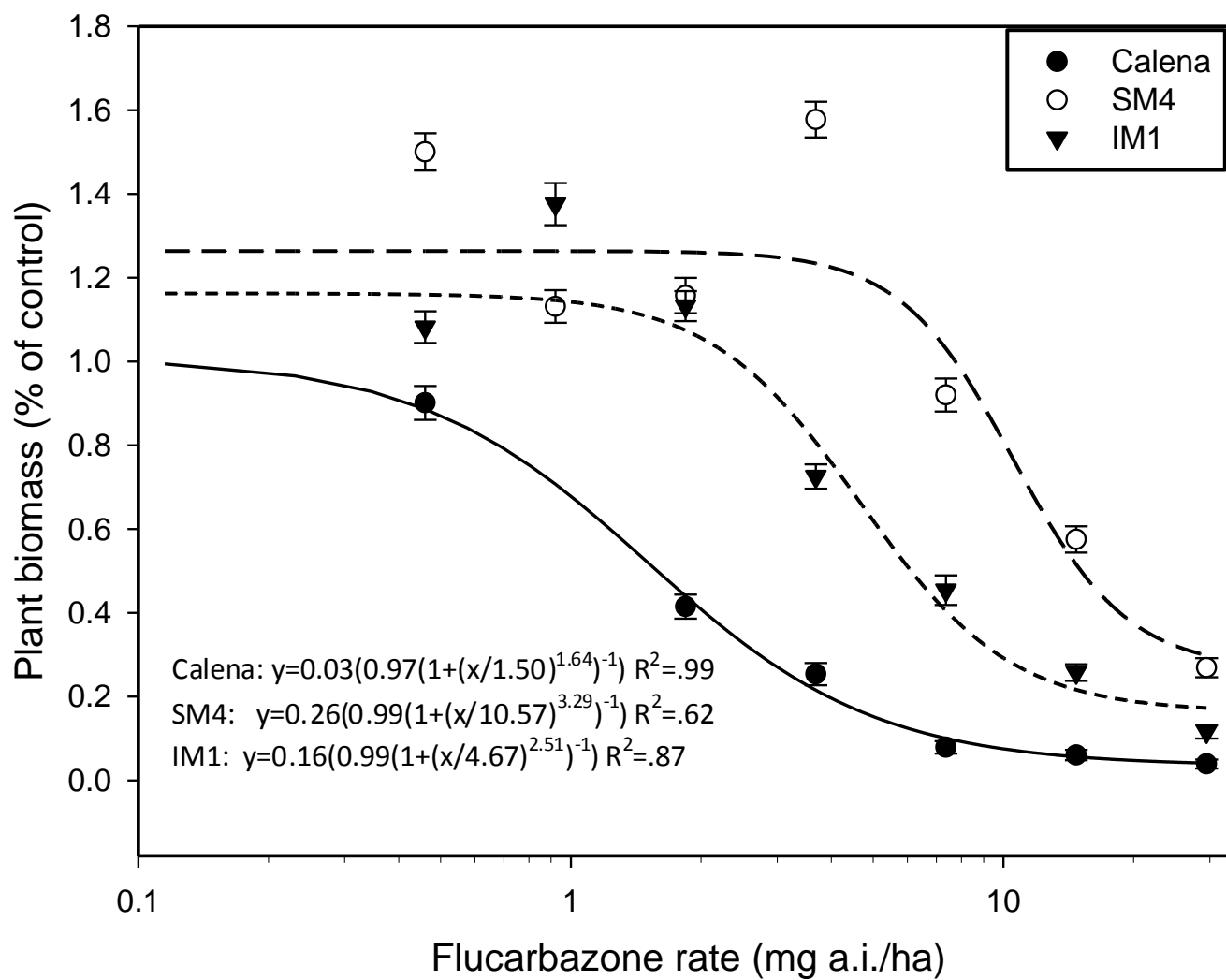


Figure 5: Reduction of ALS enzyme activity in the presence of different concentrations of imazethapyr.

