# GENETICS OF GROUP 14 HERBICIDE TOLERANCE IN LENTIL

A Thesis Submitted to the
College of Graduate and Postdoctoral Studies
In Partial Fulfilment of the Requirements
For the Degree of Doctor of Philosophy
In the Department of Plant Science
University of Saskatchewan
Saskatoon

By

Vladimir Pajic

## PERMISION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

## DISCLAIMER

This thesis was created to meet the thesis requirements for the degree of Doctor of Philosophy at the University of Saskatchewan. Reference in this thesis to any specific commercial products, process, or service by trade name, trademark, manufacturer, or otherwise, does not constitute or imply its endorsement, recommendation, or favoring by the University of Saskatchewan. The views and opinions of the author expressed herein do not state or reflect those of the University of Saskatchewan, and shall not be used for advertising or product endorsement purposes.

Requests for permission to copy or to make other uses of materials in this thesis in whole or part should be addressed to:

Head of the Plant Science Department
College of Agriculture and Bioresources
4D36 Agriculture Building, 51 Campus Drive
University of Saskatchewan
Saskatoon, Saskatchewan S7N 5A8
Canada

OR

Dean
College of Graduate and Postdoctoral Studies
University of Saskatchewan
116 Thorvaldson Building, 110 Science Place
Saskatoon, Saskatchewan S7N 5C9
Canada

## ABSTRACT

Extremely weak competition with weeds and limited herbicide options dictate the need to explore new avenues in weed control for lentil. Sulfentrazone and fluthiacet methyl are two herbicides of group 14 which have not previously been used in lentil production.

Tolerance to these two products was investigated through a set of field trials in combination with electrolyte leakage assays. The result of field trials showed that sulfentrazone has a more significant effect on the yield. The electrolyte leakage assay results confirm existence of variable levels of herbicide tolerance.

A set of 110 diverse lentil genotypes was then used in multi-environment trials to test the association between genetic markers and traits related to damage due to exposure to fluthiacet methyl. Several groups of genes were detected; among the candidate genes were cytochrome P450s, glutathione-S-transferases, some stress-related genes and50 genes involve in plant growth and development. This suggests that a non-target site resistance mechanism in combination with plant regrowth is the main cause for the observed differences in tolerance. A follow-up study on the importance of cytochrome P450s and glutathione-S-transferase demonstrated that cytochrome P450s play an important role in tolerance to both herbicides, while glutathione-S-transferase plays more sporadic role.

As a target site of sulfentrazone and fluthiacet methyl, protoporphyrinogen oxidase genes were sequenced across multiple lentil genotypes. Several single nucleotide polymorphisms (SNPs) were detected to cause amino acid substitution in target enzymes. Based on single marker analysis, one SNP was found to play a role in tolerance to fluthiacet methyl. Finally, mutation breeding was used to generate material with a higher level of tolerance to both herbicides. After initial screening, several mutant lines were selected for further validation. In the case of sulfentrazone no lines had higher levels of tolerance, but for fluthiacet methyl, two mutant lines showed promise.

The potential for use of sulfentrazone and fluthiacet methyl in lentil production exists, but it requires extensive breeding effort to produce genetic material with herbicide resistance. The knowledge collected in this research creates the blueprint for future work and development of herbicide-resistant lentil varieties.

## ACKNOWLEDGEMENTS

I would like to acknowledge the thoughtful guidance and support of my supervisor, Dr. Kirstin Bett. Although road was all but straightforward, you were persistent and always encouraging for which I will be always grateful. I am also very thankful for the contributions and advice offered by the members of my committee, Dr. Albert Vandenberg, Dr. Christian Willenborg, Dr. Curtis Pozniak and Dr. Chris Todd.

I am incredibly grateful to the entire Pulse team of the Crop Development Centre. I would not be able to complete my field and lab work without the support of Brent Barlow, Scott Ife, Thiago Prado, Kevin Mikituk, Jaret Horner, Stacey Wagenhoffer and Devini DeSilva.

I would like to express my deepest appreciation for the Syngenta Graduate Research Award in Pulse Production, the Roderick Alan McLean Memorial Award, the Saskatchewan Ministry of Agriculture and the Saskatchewan Pulse Growers for funding my research and education.

Finally, thank you to my family for their constant support and endless love.

## TABLE OF CONTENTS

PERMISION TO USE ..... i
ABSTRACT ..... ii
ACKNOWLEDGEMENTS ..... iii
LIST OF TABLES ..... viii
LIST OF FIGURES ..... xiii
LIST OF EQUATIONS ..... xviii
LIST OF ABREVIATIONS AND ACRONYMS ..... xix
1. INTRODUCTION ..... 1
2. LITERATURE REVIEW ..... 4
2.1 A History of Lentil (*Lens culinaris*) ..... 4
2.1.1 Lentil Production in Canada ..... 4
2.2 Weed Management Practices in Lentil Production ..... 6
2.3 Herbicides of Group 14 (PPO Inhibitors; Peroxidizing Herbicides) ..... 11
2.3.1 Sulfentrazone ..... 12
2.3.2 Fluthiacet Methyl ..... 14
2.4 Tetrapyrrole Pathway in Plants ..... 15
2.5 Mechanism of Action of Group 14 Herbicides ..... 19
2.6 Understanding Herbicide Tolerance and Developing Herbicide Resistance ..... 20
Prologue to Chapter 3 ..... 26
3.1 Introduction and Objectives ..... 27
3.2. Materials and Methods ..... 28
3.2.1. Plant Material ..... 28
3.2.2. Site Description and Meteorological Data ..... 28
3.2.3 Experimental Design and Herbicide Application ..... 30
3.2.4 Data Collection and Statistical Analysis ..... 30
3.2.5 Electrolyte Leakage Assay (ELA) ..... 31
3.2.6 Dose Response to Fluthiacet Methyl ..... 33
3.3.1 Electrolyte leakage assay ..... 45
3.3.2 Dose response ..... 48
3.4 Discussion ..... 50
Prologue to Chapter 4 ..... 56

4. GENOME WIDE ASSOCIATION MAPPING OF FLUTHIACET METHYL TOLERANCE IN LENTIL GERMPLASM ..... 57
4.1 Introduction ..... 57
4.2 Material and Method ..... 59
4.2.1 Plant Material ..... 59
4.2.2 Genotypic Analyses ..... 62
4.2.3 Phenotypic Analyses ..... 63
4.2.4 Statistical Analyses ..... 64
4.2.5 Association Analysis ..... 64
4.3 Results ..... 65
4.3.1 Linkage Disequilibrium and Population Structure ..... 65
4.3.2 Phenotypic Data ..... 68
4.3.3 Genotype-Phenotype Associations ..... 75
4.4 Discussion ..... 82
Prologue to Chapter 5 ..... 91
5. IMPACT OF CYTOCHROME P450s AND GLUTATHIONE-S-TRANSFERASES ON THE TOLERANCE OF LENTIL TO SULFENTRAZONE AND FLUTHIACET METHYL ..... 92
5.1 Introduction and Objectives ..... 92
5.2 Materials and Methods ..... 93
5.3 Data Analysis ..... 94
5.4 Results ..... 94
5.5 Discussion ..... 98
Prologue to Chapter 6 ..... 100
6. SEQUENCE DIVERSITY OF PROTOPORPHYRINOGEN IX OXIDASE GENES, TARGET SITES OF GROUP 14 HERBICIDES, AMONG LENTIL GENOTYPES ..... 101
6.1 Introduction and Objectives ..... 101
6.2 Materials and Methods ..... 102
6.2.1 Identification of PPOX genes in the lentil genome ..... 102
6.2.2 Plant material, genomic and phenotypic data ..... 102
6.3 Results ..... 103
6.4 Discussion ..... 110
Prologue to Chapter 7 ..... 113

7. INCREASING TOLERANCE OF LENTIL TO SULFENTRAZONE AND FLUTHIACET METHYL THROUGH MUTATION BREEDING ..... 114
7.1 Introduction ..... 114
7.2 Material and Methods ..... 115
7.2.1 Plant Material ..... 115
7.2.2 Initial Field Screening of M3 Lines with High Rates of Sulfentrazone and Fluthiacet Methyl ..... 115
7.2.3 Field Testing of Selected M4 Lines with Sulfentrazone ..... 116
7.2.4 Dose Response of Selected M4 Lines to Fluthiacet Methyl ..... 117
7.2.5 Electrolyte Leakage Assay of Selected Individual Mutant Plants and Selected M4 Lines with Sulfentrazone and Fluthiacet Methyl ..... 117
7.2.6 Statistical Analysis ..... 118
7.3 Results ..... 119
7.3.1 Results of the Initial Screening of M3 Lines with Sulfentrazone and Fluthiacet Methyl ..... 119
7.3.2 Results of Field Testing of Selected M4 Lines with Sulfentrazone ..... 123
7.3.3. Results of Electrolyte Leakage Assay of Individually Selected M4 Plants Treated with Sulfentrazone ..... 124
7.3.4 Results of Electrolyte Leakage Assay Performed on Selected M4 Lines Treated with Fluthiacet Methyl ..... 127
7.3.5 Fluthiacet Methyl Dose Response of Selected M4 Lines ..... 128
7.4 Discussion ..... 130
8. GENERAL DISCUSSION ..... 134
8.1 Future work ..... 138
LITERATURE CITED ..... 140
APPENDIX A: List of lentil accessions used in genome wide association study ..... 162
APPENDIX B: Linkage disqulibrium for each lentil chromosome ..... 165
APPENDIX C: List of significant associations in the control treatment ..... 166
APPENDIX D: Model parameters for electrolyte leakage assay of inhibitors of cytochrome P450 and glutathione-S-transferase ..... 171
APPENDIX E: Polymorphisms found on chloroplast and mitochondria PPOX genes among lentil genotypes ..... 172
APPENDIX F: Indoor pre-screening of mutagenized lines with fluthiacet methyl ..... 174
APPENDIX G: Field screening of selected mutagenized lines with sulfentrazone ..... 175

APPENDIX H: Correlation between NDVI values and dry biomass ..... 177
APPENDIX I: File containg SNP data used in genome wide association study ..... 178
APPENDIX J: Single nucleotide polymorphisms located on the chloroplast protoporphyrinogen oxidase gene ..... 179
APPENDIX K: Single nucleotide polymorphisms located on the mitochondria protoporphyrinogen oxidase gene ..... 180

## LIST OF TABLES

Table 2.1 Herbicides registered for lentil production use in Western Canada……..……10

Table 3.1 Lentil varieties used in testing levels of tolerance to sulfentrazone and fluthiacet methyl both under field conditions (Saskatoon and Scott locations in 2011, 2012 and 2016) and used for electrolyte leakage assay……28

Table 3.2 Soil characteristics at Saskatoon (Kernen Crop Research Farm) and Scott (Agriculture and Agri-Food Canada Scott Research Farm) locations…….29

Table 3.3 Average monthly temperature and precipitation at Saskatoon and Scott locations in 2011, 2012 and 2016 during the April to September periods……29

Table 3.4 F-values from analysis of variance for the effect of genotypes and herbicide treatment on injury ratings at Saskatoon location in 2011, 2012 and 2016 for seven selected lentil varieties……..…….....34

Table 3.5 F-values from analysis of variance for the effect of genotypes and herbicide treatment on injury ratings at Scott location in 2011, 2012 and 2016 for seven selected lentil varieties……..…… 35

Table 3.6 F-values (with significance level) from the analysis of variance of the effects of herbicide and genotype on yield at Saskatoon and Scott in 2011, 2012 and 2016 for seven lentil varieties…40

Table 3.7. $T_{50}$ value and standard error (SE) of electrolyte leakage assay for each of seven lentil varieties (CDC Impala, CDC Rosetown, CDC Maxim, CDC KR-1, CDC Improve, CDC Sedley and CDC Redberry) treated with 150 µM of sulfentrazone. T-value and significance level of paired t-tests between all tested varieties treated with 150 µM of sulfentrazone……..46

Table 3.8 $T_{50}$ value and standard error (SE) of electrolyte leakage assay for each of seven lentil varieties (CDC Impala, CDC Rosetown, CDC Maxim, CDC KR-1, CDC Improve, CDC Sedley and CDC Redberry) treated with 25 µM of fluthiacet methyl. T-value and significance level of paired t-tests between all tested varieties treated with 25 µM fluthiacet methyl.....48

Table 3.9 Parameters and standard errors (SE) from a three-parameter log-logistic model of dry weight of CDC Impala and CDC Improve treated with 12 different doses (0-256 g.a.i $ha^{-1}$) of

fluthiacet methyl. B- slope; D- upper limit; E- dose resulting in 50% response i.e. $ED_{50}$……..…….…….……49

Table 4.1 Meteorological data (average temperature and precipitation) during growing seasons of 2015 and 2016 and historical averages for the period of 1980-2010 at Elrose and Saskatoon locations……...……..61

Table 4.2 Length (Mbp) of lentil chromosomes in the v1.2 assembly (obtained from KnowPulse website), number of SNPs per individual chromosome and estimated linkage disequilibrium for each chromosome based on set of 6,779 SNPs used in this study……...…….65

Table 4.3 F-values from the analysis of variance of the lentil injury ratings resulting from application of 16 g.a.i $ha^{-1}$ fluthiacet methyl at 4-6 leaf stage. Ratings were collected 3 and 7 days after treatment (DAT) at the Saskatoon and Elrose locations in 2015 and 2016.……. 68

Table 4.4 F-values from the analysis of variance of NDVI data collected 7 days after treatment (DAT), 14 DAT, 21 DAT, and 28 DAT, for both control and 16 g.a.i $ha^{-1}$ fluthiacet methyl treatment in 2015 and 2016 at Saskatoon and Elrose ……..…70

Table 4.5 F-values from the analysis of variance (ANOVA) of dry biomass resulting from control and fluthiacet methyl treatments in a controlled environment collected 14 days after treatment……73

Table 4.6 Intraclass coefficient calculated for injury 3 and 7 days after treatment (DAT); NDVI 7, 14, 21 and 28 days after treatment (DAT) and dry biomass following a fluthiacet methyl (16 g.a.i $ha^{-1}$) treatment or a control (untreated) at Saskatoon and Elrose in 2015 and 2016 or under controlled environment conditions…….75

Table 4.7 Name and position of single-nucleotide polymorphism markers found to be associated with injury rating data of fluthiacet methyl (16 g.a.i $ha^{-1}$) treatment collected at 3 DAT and 7 DAT at Saskatoon and Elrose in 2015 and 2016), marker information include significance level (p value) and $R^2$ of MLM model; and name with position of candidate gene identified in proximity of marker……...….77

Table 4.8 Name and position in lentil genome of single-nucleotide polymorphism markers found to be associated with NDVI data of fluthiacet methyl (16 g.a.i $ha^{-1}$) treatment collected 7 DAT at

Saskatoon and Elrose in 2015 and 2016; marker information include significance level (p value) and $R^2$ of MLM model; and name with positon of candidate gene identified in proximity of marker……...…….79
Table 4.9 Name and position in lentil genome of single-nucleotide polymorphism markers found to be associated with NDVI data of fluthiacet methyl (16 g.a.i $ha^{-1}$) treatment collected 14 DAT at Saskatoon and Elrose in 2015 and 2016; marker information include significance level (p value) and $R^2$ of MLM model; and name with positon of candidate gene identified in proximity of marker……...…..79
Table 4.10 Name and positon in lentil genome of single-nucleotide polymorphism markers found to be associated with NDVI data of fluthiacet methyl (16 g.a.i $ha^{-1}$) treatment collected 21 DAT at Saskatoon and Elrose in 2015 and 2016; marker information include significance level (p value) and $R^2$ of MLM model; and name with positon of candidate gene identified in proximity of marker……...….80
Table 4.11 Name and positon in lentil genome of single-nucleotide polymorphism markers found to be associated with NDVI data of fluthiacet methyl (16 g.a.i $ha^{-1}$) treatment collected 28 DAT at Saskatoon and Elrose in 2015 and 2016; marker information include significance level (p value) and $R^2$ of MLM model; and name with positon of candidate gene identified in proximity of marker……81
Table 4.12 Name and position in lentil genome of single-nucleotide polymorphism markers found to be associated with dry biomass of fluthiacet methyl (16 g.a.i $ha^{-1}$); experiment conducted in controlled environment (repeated two times); marker information include significance level (p value) and $R^2$ of MLM model; and name with positon of candidate gene identified in proximity of marker……....81
Table 5.1 $T_{50}$ values (in hours) of ELA with standard error (SE) for sulfentrazone and fluthiacet methyl treatment with and without inhibitors of cytochrome P450s (1-aminobenzotriazole) and glutathione-S-transferases (tridiphane or ellagic acid) among four lentil varieties.....…...95
Table 5.2 T-values stemming from the comparison of $T_{50}$ values through the t-test of herbicide treatments without inhibitors (sulfentrazone 200 µM and fluthiacet methyl 25 µM) and herbicide treatments with inhibitors of cytochrome P450s (1-aminobenzotriazole) and glutathione-S-transferases (tridiphane or ellagic acid)…….…… 97

Table 6.1 Single-marker analysis (ANOVA) of Lcu_Ch_PPOX1 allele state association with NDVI data for three herbicide treatments (Control (unsprayed), Sulfentrazone (280 g.a.i $ha^{-1}$), Fluthiacet methyl (16 g.a.i $ha^{-1}$)) collected at Saskatoon and Elrose locations in 2016 over four weekly time points post herbicide application.....….105

Table 6.2 Single-marker analysis (ANOVA) of Lcu_Ch_PPOX2 allele state association with NDVI data for three herbicide treatments (Control (unsprayed), Sulfentrazone (280 g.a.i $ha^{-1}$), Fluthiacet methyl (16 g.a.i $ha^{-1}$)) collected at Saskatoon and Elrose locations in 2016 over four weekly time points post herbicide application……..…106

Table 6.3 Single-marker analysis (ANOVA) of Lcu_Mh_PPOX1 allele state association with NDVI data for three herbicide treatments (Control (unsprayed), Sulfentrazone (280 g.a.i $ha^{-1}$), Fluthiacet methyl (16 g.a.i $ha^{-1}$)) collected at Saskatoon and Elrose locations in 2016 over four weekly time points post herbicide application …….....106

Table 6.4 Single-marker analysis (ANOVA) of Lcu_Mh_PPOX2 allele state association with NDVI data for three herbicide treatments (Control (unsprayed), Sulfentrazone (280 g.a.i $ha^{-1}$), Fluthiacet methyl (16 g.a.i $ha^{-1}$)) collected at Saskatoon and Elrose locations in 2016 over four weekly time points post herbicide application……..……107

Table 7.1 F-values from the analysis of variance (ANOVA) of dry biomass and NDVI data across 22 selected mutagenized lines and CDC Redberry tested with 280 g.a.i $ha^{-1}$ sulfentrazone in the field at Saskatoon in 2015……...…..123

Table 7.2 $T_{50}$ values of electrolyte leakage assay and t values with significance of paired t-tests between $T_{50}$ values of CDC Redberry and ten selected mutants treated with 150 µM of sulfentrazone …….……..123

Table 7.3 $T_{50}$ values of electrolyte leakage assay and standard error for selected mutagenized lines (Lot2-11, Lot2-418, Lot2-457, Lot2-498) and CDC Redberry, and t values with significance of paired t-tests between tested genotypes treated with electrolyte leakage assay at 25 µM concentration of fluthiacet methyl ……..…….126

Table 7.4 Effective dose 50 ($ED_{50}$) calculated for selected mutagenized lines (Lot2-11; Lot2-418; Lot2-457; Lot2-498) and CDC Redberry, and multiple comparison of the $ED_{50}$ between examined material……..…..129

Table A.1. List of lentil accessions used in association mapping with country of origin and their STRUCTURE sub-group assignment……..162

Table C.1. Name and position in lentil genome of single-nucleotide polymorphism markers found to be associated with NDVI data of control (unsprayed) treatment collected at 7 DAT; 14 DAT; 21 DAT and 28 DAT at Saskatoon and Elrose in 2015 and 2016……..……166

Table C.2. Name and position in lentil genome of single-nucleotide polymorphism markers found to be associated with dry biomass of control (unsprayed) treatment…….170

Table D.1. Model parameters of Weibull 2 model with standard error (SE) for all treatments..…171

Table E.1. List of 86 diverse lentil genotypes with allele calls of two SNPs (Lcu_Ch_PPOX1 and Lcu_Ch_PPOX2) located on chloroplast PPOX gene and two SNPs (Lcu_Mh_PPOX1 and Lcu_Mh_PPOX2) located on mitochondria PPOX gene……..172

Table G.1. Mean of dry biomass and standard deviation (SD) with Tukey's (HSD) grouping for 22 selected M4 lentil lines and CDC Redberry (check) for control (unsprayed) and sulfentrazone (280 g.a.i $ha^{-1}$) treatment at Saskatoon in 2015……..…175

Table G.2. Mean of NDVI values and standard deviation (SD) with Tukey's (HSD) grouping for 22 selected M4 lentil lines and CDC Redberry (check) for control (unsprayed) and sulfentrazone (280 g.a.i $ha^{-1}$) treatment at Saskatoon in 2015…….176

## LIST OF FIGURES

Figure 2.1. Chemical structure of sulfentrazone……....13

Figure 2.2. Chemical structure of fluthiacet-methyl……..…….15

Figure 2.3. Enzymatic transformation of protoporphyrinogen IX into protoporphyrin IX mediated by PPOX…….16

Figure 2.4. Overview of the tetrapyrrole pathway…….17

Figure 2.5. Example of feedback loops that down-regulate 5-aminolaevulinic acid (ALA) production……..…18

Figure 2.6. Localization of PPO inhibitors' effects in the cell…….…...19

Figure 3.1. Injury ratings (scale: 0- white color to 100-dark purple) of seven lentil varieties subjected to five herbicide treatments at Saskatoon in 2011. A- 21 days after seeding (DAS); B- 41 DAS; C- 49 days DAS; D- 66 DAS..…….…….36

Figure 3.2. Injury ratings (scale: 0- white color to 100-dark purple) of seven lentil varieties and five herbicide treatments at Saskatoon location in 2012 collected: A- 30 days after seeding (DAS); B- 43 DAS; C- 55 days DAS…..…...37

Figure 3.3. Injury ratings (scale: 0- white color to 100-dark purple) of seven lentil varieties and five herbicide treatments at Saskatoon location in 2016 collected: A- 29 days after seeding (DAS); B- 102 DAS.…….…….…….38

Figure 3.4. Injury ratings (scale: 0- white color to 100-dark purple) of seven lentil varieties and five herbicide treatments at Scott location in 2011 collected: A- 35 days after seeding (DAS); B- 49 DAS; C- 54 days DAS…….……..38

Figure 3.5. Injury ratings (scale: 0- white color to 100-dark purple) of seven lentil varieties and five herbicide treatments at Scott location in 2012 collected: A- 30 days after seeding (DAS); B- 37 DAS……...39

Figure 3.6. Injury ratings (scale: 0- white color to 100-dark purple) of seven lentil varieties and five herbicide treatments at Scott location in 2016 collected: A- 35 days after seeding (DAS); B- 47 DAS; C- 72 days DAS…….....39

Figure 3.7. Yield (g/m$^2$) of seven lentil varieties (CDC Impala; CDC Improve; CDC KR-1; CDC Maxim; CDC Redberry; CDC Rosetown; CDC Sedley) grown at Saskatoon in 2011, and treated with fluthiacet methyl at 4 and 8 g.a.i ha$^{-1}$, sulfentrazone at 140 and 280 g.a.i ha$^{-1}$ and unsprayed control……41

Figure 3.8. Yield (g/m$^2$) of seven lentil varieties (CDC Impala; CDC Improve; CDC KR-1; CDC Maxim; CDC Redberry; CDC Rosetown; CDC Sedley) grown at Saskatoon in 2012, and treated with fluthiacet methyl at 4 and 8 g.a.i ha$^{-1}$, sulfentrazone at 140 and 280 g.a.i ha$^{-1}$ and unsprayed control……...….42

Figure 3.9. Yield (g/m$^2$) of seven lentil varieties (CDC Impala; CDC Improve; CDC KR-1; CDC Maxim; CDC Redberry; CDC Rosetown; CDC Sedley) grown at Saskatoon in 2016, and treated with fluthiacet methyl at 4 and 8 g.a.i ha$^{-1}$, sulfentrazone at 140 and 280 g.a.i ha$^{-1}$ and unsprayed control……..……..43

Figure 3.10. Yield (g/m$^2$) of seven lentil varieties (CDC Impala; CDC Improve; CDC KR-1; CDC Maxim; CDC Redberry; CDC Rosetown; CDC Sedley) grown at Scott in 2011, and treated with fluthiacet methyl at 4 and 8 g.a.i ha$^{-1}$, sulfentrazone at 140 and 280 g.a.i ha$^{-1}$ and unsprayed control……...….44