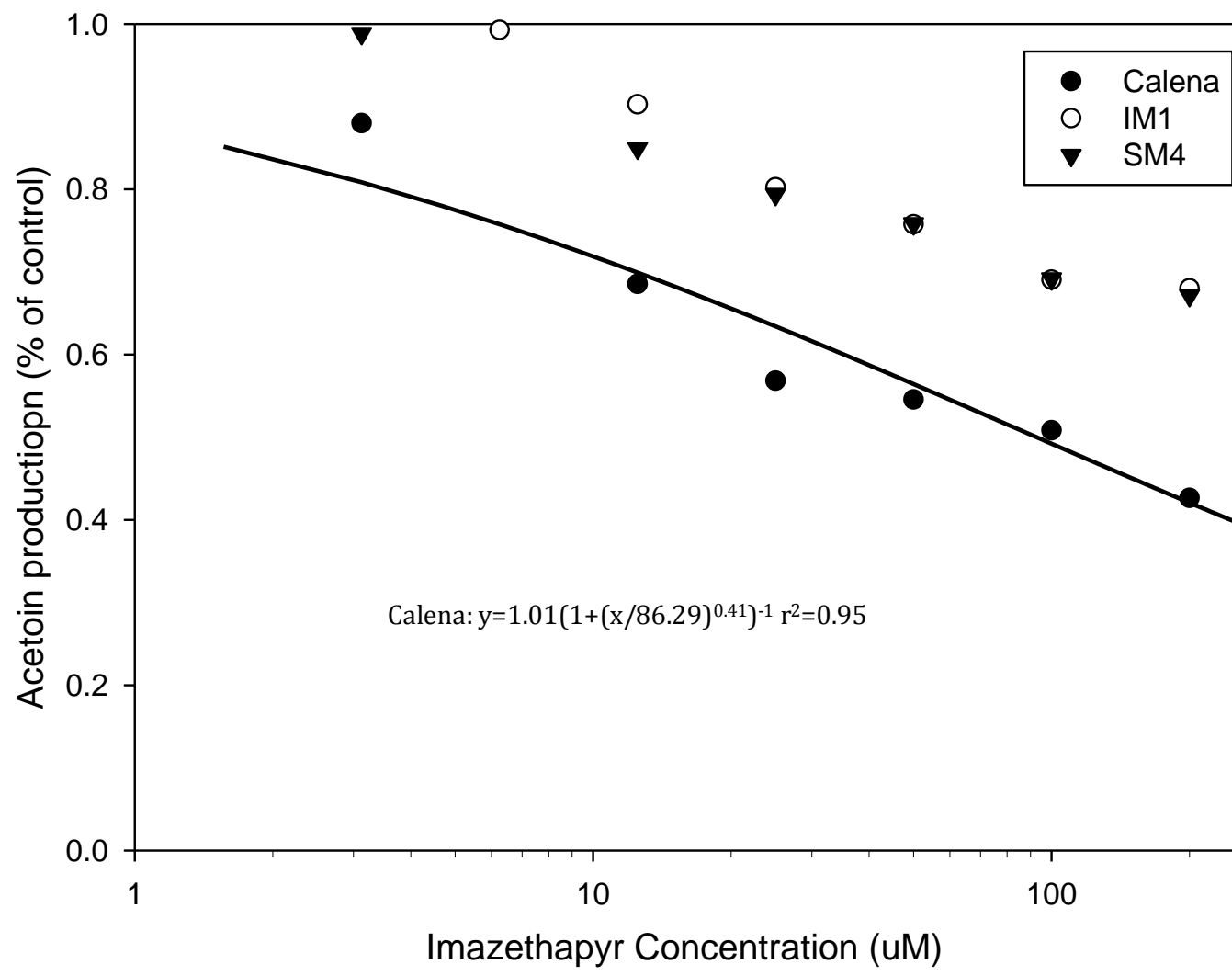


Figure 6: Reduction of ALS enzyme activity in the presence of different concentrations of sulfosulfuron.

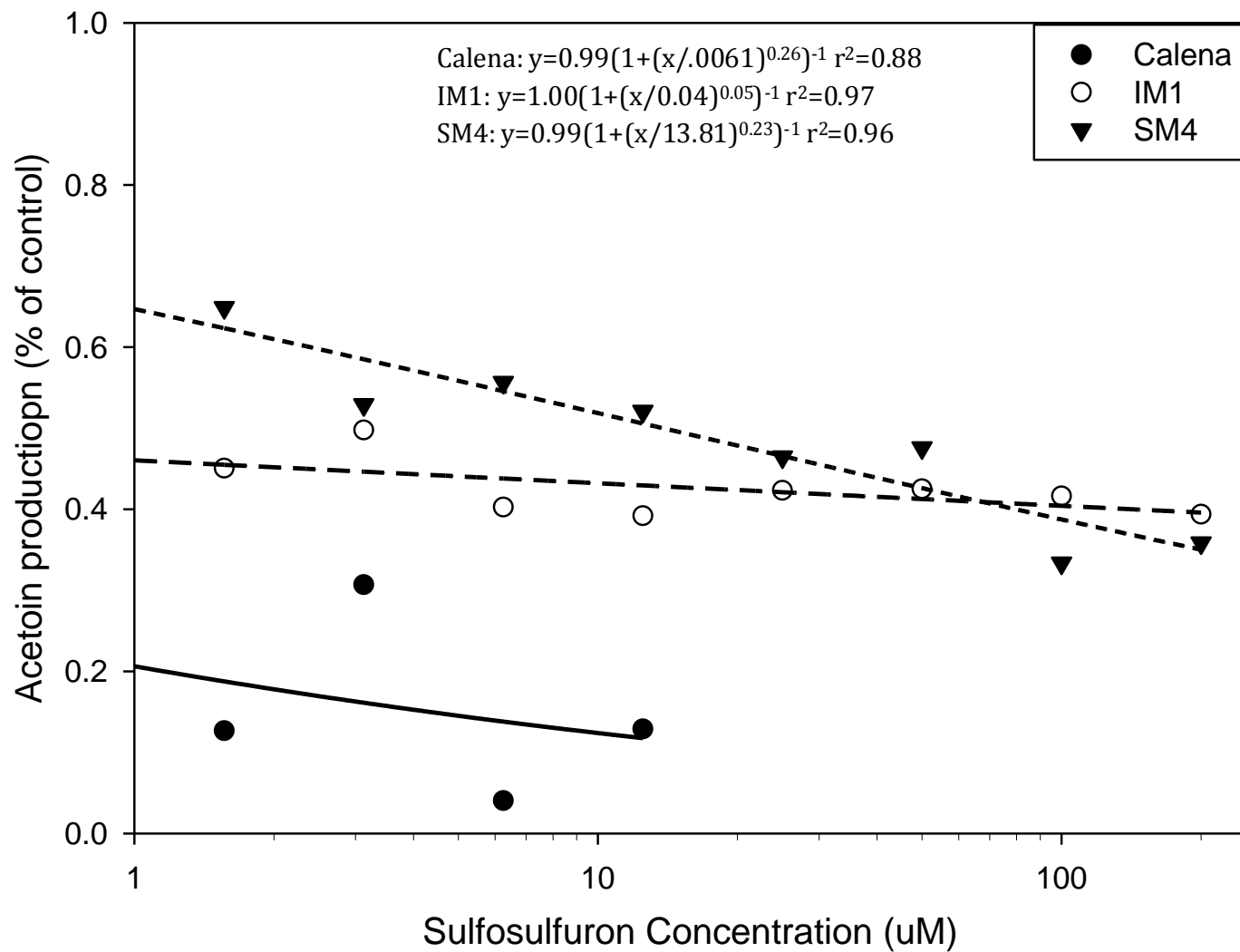