Figure 3.11 Yield (g/m$^2$) of seven lentil varieties (CDC Impala; CDC Improve; CDC KR-1; CDC Maxim; CDC Redberry; CDC Rosetown; CDC Sedley) grown at Scott in 2016, and treated with fluthiacet methyl at 4 and 8 g.a.i ha$^{-1}$, sulfentrazone at 140 and 280 g.a.i ha$^{-1}$ and unsprayed control……45

Figure 3.12. Percent electrolyte leakage of tissue samples of seven lentil varieties (CDC Sedley, CDC Redberry, CDC Rosetown, CDC Impala, CDC Improve, CDC KR-1 and CDC Maxim) for 96h post exposure to 150 µM sulfentrazone……...……..…..46

Figure 3.13. Percent electrolyte leakage of tissue samples of seven lentil varieties (CDC Sedley, CDC Redberry, CDC Rosetown, CDC Impala, CDC Improve, CDC KR-1 and CDC Maxim) for 96h post exposure to 25 µM fluthiacet methyl……47

Figure 3.14 Responses of CDC Impala (black) and CDC Improve (red) to application of different rates of fluthiacet methyl (ranging from 0 to 256 g.a.i $ha^{-1}$) expressed as above-ground dry mass collected 14 days after treatment……49

Figure 4.1. Relationship among 110 lentil accessions selected to represent global diversity of lentil germplasm (STRUCTURE plot and Neighbor-joining tree)……..67

Figure 4.2. Distributions of injury ratings (on a scale of 1-no injury to 10-plant death) following fluthiacet methyl (16 g.a.i $ha^{-1}$) treatment across 110 lentil accessions, in 2015 and 2016 at Saskatoon and Elrose location A) 3 days and B) 7 days after treatment…….69

Figure 4.3 Distribution of NDVI values for control treatment (blue, left side) and fluthiacet methyl (16 g.a.i $ha^{-1}$) treatment (red, right side) at Elrose, A) in 2015 and B) in 2016, collected 7 days after treatment (DAT), 14 DAT, 21 DAT and 28 DAT……..71

Figure 4.4 Distribution of NDVI values for control treatment (blue, left side) and fluthiacet methyl (16 g.a.i $ha^{-1}$) treatment (red, right side) at Saskatoon, A) in 2015 and B) in 2016, collected 7 days after treatment (7 DAT), 14 DAT, 21 DAT and 28 DAT…….……..72

Figure 4.5. Distribution of dry biomass (mg) across 110 lentil accessions in the first run of the experiment and in the repetition under the same conditions……..74

Figure 5.1. Non-linear curves representing ELA results for SF200 (sulfentrazone 200µM), FM25 (fluthiacet methyl 25µM), P450SF200 (sulfentrazone 200µM + inhibitor cytochrome P450), P450FM25 (fluthiacet methyl 25µM + inhibitor cytochrome P450), GSHSF200 (sulfentrazone 200µM + inhibitors glutathione-S-transferase), and GSHFM25 (fluthiacet methyl 25µM + inhibitors glutathione-S-transferase) treatments, A) CDC Redberry, B) CDC Improve, C) CDC Impala, D) CDC Rosetown…….96

Figure 6.1 The structure of lentil *Lcu_Ch_PPOX* (Lc04510, lentil genome v 1.2) with exon length given in base pairs (bp)…….103

Figure 6.2 The structure of lentil *Lcu_Mh_PPOX* (Lc25711, lentil genome v 1.2) with exon length given in base pairs (bp)…….…...104

Figure 6.3 Alignment of the amino acid sequences of chloroplast PPOX enzymes among *L. culinaris* (CDC Redberry); *L. ervoides*; *L. lamottei* and *L. odemensis*…….108

Figure 6.4 Alignment of the amino acid sequences of mitochondria PPOX enzymes among *L. culinaris* (CDC Redberry); *L. ervoides*; *L. lamottei* and *L. odemensis*…….109

Figure 7.1. Results of initial field screening in 2014 where mutagenized lentil lines and set of five checks were exposed to 560 g.a.i $ha^{-1}$ sulfentrazone. (A) Representative subset of plots, picture taken seven weeks after seeding. (B) Distribution of the number of germinated plants per plot for mutagenized line two weeks after seeding (seeding rate was 100 seeds per microplot). (C) Number of germinated plants per plot for each check, collected two weeks after seeding…….120

Figure 7.2. Examples of selected individual mutants from the 2014 sulfentrazone (560 g.a.i $ha^{-1}$) field screening at Saskatoon (SPG) location. (A) Selected plant MLA282. (B) Selected plant MLA382. (C) Selected plant MLA591. (D) Selected plant MLA707……..….121

Figure 7.3. Results of 2014 field trial where mutagenized lentil lines and set of five checks were exposed to 32 g.a.i $ha^{-1}$ of fluthiacet methyl. (A) Representative subset of plots, picture taken seven weeks after seeding. (B) Distribution of injury ratings across all microplots three days after fluthiacet methyl spraying. (C) Distribution of injury ratings across five checks three days after fluthiacet methyl spraying……...…….122

Figure 7.4. Response curves of electrolyte leakage assays fitted with three-parameter Weibull type 2 model for 10 selected mutant plants and CDC Redberry, all treated with 150 µM of sulfentrazone……125

Figure 7.5. Response curves of electrolyte leakage assay for four mutagenized lines (Lot2-11; Lot2-418; Lot2-457; Lot2-498) and CDC Redberry, treated with 25 µM of fluthiacet methyl…127

Figure 7.6. Response of selected mutagenized lines (Lot2-11; Lot2-418; Lot2-457; Lot2-498) and the CDC Redberry check to the application of fluthiacet methyl, expressed with dry biomass….129

Figure B.1. Plots of LD decay based on set of 6,779 markers given by chromosomes, A) chromosome 1; B) chromosome 2; C) chromosome 3; D) chromosome 4; E) chromosome 5; F) chromosome 6; G) chromosome 7……...…….165

Figure F.1. Selected M4 lentil lines and two checks (CDC Improve and CDC Rosetown) tested in controlled environment with 8 g.a.i ha-1 fluthiacet methyl, above ground dry biomass presented as percentage of control……174

Figure H.1. Correlation between dry biomass and NDVI of 22 selected M4 lines and CDC Redberry, dry biomass and NDVI data was collected 21 days after seeding at Saskatoon during 2015…….177

## LIST OF EQUATIONS

Equation 3.1 Electro-conductivity formula……..……..32

Equation 3.2 Four-parameter log-logistic model……..……..32

Equation 3.3.Three-parameter log-logistic model……..33

Equaiton 4.1 Interclass Correlation Coefficient…….…….64

Equaiton 5.1 Three-parameter Weibull Type 2 Model……...……94

## LIST OF ABREVIATIONS AND ACRONYMS

| | |
|---|---|
| **AHAS** | Acetohydroxyyacid Synthase |
| **ALA** | 5-aminolaevulinic acid |
| **ANOVA** | Analysis of Variance |
| **CDC** | Crop Development Centre |
| **CDS** | Coding DNA Sequence |
| **DAT** | Days after Treatment |
| **DPE** | Diphenyl Esters |
| **ELA** | Electrolyte Leakage Assay |
| **EMS** | Ethyl Methanesulfonate |
| **FAO** | Food and Agriculture Organization of the United Nations |
| **GluTR** | Glutamyl-tRNA Reductase |
| **$I_{50}$** | Half maximal inhibitory concentration |
| **ICC** | Intraclass Coefficient |
| **LD** | Linkage Disequilibrium |
| **NDVI** | Normalized Difference Vegetation Index |
| **NTSR** | Non-target Site Resistance |
| **PPO Inhibitors** | Protoporphyrinogen Oxidase Inhibitors |
| **PPOX** | Protoporphyrinogen IX oxidase |
| **ROS** | Reactive Oxygen Species |
| **SNP** | Single Nucleotide Polymorphism |
| **TSR** | Target Site Resistance |
| **WSSA** | Weed Science Society of America |

# 1. INTRODUCTION

Malnutrition and obesity are two terms that are increasingly used when describing the modern world. Promoting and increasing the production of healthy and nourishing foods which are widely available at low cost should be the goal of modern agriculture. Lentil has been a part of the human diet since the Paleozoic era, as it is an excellent source of protein (20–30 g per 100g serving) and carbohydrates (40-60 g per 100g serving) along with dietary fiber and a range of micronutrients (Siva et al., 2017). Lentil is a cool season grain legume that is grown worldwide, but it is best adapted to the cooler temperate zones of the world, or the winter season in Mediterranean climates (Khazaei et al., 2016). Among pulse crops, lentil ranks sixth in global production behind common bean, pea, chickpea, faba bean and cowpea. World production of lentil in 2016 was estimated 6.32 Mt with Canada being top producer (FAO, 2019). Over the last ten years, the share of Canadian lentil production has gradually increased from 22% to 51% of global lentil production (FAO, 2019).

Among cultivated crops in western Canada, lentil is considered to be among the least competitive (Blackshaw et al., 2002). The reason for such poor competitiveness can be found in the architecture of the plant (relatively short stature), its slower rate of development, and relatively slow rate of canopy closure (Fedoruk et al., 2011). Estimates made by Swanton and his colleagues suggest that in Saskatchewan, for the period of 1985-1989, lentil yield loss due to weeds was 14%. Overall 84% of the total yield loss that occurred in field production of the Western Canada was estimated to be due to weeds (Swanton et al., 1993).

Overdependence of current lentil production on group 2 herbicides presents a bottleneck for maintaining and increasing lentil production in Canada. The number of weeds resistant to group 2 herbicides in western Canada, and globally, has been growing exponentially, reaching 160 different weed species in 2018 (Heap, 2018). Finding alternatives to this class of herbicides is critical for the future of lentil production in Saskatchewan. The group 14 herbicides (PPO inhibitors) is an interesting alternative; even after almost 40 years of commercial usage, the number of the resistant weeds is still relatively low - just 13 weed species in 2018 (Heap, 2018). In

experimental trials, group 14 herbicides have shown very good control of the most problematic weeds in Saskatchewan, including kochia (*Kochia scoparia*), wild buckwheat (*Polygonum convolvulus*) and redroot pigweed (*Amaranthus retroflexus*).

A number of group 14 of herbicide are registered for many legume species, but the mode or source of tolerance to some products is still unknown. For lentil production, a limited number of herbicides are registered and group 14 herbicides are sporadically used in lentil production. Sulfentrazone and fluthiacet methyl are two active ingredients classified as group 14; neither are currently used in lentil production. Sulfentrazone causes serious injuries to lentil and has a re-cropping restriction of 24 months. Fluthiacet methyl is an unfamiliar product in western Canada, but it is regularly used in soybean production in the USA.

Herbicide resistance can be the result of simple target site alteration or could be a complex process involving multiple metabolic pathways. Target site resistance commonly occurs in nature where, due to spontaneous alteration of the genetic code by which a target site change occurs which then influences the interaction between the herbicide and the target molecule, neutralizing its effect. This mechanism is widely studied among weed species, but it is also employed in developing crops with herbicide resistance. On the other hand, metabolic or non-targeted resistance, is complex and not generally researched in detail. Non-targeted resistance could have multiple sources and often they interact to produce a cascade of events that enables a plant to nullify or mitigate the effect of herbicide (Yuan et al., 2007).

This thesis describes study of the effects of sulfentrazone and fluthiacet methyl on lentil plants, examines the possible sources for differential responses among lentil genotypes and sets the ground for increasing resistance to these herbicides through mutation breeding.

Three hypotheses were established:

- Differential responses to the application of sulfentrazone and fluthiacet methyl exist within lentil germplasm under both field conditions and in a controlled environment.
- Focusing on both target site and non-target site resistance, genetic sources of natural variation to the effects of sulfentrazone and fluthiacet methyl can be identified.
- By selecting within a mutagenized lentil population under high rates of sulfentrazone or fluthiacet methyl it is possible to single out genotypes with herbicide resistance.

The research objectives were to:

- Investigate effects of different rates of sulfentrazone and fluthiacet methyl in multi environment field settings and test the same set of genotypes using an electrolyte leakage assay to assess levels of tolerance among selected genotypes.
- Characterise and estimate level of genetic diversity of protoporphyrinogen oxidase genes among *Lens* species and among genotypes of cultivated lentils.
- Use an association mapping approach to identify genetic regions responsible for controlling the level of tolerance to fluthiacet methyl in a diverse set of lentil genotypes.
- Test the role of cytochrome-P450 and glutathione-S-transferase in herbicide tolerance using their inhibitors in an electrolyte leakage assay.
- Select and validate mutagenized lentil lines for tolerance to sulfentrazone and fluthiacet methyl under field conditions.

# 2. LITERATURE REVIEW

## 2.1 A History of Lentil (*Lens culinaris*)

*Lens culinaris* (Medikus) is one of the earliest domesticated grain legumes in the world. Archaeological records date lentil domestication to 8500-7500 BC (Alo et al., 2011), although some researchers suggest that humans were using lentil as food as early as 11000 BC (Sandhu & Singh, 2007). Along with emmer wheat, einkorn wheat, barley, pea, flax, and to a lesser extent chickpea, lentil is considered a "founder crop" of prehistoric agriculture. These crops were the backbone of the first civilizations in the Mediterranean Basin, the Nile Valley, temperate Europe and South-west Asia (Zohary, 1999). Today's southern Turkey and northern Syria are the most likely locale of modern lentil domestication (Cubero et al., 2009). Once domesticated, lentil spread west across ancient Greece and along the Danube river, south along the Nile Delta, and east towards India (Cubero et al., 2009). Today lentil is grown across the globe, with the largest areas of production now in North America, South Asia and Australia.

### 2.1.1 Lentil Production in Canada

In Canada, crop cultivation of lentil started in 1969. In the decade that followed, the area under lentil production was modest, with fewer than 500 ha (Morrall, 1997). By the end of the 1970s the crop had started to gain a footing in Western Canada due to the efforts of the Crop Development Centre (CDC) at the University of Saskatchewan. The year 1978 marked the release of the first Canadian variety of lentil—Laird; a large green type developed through selection of lentil lines originating in the US Pacific Northwest (Slinkard & Bhatty, 1979). At this time lentil covered about 9,000 ha in Western Canada but only two years later, in 1980, the area under lentil swelled to 44,000 ha (Slinkard & Bhatty, 1979; Slinkard, 1981). Since Laird, the CDC has released over 79 lentil varieties across 7 different market classes (Groenewegen & Thompson, 2016). Areas seeded with lentil expanded dramatically, reaching the historical maximum of 2.37 million ha in 2016. In Saskatchewan, lentil is discussed in the same breath as traditional major crops. According

to the Western Producer: "[Saskatchewan]'s lentil exports were valued at $2.5 billion [in 2015] and it is same or slightly larger than the worth of canola seed sales and wheat shipments" (Pratt, 2016). In 2017 seeded area retreated somewhat to 1.78 million ha (Statistics Canada, 2018).

### 2.1.2 Lentil Genepool and Classification

Lentil belongs to the genus *Lens* and the tribe *Vicieae*, which also includes the genera *Pisum, Vicia* and *Lathyrus* (Davies et al., 2007). Taxonomy of the *Lens* genus has changed over the years from the assumption that it consisted of four different species to the view that it comprised of two, then back to four, then to six, and finally to the most recent consensus of seven species (Ladizinsky, 1979; Cubero et al., 2009; Wong et al., 2015). Conflicting results of historical taxonomy studies stem from the evolutionary process in the *Lens* genus. All *Lens* species share common structural and biochemical features, but selection of particular molecular markers or accessions (genotypes) can significantly influence a phylogenetic study (Cubero et al., 2009). The current categorization into seven species is based on molecular markers: *L. culinaris*, *L. orientalis*, *L. odemensis, L. tomentosus, L. ervoides, L. lamottei, and L. nigricans,* (Wong et al., 2015). The number of accessions of *Lens* in the gene bank collections around the world was estimated to be 58,407 (Tullu et al., 2011). The primary gene pool of cultivated lentil consists of *L. orientalis* and *L. tomentosus*; *L. odemensis* and *L. lamottei* belong to the secondary gene pool; *L. ervoides* comprises the tertiary gene pool, and *L. nigricans* belongs to quaternary gene pool (Wong et al., 2015). Crosses of the cultivated species with members outside the primary genepool often fail because the hybrid embryos abort. It is possible to obtain a viable hybrid through embryo rescue techniques for some combinations (Davies et al., 2007). Lentil and other members of the *Lens* genus all have the same number of chromosomes (2n=14), and their karyotype has three pairs of sub-metacentric chromosomes, three pairs of acrocentrics and one pair of metacentrics with a secondary constriction very close to the centromere (Ladizinsky & Abbo, 1993). Members of the *Lens* genus are self-pollinating and have a low outcrossing rate, varying between 1% and 6% (Erskine & Muehlbauer, 1991; Skibinski et al, 1984).

World-renowned botanist Helena Barulina (1930) classified two types of *Lens culinaris*: *macrosperma* (large-seeded) with 6-9 mm seed diameter, and *microsperma* (small-seeded) with 2-6 mm seed diameter.

## 2.2 Weed Management Practices in Lentil Production

Weed management practices include any procedure that reduces the negative impact of weeds on crop growth and yield. These practices enable the crop to use all available resources in the field leading to maximal yield potential (Hager, 2009). Lentil competes poorly with weeds due to its short stature, relatively shallow root system, and open growth habit which permits emergence and establishment of many weed species (Smitchger et al., 2012; Yenish et al., 2009). Compared to other crops grown in Western Canada, lentil is the least weed-competitive (Swanton, Harker & Anderson, 1993). As a result, lentil requires special attention in weed management practices. The critical period for weed control in lentil in Western Canada starts at the 5-node stage and ends at the 10-node stage, which generally coincides with canopy closure (Fedoruk et al., 2011).

Weed management approaches in lentil production can be categorized into cultural, preventive, mechanical, biological and chemical (Yenish et al., 2009). Cultural weed management involves techniques commonly used for good crop management, such as crop rotation, seeding density, varietal selection, fertilization, etc. (Yenish et al., 2009). Crop rotation is the process of growing different crops in a systematic and recurring sequence on the same land and it is one of the most common types of cultural weed management (Liebman & Dyck, 1993). Crop rotation has a strong effect on the composition of the weed seed bank and can thereby reduce populations of noxious weed species (Ball, 1992). Seeding density of lentil can play a significant role in reducing weed biomass in the field. Increasing seed density from 130 plants per $m^2$ to 229 plants per $m^2$ leads to 26% reduction in biomass of natural weed population in the field (Baird et al., 2009). Proper use of fertilization is also considered a cultural weed management approach, as heavy application of fertilizers leads to increased weed pressure. Cultivar selection can also play a role in weed management. Although lentil generally competes poorly with weeds, cultivars vary in growth habit and morphology, and show different levels of competition with weeds (Yenish et al., 2009). Many of these techniques are applied in current lentil production systems, but some are less attractive to producers because they can increase the cost of production.

Preventive weed management is the most basic weed management method (Walker, 1995) and it encompasses a number of different techniques. Using weed-free seed material is essential. While commercial seed tends to be pre-cleaned, producers' use of seed from non-commercial sources

can lead to higher weed occurrence in fields. Regular cleaning and maintenance of farm equipment prevents it from becoming a source (or a means) of weed dispersal (Yenish et al., 2009). Irrigation water is also a potential source of weed seed distribution in fields. If allowed to naturally disperse, patches of weeds tend to move across an area, making control of encroaching populations of weeds an important preventive weed management technique (Yenish et al., 2009). Most lentil producers use some preventive weed management techniques, since they are part of good general farming practices.

Biological weed management refers to the use of living organisms, or their products, to diminish weed growth and reproduction (Cardina, 1995). Different animals can be used to control weeds: from grazing animals, such as cattle, sheep, and goats, to insects that feed on weed plants. Literature abounds with examples of insects used for successful biological control of weeds, like the application of *Dactylopius opuntiae* to control the prickly pear, and *Chrysolina quadrigemina* to control of St. John's wort (Kalamath weed) (Zimdahl, 2007). There are numerous successful strategies that use insects in weed control. For example, enhancement strategies involve increasing damage to weed plants by changing the number of insects in the existing insect population, and introducing insects to weed-abundant areas where they can cause significant damage to the weed population (Cardina, 1995). Similar strategies can be used with plant pathogens, like fungi and bacteria. Although crops do compete with weeds and can curb weed development, they are not usually seen as biological control. Those rare instances of crop plant involvement in biological control include cover crops, companion crops and allelopathic crops. In general, biological weed control is perceived as challenging because of the complexity involved, requisite long-term planning, application of multiple strategies, and manipulation of the cropping system (Cardina, 1995). This approach to weed management is not currently used in lentil production, but it is an option that should be further studied and potentially considered in the lentil production model.

Mechanical weed management has a long tradition in agriculture and is still the preferred method in small scale agriculture and agriculture in the developing countries (Zimdahl, 2007). This approach entails physical action focused on disturbing weed emergence and development, such as pulling plants, tilling before and after weed emergence, and mowing (Hager, 2009). The role of mechanical weed control in lentil production varies across the world and production systems. Hand-pulling and hand-hoeing are the most labor-intensive operations in weed control. Tillage,

which involves disturbing, cultivating, or mixing the soil through the use of ploughs, discs or cultivators, is the most widely used mechanical operation (Zimdahl, 2007). Depending on the development stage of the weeds when tillage is performed, it can lead to physical destruction of the plants, suppression of emerging seedlings, or it can cause burying of weed seeds deeper in the soil.

Between row cultivation is another very popular method of mechanical weed management, but one that is not used in lentil production due to possible negative effects on the lentil plants themselves (Yenish et al., 2009). In North America, lentil is usually produced in no-till or reduced till systems which limit the application of mechanical methods. A relatively new approach called harvest weed seed control relies on mechanically damaging and destroying weed seeds during commercial grain harvest, leading to decline of the seed bank (Harrington & Powles, 2012; Walsh, Newman & Powles, 2013). Other approaches, such as flaming, weed blowing, and application of robotized or unmanned vehicles are being actively investigated (Van Der Weide et al., 2008). Although mechanical weed management can be costly and is not applicable in all production systems, it can be combined with other approaches to ensure good weed control in lentil.

Chemical weed management is the most widely applied weed control practice in agriculture and it has revolutionized crop production systems. It uses herbicides for successful control of weeds. A herbicide is a chemical substance or cultured organism used to kill or suppress the growth of plants (Vencill, 2002). Written mentions of chemicals used to hinder plant development date back to the ancient Greeks, but the chemical era in modern agriculture did not start until the early 20th century (Zimdahl, 2007). The discovery of the process for synthesizing 2,4 dichlorophenoxy acetic acid (2,4-D) in the 1940s turned the compound into an example of the opportunities that chemical control of weeds could create for agricultural production (Rao, 2000; Zimdahl, 2007). Its success led to the discovery of many of the key compounds in contemporary chemical weed management.

Several methods for classifying herbicides have been developed by considering chemical families used in the field or by site of action (Ashton & Crafts, 1981; Zimdahl, 2007). The most widely used method is based on the mechanism (site) of action. According to the Weed Science Society of America (WSSA) there are 17 classes and 30 subclasses of herbicides. While WSSA uses numeric designation for subclasses, other organizations use alphanumeric designations, making global classification excessively complex.

Another common herbicide classification is based on the method of application. In this context herbicides are classified as soil-applied or foliage-applied (Rao, 2000). Focusing on the timing of the application leads to classification of herbicides as: pre-planting, pre-emergence and post-emergence. Pre-planting herbicides are applied before the crop is planted, and usually sprayed on soil or incorporated into the soil. Pre-emergence herbicides are applied after the crop is planted but before it emerges; and the post-emergence herbicides application period is self-explanatory (Rao, 2000; Zimdahl, 2007). Systemic herbicides are able to translocate from the site of the application throughout the plant, while contact herbicide activity remains limited to the point of application (Rao, 2000).

Selectivity is a feature of herbicides that is crucial for their commercial success. It means that under a given set of conditions, herbicide application leads to serious damage or death of certain plants (namely weeds), while crops remain uninjured (Ashton & Crafts, 1981). Selectivity is relative rather than absolute, since an herbicide is selective to specific crops only in a particular range of the applied rate of product (Ashton & Crafts, 1981). Factors that influence selectivity include: plant age, plant morphology, absorption, translocation, time and method of application, herbicide formulation, and environmental conditions (Zimdahl, 2007).