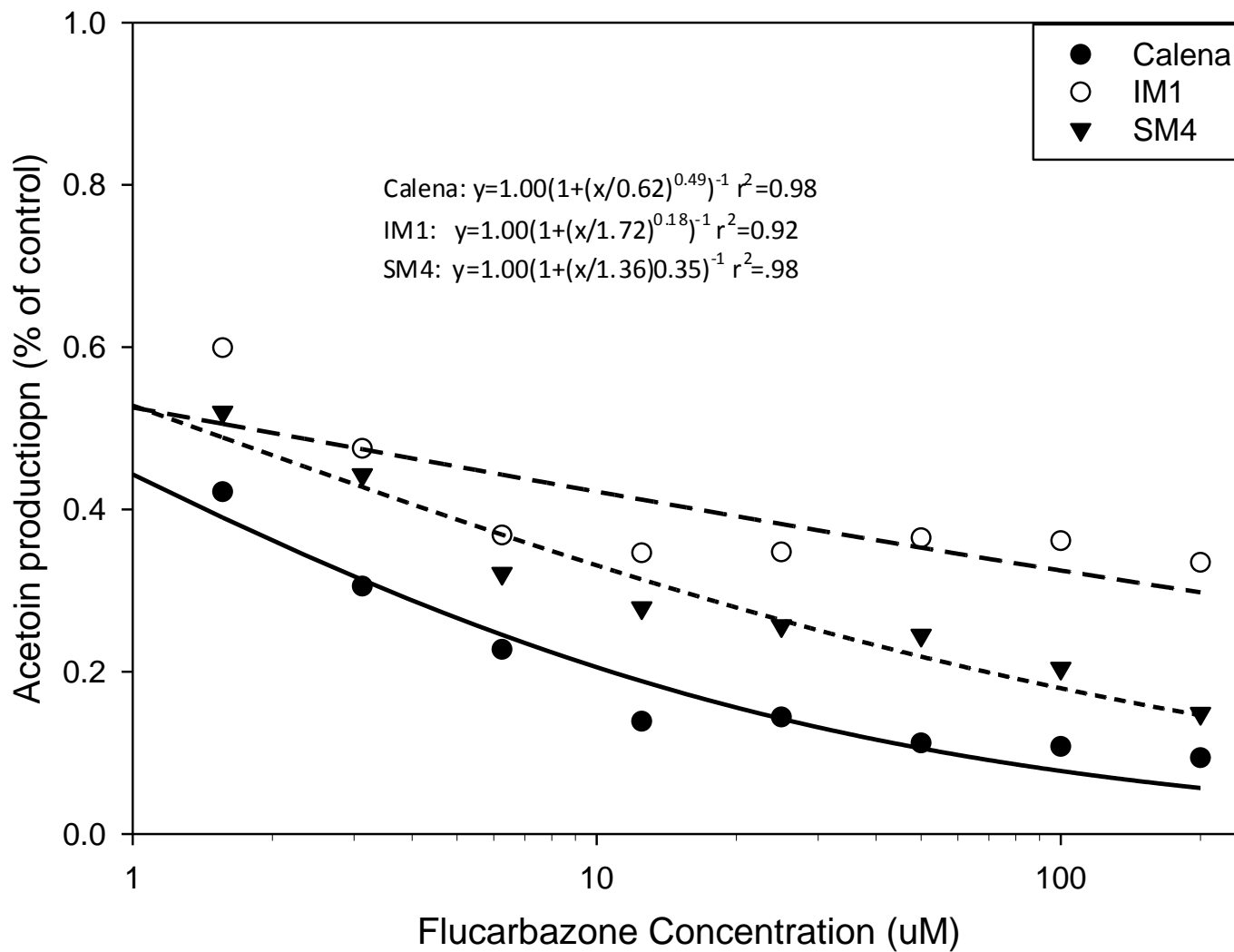