Because the use of herbicides is heavily regulated, their availability varies across production regions (Yenish et al., 2009). Factors like registration process, scale of lentil production, weed spectra, and industry interests also influence the range of herbicides available to lentil producers. Extreme variability in viable options makes it is impossible to generalize chemical weed management practices in the context of global lentil production. In Western Canada, and beyond, relatively few herbicides are registered for use in lentil, limiting options for chemical weed control. These options include: ethalfluralin (Edge™) and trifluralin (Rival™, Bonanza™) registered for fall application only; glyphosate, saflufenacil (Heat™), carfentrazone (Aim™), as well as tank mixes of these products with glyphosate, for pre-planting applications; and limited post-emerging options in the form of metribuzin for general use, and some "IMI" or group 2 products for Clearfield™ lentil varieties (see Table 2.1; Government of Saskatchewan, 2017).

Table 2.1 *Herbicides Registered for Lentil Production Use in Western Canada as of 2018*

| Active ingredient | Commercial names | Herbicide group | Weed control | Remark |
|---|---|---|---|---|
| *Clethodim* | Select™; Centurion™; Arrow™; Shadow™ | 1 | Grassy weeds | |
| *Ethalfluralin* | Edge Granular™ | 3 | Broadleaf and Grassy weeds | Only fall application |
| *Metribuzin* | Sencor 75™; TriCor 75™ | 5 | Broadleaf and Grassy weeds | |
| *Quizalofop* | Assure II™; Yuma GL™ | 1 | Grassy weeds | |
| *Imazamox* | Solo™ | 2 | Broadleaf and Grassy weeds | Only Clearfield lentil |
| *Trifluralin* | Rival™; Bonanza™ | 3 | Broadleaf and Grassy weeds | Only fall application |
| *Imazamox Imazethapyr* | Odyssey™ | 2 | Broadleaf and Grassy weeds | Only Clearfield lentil |
| *Imazamox, Imazethapyr, Sethoxydim* | Odyssey Ultra™ | 2 & 1 | Broadleaf and Grassy weeds | Only Clearfield lentil |
| *Sethoxydim* | Poast Ultra™ | 1 | Grassy weeds | |
| *Imazamox Imazapyr* | Ares™ | 2 | Broadleaf and Grassy weeds | Only Clearfield lentil |
| *Glyphosate Carfentrazone* | CleanStart™ | 9 & 14 | Broadleaf and Grassy weeds | Pre-planting application |
| *Glyphosate* | RoundUp™; Matrix™; Maverick III™ | 9 | Broadleaf and Grassy weeds | Pre-planting application |
| *Carfentrazone* | Aim™ | 14 | Broadleaf weeds | Pre-planting application, tank mix with Glyphosate |
| *Saflufenacil* | Heat™ | 14 | Broadleaf weeds | Pre-planting application, tank mix with Glyphosate |
| *MCPA* | MCPA amine™ | 4 | Broadleaf weeds | Pre-planting application, tank mix with Glyphosate |

*Note.* Adapted from http://www.publications.gov.sk.ca/details.cfm?p=77706.

## 2.3 Herbicides of Group 14 (PPO Inhibitors; Peroxidizing Herbicides)

The original herbicides of what is now known as group 14 were p-Nitrodiphenyl ethers (including nitrofen, acifluorfen, and oxyfluorfen). They were first used in the 1960s in Japan for weed control in rice cultivation (Matsunaka, 1999). The exact mode of action for this group of herbicides was unknown for almost two decades following its initial commercial use. These herbicides are also known as peroxidizing herbicides because they cause rapid membrane lipid peroxidization (Dayan & Duke, 1997).

The number of chemicals classified as peroxidizing herbicides has grown over time. The first generation was developed in the 1965-1985 period and included diphenyl esters (DPEs) and cyclic imides, followed by a second generation developed between 1985 and 1995 (Wakabayashi & Böger, 1999). More than 700 different kinds of DPEs were patented between 1980 and 1997 (Hirai, 1999). Oxidazon was the first non-DPE herbicide available for weed control (Dayan & Duke, 1997). Herbicides of group 14 have been categorized into two subclasses: 1) p-nitrodiphenyl ethers, including acifluorfen, fluoroglycocofen ethyl, fomesafen, oxyfluorfen, fluorodifen, nitrofen, chlomethoxyfen, and bifenox, and 2) heterocylic-subsituted benzenes, encompassing triazolinones, carfentrazone ethyl, sulfentrazone, thiadiazolidinones, fluthiacet methyl, tetrazolinones, tetrahydrophthalimides, flumicpropyn, flumiclorrac pentyl, oxadiazoles, oxadiazon, and azafenidin (Aizawa & Brown, 1999).

Development of many compounds initially considered to be promising weed control candidates hit a dead end at the beginning of the 1990s. A limited number of commercial products made it to market, but they never captured large market shares. A major hurdle in product development of both pre-emergent and post-emergent compounds was clear selectivity among compounds with good biological activity (Theodoridis, 2007).

Matsunaka (1999) credits Arai et al. (1966) with being the first to uncover the light requirement phenomena of peroxidizing herbicides. Arai and colleagues soaked barnyard grass seeds in a nitrofen (herbicide of group 14) solution and, following incubation in the dark, placed the seeds into three different light conditions: dark, room light, and direct sunlight. They learned that nitrofen exhibits its highest herbicidal activity in direct sunlight light, lower activity in room light, and no herbicidal activity in the dark (Ariel et al., 1966, as cited in Matsunaka, 1999).

One of the early explanations of the mode of action of group 14 herbicides was the "yellow pigment theory" (Matsunaka, 1969). Matsunaka studied rice albino mutants: artificial, white mutants of rice, tolerant to nitrofen, and a yellow rice mutant, susceptible to nitrofen. Matsunaka concluded that xanthophyll, the dominant pigment in the yellow mutant, plays a crucial role in the photo activation of herbicides group 14. However, this theory was soon dismissed, as both white and albino mutants were shown to exhibit injuries from herbicide under high light intensity (Matsunaka, 1999). While a number of alternative theories emerged, it was not until 1989 that the real target of herbicides of group 14 was discovered. Through a number of comprehensive studies, the target site was determined to be protoporphyrinogen oxidase (Matringe, Camadro, Labbe & Scalla, 1989; Witkowski & Halling, 1989; Duke et al., 1991; Camadro, Matringe, Scalla & Labbe, 1991; Matringe et al., 1992; Lee & Duke, 1994). Investigations into herbicides of group 14 peaked in the early 1990s but declined soon thereafter when glyphosate-resistant crops gained market share (Theodoridis, 2007).

### 2.3.1 Sulfentrazone

Sulfentrazone (2′,4′-dichloro-5′-(4-difluoromethyl-4,5-dihydro-3-methyl-5-oxo-1*H*-1,2,4-triazol-1-yl) methanesulfonanilide) is an aryl triazolinone selective herbicide (Figure 2.1) (Aizawa & Brown, 1999). It is applied either to the soil before emergence, or as a pre-plant incorporated treatment (Nagano 1999; Szmigielski et al., 2012). The half-life of sulfentrazone in soil depends on the type of soil and environmental conditions, and it varies greatly, from just a few weeks to almost a year. The main source of degradation of sulfentrazone in soil is microbial activity, primarily through degradation of the metabolite 3-hydroxymethyl sulfentrazone, which further oxidizes to sulfentrazone-3-carboxylic acid (Aizawa & Brown, 1999). Sulfentrazone in soil is resistant to hydrolysis and is not susceptible to photo degradation after soil application (Aizawa & Brown, 1999). In soil it is moderately mobile, but its mobility is highly dependent on the soil type and its pH level (Grey et al., 1997). Both soil pH and organic matter are limiting factors in the application of sulfentrazone, and according to recommendations, soils with organic matter higher than 6% or with a pH of 7.8 or greater are not suitable for sulfentrazone application (Government of Saskatchewan, 2017).

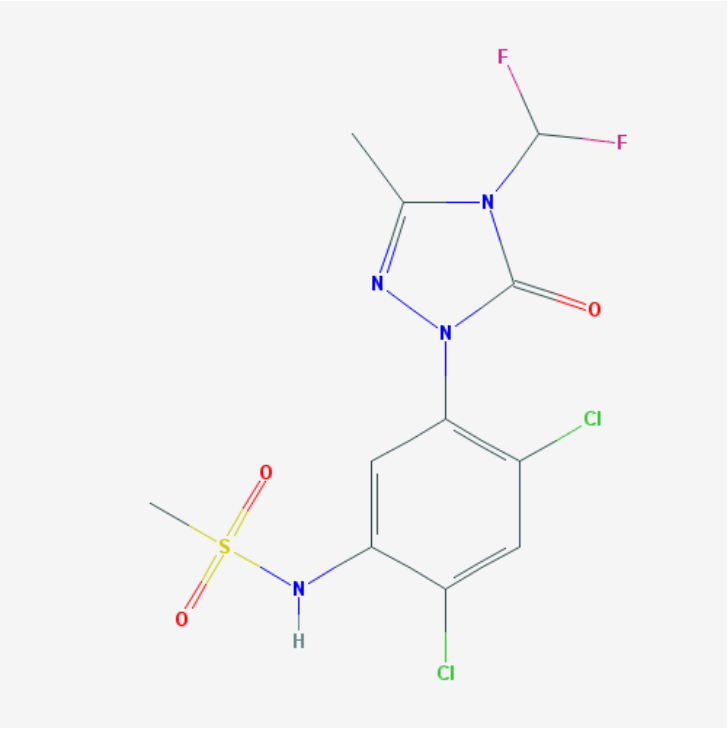



Figure 2.1. Chemical structure of sulfentrazone. From National Center for Biotechnology Information PubChem Compound Database. Retrieved from https://pubchem.ncbi.nlm.nih.gov/compound/86369 (accessed January 26, 2019).

Root uptake of sulfentrazone heavily depends on the pH level of the soil. Sulfentrazone is a weak acid and as such exists in either ionic or neutral (non-ionic) form. The neutral form is absorbed into roots mostly by ion trapping, which happens because the non-ionic molecules are more lipophilic than their ionic forms and thus diffuse more easily through the plant membranes (Ferrell, Witt & Vencill, 2003). In lower pH soils, sulfentrazone is present in a neutral form and is more readily absorbed by roots. Studies show that uptake and translocation is not the primary reason for increased plant tolerance to this chemical (Dayan et al., 1997; Thomas et al., 2005). Metabolism of sulfentrazone in plant tissue happens through a stepwise oxidation of the methyl group on the triazolinone ring and leads to formation of 3-hydroxymethyl-sulfentrazone and 3-carboxylic-sulfentrazone (Dayan, Armstrong & Weete, 1998). Further metabolism yields a free triazole ring and its N-glycoside. The free aromatic ring and further metabolism produces an unextractable fraction (Aizawa & Brown, 1999).

Crops registered for sulfentrazone application in Canada include chickpea, pea, common bean, soybean, flax and sunflower (FMC Canada, 2017). The labeled pests include: nightshade, kochia, lambsquarters, redroot pigweed, seedling Canada fleabane, water hemp, wild buckwheat and suppression of cleavers (FMC Canada, 2017).

### 2.3.2 Fluthiacet Methyl

Fluthiacet methyl (methyl{2-chloro-4-fluoro-5-[(EZ)-5,6,7,8-tetrahydro-3-oxo-1H,3H-[1,3,4]thiadiazolo[3,4-a]pyridazin-1-ylideneamino]phenylthio}acetate) (Figure 2.2) is an isourazole-type herbicide developed in the mid-1990s (Aizawa & Brown, 1999). It is a foliar applied post-emergence herbicide that offers selective broad-spectrum control of broadleaf weeds in corn and soybean production (Miyazawa et al., 1993). The rate needed to achieve good weed control is quite low, ranging between 5 to 10 g.a.i $ha^{-1}$, thus limiting environmental effects on agroecosystems (Shimizu et al., 1995). The soil half-life of fluthiacet methyl is two days, consistent with the notion of its low environmental impact (Shaner, 2014). Soil degradation of fluthiacet methyl is not yet fully understood, but it is known that the major metabolite of degradation is urazole (isomer of fluthiacet methyl) (Aizawa & Brown, 1999).

Absorption, translocation and metabolism of fluthiacet methyl in plants is well studied and understood. Absorption of this herbicide was considered one of its selectivity factors. Studies have shown that 50-79% of fluthiacet methyl gets absorbed within just 2 h after application (Fausey, Penner & Renner, 2000). Translocation of fluthiacet methyl through the plant is limited. Injuries can be seen just a few hours after application, and the amount of fluthiacet methyl translocated within 2 and 12 h is almost the same (Fausey et al., 2000). Metabolism of fluthiacet methyl in plants involves isomerisation to urazole. This reaction involves glutathione-S-transferase, a plant enzyme that can also play role in plant detoxification of several herbicides (Aizawa & Brown, 1999). Urazol derived from fluthiacet methyl is more effective in inhibiting protoporphyrinogen IX oxidase and thereby causes more damage than fluthiacet methyl itself (Aizawa & Brown, 1999). Isomerisation of fluthiacet methyl can occur even without glutathione-S-transferase by nucleophilic reaction with a glutathione thiol anion (Aizawa & Brown, 1999).

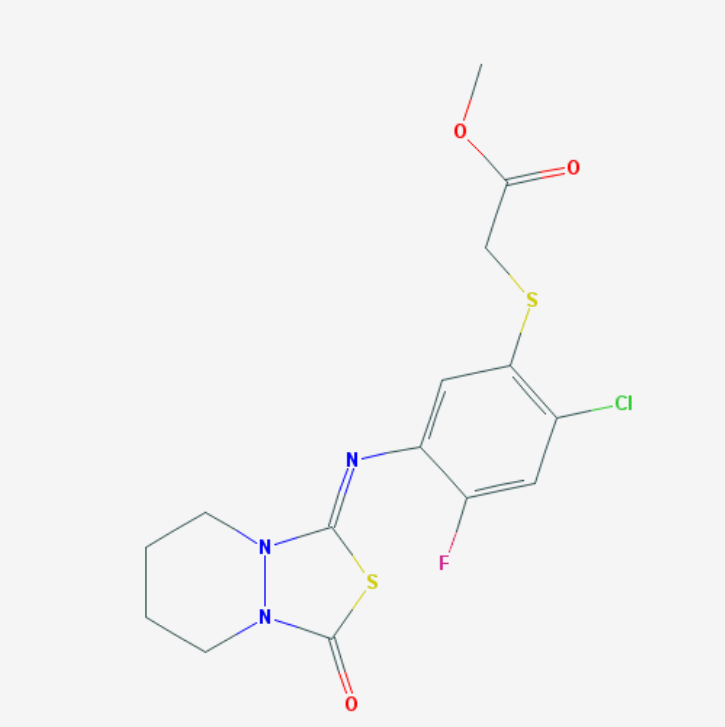



Figure 2.2. Chemical structure of fluthiacet-methyl. From National Center for Biotechnology Information PubChem Compound Database. Retrieved from https://pubchem.ncbi.nlm.nih.gov/compound/93542 (accessed January 26, 2019).

Fluthiacet methyl is not registered for use in Canada, while in the USA its registration applies only to corn and soybean (FMC Agricultural Solution, 2017). Its labeled pests include kochia, waterhemp, velvetleaf, lambsquarters, redroot pigweed, smooth pigweed, spiny pigweed, and wild buckwheat, among others. (FMC Agricultural Solution, 2017).

### 2.4 Tetrapyrrole Pathway in Plants

Protoporphyrinogen IX oxidase (PPOX; PPO; PPG; PPX; Protox; EC 1.3.3.4) is a membrane-bound enzyme involved in tetrapyrrole biosynthesis of chlorophyll, heme, siroheme, and phytochromobilin (Poulson & Polglase, 1975; Tanaka & Tanaka, 2007; Watanabe et al., 2000; Moulin & Smith, 2005). PPOX extracts six electrons from protoporphyrinogen IX to form protoporphyrin IX (Figure 2.3). Plant-type PPOX is a FAD-containing oxidase (Tanaka & Tanaka, 2007) and it is the final enzyme in the common branch of the chlorophyll and heme biosynthetic pathways in plants (Camadro et al., 1999; Cornah et al., 2003). Early enzymes of tetrapyrrole

synthesis are detected exclusively in chloroplasts (Lermontova et al., 1997). PPOX has been detected in both chloroplast and mitochondria of tobacco (*Nicotiana tabacum* cv. Samsun NN), and subsequently labeled as PPOX-I (plastid form), with 548 amino acid residues, and PPOX-II (mitochondrial form), with 504 amino acid residues. However, further studies showed that both forms represent nuclear-encoded proteins (Lermontova et al., 1997; Watanabe et al., 2000). These two isoforms share less than 30% amino acid sequence identity (Heinemann et al., 2007; Camadro et al., 1999). Research in spinach uncovered three isoforms: PPOX-I; PPOX-IIS; PPOX-IIL, with slight variations in the two isoforms of PPOX-II as a result of alternative translation initiation from a single mRNA species (Watanabe et al., 2000; Tanaka & Tanaka, 2007). Mutating two positions, $Leu^{356}$ and $Leu^{372}$, to valine could increase catalysis up to 100-fold, and by mutating only $Leu^{372}$ to valine, the enzyme was able to decrease binding of the substrate protogen 100-fold (Heinemann et al. 2007). So far, no study has illuminated the specifics of the number and structure of PPOX in lentil plants.

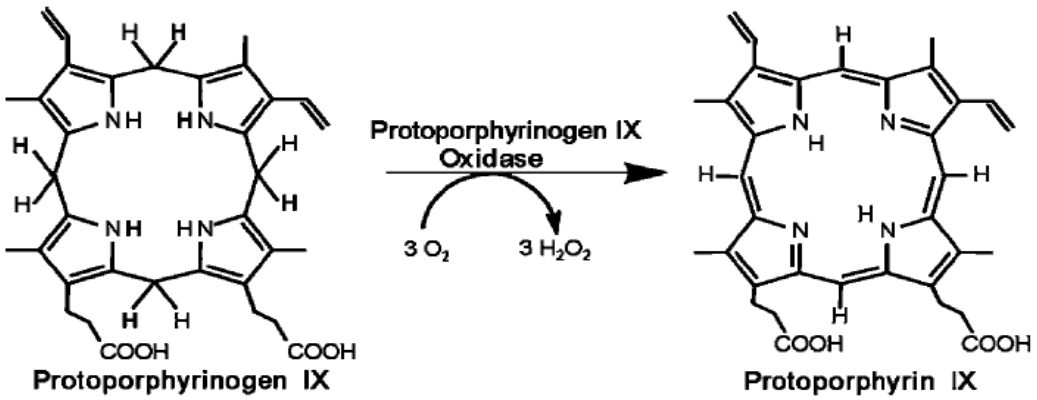



Figure 2.3. Enzymatic transformation of protoporphyrinogen IX into protoporphyrin IX mediated by PPOX. Reprinted with permission from "Functional definition of the tobacco protoporphyrinogen IX oxidase substrate-binding site", by I. Heinemann et al., 2007, *Biochemical Journal, 402*(3), p.576 Copyright [2007] by the Biochemical Journal.

PPOX-mediated transformation of protoporphyrinogen IX to protoporphyrin IX represents the seventh step in the tetrapyrrole pathway (Figures 2.3 and 2.4). The pathway starts with transformation of glutamyl-tRNA to an initial precursor, 5-aminolaevulinic acid (ALA). Through multiple stages of transformation, eight molecules of ALA are used to form uroporphyrinogen III, the tetrapyrrole primogenitor. At this point the first branching of this biopathway takes place and

uroporphyrinogen III is either oxidized and $Fe^{2+}$ inserted to form siroheme, or it is oxidatively decarboxylated (by three different enzymes, including PPOX) to form protoporphyrin IX. In this phase, two major branches diverge: one for $Mg^{2+}$ insertion in to the protoporphyrin IX to form chlorophyll, and the other for $Fe^{2+}$ insertion for heme production and later for phytochromobilin production. All tetrapyrroles are synthesized in the chloroplast and plastids, with the last phase of heme synthesis taking place in mitochondria (Moulin & Smith, 2005).

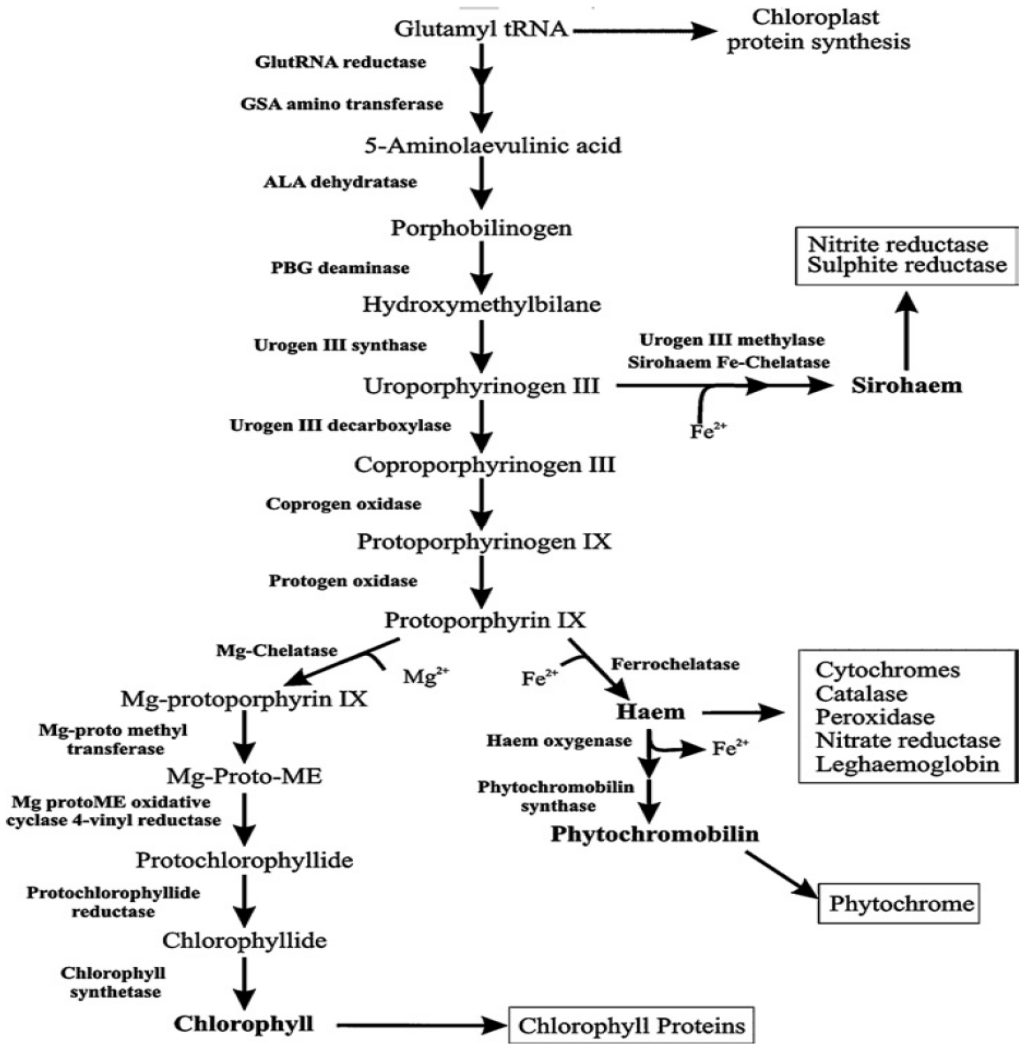



Figure 2.4. Overview of the tetrapyrrole pathway. Reprinted with permission from "Regulation of tetrapyrrole biosynthesis in higher plants", by M. Moulin and A. G. Smith, 2005, *Biochemical Society Transactions, 33*(4), p.738 Copyright [2005] by the Biochemical Society.

The tetrapyrrole pathway is extremely important due to its end products, but most of its intermediate products can also cause photo-oxidative damage in the cell. Details of how this pathway is regulated are still unclear but the initial precursor, 5-aminolaevulinic acid (ALA), was identified as a major regulatory point (Moulin & Smith, 2005; Czarnecki & Grimm, 2012). Two different regulatory feedback loops can down-regulate production of ALA and, therefore, down-regulate the complete pathway (Figure 2.5). The first feedback loop is heme-regulated. While its mechanism of action is still uncertain, some have suggested that degradation of glutamyl-tRNA reductase (GluTR) is at its base (Czarnecki & Grimm, 2012). The second feedback loop is regulated by the FLU protein, first identified in the *flu* mutant of *Arabidopsis thaliana*. In wild-type plants, the switch from light to dark phase was followed by reduction in ALA concentration, but the *flu* mutant continued to accumulate ALA and other products of the tetrapyrrole pathway (Kauss et al., 2012; Meskauskiene et al., 2001). It was reported that FLU also interacts with GluTR, but heme and FLU act independently on the same target (Kauss et al., 2012).

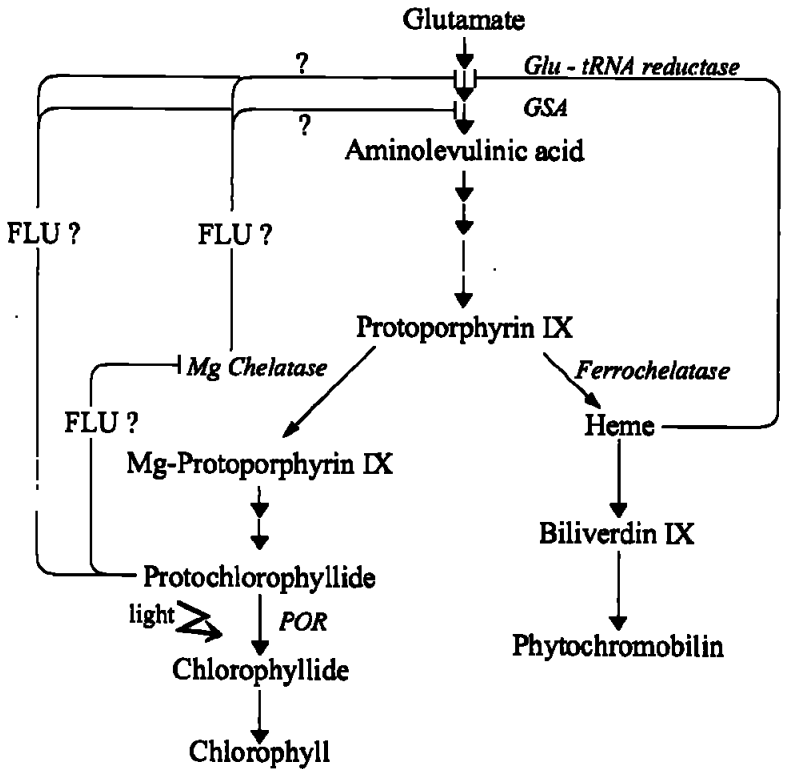



Figure 2.5. Example of feedback loops that down-regulate 5-aminolaevulinic acid (ALA) production. Reprinted with permission from "FLU-A negative regulator of chlorophyll biosynthesis in *Arabidopsis thaliana*", by Meskauskiene et al, *Proceedings of the National Academy of Sciences, 98*(22), p.12830. Copyright [2001] by the National Academy of Sciences.

## 2.5 Mechanism of Action of Group 14 Herbicides

Herbicides of group 14, today commonly referred to as PPO inhibitors, competitively inhibit protoporphyrinogen IX oxidase and terminate normal transformation of protoporphyrinogen IX to protoporphyrin IX. PPOX has extremely high affinity to PPO inhibitors, with $I_{50}$ concentration for most of the PPO inhibitors in the nanomolar range (Devine et al., 1993). With rapid accumulation of protoporphyrinogen IX in chloroplasts some of it leaks into the cytoplasm. Presence of protoporphyrinogen IX in the cytoplasm leads to its uncontrolled auto-oxidization to protoporphyrin IX (Duke et al., 1991). Since protoporphyrin IX is formed outside of its native environment, the cytoplasm lacks the necessary enzymes to prevent its accumulation and stop the negative effects (Figure 2.6). Protoporphyrin IX is a photoactive compound. When absorbing light, it transforms into the triplet state which reacts with oxygen and creates singlet oxygen. Both protoporphyrin IX in the triplet state and singlet oxygen can initiate a chain reaction of lipid peroxidation.

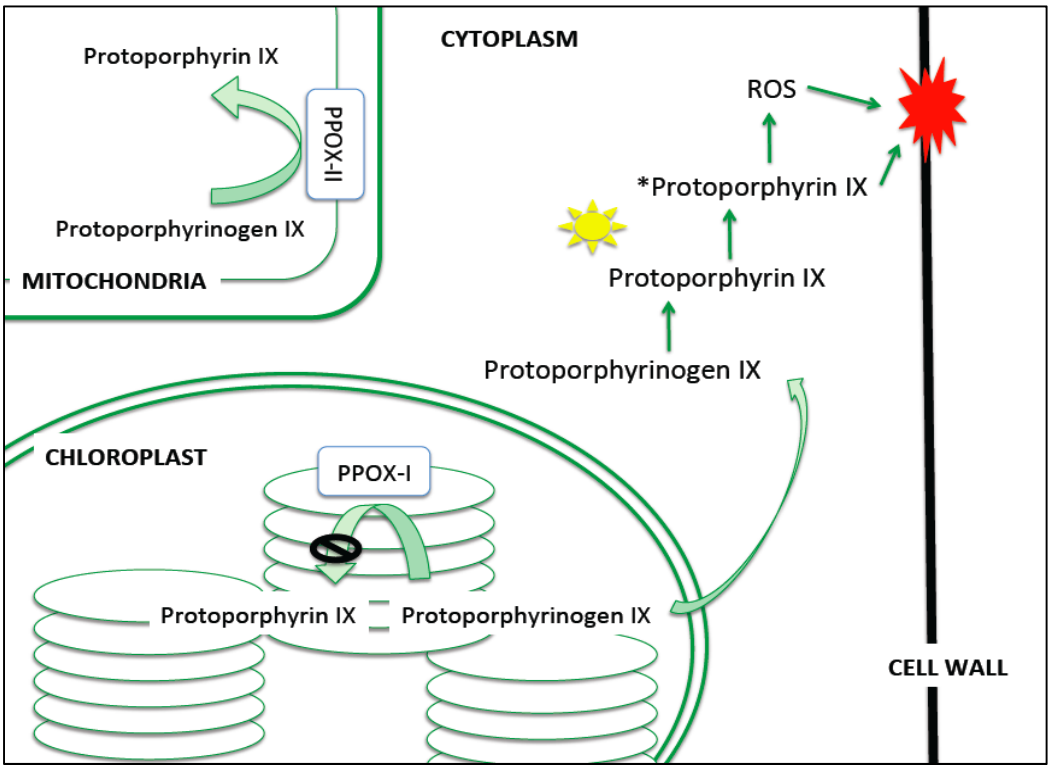



Figure 2.6. Localization of PPO inhibitor effects in the cell. Adapted with permission from "Development of Protoporphyrinogen Oxidase as an Efficient Selection Marker for *Agrobacterium tumefaciens*-Mediated Transformation of Maize", by X. Li et al., 2003, *Plant Physiology, 133*(2), p.737.

Symptoms of injuries caused by PPO inhibitors include leaf cupping, crinkling, bronzing (discoloration of the foliage with reddish-brown appearance) and tissue necrosis. PPO inhibitors are a fast-acting type of herbicide and the first injuries are visible within a few hours of application. Symptoms progress from the appearance of water-soaked spots; through color change from green to yellow, brown and black, wilting and desiccation (within 24 hours); to necrotic lesions on the leaves, and chlorosis of the leaf (Dayan & Duke, 1997). As with most herbicides, PPO inhibitors inflict more damage on young plant tissues.

### 2.6 Understanding Herbicide Tolerance and Developing Herbicide Resistance

The wider plant science community often uses the terms 'herbicide tolerance' and 'herbicide resistance' interchangeably.

According to Weed Science Society of America, herbicide *tolerance* is defined as:

> "The inherent ability of a species to survive and reproduce after herbicide treatment. This implies that there was no selection or genetic manipulation to make the plant tolerant; it is naturally tolerant." (Weed Technology, 1998, p. 789)

While herbicide *resistance* is defined as:

> "The inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type. In a plant, resistance may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis." (Weed Technology, 1998, p. 789).

Molecular and genetic causes of herbicide tolerance have not been studied in depth. For example, 2,4-D is one of the oldest weed control compounds, used on a large scale, yet the underlying molecular mechanism that makes this herbicide selectively kill dicots and spare monocots remains unclear (Song, 2014). A shortage of research into the specific mechanisms of herbicide tolerance was partly due to the high cost of studies. However, recent advances in several "omics" platforms have lowered the cost of potential studies and opened the door to the discovery of the underlying factors of herbicide tolerance. The onus is now on the scientific community to develop strategies that leverage herbicide tolerance for further advancement of crop protection.

Most herbicide resistant crops currently available have been developed through mutagenesis or genetic engineering. Mutagenesis breeding is used in cases of scarce genetic diversity, where bottlenecks of genetic material had been created (Sikora et al., 2011). According to the FAO

Mutant Variety Database (2018), a total of 3,234 different varieties were registered as developed through mutation breeding across all agronomically important crops; lentil accounts for 13 of these entries.

Physical and chemical agents used to cause changes in genetic information and increase mutation rates above background levels are called mutagens. Mutagens are usually classified into physical or chemical. Radiation is a physical mutagen. Several different radiation types are used in practice, includingelectromagnetic radiation (X-ray, gamma ray or ultraviolet radiation), corpuscular radiation (thermal and fast neutrons) and ion and electron beams (Roychowdhury & Tah, 2013). The most frequently used types of radiation are: gamma rays, which cause small deletions and point mutations, and fast neutrons, which usually cause translocations, chromosome losses, and large deletions (Sikora et al., 2011). Chemical mutagens usually cause more limiting modifications of genetic material than physical mutagens, and their implementation does not require complicated equipment (Oladosu et al., 2016). The most widely used chemical mutagens are base analogues (5-bromouracil, 5-bromodeoxyuridine, 2-aminopurine), alkylating (ethylmethane sulfonate (EMS), diethyl sulfonate (DES), sodium azide, diazomethane, ethylene oxide), intercalating agents (acridine orange, proflavin, ethidium bromide) and chemicals that directly modify DNA structure (Mba, 2013). Most of the reported varieties developed through mutation were induced by using alkylating agents, primarily ethylmethane sulfonate (EMS) (Sikora et al., 2011). Although EMS exhibits a bias that causes mainly G/C-to-A/T transitions, it is used extensively in legume and lentil studies (Mohd-Yusoff et al., 2015). Another chemical mutagen, widely employed among legumes researchers, is sodium azide ($NaN_3$). Sodium azide causes A/T-to-G/C transitions about three times more often than G/C-to-A/T (Gruszka et al., 2012).

The purpose of mutation breeding in the development of herbicide resistant crops is to create new genetic diversity which may reduce or eliminate the negative effects of specific herbicides. Mutations can lead to modifications of specific enzymes crucial in herbicide interactions with the plant. Modification of the target sites for a herbicide is the most common approach in the development of herbicide resistant crops. Commercially available herbicide resistant crops are usually developed through the application of one of the three key enzymes: acetohydroxyacid synthase (also known as acetolactate synthase), acetyl-CoA carboxylase, and D1 protein of photosynthesis II (Tan & Bowe, 2012). Acetohydroxyyacid synthase (AHAS) is part of the

biosynthesis of branched amino acids, and it is the target of group 2 herbicides. *AHAS* genes and enzymes have been examined in many studies, and five mutation points ($Ala^{122}$, $Pro^{197}$, $Ala^{205}$, $Trp^{574}$ and $Ser^{653}$) have been shown to be crucial for resistance leading to development of herbicide resistant crops (Tan & Bowe, 2012). In addition to these five sites, three critical mutation points ($Asp^{376}$, $Arg^{377}$, $Gly^{654}$) have been discovered among weed species (Tranel, Wright & Heap 2018). At this time crops resistant to group 2 herbicides are developed only for imidazolinone and sulfonylurea products. Crops with imidazolinone resistance include maize, rice, wheat, rapeseed, sunflower and lentil (Tan et al., 2005; Green, 2007; Pozniak et al., 2004).

Imidazolinone resistant lentil now represents the backbone of chemical weed management in lentil production in Canada. Mutation that leads to imidazolinone resistance was created through application of ethyl methane sulfonate (EMS), and it resulted in amino acid substitution at $Ala^{205}$ to $Val^{205}$ (Slinkard et al., 2007; Thompson, 2013). Today, over 16 different lentil varieties carry this mutation and they are sold under the Clearfield™ brand (Government of Saskatchewan, 2018). At the end of the 20th century, genetic engineering became the key tool for development of herbicide resistant crops. The first genetic engineering successes included the development of crops resistant to glufosinate, glyphosate and bromoxynil. The first herbicide resistant crop available to farmers was bromoxynil resistant cotton, but it never captured much of the market share (Duke, 2005). Glufosinate and glyphosate resistant traits currently dominate seed markets. Glyphosate resistance was developed by introducing 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) gene from *Agrobacterium spp*. strain CP4 into the crop (Padgette et al., 1995. To ensure glufosinate resistance, a gene from *Streptomyces sp.* was introduced into the crop. This gene encodes acetyl transferase which can metabolize glufosinate into non-toxic compounds (Dröge et al., 1992). Both glufosinate and glyphosate herbicide resistance had profound effect on weed management, and agriculture in general. Large amount of herbicide resistance research has been conducted, with variable success. Although technology to develop new herbicide resistant crops exists, the looming problem of herbicide resistant weeds can hinder success. Efforts to develop new herbicide resistance and understand sources of herbicide tolerance are quickly becoming a Sisyphean task, as the number of herbicides able to control weeds continues to decline due to the increase in herbicide resistant weed species (Harker et al., 2012).

### 2.6.1 Modes of Tolerance/Resistance to PPO Inhibitors

Since the initial use of the PPO inhibitors in the 1960s, only six species of weeds have been reported as resistant to this group of herbicides. In contrast, 160 weed species have developed developed resistance to ALS inhibitors and 49 weed species are resistant to EPSP synthase inhibitors. Resistance to PPO inhibitors among weeds is surprisingly rare (Heap, 2018).

Plants become resistant to herbicides through one of three general mechanisms: target site modification, metabolic degradation of herbicides, or prevention of intact herbicides from reaching target sites (Dayan & Duke, 1997). The PPO inhibitor mode of action in plants is complex. Asami and Yoshida (1999) suggest four different scenarios for resistance to PPO inhibitors. In the first scenario, resistance can be obtained through modification of PPOX genes, and in the second, through overexpression of PPOX genes. Their third scenario requires genetic manipulation and introduction of PPOX from foreign species (e.g. from a bacterial or fungal source) unaffected by PPO inhibitors. The final fourth scenario requires that the plant cell be able to digest protoporphyrinogen IX located in cytoplasm after inhibition of PPOX.

Dayan and Duke (1997), however, have given a more detailed scheme of possible resistance to PPO inhibitors, which provides a better tool for exploration of tolerance in lentil. They determined six potential mechanisms of resistance:

1. Inhibition of uptake or sequestration of the herbicide,
2. Rapid metabolic degradation of the herbicide,
3. Herbicide-resistant PPOX enzyme,
4. Degradation of extra-plastidic protoporphyrinogen IX and protoporphyrin IX,
5. Inactivated, herbicide-resistant, extra-plastidic PPOX, and
6. Quenching of singlet oxygen and other toxic oxygen species.

An additional source of resistance was discovered in the *Arabidopsis thaliana* mutation designated *aci5-3* and located in the *CHLI-1* gene which codes for one of the three subunits of Mg chelatase (Soldatova et al., 2005). This mutation leads to the reduction in the synthesis of 5-aminolevulinate and to reduction in herbicide damage from PPO inhibitors. Sherman et al. (1991) found that by adding 5-aminolevulinate along with the herbicide increases accumulation of protoporphyrinogen IX and higher herbicidal activity among mustard (*Sinapis alba)* plants. They concluded that the

reduced effect of PPO inhibitors in mustard plants can be attributed to the down-regulation of the tertrapyrrole pathway.

*Amaranthus tuberculatus* (waterhemp) is one of the six weeds known to possess resistance to PPO inhibitors. A codon deletion in the gene that codes for *PPOX* is responsible for the resistance (Patzoldt et al., 2006). Genes that encode PPOX enzyme have been identified and labeled as: *PPOX1, PPOX2*, and *PPOX2L* (the last two share more than 98% amino acid identity). *PPX2L* has a three base pair deletion in resistant waterhemp causing a mutation in the glycine residue at position 210 (Lee & Tranel, 2008; Thinglum et al., 2011). The $Gly^{210}$ indel in *PPOX2L* is part of a bi-GTG repeat (or a bi-TGG repeat) (Patzoldt et al., 2006). Waterhemp plants with this codon deletion were able to survive 31 to 53-fold higher doses of herbicides than wild type plants (Patzoldt et al., 2006).

The closest relative of lentil whose interaction with PPO inhibitors has been studied extensively is *Glycine max* (soybean). A few other legume crops (including chickpea, peanut, and pea) also show some degree of tolerance to PPO inhibitors. According to Fausey et al. (2000), soybean has increased tolerance to two PPO inhibitors (flumiclorac and fluthiacet methyl) due to decreased herbicide retention and increased herbicide metabolism. A comparative study of soybean showed no difference between tolerant and less tolerant genotypes with respect to absorption of herbicide, metabolism of herbicide, or even affinity of PPOX to the herbicide (Dayan et al., 1997). The conclusion was that those soybean plants that can cope with oxidative stress caused by herbicides tend to be more tolerant of PPO inhibitors (Dayan et al., 1997). The mechanism managing this oxidative stress is not well understood and requires further analysis.

Recent research divides herbicide resistance in two categories: target site resistance, and non-target site resistance. Target site resistance (TSR) encompasses modifications in the gene encoding the protein target leading to the reduction in herbicide efficacy (Délye, 2012). Non-target site resistance (NTSR) refers to all mechanisms other than TSR. These other mechanisms are not as well understood or studied (Délye, 2012). Several groups of enzymes have been identified as key players, including cytochrome P450, glutathione-S-transferases, glycosyltransferases, and ABC transporters (Yuan et al., 2007). Dayan et al. (1997) suggest that oxidative degradation of herbicides as a detoxification mechanism is associated with cytochrome P450 monooxygenase activity. Compounds that inhibit cytochrome P450 monooxygenase can be used to determine

importance of cytochrome P450 monooxygenase to the PPO inhibitor tolerance (Werck-Reichhart & Feyereisen 2000). For PPO inhibitors, both TSR and NTSR mechanisms feature in different plant species, suggesting multiple possible avenues for development of increased herbicide resistance.

## Prologue to Chapter 3

The subsequent research chapter explores the effects of sulfentrazone and fluthiacet methyl on selected lentil varieties. The set of field experiments was performed at two locations for three years to determine levels of damage and yield impact among selected lentil varieties. The field experiments were conducted by Ken Sapsford and Eric Johnson. As a follow up to field experiments, electrolyte leakage assays were conducted to assess the speed of leaf tissue decay caused by sulfentrazone and fluthiacet methyl. The final portion of the research chapter reports a dose response study of fluthiacet methyl, as previous experiments in this chapter did not produce corresponding results. Two lentil varieties were selected to perform dose response experiments in a controled environment to determine if they exhibit different levels of tolerance to fluthiacet methyl.

# 3. EFFECTS OF SULFENTRAZONE AND FLUTHIACET METHYL ON SELECTED LENTIL VARIETIES

## 3.1 Introduction and Objectives

Lentil production in Western Canada is hindered by poor competitiveness with weeds and by limited herbicide weed control options. Discovery of new herbicides is slow and costly, so researchers tend to opt for testing existing chemistries in search of a match for lentil. Among existing herbicide options, Group 14 herbicides hold the greatest promise. Many legume species have already registered many products ofthis group of herbicides (Government of Saskatchewan, 2017). There is limited understanding of the mechanisms of this tolerance due to the lack of research. Increasing tolerance to Group 14 in lentil first requires an examination of the extent of the existing variability in phenotypic response to these herbicides.

A preliminary study into the possible use of Group 14 herbicides in lentil production started in 2008 (Holm et al., 2012). Two Group 14 herbicides—sulfentrazone and fluthiacet methyl—were selected for this research, and possible subsequent implementation in lentil production. Focus on these compounds was warranted by the existing research into their effects. Earlier studies had shown genetic variation in response to these two herbicides (Holm et al., 2012). Sulfentrazone already provides excellent control of some noxious weeds in Western Canada, such as kochia (*Kochia scoparia*), lambsquarters (*Chenopodium album*), wild buckwheat (*Polygonum convolvulus*), redroot pigweed (*Amaranthus retroflexus*), and others (Government of Saskatchewan, 2017). Fluthiacet methyl has also proven efficient in dealing with some herbicide resistant weeds. For example, it provided 90% control of glyphosate-resistant common waterhemp (*Amaranthus rudis*) (Jhala et al., 2017).

Research presented in this chapter examines phenotypic variability resulting from the application of sulfentrazone or fluthiacet methyl on lentil varieties representing all major market classes of lentil grown in Western Canada. The first experiment investigated the effects of the herbicides under field conditions, using injury ratings and yield as indicators of herbicide damage across multiple environments. The second experiment measured the level of damage caused by the two herbicides through an electrolyte leakage assay. The final experiment dealt solely with the effect

of fluthiacet methyl on the two varieties that had previously demonstrated contrasting reactions to the application of the herbicide.

## 3.2. Materials and Methods

### 3.2.1. Plant Material

The seven lentil varieties selected for testing are described in Table 3.1. They were selected as they represent major market classes of lentil grown in Western Canada. CDC Impala, CDC Maxim and CDC Improve, possess tolerance to imidazolinone (Clearfield™ trait).

Table 3.1 *Lentil varieties used in testing levels of tolerance to sulfentrazone and fluthiacet methyl both under field conditions (Saskatoon and Scott location in 2011, 2012 and 2016) and used for electrolyte leakage assay*

| Name | Type | Seed weight (g/1000 seeds) | Release year |
|---|---|---|---|
| **CDC Rosetown** | Red extra small | 31 | 2005 |
| **CDC Impala** | Red extra small | 31 | 2007 |
| **CDC Redberry** | Red small | 42 | 2003 |
| **CDC Maxim** | Red small | 40 | 2007 |
| **CDC KR-1** | Large red | 56 | 2009 |
| **CDC Sedley** | Large green | 68 | 2001 |
| **CDC Improve** | Large green | 67 | 2006 |

*Note*. Data extracted from http://saskseed.ca/images/varieties2011.pdf (accessed Jan. 26, 2019). Copyright (2011) by Government of Saskatchewan.

### 3.2.2. Site Description and Meteorological Data

Field experiments were conducted in 2011, 2012, and 2016 at Kernen Crop Research Farm (52°16' N, 106°51' W) near Saskatoon (henceforth referred to as "Saskatoon"), and at Agriculture and Agri-Food Canada Scott Research Farm (52°36' N, 108°84' W; henceforth referred to as "Scott"). The soil at Saskatoon is Sutherland series clay loam (Orthic Dark Brown Chernozem), while at Scott it is loam (Dark Brown Chernozem). Detailed soil characteristics are provided in Table 3.2, while meteorological data can be found in Table 3.3.

Table 3.2 *Soil characteristics at Saskatoon (Kernen Crop Research Farm) and Scott (Agriculture and Agri-Food Canada Scott Research Farm) locations*

| Location | % Sand | % Silt | % Clay | % Organic material | Soil pH | Texture |
|---|---|---|---|---|---|---|
| **Saskatoon** | 19 | 36 | 45 | 5.2 | 7.2 | Silty loam clay |
| **Scott** | 38 | 43 | 17 | 3.3 | 6 | Loam |

Table 3.3 *Average monthly temperature and precipitation at Saskatoon and Scott locations in 2011, 2012 and 2016 during the April to September periods*

| | | Saskatoon | | | | Scott | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 2011 | 2012 | 2016 | Historical 1980-2010 | 2011 | 2012 | 2016 | Historical 1980-2010 |
| **April** | Average temperature (C°) | 3.1 | 4.4 | 5.5 | 5.2 | 2.2 | 3.8 | 5.9 | 3.8 |
| | Precipitation (mm) | 1.6 | 29.3 | 3.0 | 21.8 | 10.4 | 38.4 | 1.9 | 21.6 |
| **May** | Average temperature (C°) | 10.9 | 10.1 | 13.7 | 11.8 | 10.8 | 9.9 | 12.4 | 10.8 |
| | Precipitation (mm) | 18.1 | 120.5 | 41.6 | 36.5 | 26.2 | 50.8 | 64.8 | 36.3 |
| **June** | Average temperature (C°) | 15.5 | 15.8 | 17.4 | 16.1 | 14.6 | 15.2 | 15.8 | 15.3 |
| | Precipitation (mm) | 96.7 | 123.5 | 49.7 | 63.6 | 81.6 | 200 | 20.8 | 61.8 |
| **July** | Average temperature (C°) | 18.4 | 19.7 | 18.7 | 19.0 | 17.2 | 18.7 | 17.8 | 17.1 |
| | Precipitation (mm) | 69.4 | 81.9 | 58.6 | 53.8 | 68 | 87.6 | 88.1 | 72.1 |
| **Aug** | Average temperature (C°) | 17.2 | 17.3 | 16.9 | 18.2 | 16.5 | 17.1 | 16.1 | 16.5 |
| | Precipitation (mm) | 17.1 | 48.9 | 70.2 | 44.4 | 60.4 | 45 | 48.3 | 45.7 |
| **Sept** | Average temperature (C°) | 14.7 | 13.0 | 11.8 | 12.0 | 14.1 | 12.7 | 10.9 | 10.4 |
| | Precipitation (mm) | 6.2 | 0.8 | 24.1 | 38.1 | 3.8 | 22.8 | 22.2 | 36.0 |

*Note*. Data extracted from http://climate.weather.gc.ca/ (accessed Jan. 26, 2019). Copyright (2017) by Government of Canada.