Figure 7: Reduction of ALS enzyme activity in the presence of different concentrations of flucarbazone.



Registration of Two Camelina (*Camelina sativa* L.) Genotypes with Resistance to Several ALS Inhibitor Herbicides

Dustin T Walsh, Ian C. Burke, and Scot H. Hulbert²

Abstract

Camelina (*camelina sativa* L.) is highly susceptible to soil residual levels of many acetolactate synthesis (ALS) inhibitor herbicides. Other crops with resistance to these herbicides have been developed through mutation. Development of camelina with this resistance would allow greater flexibility for the crop in rotation with other crops. Four mutants (IM1, IM6, IM10, and IM18) were selected for increased resistance to imazethapyr and one mutant (SM4) was selected for resistance to sulfosulfuron was identified. The mutants appear to be controlled by a single codominant gene. Plants treated with imazethapyr, sulfosulfuron, and flucarbazone were evaluated visually and for biomass accumulation. All mutants were visually more resistant to all three herbicides than the wild type, and SM4 appeared significantly more resistant to all three herbicides than the IM mutants. SM4 showed the highest level of resistance to both imazethapyr, sulfosulfuron and flucarbazone. These mutant lines are available to industry and public breeders to create camelina varieties with increased resistance to these ALS herbicides.

² Graduate Student, Assistant Professor, and Professor respectively, Department of Crop Sciences, Johnson Hall 201, Washington State University, Pullman, WA 99164, Corresponding author's email: dtwalsh@wsu.edu

Abbreviations: EMS, ethyl methane sulfonate; ALS, acetolactate synthase; DAT, days after treatment;

Acetolactate synthase (ALS) inhibitor herbicides target the acetolactate synthase enzyme (also known as acetoxyacid synthase) in higher plants and are very popular herbicides because they are effective at controlling a wide range of grass and broadleaf weeds and have selectivity for many crops. Some of these herbicides have long term soil residual activity which can damage sensitive crops, such as camelina (*Camelina sativa* L.; Hanson and Thill 2001). Plants with herbicide resistance have been obtained through mutagenesis in many crop species, including maize (*Zea mays* L.; Newhouse et al. 1991), *Arabidopsis thaliana* L. (Haughn and Somerville 1986), sugar beet (*Beta vulgaris* L.; Wright and Penner 1998; Hart et al. 1992), canola (*Brassica napus* L.; Swanson et al. 1989), soybean (*Glycine max* L.; Sabastian et al. 1989), tobacco (*Nicotiana tabacum* L.; Chaleff and Ray 1984), cotton (*Gossypium hirsutum* L.; Rajasekaran et al. 1996) rice (*Oryza sativa* L.; Croughan 1998) and wheat (*Triticum spp*; Ponziak and Huci 2004). Some crop cultivars have been released with resistance to members of this herbicide family (Devine 2005). The most popular commercialized cultivars have been Clearfield® (BASF, N. Carolina) varieties which are tolerant to imidazolinone herbicides and include varieties of corn, wheat, rice, and canola (Tan et al. 2005). Increased resistance in these crops is most commonly due to a form of ALS enzyme that is less sensitive to the herbicides.

Imidazolinone and sulfonylurea herbicide degradation in the soil is dependent on soil temperature, moisture, and pH. If conditions are not optimal, the herbicides may remain in the soil for long periods of time (Hanson and Thill 2001). Many crops from the mustard family are highly sensitive to Imidazolinone and sulfonylurea herbicide residual levels, including canola and camelina. Camelina varieties with increased resistance would avoid damage by residual herbicides. Development of lines of camelina with increased resistance to ALS herbicides would be useful as germplasm or released cultivars. The objective of this research was to select and characterize camelina plants with resistance to ALS inhibitor herbicides from a mutagenized population.

Methods

Several experiments were conducted to treat camelina seed with EMS. In experiment 1, seven batches of 10g of camelina (cv. Cheyenne) was treated with 0%, 0.05%, 0.1%, 0.15%, 0.2%, 0.25%, and 0.3% EMS in a 0.1M phosphate buffer, pH 7.5. On April 14, 2007 the batches were broadcast and raked in a 7.62m by 29.26m field plot. In experiments 2, 3, and 4, 110g batches of camelina seed was treated with 1L of 0.3% EMS in a 0.1M phosphate buffer, pH 7.5. Experiment 2 had four batches of Calena and three batches of Cheyenne, experiment 3 had six batches of Cheyenne, and experiment 4 had three batches of Calena. Seed from these experiments was planted on the last week of April, 2008, in a 58 m by 16 m plot by broadcasting and raking. All plots were at the WSU Plant Pathology Farm in a Palouse silt loam soil. On September 8, 2008 plots were harvested in 15 separate bulks. Two 350 g subsamples of M₂ seed from each of the 15 bulks were taken. One set of subsamples was planted in 5 m by 10 m plots on September 10, 2008. The plots were treated with 52.5g ai/ha imazethapyr in solution with 0.25% non-ionic surfactant on September 26, 2008. The second set of subsamples was planted in 3.86 m by 15.54m plots on February 3, 2009 and were treated with 17.5g ai/ha sulfosulfuron and 0.25% non-ionic surfactant on February 18, 2009. Plants that survived the herbicide treatments were moved into pots and raised in the greenhouse.