### 3.2.3 Experimental Design and Herbicide Application

A two-factor (lentil genotype, herbicide application) experiment was set up using a split-plot design with four replications. Lentil varieties were randomized across whole plots, while herbicide treatments were assigned to subplots, and included: untreated, fluthiacet-methyl at 4 and 8 g.a.i $ha^{-1}$, and sulfentrazone at 140 and 280 g.a.i $ha^{-1}$. Herbicides were applied using a field-scale tractor mounted sprayer equipped with an Airmix™ 100015 flat nozzle calibrated to deliver 100 L/ha at 275 kPa. The length of individual plot was 6 m with width of plot being 2.25 m. The row spacing was set at 23.5 cm and the seeding rate was 125 seeds per $m^2$. Tilage system used was minum till, and all plots were sprayed with ethalfluralin (Edge™) previous fall or pre-seeding for weed control,

The 140 g.a.i $ha^{-1}$ rate for sulfentrazone was selected due to its ability to control numerous weed species well, including kochia, redroot pigweed, lambsquarters, wild buckwheat, and to suppress cleavers (FMC of Canada, 2016). Timing of sulfentrazone application varied across site-years, from pre-seeding to post seeding, but the application always took place before emergence. Since fluthiacet methyl is not yet registered for use on pulses in Canada, application herbicide rate was determined using corn and soybean production guidelines established in the United States. FMC of Canada (2011) recommends a stand-alone application of fluthiacet methyl in the 4.41-6.77 g.a.i $ha^{-1}$ range. Fluthiacet-methyl was applied post-emergence, to 4-6 leaf stage lentils. Agral 90, a non-ionic surfactant, was added to fluthiacet-methyl at 0.25% v/v.

### 3.2.4 Data Collection and Statistical Analysis

Phenotypic data collected during this trial included injury rating and yield. A visual scale of 0 (no injury) to 100 (complete plant death) was used for herbicide injury ratings, which were collected multiple times across all site years on a plot basis. Yield ($g/m^2$) was collected on a plot basis.

Injury ratings and yield data were tested for normality and homogeneity of variance using Shapiro–Wilk test and Levene's test with SAS software package (SAS 2013, Version 9.4). Yield data displayed heterogeneity of variance across all combined site-year datasets. Since normality and heterogeneity did not improve using box cox, square-root and log-transformation, site-year datasets were analyzed individually. Injury rating data across site-years were collected at different

time points, ranging from 21 to 106 days after seeding, compelling an independent analysis of each injury data set. Injury ratings and yield data were examined using an analysis of variance (ANOVA) with the Proc Mixed function in SAS 9.4. Herbicide treatment, genotype, and herbicide treatment by genotype interaction were fixed effects, and replication was considered as a random effect. Analysis of variance of injury ratings was deepened by using the 'SLICE' function (SAS Institute Inc. 2011) which provided additional simple effects analysis, or partitioned analysis of the least squares means of the interaction between herbicide treatment and genotype. Simple effects testing typically follows a determination that an interaction is significant, and this was used to establish under which circumstances the genotype variable had an effect significantly different from zero, rather than illuminate the interaction itself.

### 3.2.5 Electrolyte Leakage Assay (ELA)

The same set of seven lentil varieties (Table 3.1) was used in an electrolyte leakage assay (ELA). Plants were grown in a growth room at University of Saskatchewan (Conviron by Controlled Environments Limited) with soilless mix (2:1 Sunshine #3: Perlite, Sun Gro Horticulture, Canada) under the following conditions: day/night temperatures set at 21 °C/15 °C, day length of 18 h, with light intensity of 350 μmol/$m^2$s. Plant leaflets were harvested two weeks after seeding, when lentil plants were at the 5-6 leaf stage. Leaflets were individually cut and weighed to ensure a sample size of approximately 250 mg. Only whole leaflets without any injured tissue (no physical damage or insect injury) were included in the sample. After cutting and weighing, the leaflets were washed with a base buffer solution for 1 min to ensure that dirt particles were removed from leaflet surfaces. The base buffer solution was a mix of 1% sucrose and 1 mM of MES (4-morpholineethanesulfonic acid) adjusted to pH 6.5 with concentrated NaOH. The experiment had three treatments: control (base buffer solution), sulfentrazone (at 150 μM concentration) and fluthiacet methyl (at 25 μM concentration). Sulfentrazone and fluthiacet methyl concentrations were selected based on a set of preliminary experiments, where the range of concentration varied from 1 μM to 1000 μM for both herbicides. Sulfentrazone solution was prepared using the commercial product—Authority™ (480g/l, FMC Corporation), while fluthiacet methyl solution was prepared with Cadet™ (10.3%, FMC Corporation). Once washed, leaf material was placed in 30 ml of solution and measured for the first electro-conductivity value ($EC_{start}$). Samples were then

placed in a dark growth chamber at room temperature for 24 h before electro-conductivity was measured again ($EC_x$). Subsequently, the samples were placed in the growth chamber under continuous light conditions (500 µmol/$m^2$s at 22°C) and electro-conductivity was measured every 12 h for the subsequent 72 h ($EC_x$ to $EC_y$). Finally, following the 96-h measurement period, the samples were placed in a 95°C water bath for 1.5 h before the final electro-conductivity measurement was taken ($EC_{final}$).

Electro-conductivity was used to calculate percent leakage as an indirect measure of tissue damage. The following formula was used to determine percent leakage:

$$\%Leakage_x = \frac{EC_{Start} - EC_x}{EC_{Start} - EC_{Final}} \quad \text{(3.1)}$$

The experiment was performed twice under these conditions.

A homogeneity of variance test did not show significant differences in residual variance between the two repetitions of the experiment, so the data were merged. Non-linear regression was used to fit experimental data to several possible models. Relative quality of different models was evaluated and compared using the Akaike information criterion (AIC), and the model with the lowest AIC score was selected. Model comparisons were conducted in R (R Development Core Team 2014) using the mselect function (Ritz and Streibig, 2005). Sulfentrazone and fluthiacet methyl data sets were each fitted to a four-parameter log-logistic model (Seefeldt et al., 1995):

$$Y = C + \frac{D - C}{(1 + \exp(B(\log(X) - \log(E))))} \quad \text{(3.2)}$$

where Y is the response (i.e. % leakage), C is the lower limit, D is the upper limit (fixed at 100), B is the slope of the line, E is the dose resulting in a 50 % response, and X is time in hours. Time until 50% total leakage ($T_{50}$) is a coefficient use to describe in simplified form the level of tolerance observed in the sample. $T_{50}$ is the time needed for one sample to reach 50% of maximal leakage. In the above four-parameter log-logistic model; it corresponds to parameter E. A low $T_{50}$ value suggests high sample susceptibility to the herbicide, as half maximal leakage is reached more quickly, while a large $T_{50}$ reflects longer time necessary for the herbicide to cause 50% leakage.

### 3.2.6 Dose Response to Fluthiacet Methyl

Two lentil varieties: CDC Improve, representing lines tolerant to sulfentrazone, and CDC Impala representing susceptible lines, were selected for an indoor rate study. Plants were grown in a growth room at University of Saskatchewan (Conviron by Controlled Environments Limited) with soilless mix (2:1 Sunshine #3: Perlite, Sun Gro Horticulture, Canada) in 10 $cm^2$ square pots under the following conditions: day/night temperatures set at 21 °C/15 °C, day length of 18 h, with light intensity of 350 μmol/$m^2$s. Plants were watered every fourth day; fertilization was done 12 d after seeding using water soluble 20-20-20 N-P-K fertilizer (Plant-Prod, Canada). Plants were sprayed 14 days after seeding (5-leafstage) using twelve rates of fluthiacet methyl: 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128 and 256 g.a.i $ha^{-1}$. The spraying was done in the spray cabinet with a single Even-Spray nozzle 8001 delivering 109 L/ha at 275 kPa. Plants were cut off at the soil surface 14 d after herbicide treatment and dried at 80 °C for 48 h before weighing. The experiment was repeated a second time.

Data from the two repeats were combined, as a test of homogeneity of variance did not reveal significant differences in residual variances between the two repeats of the experiment. Dry weight was regressed over herbicide rates using the three-parameter log-logistic model (Finney, 1971)

$$Y = \frac{D}{(1+\exp(B(\log(X)-\log(E))))} \quad \text{(3.3)}$$

where Y is the response (dry weight), D is the upper limit of the model, B is the slope of the line, E is the dose resulting in a 50% response (i.e. rate causing 50% reduction of biomass; also known as $ED_{50}$), and X is the herbicide dose (Knezevic et al., 2007). Analysis was performed using the drc package within the statistical software R. Comparison of $ED_{50}$ values was done using the EDcomp function.

### 3.3 Results

Results of the mixed model analysis of herbicide and genotype effects on the injury ratings are provided in Table 3.4 and 3.5. Herbicide treatment had a significant effect ($p<0.001$) on injury ratings for all six site-years. The effect of varieties was less clear. At the Saskatoon location, varieties were significantly different ($p<0.05$ to $p<0.001$). At the Scott location, varieties were significantly different ($p<0.05$) only for the initial injury rating, but not for the second or third, in two of the three years (2012 and 2016). The interaction effect between herbicide and genotype was similarly influenced by the location. At the Saskatoon site, the interaction was highly significant ($p<0.001$) in all years except 2011, while at the Scott location it was not significant for injury ratings, except in 2012.

Table 3.4 *F-values from analysis of variance for the effect of genotypes and herbicide treatment on injury ratings at Saskatoon location in 2011, 2012 and 2016 for seven selected lentil varieties. Injury ratings are labeled based on days after seeding (DAS) when they were collected.*

| | | INJURY RATING | | | |
|---|---|---|---|---|---|
| | | **21 DAS** | **41 DAS** | **49 DAS** | **66 DAS** |
| **Saskatoon 2011** | **Genotypes** | 4.22** | 4.4** | 3.72* | $3.17^{NS}$ |
| | **Herbicides** | 210.3*** | 97.23*** | 180.32*** | 160.55*** |
| | **Genotypes*Herbicides** | 3.47*** | $1.37^{NS}$ | $1.3^{NS}$ | 2.28** |
| | | **30 DAS** | **43 DAS** | **55 DAS** | |
| **Saskatoon 2012** | **Genotypes** | 4.41** | 3.17* | 3.9* | |
| | **Herbicides** | 82.02*** | 104.91*** | 129.73*** | |
| | **Genotypes*Herbicides** | 2.64*** | 2.05** | 3.12*** | |
| | | **29 DAS** | | | **102 DAS** |
| **Saskatoon 2016** | **Genotypes** | 20.42*** | | | 7.81*** |
| | **Herbicides** | 211.97*** | | | 60.1*** |
| | **Genotypes*Herbicides** | 2.52*** | | | 7.79*** |

***p <0.001; **p <0.01;*p<0.05; $^{NS}$, not significant

Table 3.5 *F-value from analysis of variance for the effect of genotypes and herbicide on injury ratings at Scott location in 2011, 2012 and 2016 for seven selected lentil varieties. Injury ratings are labeled based on days after seeding (DAS) when they were collected.*

| | | INJURY RATING | | |
|---|---|---|---|---|
| | | **35 DAS** | **49 DAS** | **54 DAS** |
| **Scott 2011** | **Genotypes** | $2.06^{NS}$ | $0.44^{NS}$ | $0.63^{NS}$ |
| | **Herbicides** | 39.55*** | 11.64*** | 6.27*** |
| | **Genotypes*Herbicides** | $0.45^{NS}$ | $0.68^{NS}$ | $0.49^{NS}$ |
| | | **30 DAS** | **37 DAS** | |
| **Scott 2012** | **Genotypes** | 4.28** | $2.06^{NS}$ | |
| | **Herbicides** | 169.58*** | 39.55*** | |
| | **Genotypes*Herbicides** | $1.49^{NS}$ | 0.45** | |
| | | **35 DAS** | **47 DAS** | **72 DAS** |
| **Scott 2016** | **Genotypes** | 4.77** | $1.03^{NS}$ | $2.68^{NS}$ |
| | **Herbicides** | 117.42*** | 34.77*** | 25.18*** |
| | **Genotypes*Herbicides** | $1.18^{NS}$ | $0.88^{NS}$ | $1.45^{NS}$ |

***p <0.001; **p <0.01; $^{NS}$, not significant

Injury ratings for all six site-years are represented by heat maps in Figures 3.1-3.6. Visual inspection of the results makes it clear that herbicide effects are highly dependent on environmental factors. While sulfentrazone caused injuries at the Scott location, the effect varied greatly across years. Results were more consistent at the Saskatoon site. In 2011 at the Scott location, herbicide damage was observed prior to flowering and it was at a much lower level than in Saskatoon. In 2012, sulfentrazone treatments caused high injuries to the plants at both locations. In 2016, injury ratings changed significantly over the course of plant growth at both locations. Injuries caused by fluthiacet methyl treatments declined over time at all site years, while sulfentrazone treatments caused more persistent injuries visible over the entire season.

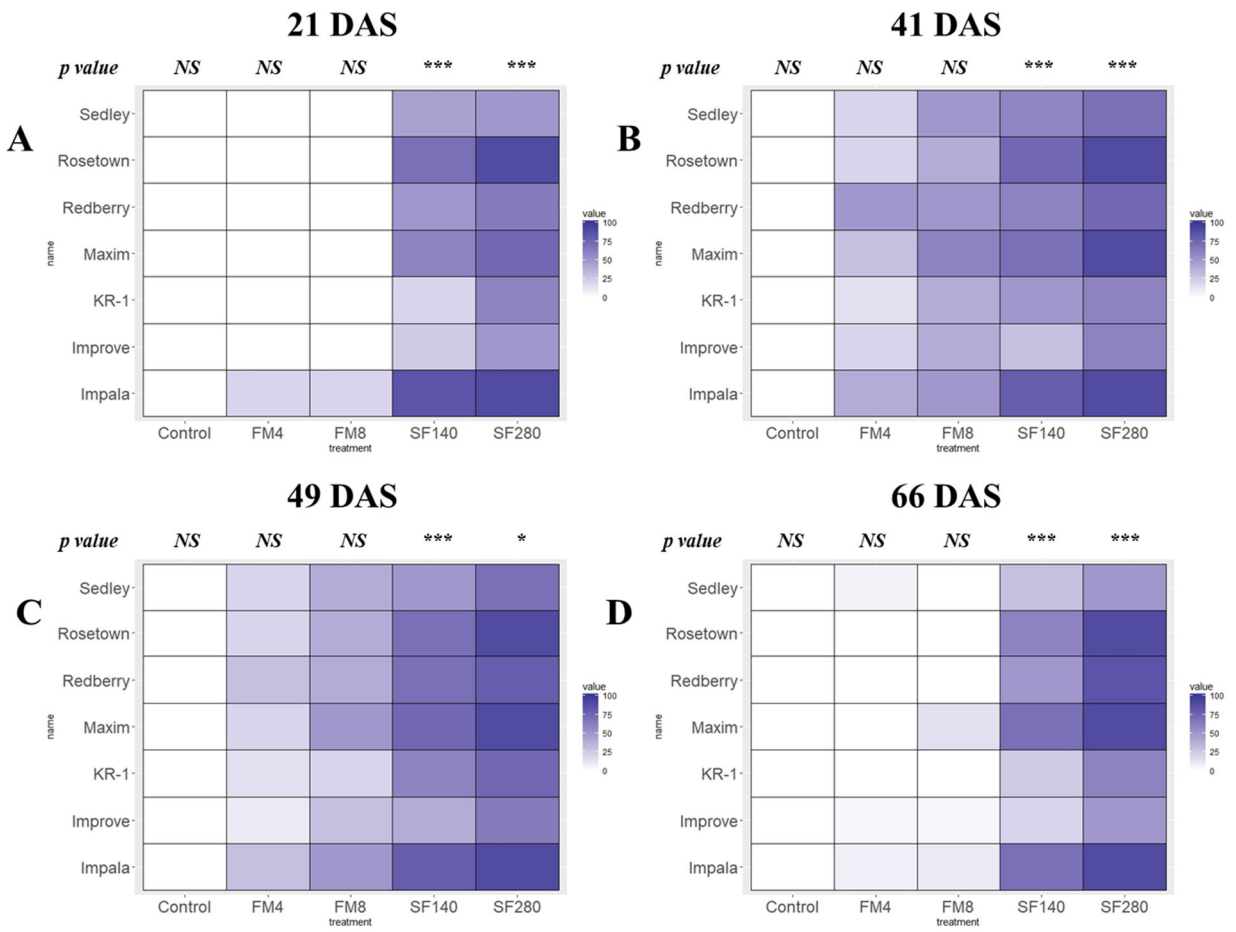



Figure 3.1. Injury ratings (scale: 0- white color to 100-dark purple) of seven lentil varieties subjected to five herbicide treatments at Saskatoon in 2011. A- 21 days after seeding (DAS); B- 41 DAS; C- 49 days DAS; D- 66 DAS. Treatments included Control: unsprayed; FM4: fluthiacet methyl 4 g.a.i $ha^{-1}$; FM8: fluthiacet methyl 8 g.a.i $ha^{-1}$; SF140: sulfentrazone 140 g.a.i $ha^{-1}$; SF280: sulfentrazone 280 g.a.i $ha^{-1}$.

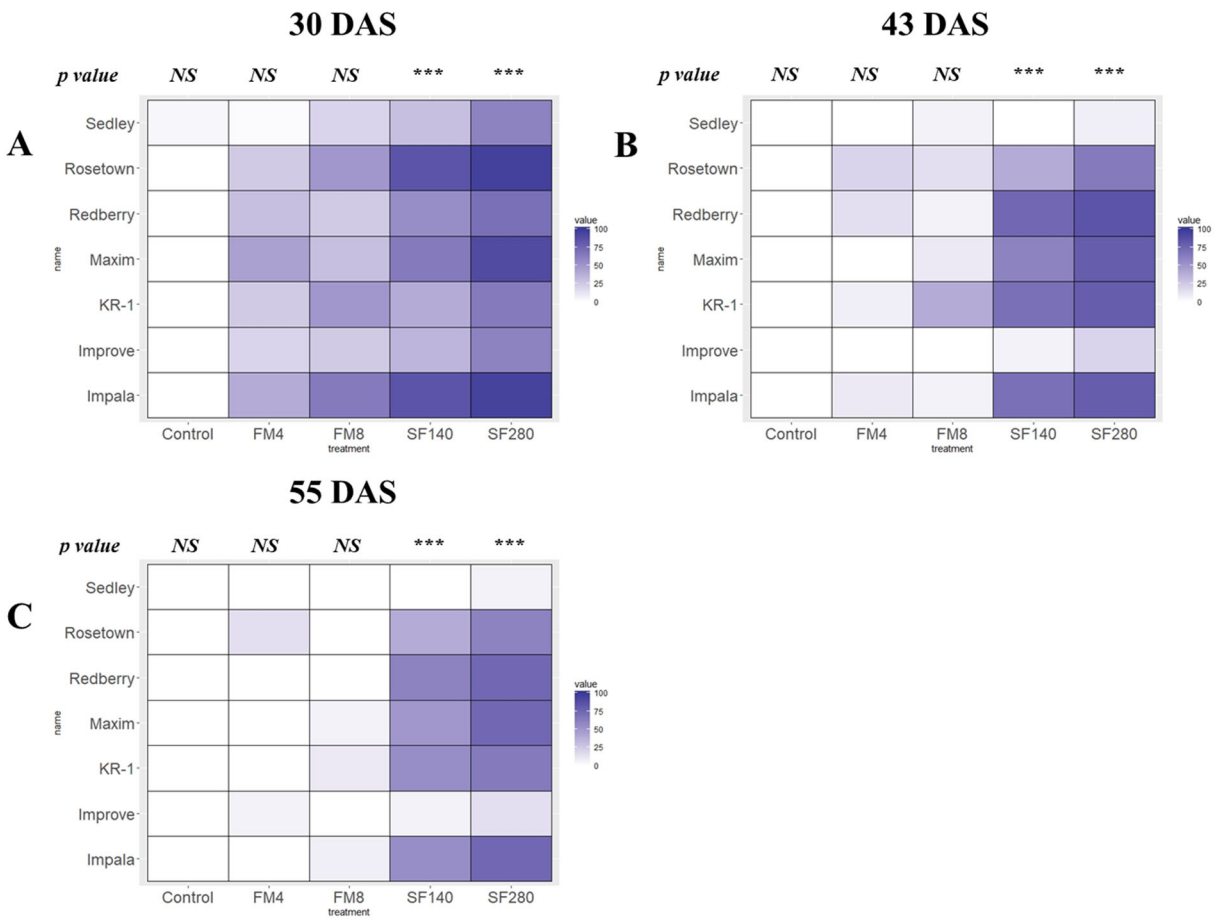



Figure 3.2. Injury ratings (scale: 0- white color to 100-dark purple) of seven lentil varieties and five herbicide treatments at Saskatoon location in 2012 collected: A- 30 days after seeding (DAS); B- 43 DAS; C- 55 days DAS. Treatments included: Control- unsprayed; FM4- fluthiacet methyl 4 g.a.i $ha^{-1}$; FM8- fluthiacet methyl 8 g.a.i $ha^{-1}$; SF140- sulfentrazone 140 g.a.i $ha^{-1}$; SF280- sulfentrazone 280 g.a.i $ha^{-1}$.

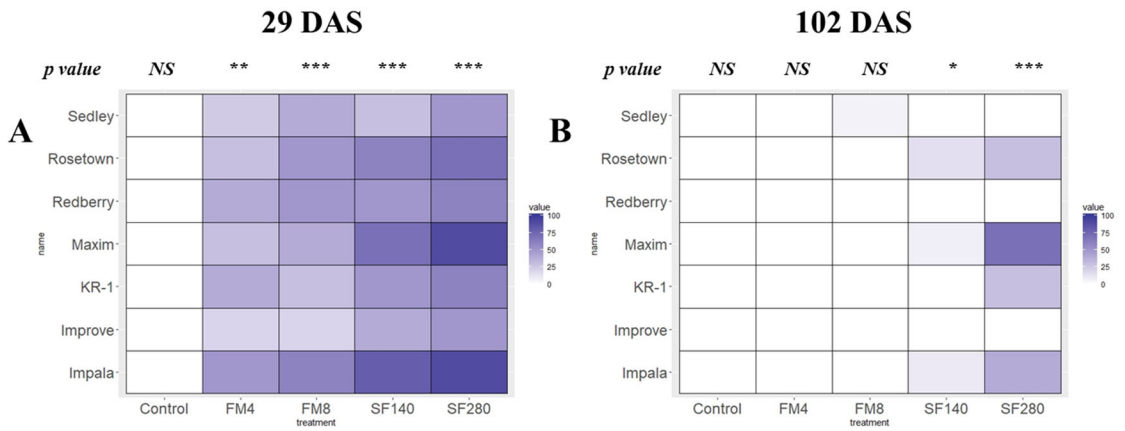



Figure 3.3. Injury ratings (scale: 0- white color to 100-dark purple) of seven lentil varieties and five herbicide treatments at Saskatoon location in 2016 collected: A- 29 days after seeding (DAS); B- 102 DAS. Treatments included: Control- unsprayed; FM4- fluthiacet methyl 4 g.a.i ha$^{-1}$; FM8- fluthiacet methyl 8 g.a.i ha$^{-1}$; SF140- sulfentrazone 140 g.a.i ha$^{-1}$; SF280- sulfentrazone 280 g.a.i ha$^{-1}$.

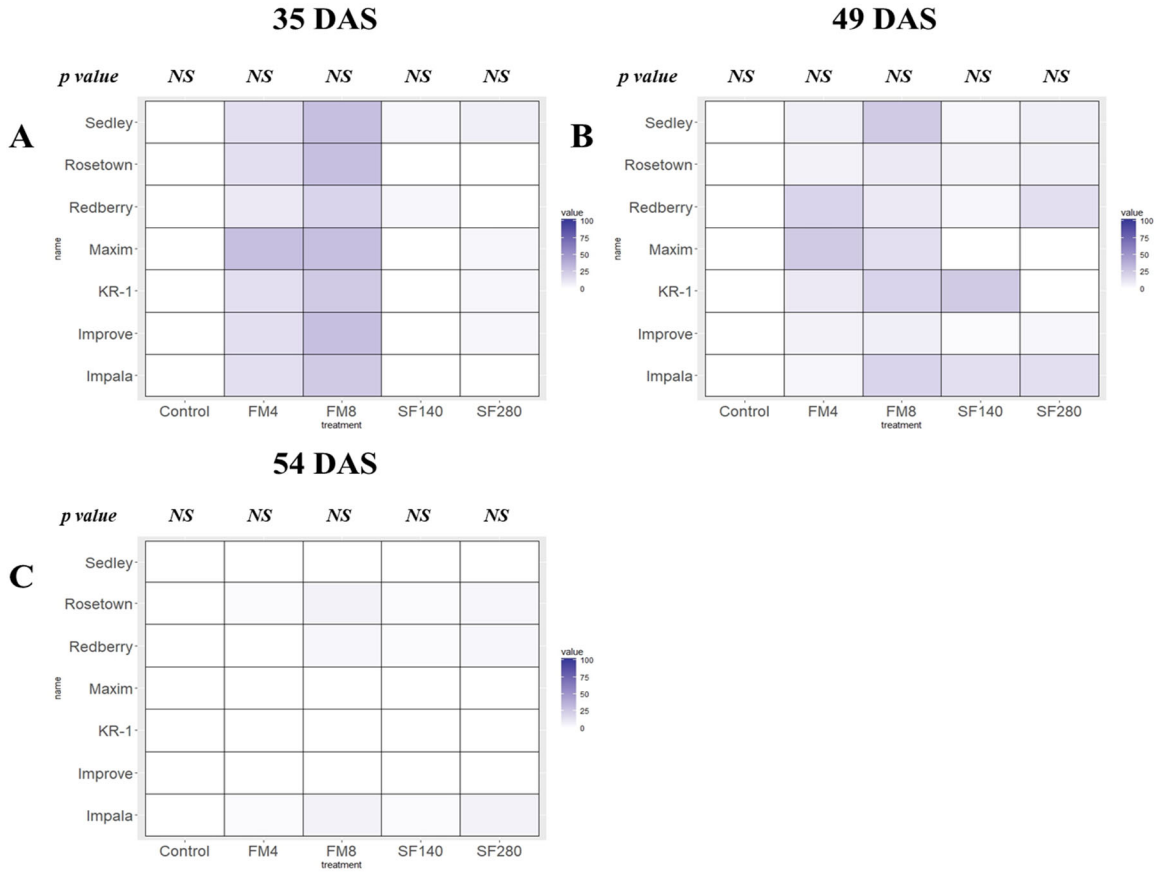



Figure 3.4. Injury ratings (scale: 0- white color to 100-dark purple) of seven lentil varieties and five herbicide treatments at Scott location in 2011 collected: A- 35 days after seeding (DAS); B- 49 DAS; C- 54 days DAS. Treatments included: Control- unsprayed; FM4- fluthiacet methyl 4 g.a.i ha$^{-1}$; FM8- fluthiacet methyl 8 g.a.i ha$^{-1}$; SF140- sulfentrazone 140 g.a.i ha$^{-1}$; SF280- sulfentrazone 280 g.a.i ha$^{-1}$.

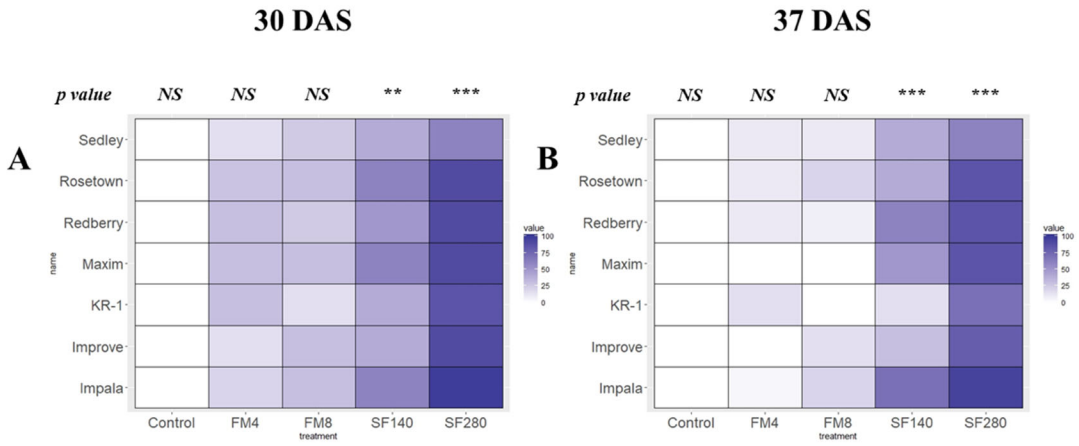



Figure 3.5. Injury ratings (scale: 0- white color to 100-dark purple) of seven lentil varieties and five herbicide treatments at Scott location in 2012 collected: A- 30 days after seeding (DAS); B- 37 DAS. Treatments included: Control- unsprayed; FM4- fluthiacet methyl 4 g.a.i $ha^{-1}$; FM8- fluthiacet methyl 8 g.a.i $ha^{-1}$; SF140- sulfentrazone 140 g.a.i $ha^{-1}$; SF280- sulfentrazone 280 g.a.i $ha^{-1}$.

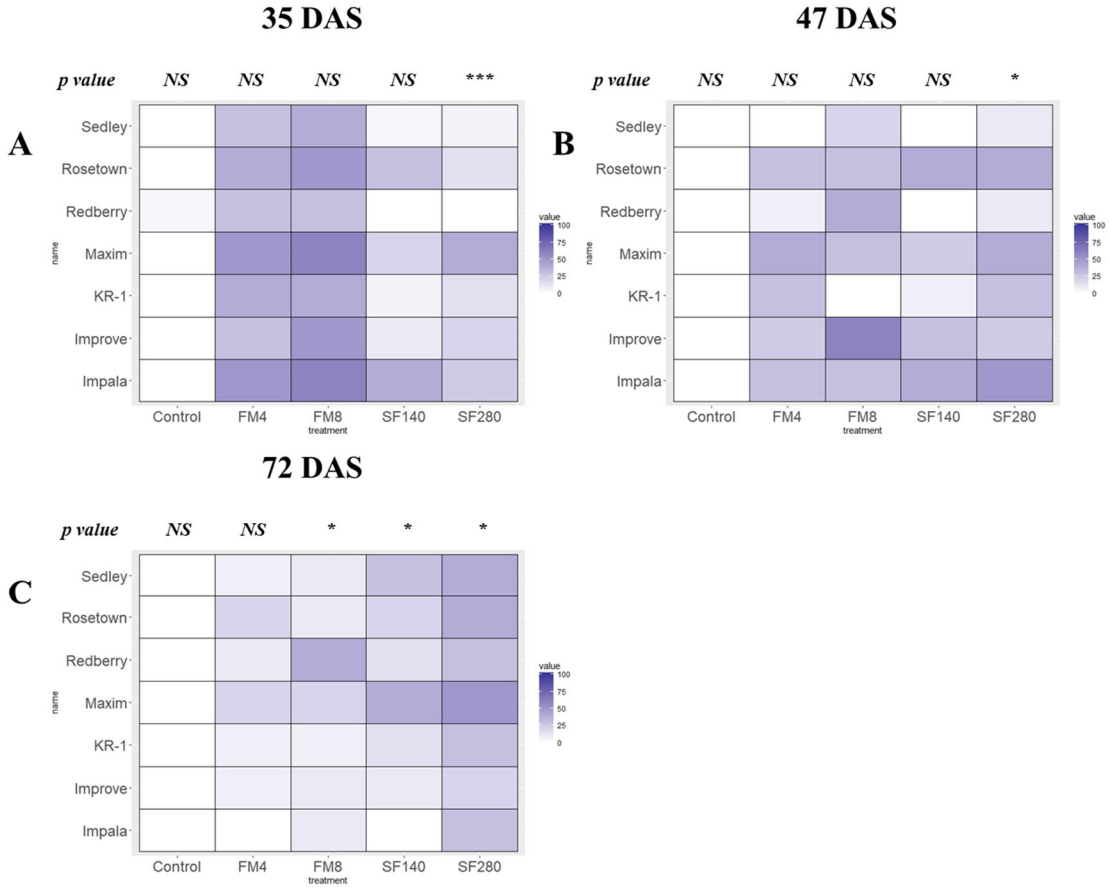



Figure 3.6. Injury ratings (scale: 0- white color to 100-dark purple) of seven lentil varieties and five herbicide treatments at Scott location in 2016 collected: A- 35 days after seeding (DAS); B- 47 DAS; C- 72 days DAS. Treatments included: Control- unsprayed; FM4- fluthiacet methyl 4 g.a.i $ha^{-1}$; FM8- fluthiacet methyl 8 g.a.i $ha^{-1}$; SF140- sulfentrazone 140 g.a.i $ha^{-1}$; SF280- sulfentrazone 280 g.a.i $ha^{-1}$.

A simple effects test expectedly revealed that the control treatment did not have a statistically significant effect on varieties in any site-year (Figures 3.1-3.6). For the most part, fluthiacet methyl treatments did not significantly affect varieties, with a single exception of early injury ratings at the Saskatoon location in 2016. However, the effect of sulfentrazone treatments on varieties was statistically significant ($p<0.05$) at the Saskatoon location in all tested years, and at Scott in 2012 and 2016. In the cases where sulfentrazone treatments had a statistically significant effect on varieties, CDC Impala and CDC Rosetown consistently had the highest injury ratings, while CDC Improve and CDC Sedley displayed the lowest injury. In those few cases of fluthiacet methyl treatments significantly affecting varieties, CDC Impala always had the highest injury rating.

Testing homogeneity of variance for the yield variable across site-years showed that residual variances were not the same in all site-years, requiring independent analyses of individual site-years. Table 3.6 shows the results of the mixed model ANOVA of genotype, herbicide treatment, and interaction effect between genotype and herbicide treatment on yield. Differences among varieties were highly significant in 2011 and 2016 at the Saskatoon location, and in 2011 at the Scott location. Differences between herbicide treatments were highly significant in all three years at the Saskatoon location, but only in 2016 at the Scott location. The interaction effect between genotype and herbicide treatment was highly significant in all three years at the Saskatoon location, but was not significant at the Scott location in either year.

Table 3.6 *F-values (with significance level) from the analysis of variance of the effects of herbicide and genotype on yield at Saskatoon and Scott in 2011, 2012 and 2016 for seven lentil varieties*

| | **Saskatoon** | | | **Scott** | | |
|---|---|---|---|---|---|---|
| | **2011** | **2012** | **2016** | **2011** | **2012** | **2016** |
| **Genotype** | 4.07** | $1.6^{NS}$ | 6.39*** | 4.17** | NA | $1.83^{NS}$ |
| **Herbicide** | 70.65*** | 112.03*** | 17.78*** | $1.11^{NS}$ | NA | 3.3* |
| **Genotype*Herbicide** | 2.89*** | 2.92*** | 3.45*** | $0.84^{NS}$ | NA | $1.14^{NS}$ |

***p <0.001; **p <0.01; p* < 0.05; $^{NS}$, not significant.

The effect of herbicide treatment on genotype was studied through a simple effects analysis. In all tested years at the Saskatoon location, fluthiacet methyl did not cause a significant decrease in yield among the tested varieties regardless of the rate (Figures 3.7-3.11). However, sulfentrazone

caused statistically significant yield decreases at the Saskatoon location. At 280 g.a.i $ha^{-1}$ of sulfentrazone, all tested varieties experienced decreased yield in 2011 and 2012 (Figure 3.7 and Figure 3.8), while in 2016 most varieties did decrease, with the exception of CDC Improve and CDC KR-1 (Figure 3.9).

CDC Improve was also the only genotype that did not experience a decrease in yield at the 140 g.a.i $ha^{-1}$ sulfentrazone rate in any of the three years tested at the Saskatoon location (Figures 3.7-3.9). Overall, effects of the 140 g.a.i $ha^{-1}$ sulfentrazone treatment varied across tested varieties in all three years at the same location, causing significant reduction in some cases, and not showing a decrease in yield relative to the control treatment in others. The largest decrease of yield at the Saskatoon location was observed in CDC Impala, while CDC Improve demonstrated the smallest. No significant decrease in yield was observed among different herbicide treatments at the Scott location in either 2011 or 2016, while in 2012 heavy rain and flooding led to the loss of experiment.

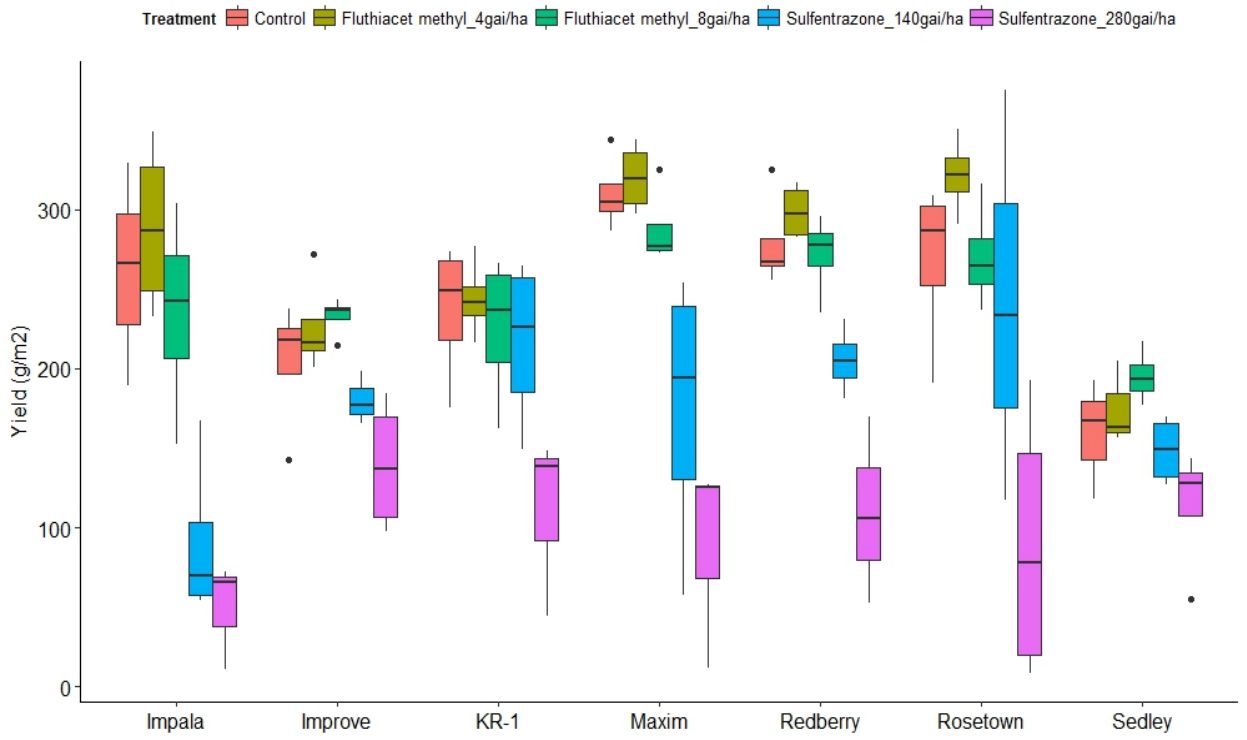



Figure 3.7. Yield ($g/m^2$) of seven lentil varieties (CDC Impala; CDC Improve; CDC KR-1; CDC Maxim; CDC Redberry; CDC Rosetown; CDC Sedley) grown at Saskatoon in 2011 and treated with fluthiacet methyl at 4 and 8 g.a.i $ha^{-1}$, sulfentrazone at 140 and 280 g.a.i $ha^{-1}$ and unsprayed control.

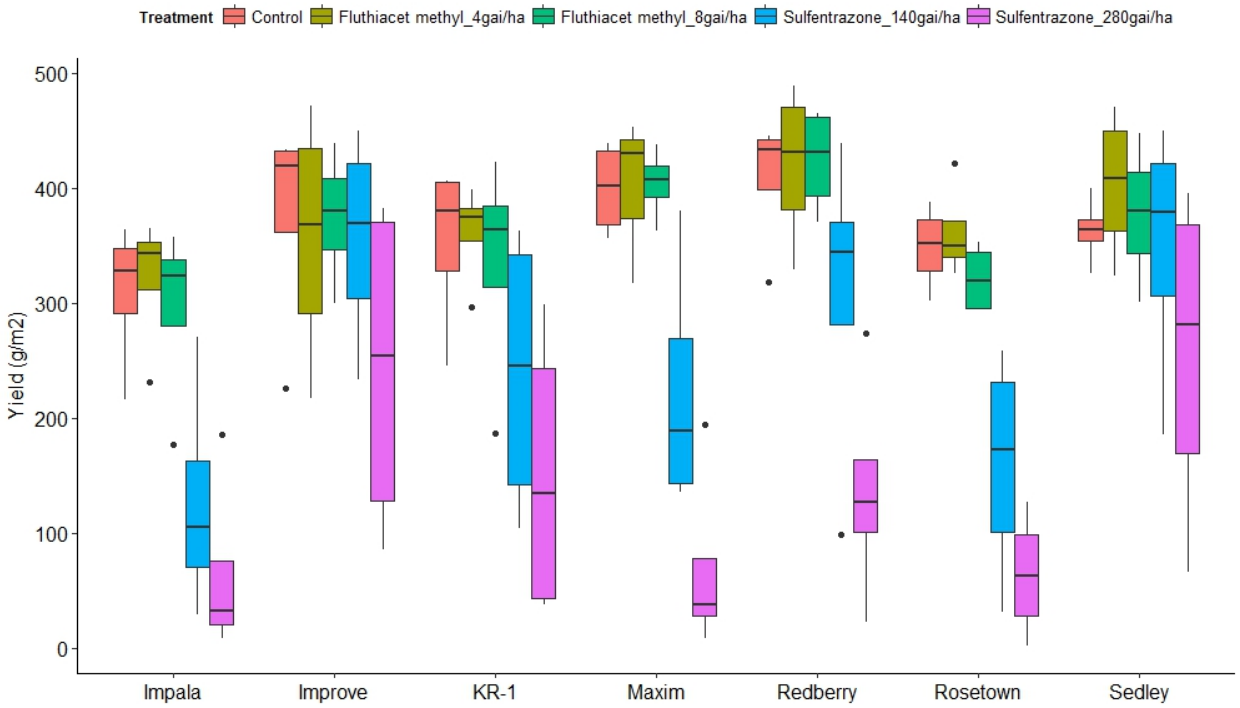



Figure 3.8. Yield ($g/m^2$) of seven lentil varieties (CDC Impala; CDC Improve; CDC KR-1; CDC Maxim; CDC Redberry; CDC Rosetown; CDC Sedley) grown at Saskatoon in 2012 and treated with fluthiacet methyl at 4 and 8 g.a.i $ha^{-1}$, sulfentrazone at 140 and 280 g.a.i $ha^{-1}$ and unsprayed control.

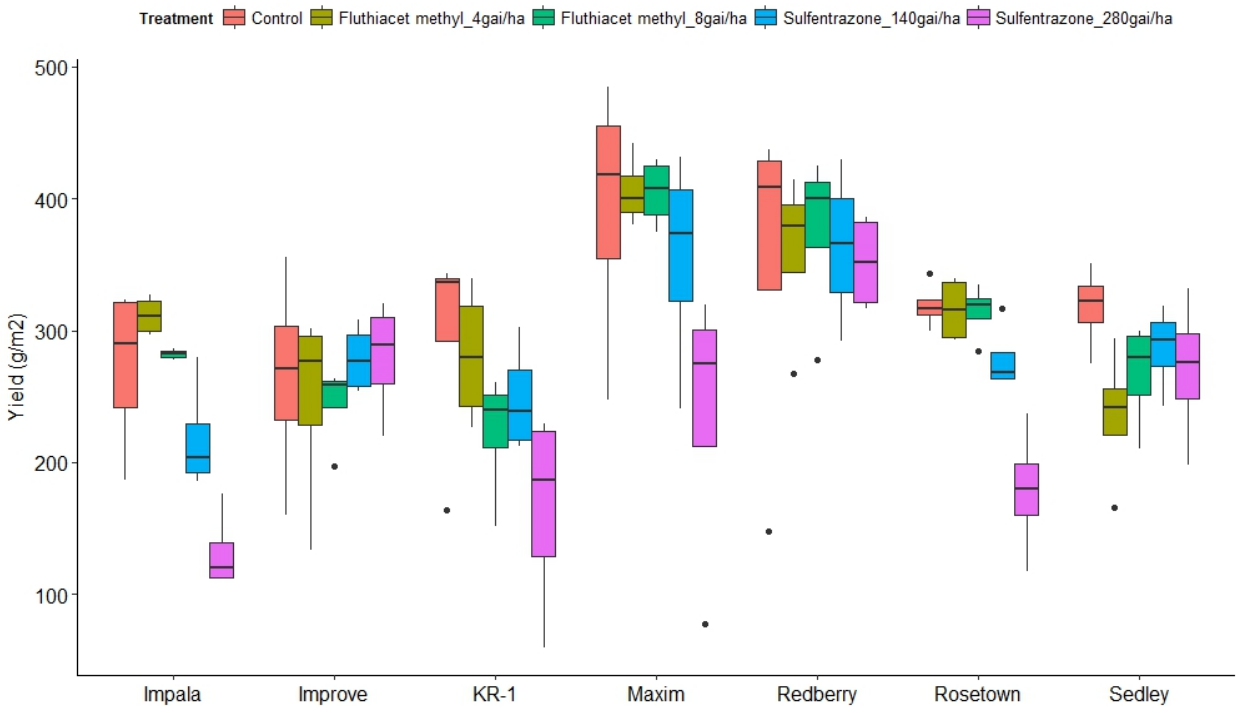



Figure 3.9. Yield ($g/m^2$) of seven lentil varieties (CDC Impala; CDC Improve; CDC KR-1; CDC Maxim; CDC Redberry; CDC Rosetown; CDC Sedley) grown at Saskatoon in 2016 and treated with fluthiacet methyl at 4 and 8 g.a.i $ha^{-1}$, sulfentrazone at 140 and 280 g.a.i $ha^{-1}$ and unsprayed control.

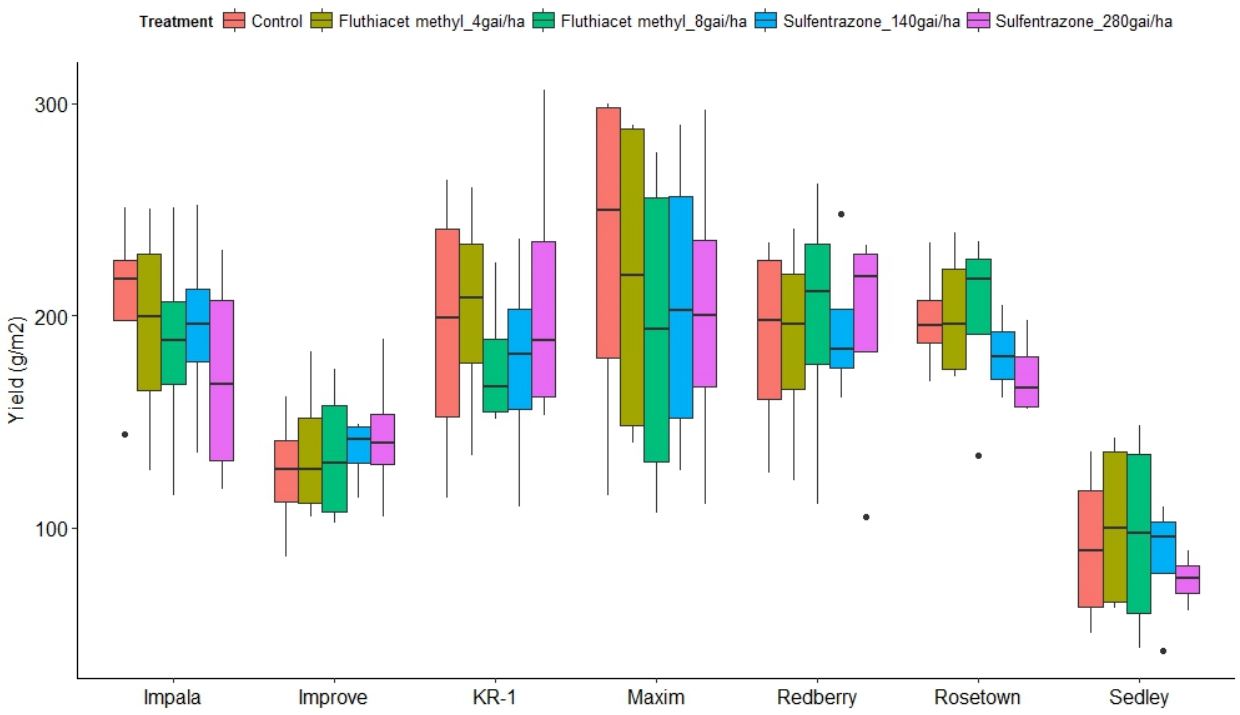



Figure 3.10. Yield (g/m$^2$) of seven lentil varieties (CDC Impala; CDC Improve; CDC KR-1; CDC Maxim; CDC Redberry; CDC Rosetown; CDC Sedley) grown at Scott in 2011 and treated with fluthiacet methyl at 4 and 8 g.a.i ha$^{-1}$, sulfentrazone at 140 and 280 g.a.i ha$^{-1}$ and unsprayed control.