Either 500 cm³ square pots or flats filled with 59 cm³ cells were used for all greenhouse experiments. Plants were grown in commercial potting media (sunshine mix #1; Sun Grow Horticulture Inc., Bellevue). Seeds were planted 0.25cm deep. Plants were grown under a 32/25 C (\pm 3 C) day/night temperature regime with a 16-h photoperiod supplemented with overhead sodium vapor lighting at 980 $\mu\text{mol}/\text{m}^2/\text{s}$. When assaying for resistance, seedlings were treated with herbicides using a moving-nozzle cabinet sprayer equipped with a flat-fan nozzle tip calibrated to deliver 168 L ha⁻¹ of spray solution at 206 kPa in a single pass over the foliage. Plants treated with herbicides were visually evaluated or harvested for analysis 21 days after treatment (DAT).

M₃ seed from field selections were planted and treated with 52.5g ai/ha imazethapyr or 23.4g ai/ha sulfosulfuron (according to what the lines were originally selected with) in order to confirm heritable resistance to these herbicides. Confirmed resistant mutants were designated imazethapyr mutants (IM) or sulfosulfuron mutants (SM) based on which chemical treatment they were selected under.

In order to evaluate the relative resistance of wild type camelina (cv. Calena), IM1, IM6, IM10, IM18, and SM4 to applications of three different classes of ALS inhibitor herbicides flats of 500 cm³ pots were planted with three pots of each line. Prior to treatment plants were thinned to six per pot. One flat was treated with imazethapyr at 52.5g ai/ha, one with sulfosulfuron at 23.4g ai/ha, one with flucarbazone at 29.47g ai/ha, and one was left untreated. The whole experiment was repeated in another greenhouse. Flats were treated with herbicide ten days after planting and were visually evaluated for stunting and discoloration at 7, 14, and 21 DAT. Three plants were randomly selected from each pot and cut at soil level and fresh weights were taken 21DAT. The samples were then dried at 50C for 4d, and weighed. SAS was then used to compute means and significant differences between the mutants for each herbicide.

Mutant lines were crossed to the susceptible cultivars to examine their inheritance and to create stocks with fewer mutant alleles at non-target loci. Mutants IM1, IM6, and SM4 were created in the Cheyenne background and were crossed to Calena, While mutants IM10 and IM18 were generated in the Calena background and were crossed to Cheyenne. All plants were grown in four liter pots and crosses were performed by emasculating the female flower from the wild type and pollinating with anthers from the mutant lines. The F₁ generation was untreated and the seed harvested to produce F₂ families. F₂ plants were grown in 500 cm³ square pots and thinned to six plants per pot just prior to treatment with imazethapyr. For segregation assays, seedlings were treated at the two- to three-leaf

stage with a 52.5g ai/ha dose of imazethapyr in solution with 0.25% non-ionic surfactant. Plants were categorized into three categories; 1) resistance similar to the resistant parent 2) dead or severely discolored with no signs of growth, similar to the susceptible parent and 3) intermediate between the two parents, green and growing slowly. The experiment was performed twice. Chi square goodness of fit tests were used to determine if segregation fit expected Mendelian ratios.

Characteristics

Four mutants from plots treated with imazethapyr, designated IM1, IM6, IM10, and IM18 (Imazethapyr Mutant), were confirmed to have heritable resistance by testing progeny seedlings. One mutant from plots treated with sulfosulfuron, designated SM4 (Sulfosulfuron Mutant), was confirmed to have heritable resistance. A total of 48 plants were tested for each mutant. Susceptible progeny were not observed in any of these tests, indicating that the M₂ plant selected in the field was homozygous for the resistant form of ALS.