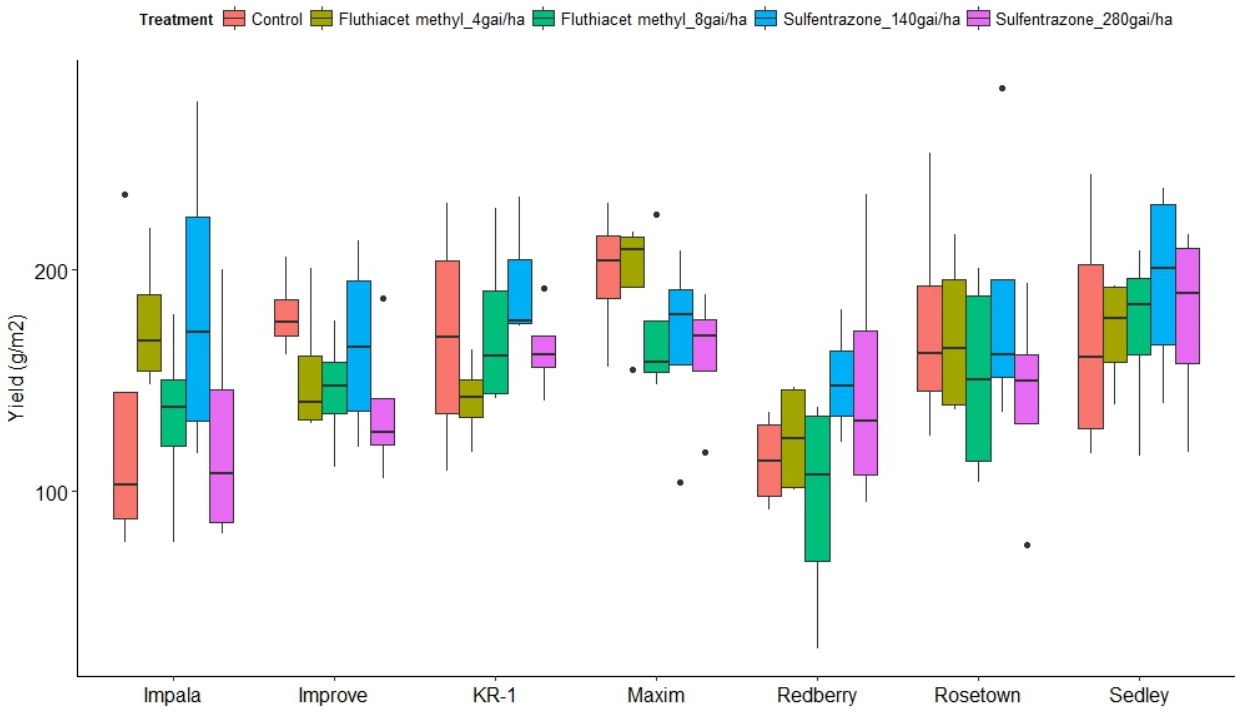



Figure 3.11. Yield (g/m$^2$) of seven lentil varieties (CDC Impala; CDC Improve; CDC KR-1; CDC Maxim; CDC Redberry; CDC Rosetown; CDC Sedley) grown at Scott in 2016 and treated with fluthiacet methyl at 4 and 8 g.a.i ha$^{-1}$, sulfentrazone at 140 and 280 g.a.i ha$^{-1}$ and unsprayed control.

### 3.3.1 Electrolyte leakage assay

The lack of fit test for the ELA data from the sulfentrazone treatment indicated that the four-parameter log-logistic model is suitable for describing the data ($p = 0.284$). Calculated curves are shown in Figure 3.12, and clearly show an increase in leakage starting 48 h after the beginning of the experiment. Estimated $T_{50}$ values ranged between 74.2 and 92.1 h. The lowest $T_{50}$ value was for CDC Impala, and the highest was for CDC Redberry. The t-test of $T_{50}$ values between varieties shows that differences between CDC Impala and CDC Rosetown are not statistically significant, the same was observed among CDC KR-1, CDC Sedley and CDC Improve (Table 3.7). In all other cases differences between varieties were statistically significant ($p< 0.01$).

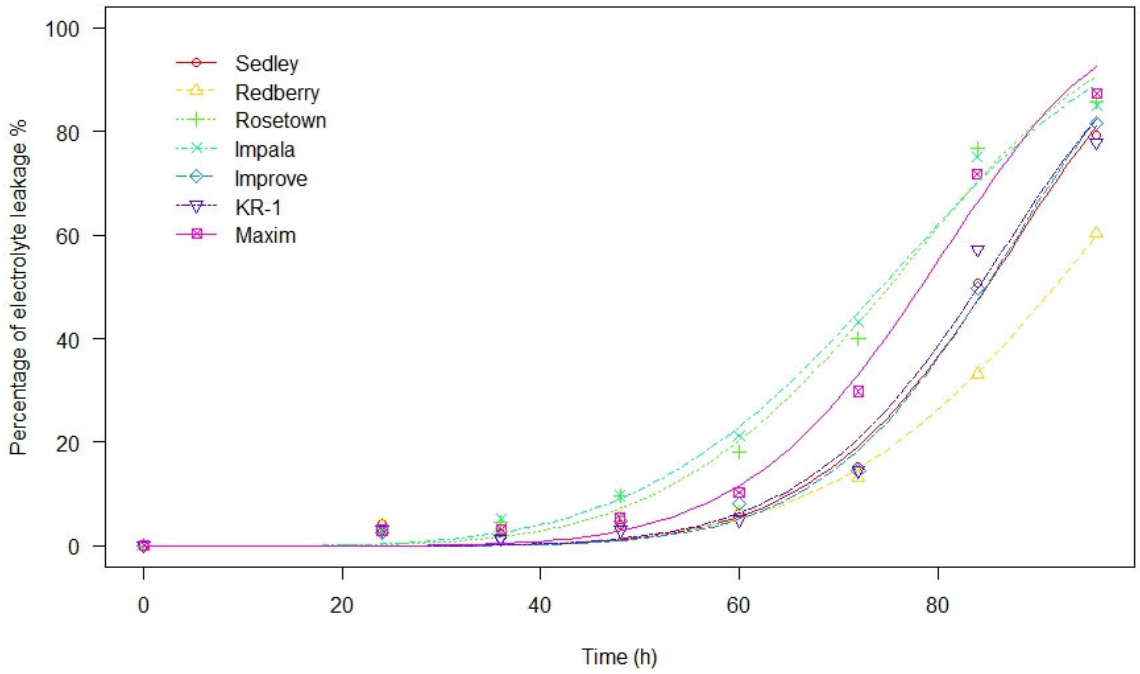



Figure 3.12. Percent electrolyte leakage of tissue samples of seven lentil varieties (CDC Sedley, CDC Redberry, CDC Rosetown, CDC Impala, CDC Improve, CDC KR-1 and CDC Maxim) for 96 h post exposure to 150 uM sulfentrazone.

Table 3.7 *$T_{50}$ value and standard error (SE) of electrolyte leakage assay for each of seven lentil varieties (CDC Impala, CDC Rosetown, CDC Maxim, CDC KR-1, CDC Improve, CDC Sedley and CDC Redberry) treated with 150 µM of sulfentrazone. T-value and significance level of paired t-tests between all tested varieties treated with 150 µM of sulfentrazone*

| | **CDC Impala** | **CDC Rosetown** | **CDC Maxim** | **CDC KR-1** | **CDC Improve** | **CDC Sedley** | **CDC Redberry** |
|---|---|---|---|---|---|---|---|
| **$T_{50}$ (SE)** | 74.2 (0.88) | 75.1 (0.81) | 78.1 (0.69) | 83.4 (0.69) | 84.6 (0.67) | 84.6 (0.7) | 92.1 (0.97) |
| **CDC Impala** | | -7.19$^{NS}$ | -3.53*** | -8.57*** | -9.81*** | -9.70*** | -1.53*** |
| **CDC Rosetown** | | | 2.76** | 7.33*** | 8.38*** | -9.33*** | 1.22*** |
| **CDC Maxim** | | | | 5.30*** | 6.52*** | -6.95*** | -1.32*** |
| **CDC KR-1** | | | | | 1.25$^{NS}$ | -1.25$^{NS}$ | -7.84*** |
| **CDC Improve** | | | | | | 3.87$^{NS}$ | -6.73*** |
| **CDC Sedley** | | | | | | | 6.11*** |
| **CDC Redberry** | | | | | | | |

***p <0.001; **p<0.01; $^{NS}$, not significant.

ELA data for the fluthiacet methyl treatment also fit a four-parameter log-logistic model, whose p-value, calculated in the lack of fit test, was 0.744. The response curves show an increase of leakage 40 h after the beginning of the experiment (Figure 3.13).

Calculated $T_{50}$ values among tested varieties varied between 61.7 and 76.6 h (Table 3.8). The lowest $T_{50}$ values were found in CDC Impala and the highest in CDC KR-1. CDC Impala, CDC Rosetown and CDC Sedley had no significant difference in their $T_{50}$ values and they grouped as a cluster of susceptible lines. CDC Improve and CDC Maxim clustered in a second group, with slightly higher $T_{50}$ than the first group; showing more tolerance than the first group. CDC KR-1 and CDC Redberry had the highest $T_{50}$ values of all tested varieties and as such had the highest level of tolerance.

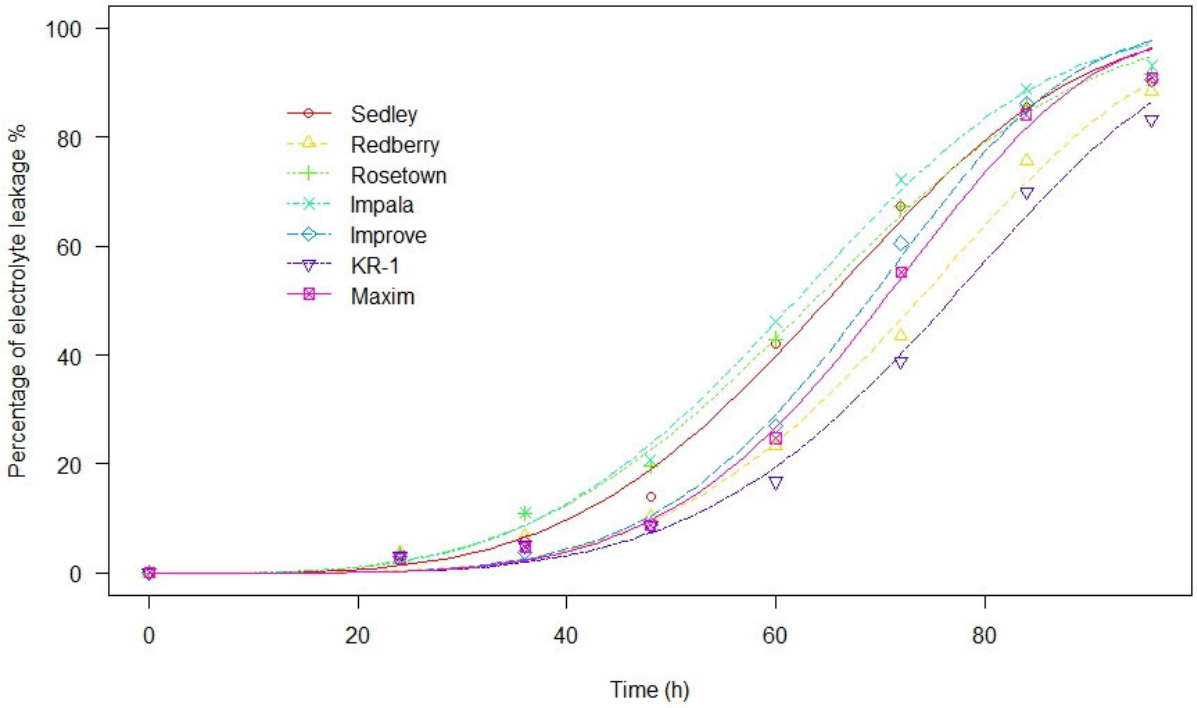



Figure 3.13. Percent electrolyte leakage of tissue samples of seven lentil varieties (CDC Sedley, CDC Redberry, CDC Rosetown, CDC Impala, CDC Improve, CDC KR-1 and CDC Maxim) for 96 h post exposure to 25 uM fluthiacet methyl.

Table 3.8 *$T_{50}$ value and standard error (SE) of electrolyte leakage assay for each of seven lentil varieties (CDC Impala, CDC Rosetown, CDC Maxim, CDC KR-1, CDC Improve, CDC Sedley and CDC Redberry) treated with 25 µM of fluthiacet methyl. T-value and significance level of paired t-tests between all tested varieties treated with 25 µM fluthiacet methyl*

| | **CDC Impala** | **CDC Rosetown** | **CDC Sedley** | **CDC Improve** | **CDC Maxim** | **CDC Redberry** | **CDC KR-1** |
|---|---|---|---|---|---|---|---|
| **$T_{50}$ (SE)** | 61.7 (0.98) | 63.5 (1.03) | 64.2 (0.93) | 68.4 (0.81) | 70.0 (0.83) | 73.9 (0.92) | 76.6 (0.90) |
| **CDC Impala** | | -1.28$^{NS}$ | -1.91$^{NS}$ | -5.53*** | -6.84*** | -9.77*** | -1.22*** |
| **CDC Rosetown** | | | -5.29$^{NS}$ | 3.60** | 4.65*** | 6.84*** | 8.52*** |
| **CDC Sedley** | | | | 3.32*** | 4.46*** | 6.84*** | 8.69*** |
| **CDC Improve** | | | | | -1.39$^{NS}$ | -4.60*** | -7.15*** |
| **CDC Maxim** | | | | | | -3.19** | 5.15*** |
| **CDC Redberry** | | | | | | | 2.08* |
| **CDC KR-1** | | | | | | | |

***p <0.001; **p <0.01; p* <0.05; $^{NS}$, not significant.

### 3.3.2 Dose response

Based on a lack of fit test, the four-parameter log-logistic model provided an acceptable description of the dose response data (p = 0.051). Figure 3.14 shows the dose response curves for both CDC Impala and CDC Improve. Parameters of the model with standard error values are provided in Table 3.9. The slopes, or B values, for the CDC Impala and CDC Improve were not statistically significant different. The $ED_{50}$ value for CDC Impala was calculated to be 7.17 g.a.i $ha^{-1}$ (SE = 0.92) of fluthiacet methyl, and the $ED_{50}$ value of CDC Improve was 20.38 g.a.i $ha^{-1}$ (SE = 2.39). According to a paired t-test these $ED_{50}$ values were significantly different from each other (p<0.001).

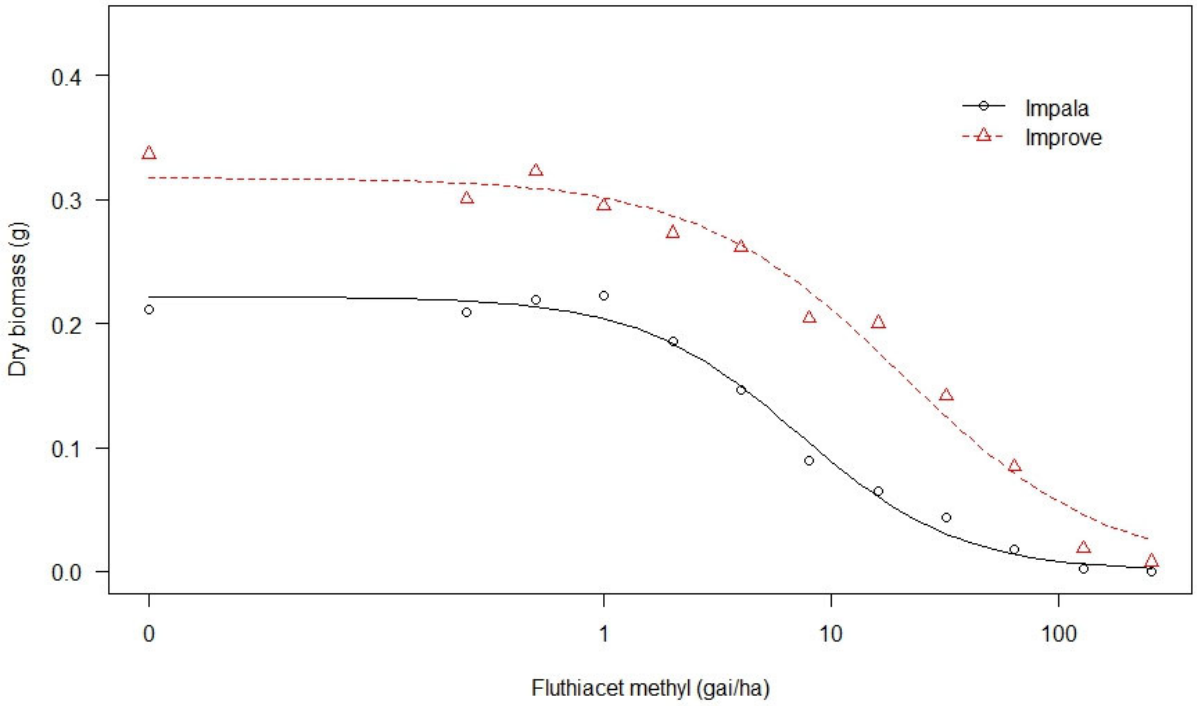



Figure 3.14. Responses of CDC Impala (black) and CDC Improve (red) to application of different rates of fluthiacet methyl (ranging from 0 to 256 g.a.i $ha^{-1}$) expressed as above-ground dry mass collected 14 days after treatment.

Table 3.9 *Parameters and standard errors (SE) from a three-parameter log-logistic model of dry weight of CDC Impala and CDC Improve treated with 12 different doses (0-256 g.a.i $ha^{-1}$) of fluthiacet methyl. B- slope; D- upper limit; E- dose resulting in 50% response i.e. $ED_{50}$*

| | **B (± *SE*)** | **D (± *SE*)** | **E (± *SE*)** |
|---|---|---|---|
| **CDC Impala** | 1.24 (0.15) | 0.22 (0.01) | 7.17 (0.92) |
| **CDC Improve** | 0.97 (0.09) | 0.32 (0.01) | 20.38 (2.39) |

### 3.4 Discussion

The combination of field testing and electrolyte leakage assay was performed to determine the existence and extent of the variation of the effect of sulfentrazone and fluthiacet methyl on a set of seven lentil varieties. The selected lentil varieties are some of the most commonly grown in Western Canada and represent major market classes of lentil (i.e. extra small red, small red, large red and large green). In the field experiment, the focus was on the effect of herbicide on the whole plant under field conditions in multiple environments. The focus of the electrolyte leakage assay was on the effects of the herbicides under controlled conditions on leaf tissue. In addition to these two experiments, a dose-response study with fluthiacet methyl was conducted, with a goal to clarify the field results.

Injury ratings (Figures 3.1-3.6) indicated that sulfentrazone (at 140 and 280 g.a.i $ha^{-1}$) can cause high levels of injury on all tested varieties. In the case of fluthiacet methyl (at 4 and 8 g.a.i $ha^{-1}$), initial injury ratings showed high levels of damage followed by recovery and a subsequent decrease in injury. The regrowth among damaged plants did lead to slight delay in lentil development. The recovery was more pronounced in the fluthiacet methyl treatment than in the sulfentrazone treatment, due to a shorter half-life. Differences in injury ratings between the varieties tested were more pronounced following sulfentrazone treatment, in contrast to the fluthiacet methyl treatments. The typical effect of sulfentrazone is damage to seedlings which can lead to delayed emergence and a reduced stand count (Taylor-Lovell et al., 2001). Sulfentrazone can have a long half-life in the soil of 100 to 280 d (FMC Corporation, 1989) and thereby can have a prolonged effect on lentil plants. For these reasons, injuries caused by sulfentrazone only showed modest recovery from the initial injury rating to the last. In contrast, the highest level of injury following fluthiacet methyl application was most visible for just 3-7 d. After 7 d, lentil plants already showed signs of recovery and, under favorable growing conditions, most injuries dissipated in 2-3 weeks in all varieties tested, which corresponds to findings in sorghum research (Reddy et al., 2014). In that study, symptoms of herbicide injury disappeared 3 weeks after herbicide treatment.

In the first year at the Scott location (2011), injuries caused by sulfentrazone were extremely low across all varieties, whereas at Saskatoon they were much higher. In the subsequent study years, 2012 and 2016, the injury caused by sulfentrazone at the Scott location increased, but remained

lower than the injury levels observed at the Saskatoon location. The differences in injury levels between locations could be linked to differences in soil structure, organic matter, and sand contentwhich vary greatly between the two locations (Table 3.2). Soil at Scott location was slightly acidic where at Saskatoon soil has higher pH value, and it was found that increased soil pH value leads to accumulation of sulfentrazone in a soil solution (Grey et al. 1997). As a soil-applied herbicide, sulfentrazone activity is highly dependent on the organic matter and clay content of the soil for potency (Szmigielski et al., 2012; Tidemann et al., 2014). Sulfentrazone decreases mobility in soil with high organic matter and high clay content (Szmigielski et al. 2009). The Scott location had less organic matter and less clay, but more sand compared to the Saskatoon location, so those, in addition to pH value, are probably key factors leading to different levels of damage caused by sulfentrazone.

Of the seven lines tested, CDC Impala generally had the highest injury rating for sulfentrazone treatments across all site-years; CDC Rosetown and CDC Maxim also showed elevated injury ratings in most site-years. On the other hand, CDC Improve had the lowest injury ratings for sulfentrazone (regardless of the rate) across most site-years, while CDC KR-1 and CDC Sedley had low injury ratings across several site-years. Statistical analysis of injury rating data collected for fluthiacet methyl treatment revealed only limited difference among varieties as only one site year produced significant differences among tested varieties. CDC Impala and CDC Rosetown are close genetic relatives, as are CDC Redberry and CDC Maxim, and CDC Sedley and CDC Improve, and the levels of injury between these pairs were very similar. The more susceptible lines CDC Impala, CDC Rosetown, and CDC Maxim, are small-seeded material while the larger seeded CDC Sedley, CDC KR-1 and CDC Improve were more tolerant. This suggests that seed size may play some role in the level of tolerance to sulfentrazone and fluthiacet methyl. A possible explanation is that the large-seeded plants have more stored energy in the seeds than small-seeded plants. They can use this stored energy to recovery more vigorously after herbicide application.

Injuries caused by both fluthiacet methyl rates tested differed across site-years. In every site-year it was observed that the level of injury decreased over time, meaning that recovery after herbicide damage did happen under these environmental conditions. Inconsistencies in the timing of data collection across site-years for fluthiacet methyl treatments greatly impaired comparison among site-years. Injuries caused by fluthiacet methyl were significantly different among tested varieties

in one instance: initial injury ratings (performed three days after spraying) in 2016 at the Saskatoon location. In this case, the line most injured was CDC Impala, while the line with the lowest injury rating was CDC Improve. Because of rapid recovery from exposure to fluthiacet methyl, future studies should pay extra attention to the timing of data collection. Exposure to fluthiacet methyl, at the rates tested, did not cause any yield penalties across site-years. This result corresponds to the injury rating data since injury symptoms decreased over time. Selected rates of 4 and 8 g.a.i $ha^{-1}$ of fluthiacet methyl were not high enough to cause permanent damage or death of plants among tested varieties.