To test the relative sensitivity of the different mutants to different herbicides, biomass was measured 21 days after herbicide application. When treated with imazethapyr and sulfosulfuron, SM4 had significantly more biomass than the four IM mutants and the wild type (table 1). The four IM mutants did not have significantly different biomass accumulation than the wild type when treated with imazethapyr or sulfosulfuron, although they appeared much less damaged by the herbicides. All five mutant lines have increased resistance to flucarbazone based on biomass accumulation, and SM4 had significantly more biomass than the other four mutants. Although IM1, IM6, IM 10 and IM18 did not show a significantly different dry biomass, they were visually less damaged than the susceptible check when treated with imazethapyr and sulfosulfuron. The IM mutants were visually different because they did not die, although they were stunted by the treatment, they were still green. The four IM mutants possess a similar resistance profile and cannot be visually distinguished when treated with imazethapyr

or sulfosulfuron, which may indicate they have the same mutation. Despite not having a significantly different biomass 21DAT than the wild type, the four IM mutants were strikingly different visually. At 21DAT the susceptible check was completely yellow or brown, while the IM mutants were still green, although they did not appear to be growing rapidly. Overall SM4 had a higher biomass than any of the other mutants for all three herbicide treatments which sets it apart from the other mutants and it likely has a different mutation in the ALS gene.

When F₂ seedlings from crosses between mutants and wild type plants were treated with imazethapyr there was a range of apparent resistance. The most resistant plants appeared similar to the resistant parents, others similar to the susceptible parent or intermediate in resistance. This is consistent with single codominant gene model where the homozygous resistant types are more resistant than the heterozygotes. When seedlings from IM6 and IM18 derived families were classified into resistant, intermediate and susceptible classes, ratios of 117:279:155 and 145:354:147 were observed respectively. The ratios fit the expected 1:2:1 ratio ($\chi^2 = 5.33$, $p > 0.05$, and $\chi^2 = 5.96$, $p > 0.05$ respectively). When the resistant and intermediate classes were combined, the data for IM6 and IM18 derived seedlings also fit a 3:1 ratio at the 0.05 level of significance ($\chi^2 = 2.88$, $p > 0.05$, and $\chi^2 = 1.76$, $p > 0.05$ respectively).

When seedlings from IM1 and SM4 derived seedlings were classified into resistant, intermediate and susceptible classes ratios of 149:450:210 and 173:452:196 were observed. These ratios deviated significantly from the expected 1:2:1 ratio ($\chi^2 = 19.44$, $p < 0.001$ and $\chi^2 = 9.68$, $p < 0.01$ respectively) due to a deficiency of resistant types and an excess of intermediate types. When the resistant and intermediate classes were combined, a 599:210 ratio for IM1 families, and a 625:196 ratio for SM4 families was observed. The combined ratios fit a 3:1 ratio ($\chi^2 = 0.396$, $p = > 0.05$, $F < 3.841$, and $\chi^2 = 0.556$, $p > 0.05$ making it seem likely that the intermediate and resistant classes were not being accurately

discriminated. IM10 derived families were not examined for segregation because no crosses were successful in yielding viable seed.

Overall the segregation of the resistance indicates that for all mutants lines examined the resistance is controlled by a single codominant gene. Although it may be difficult to distinguish between homozygous resistant and heterozygous plants, using different herbicide rates may be able to increase the accuracy of classification, but no attempt was made to do this.

All mutants were stunted by direct application of recommended field rates of the herbicides herbicide treatment to various degrees. SM4 was the least effected although it was significantly stunted, SM4 continued to grow more than the four IM mutants. SM4 became swollen at the stem and the first two leaves became much larger when treated with herbicide than untreated. In all experiments IM1, IM6, IM10, and IM18 were indistinguishable visually. For the IM mutants it appeared that growth was nearly stopped for 10-14 DAT, after which secondary buds would often elongate and surpass the primary growing point. For advancing this trait and making selections, using a low rate of herbicide may result in less stunting, but still make susceptible types apparent.

These mutants would not be good candidates for treatment with the herbicide in the field because of the level of stunting they sustain, but may be useful for planting in fields with concerns about residual herbicide. The damage seen in the greenhouse at field application rates would reduce yield in the field substantially. The ALS inhibitor herbicides can be effective at very low concentrations and these mutants may be able to tolerate soil residual levels of herbicide that would significantly damage a susceptible Camelina variety. With less concern about soil residual herbicide damage, the crop would likely be included in more growers' rotations which include ALS inhibitor herbicides.

Seed Availability

A limited quantity of M_3 seed from all five mutants and F_3 seed from outcrosses of IM1 to Calena, homozygous for the mutant alleles, is available from Scot Hulbert, Department of Crop and Soil Sciences, Washington State University, Pullman, Washington.

References

- Chaleff, R. S. and T. B. Ray. 1984. Herbicide resistant mutants from tobacco culture. *Science* 223:1148-1151.
- Croughan, T. P., inventor; Board of Supervisors of Louisiana State University, assignee. 1998. Herbicide resistant rice. U.S. patent 5,773,704.
- Devine, M.D.. 2005. Why are there not more herbicide tolerant crops?. *Pest. Manag. Sci.* 61:312-317.
- Hanson, B.D. and D. C. Thill. 2001. Effects of Imazethapyr and Pendimethalin on Lentil (*Lens culinaris*), Pea (*Pisum sativum*), and a Subsequent Winter Wheat (*Triticum aestivum*) Crop Weed Technol. 15:190-194
- Hart, S. E., J. W. Saunders, and D. Penner. 1992. Chlorsulfuron resistant sugar beet: cross-resistance and physiological basis of resistance. *Weed Sci.* 40:378-383.
- Haughn, G.W. and C. R. Somerville. 1986. Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. *Mol. Gen. Genet.* 204:430-434.
- Newhouse, K. E., B. K. Singh, D.L. Shaner and M. A. Stidham. 1991. Mutations in corn (*zea mays* L.) conferring resistance to imidazolinones. *Theor. Appl. Genet.* 83:65-70.
- Ponziak, C. J. and P. J. Huci. 2004. Genetic analysis of imidazolinone resistance in mutation-derived lines of common wheat. *Crop Sci.* 44:23-30.
- Rajasekaran, K., J. W. Gula, and D. M. Anderson. 1996. Selection and characterization of mutant cotton (*Gossypium hirsutum* L.) cell lines resistant to sulfonylurea and imidazolinone herbicides. *Plant Sci.* 199:115-124.
- Sabastian, S.A., G. M. Fader, D. R. Ulrich, D. R. Forney, and R. S. Chaleff. 1989. Semidominant soybean mutation for resistance to sulfonylurea herbicides. *Crop Sci.* 29:1403-1408.

Swanson, E. B., M. J. Hargreaves, M. J. Arnoldo, M. Sippell, and R. S. V. Wong. 1989. Microspore mutagenesis and selection: canola plants with field resistance to imidazolinone. *Theor. Appl. Genet.* 78:525-530.

Tan, S., R. R. Evans, M. L. Dahmer, B. K. Singh, and D. L. Shaner. 2005. Imidazolinone-resistant crops: history, current status, and future. *Pest Management Science* 61:246-257.

Wright, T. R. and D. Penner. 1998. Cell selection and inheritance of imidazolinone resistance in sugar beet (*Beta vulgaris*). *Theor. Appl. Genet.* 96:612-620.

Tables and Figures

Table 4: Biomass of camelina mutants treated with ALS herbicides 21 days after treatment with the herbicide. Expressed as % of untreated control. Numbers with the same letter designation are not statistically different.

	Imazethapyr		Flucarbazone		Sulfonylurea	
Calena	13.2	FG	15.7	FG	13.6	FG
SM4	53.9	B	32.9	C	27.2	CD
IM1	19.4	EF	24.5	DE	15.3	FG
IM6	18.5	EF	23.6	DE	10.5	G
IM10	18.1	EF	23.7	DE	11.3	G
IM18	16.6	FG	26.2	D	11.3	G

Table 5: Segregation ratios for wild type/resistant F₂ families

F ₂	Number				Chi-sq	Chi-sq
	of Plants	Resistant	Intermediate	Susceptible	1:2:1	3:1
Calena/IM 1	809	149	450	210	19.4351*	0.39596
Cheyenne/IM 6	551	117	279	155	5.3303	2.8802
Cheyenne/IM 18	646	145	354	147	5.9628	1.7358
Calena/SM4	821	173	452	196	9.6796*	0.5558