In a study with soybean and multiple herbicides, it was found that injury ratings are not the best predictors of yield reduction (Hagood, Williams & Bauman, 1980). Instead of herbicide injury in early growth stages, a reduction of plant stand caused by herbicide exposure was a better indicator of impact on yield (Hagood et al., 1980). A similar pattern was observed here, where sulfentrazone did cause injuries, but it also caused the reduction in plant stand (data not shown) leading to yield loss, and fluthiacet methyl caused injury only in early growth stages but no yield reduction. Both sulfentrazone rates caused a significant reduction in yield for some varieties at the Saskatoon location, but not at Scott. Yield reduction caused by the application of 140 g.a.i $ha^{-1}$ sulfentrazone varied between varieties. The highest reduction was observed in CDC Impala and CDC Maxim, while CDC Improve did not show any yield reduction at that rate. At the higher rate of 280 g.a.i $ha^{-1}$, however, all varieties experienced a decrease in yield at the Saskatoon location in 2011 and 2012. This suggests that 140 g.a.i $ha^{-1}$ of sulfentrazone could potentially be used for some lentil varieties, but a rate of 280 g.a.i $ha^{-1}$ is unacceptable as it causes yield losses across all tested varieties. Those yield losses ranged from around 80% of control, in the case of CDC Impala, to 30% of control, in the case of CDC Improve and CDC Sedley. At Saskatoon in 2016, three lentil varieties (CDC Improve, CDC Sedley and CDC Redberry) did not have any yield loss for any herbicide treatment, suggesting again that the role of the environment is significant in the effectiveness of the herbicides. In 2011 and 2016, yields of the control treatments were significantly lower at Scott than at Saskatoon, this strongly implies that environmental effects played a significant role in yield reduction. In those cases, herbicide injury did not cause further impacton yield as it was already significantly reduced.

Electrolyte leakage assays are conducted under controlled conditions and are more suitable for testing the effect on specific plant organs such as roots, leaves or seedlings (Li et al., 2000). An ELA is a bioassay that has been used to test the impact of sulfentrazone on leaf tissue of soybean (Dayan et al., 1997) and effect of fluthiacet methyl on cotyledons of cotton (*Gossypium hirsutum*) and velvetleaf (*Abutilon theophrasti*) (Shimizu et al., 1995). This assessment leverages certain characteristics of group 14 herbicides which can induce rapid lipid peroxidation and disintegration of cell membranes and thereby influence the electro-conductivity of the sample. Conducting an ELA on leaf tissue determines how long leaf cells can withstand the effects of sulfentrazone or fluthiacet methyl over a given time frame. Varieties with high susceptibility to herbicides have a rapid increase in electro-conductivity, while those with higher tolerance can delay this increase in electro-conductivity. Comparing the reactions of the seven lentil varieties in the ELA helped estimate their relative levels of herbicide tolerance.

Modifications to the standard ELA were necessary for lentil, as the size of the leaflets did not allow collection of leaf disks. Instead, complete leaflets were used, and the uniformity of samples was achieved by keeping sample size constant at 250 mg. An advantage of leaflets in comparison to leaf disks is that they do not have a circumference of damaged tissue, only one point of damage where leaflets are detached. The non-linear modeling used here is a novel approach to analyzing ELA data. The time required for 50% damage ($T_{50}$) was used to efficiently assess the effects of herbicide on different varieties, and thereby compare levels of tolerance among varieties. A similar index is used in freezing-induced electrolyte leakage studies where temperature is used instead time (Lindén et al., 2000).

For sulfentrazone exposure, $T_{50}$ values ranged between 74.2 and 92.1 h. The most susceptible group included CDC Impala and CDC Rosetown. CDC Maxim was less susceptible, while CDC KR-1, CDC Improve, and CDC Sedley showed higher tolerance and CDC Redberry showed the highest tolerance of the tested varieties. Comparing ELA results to the field results shows consistency: CDC Impala and CDC Rosetown, followed by CDC Maxim proved to be the most susceptible in both experiments. CDC KR-1, CDC Improve, CDC Sedley and CDC Redberry showed signs of higher levels of tolerance in both experiments.

The ELA with fluthiacet methyl also revealed significant differences among the varieties tested, with $T_{50}$ values ranging from 61.7 to 76.6 h. The most susceptible varieties included CDC Impala,

CDC Rosetown, and CDC Sedley, while CDC Maxim and CDC Improve were somewhat less susceptible. CDC Redberry and CDC KR-1 were the most tolerant varieties. Since the field trial of fluthiacet methyl showed very limited differences among tested varieties, it is not possible to meaningfully compare those to the results of the ELA. Comparing results of the ELA conducted with sulfentrazone and fluthiacet methyl, it is possible to conclude that highly susceptible material for one herbicide is also highly susceptible for the other herbicide; i.e. CDC Impala and CDC Rosetown. CDC Redberry showed higher tolerance to both herbicides compared to the other varieties, for which no definite conclusion could be drawn. One possibility is that varieties do not share one uniform mechanism responsible for herbicide tolerance, rather they have different mechanisms which are sometimes more effective for one, but not for the other, herbicide. In the case of the most susceptible varieties, these mechanisms are absent or just not effective enough to provide meaningful tolerance to either of the herbicides.

A dose response study was performed in a controlled environment to provide insight into the levels of herbicide tolerance across two specific lentil varieties. Of the seven previously tested varieties, CDC Impala and CDC Improve were selected as contrasting outliers. Anchoring the analysis in the two extremes should make it easier to determine whether there are indeed discernable levels of tolerance between the varieties tested. The $ED_{50}$ value of CDC Impala was almost three times lower than that of CDC Improve. These results support the hypothesis that tolerance level to fluthiacet methyl varies among lentil varieties. Considering the field trial results in light of the dose response leads once again to the conclusion that rates of 4 g.a.i $ha^{-1}$ and 8 g.a.i $ha^{-1}$ fluthiacet methyl are not sufficient for effectively screening the levels of tolerance. An increase of fluthiacet methyl rate to 16 g.a.i $ha^{-1}$ should remedy this problem. This rate would likely induce a longer period of visible damage, thus potentially expanding the period for meaningful data collection. Dose response and ELA results were consistent with each other, with CDC Impala proving most susceptible to fluthiacet methyl, and CDC Improve incurring less damage.

Together, these three experiments demonstrate that phenotypic diversity to sulfentrazone and fluthiacet exposure does exist in lentil. All the tested varieties originated from a single breeding program suggesting that more extreme phenotypic reactions to herbicide treatments could perhaps be found in a wider lentil genepool. Further study is needed to better understand the underlying

causes of different reactions to both fluthiacet methyl and sulfentrazone and to unlock successful breeding of higher tolerance.

## Prologue to Chapter 4

Based on the results of the previous research chapter, where different levels of fluthiacet methyl tolerance were observed, a larger lentil collection was assembled to be studied. Results in this research chapter focus on identifying underlining causes for diverse levels of tolerance, by means of association mapping of different measures of damage caused by fluthiacet methyl and genomic data of 110 lentil genotypes. The experiments were conducted both under field conditions and in a controlled environment. In all the experiments, an unsprayed or control treatment was included and data together with results served as a reference for fluthiacet methyl results.

# 4. GENOME WIDE ASSOCIATION MAPPING OF FLUTHIACET METHYL TOLERANCE IN LENTIL GERMPLASM

## 4.1 Introduction

Lentil grown in the no-till systems of Western Canada is a weak competitor with weeds and producers rely heavily on herbicide application for successful production (Yenish et al., 2009; Fedoruk et al., 2011). Dependence on chemical weed control in current agronomic systems is becoming a serious pitfall to establishing long term sustainable lentil production in the region. Simultaneously, the number of herbicide resistant weeds is increasing at an alarming rate, putting additional pressure on the herbicides available for lentil production. Increasing the number of viable herbicide options would help mitigate these problems, especially if accompanied by the development of herbicide resistant germplasm.

Plant breeding has enabled the development of herbicide resistant crops, precipitating wide adoption by producers (Owen & Zelaya, 2005). In most cases, herbicide-resistant crops were developed through modification of a target-site (Duke, 2005). The main advantages of this approach include extremely high levels of herbicide resistance, and "simple genetic" or single gene-trait etiology, which enables easier incorporation of the resistance trait in plant breeding programs. A major downside of this approach is that a small number of herbicides are applied frequently over extended areas, increasing the negative impact on the environment and increasing the number of resistant weed species (Green & Owen, 2011). To reduce some of the negative effects of the previous generation of herbicide-resistant crops, seed companies are beginning to adopt a new approach, one involving stacking of multiple different target site resistance genes (Ainley et al., 2013).

In contrast to altered target site resistance, non-target site, or metabolic resistance is under complex genetic control (Powles & Yu, 2010). This type of resistance is used less frequently in breeding programs, largely due to its complexity and lack of understanding of the underlying mechanism.

However, several large enzyme families, such as cytochrome P450 monooxygenases and glutathione-S-transferases, are known to play a significant role in non-target site resistance (Yuan et al., 2007; Powles & Yu, 2010).

Fluthiacet methyl is a member of the group 14 herbicides (PPO inhibitors) with relatively few resistant weed species worldwide (Heap, 2018). It is a post-emergence, foliar-applied herbicide, but it is not currently registered for commercial use in Canada. Lentil genotypes have demonstrated differential response to it under field conditions (Holm et al., 2012). Analysis of the diversity of lentil cultivar responses to an herbicide could be exploited to identify genetic loci associated with the phenotypic variation. Once these loci are identified, they could be used in breeding programs to, for example, bundle a number of loci into a single genotype and thereby increase its herbicide resistance.

Regions of the genome responsible for phenotypic traits can be identified using different strategies, including bi-parental mapping and association mapping (Myles et al., 2009). Bi-parental mapping is more costly, requiring additional time to develop and establish mapping populations relative to association mapping (Myles et al., 2009). Association analysis is not commonly used in investigations of herbicide tolerance. There are only a few examples of association analysis and herbicide resistance discussed in the literature, including an exploration of glyphosate tolerance in a cotton (*Gossypiun hirsutum*) population, where shikimate accumulation and dry weight were screened across 202 accessions (Wang et al., 2016). The lack of broader interest in complex controls is due to the ready availability of solutions leveraging simple herbicide resistance traits, like Round-up Ready™, LibertyLink™ and Clearfield™.

The objective of the research described in this chapter was to investigate the genetic control of differential response to fluthiacet methyl across diverse lentil genotypes. To achieve this, multi-environment field trials were conducted, with phenotyping being based on injury ratings and normalized difference vegetation index (NDVI) measurements. The effect of fluthiacet methyl was also studied in a controlled environment using above ground dry biomass as the measurement of response. Sulfentrazone was included in the initial trials but omitted from further analysis. Due to environmental effects, like significant lack of precipitation, the efficacy of sulfentrazone in the field experiments varied dramatically over tested site-years, producing unreliable results. The indoor study did not include sulfentrazone due to technical difficulties related to uniform

application in a controlled environment. Therefore, none of the sulfentrazone results were included in the association study.

## 4.2 Material and Method

### 4.2.1 Plant Material

A total of 110 diverse lentil accessions, were used in the association studies. Among them were 33 cultivars from Crop Development Center, University of Saskatchewan, as well as 87 landraces collected across the world and provided by the USDA (United States Department of Agriculture) and ICARDA (International Center for Agricultural Research in the Dry Areas) germplasm banks. A detailed list of all accessions and countries of origin is given in Appendix A. Lentil lines were grown under field conditions in 2015 and 2016 at two locations: Saskatoon, SK (52°03'48.9"N 106°26'22.1"W) and Elrose, SK (51°17'55.2"N 107°58'47.5"W). Meteorological data for all site-years is provided in Table 4.1.

The experimental design was a split-plot, with herbicide treatment as the main plot and lentil genotypes as subplots. Genotypes were replicated four times and seeded in 1 $m^2$ micro plots. Seeding rate was 100 seeds per micro plot. Treatments were 16 g.a.i $ha^{-1}$ of fluthiacet methyl applied post-emergence at the 4-6 leaf stage using tractor sprayer, and an untreated control. The non-ionic surfactant Agral 90 was added to fluthiacet methyl spray solution at 0.25% v/v before spraying.

In the indoor experiment, the same lentil accessions were grown in a controlled environment chamber at University of Saskatchewan (Conviron, Controlled Environments Limited, Winnipeg, MB) using a split-plot design with five replications, where an experimental unit was 4 plants per 10 $cm^2$ pot filled with soilless mix (2:1 Sunshine Mix #3: Perlite, Sun Gro Horticulture, Canada). Benches on one side of the chamber were used for the control or unsprayed, and those on the other side were used for the sprayed samples. Treatments included unsprayed control and fluthiacet methyl treatment. Rate of fluthiacet methyl was 16 g.a.i $ha^{-1}$, it was mixed with an adjuvant (Agral 90 at 0.25 vol %) and application was performed with a single Even-Spray nozzle 8001 delivering 109 L/ha at 275 kPa in the spray cabinet  The chamber was set up using the following conditions: temperature of 21°C during the day phase and 18°C during the night phase; length of day was 18

h with a light intensity of 350 μmol/$m^2$s, while the night phase lasted 6 h. Seeds were scarified manually using sandpaper prior to planting to ensure uniform germination across all genotypes. Flood benches were set up to water two times a week and provide uniform watering pattern across all pots. Plants were fertilized twice: two weeks after seeding, and one week after spraying, using the recommended rate of 3 g/l 20:20:20 (N:P:K). The whole experiment was conducted twice.

Table 4.1 *Meteorological data (average temperature and precipitation) during growing seasons of 2015 and 2016 and historical averages for the period of 1980-2010 at Elrose and Saskatoon locations*

| | | Saskatoon | | | Elrose | | |
|---|---|---|---|---|---|---|---|
| | | 2015 | 2016 | Historical 1980-2010 | 2015 | 2016 | Historical 1980-2010 |
| **April** | Average temperature (°C) | 5.6 | 5.5 | 5.2 | 6.2 | 6.2 | 4.9 |
| | Precipitation (mm) | 21.1 | 3 | 21.8 | 11.6 | 0.4 | 19.5 |
| **May** | Average temperature (°C) | 10.1 | 13.7 | 11.8 | 10.3 | 13.3 | 11.3 |
| | Precipitation (mm) | 0.4 | 41.6 | 36.5 | 16.8 | 71.2 | 44.2 |
| **June** | Average temperature (°C) | 17.2 | 17.4 | 16.1 | 17.4 | 17 | 15.9 |
| | Precipitation (mm) | 13.6 | 49.7 | 63.6 | 34.9 | 108.7 | 57.1 |
| **July** | Average temperature (°C) | 19.4 | 18.7 | 19 | 18.7 | 18.2 | 18.2 |
| | Precipitation (mm) | 84.3 | 58.6 | 53.8 | 78.3 | 129.2 | 57.3 |
| **Aug** | Average temperature (°C) | 17.4 | 16.9 | 18.2 | 17.6 | 16.7 | 17.8 |
| | Precipitation (mm) | 45.2 | 70.2 | 44.4 | 53.7 | 102.5 | 41.1 |
| **Sept** | Average temperature (°C) | 11.9 | 11.8 | 12 | 12.1 | 12 | 11.5 |
| | Precipitation (mm) | 50 | 24.1 | 38.1 | 44.1 | 16.7 | 29.2 |

*Note*. Data extracted from http://climate.weather.gc.ca/ (accessed Jan. 26, 2019). Copyright (2017) by Government of Canada.

### 4.2.2 Genotypic Analyses

Single nucleotide polymorphism (SNP) genotypes of 110 accessions were extracted from a larger dataset developed by the Cook Lab at UC Davis using a restriction site associated DNA sequencing (RADSeq) protocol (Cook et al. unpublished). Briefly, Illumina sequencing libraries were prepared using genomic DNA extracted from all accessions. Two restriction enzymes—HindIII and NlaIII, were used to digest the genomic DNA. A number of steps, including ligation, cleanup of the adapters, fragment size checkup, and PCR for enrichment of libraries, were completed before sequencing. Sequencing was performed using an Illumina HiSeq400 at the University of California, Davis Genome Center. Reads were mapped to lentil assembly v0.8 using Burrows-Wheeler Aligner (BWA-MEM algorithm) with default mapping parameters (Li & Durbin, 2009). Polymorphisms were detected using the Genome Analysis Toolkit (GATK) pipeline (McKenna et al., 2010). All polymorphisms were filtered by applying hard filtering parameters of GATK Best Practices recommendations (DePristo et al., 2011; Van der Auwera et al., 2013). After quality filtering, a set of 6,779 SNPs was formed (Appendix I) and every SNP was present in 95% of lentil accessions. These were further processed, and positions of SNPs were remapped from the lentil v0.8 draft genome assembly to the v1.2 assembly, using the lentil genome position convert tool on the KnowPulse website (http://knowpulse.usask.ca/portal/posconvert).

The complete set of SNP data was sorted by chromosome location. Linkage disequilibrium (LD) was estimated for each chromosome independently. Calculation of pairwise $r^2$ values was performed using TASSEL v.5.2.40 (Bradbury et al., 2007). Linkage disequilibrium was estimated for loci on the same chromosome (intrachromosome) and for unlinked loci (interchromosome). Estimated $r^2$ values were plotted over genetic distance (kilobase pair). Locally weighted scatterplot smoothing curves were fitted using the LOESS procedure in SAS v9.4 software (SAS Institute, Cary, NC). The LOESS procedure was performed using second degree of local polynomials with the smoothing value of 0.7. The 95$^{th}$ percentile of the distribution of $r^2$ values for the unlinked markers was used to assess critical $r^2$ value (Breseghello & Sorrells, 2005). Calculation of the critical $r^2$ value was repeated five times using different sets of randomly selected unlinked loci, and an average value was calculated. The point where the LOESS curve intersects with the critical $r^2$ value for the first time was used to estimate LD. The rate of LD decay was calculated for individual chromosomes and $r^2$ values were plotted against the physical distance between

individual SNPs. LD value is defined by the intersection of the fitted LOESS curve, and the critical $r^2$ value.

STRUCTURE v 2.3.4 software (Pritchard et al., 2000) was used to evaluate population structure among lentil accessions and estimate the number of sub-populations. The analysis was performed using an admixture model. Both burn-in, and Markov Chain Monte Carlo, were set to 100,000, and number of replications was set to 5. K values ranging from 2 to 10 were tested to determine the number of sub-populations. The best K value was selected using the procedure recommended by Evanno, Regnaut, & Goudet (2005). Results of the initial K value analysis were uploaded to the STRUCTURE Harvester website (http://taylor0.biology.ucla.edu./struct_harvest/), where a ΔK value was calculated. The number of sub-populations was determined through an ad hoc statistic of ΔK. The probability of the K value for each accession was used to generate a Q matrix. In addition to the STRUCTURE analysis, a neighbor-joining tree was constructed using TASSEL v.5.2.40 (Bradbury et al., 2007). SPAGeDI software (Hardy & Vekemans, 2002) was used to generate a kinship matrix using the Loiselle et al. (1995) method. Negative values in the kinship matrix were set to zero, since the software used for association mapping requires this modification.

### 4.2.3 Phenotypic Analyses

In the field experiment, collection of phenotypic data began three days after fluthiacet methyl application. Injury ratings were collected 3 and 7 d after the treatment, using a rating scale from 1 to 10 (with 1 representing no injuries, and increments of one represent increase of 10% in damage till 10 representing plot wide plant death). NDVI data were collected using a hand-held GreenSeeker™ RT-100 (Trimble Navigation Unlimited, Sunnyvale, CA) at a 30 cm height above the canopy, recording reflectance at 660 nm and 780 nm. NDVI data were collected at four time points: NDVI_7DAT collected 7 days after fluthiacet methyl treatment (DAT); NDVI_14DAT collected 14 DAT; NDVI_21DAT collected at 21 DAT, and NDVI_28DAT collected at 28 DAT. Lentil plants with fewer than ~6-8 leaves were too small to detect NDVI values properly. The four time points were selected to ensure that both the peak of the injury and the subsequent recovery period were captured.

In the controlled environment study, phenotypic data consisted of above ground dry biomass measurements of whole plants, collected 14 days after herbicide application. Collected plant tissue

was dried at 80°C for 48 hours. Above ground dry biomass was collected at the pot level, and divided by the number of plants in each pot.

### 4.2.4 Statistical Analyses

Phenotypic data collected in the field experiments were analyzed using a mixed model in SAS v9.4 software (SAS Institute, Cary, NC), where year, location, treatment and genotype were considered fixed effects, and replication was treated as a random effect. Dry biomass collected in the indoor study was also analyzed using a mixed model, where repetition, (i.e. the first or second repetition of the indoor experiment), treatment and genotype were considered fixed effects, and replication within repetition was treated as a random effect. All phenotypic data (NDVI data, injury ratings from the field study, and dry weight data from the indoor study) were transformed using a Box-Cox transformation since they showed some deviation from normal distribution. Data transformation was conducted using the PROC TRANSREG function of SAS v9.4 (SAS Institute, Cary, NC).

Intraclass correlation coefficient (ICC) was calculated for each trait used in association mapping in order to assess the effect of sub-population on the trait. Sub-population information was that obtained using STRUCTURE. The following formula was used to calculate intraclass correlation coefficient:

$$ICC = \frac{\sigma^2(\mathrm{b})}{\sigma^2(\mathrm{b}) + \sigma^2(\mathrm{w})} \quad \ldots\ldots(4.1)$$

where $\sigma^2(\mathrm{w})$ is the pooled variance within a subpopulation, and $\sigma^2(\mathrm{b})$ is the variance of the trait between sub-populations.

### 4.2.5 Association Analysis

Trait-marker associations were performed in TASSEL v.5.2.40 (Bradbury et al., 2007) using a mixed linear model (MLM) and recorded injury ratings, NDVI values, and biomass. The MLM utilized genotypes, phenotypes, kinship matrix (K), and population structure (Q) to identify statistically significant associations. The phenotypic data of control treatment (i.e. NDVI and dry biomass) was also used as covariate in MLM model when analyzing corresponding fluthiacet methyl data. Only SNPs scored in at least 95% of the accessions, and that had a minor allele frequency (MAF) greater than 5%, were used. Association thresholds were based on Bonferroni-

corrected thresholds with α=1, α=0.5, and α = 0.05 used as the cut-offs. The threshold values for these cut-offs were 1.48 $e^{-4}$, 7.38 $e^{-5}$ and 7.38 $e^{-6}$ (or "−log10 P" values of 3.83; 4.13 and 5.13), respectively, they were calculated by dividing alpha value and the number of SNP markers used in analysis. The three alpha values (α=1, α=0.5, and α = 0.05) were used to enable investigation of even weak associations (Yang, Li & Bickel 2013; Khazaei et al., 2017).

## 4.3 Results

### 4.3.1 Linkage Disequilibrium and Population Structure

After filtering, the genotypic data consisted of 6,779 SNPs, with more than 850 SNP markers per chromosome. Chromosome 2 had the largest number of SNPs—1195, while the shortest chromosome, chromosome 3, had the fewest—865 (Table 4.2). The critical $r^2$ value in estimating LD or 95% percentile of the distribution of unlinked $r^2$ was calculated to be 0.131. It was used to assess linkage disequilibrium based on the intersection of estimated LOESS curve and the calculated critical $r^2$ value. Estimated LD ranged between 500 kbp for chromosome 1 and 1500 kbp for chromosome 5 (Table 4.2 and Appendix B).

Table 4.2 *Length (Mbp) of lentil chromosomes in the v1.2 assembly (obtained from KnowPulse website), number of SNPs per individual chromosome and estimated linkage disequilibrium for each chromosome based on set of 6,779 SNPs used in this study*

| **Chromosome** | **1** | **2** | **3** | **4** | **5** | **6** | **7** |
|---|---|---|---|---|---|---|---|
| **Size of chromosome (Mbp)** | 338.9 | 316.7 | 199.2 | 245.9 | 262.8 | 210.4 | 246.5 |
| **Number of SNPs** | 1039 | 1195 | 865 | 979 | 916 | 899 | 886 |
| **Linkage disequilibrium (Kbp)** | 500 | 700 | 800 | 1000 | 1500 | 1000 | 1000 |

The highest ΔK value from the STUCTURE analysis was reached at K=3, suggesting that there are 3 distinct sub-groups among the lentil accessions tested (Figure 4.1A). The largest subgroup (red in Figure 4.1) consisted of lines from temperate regions, including Canadian, European and South American accessions. The second largest group (blue in Figure 4.1) included Indian, Iranian,

Afghan accessions and a few of the Middle Eastern lines. The smallest group (green in Figure 4.1) was generally made up of Middle Eastern and North African lines. Seven accessions were found to be have high levels of admixtures (Appendix A). In these cases, accessions have a high proportion of two different sub-groups. The relationships among subgroups can be observed in the neighbor-joining (NJ) tree (Figure 4.1B) where branches are color coded based on the predominant subgroup.

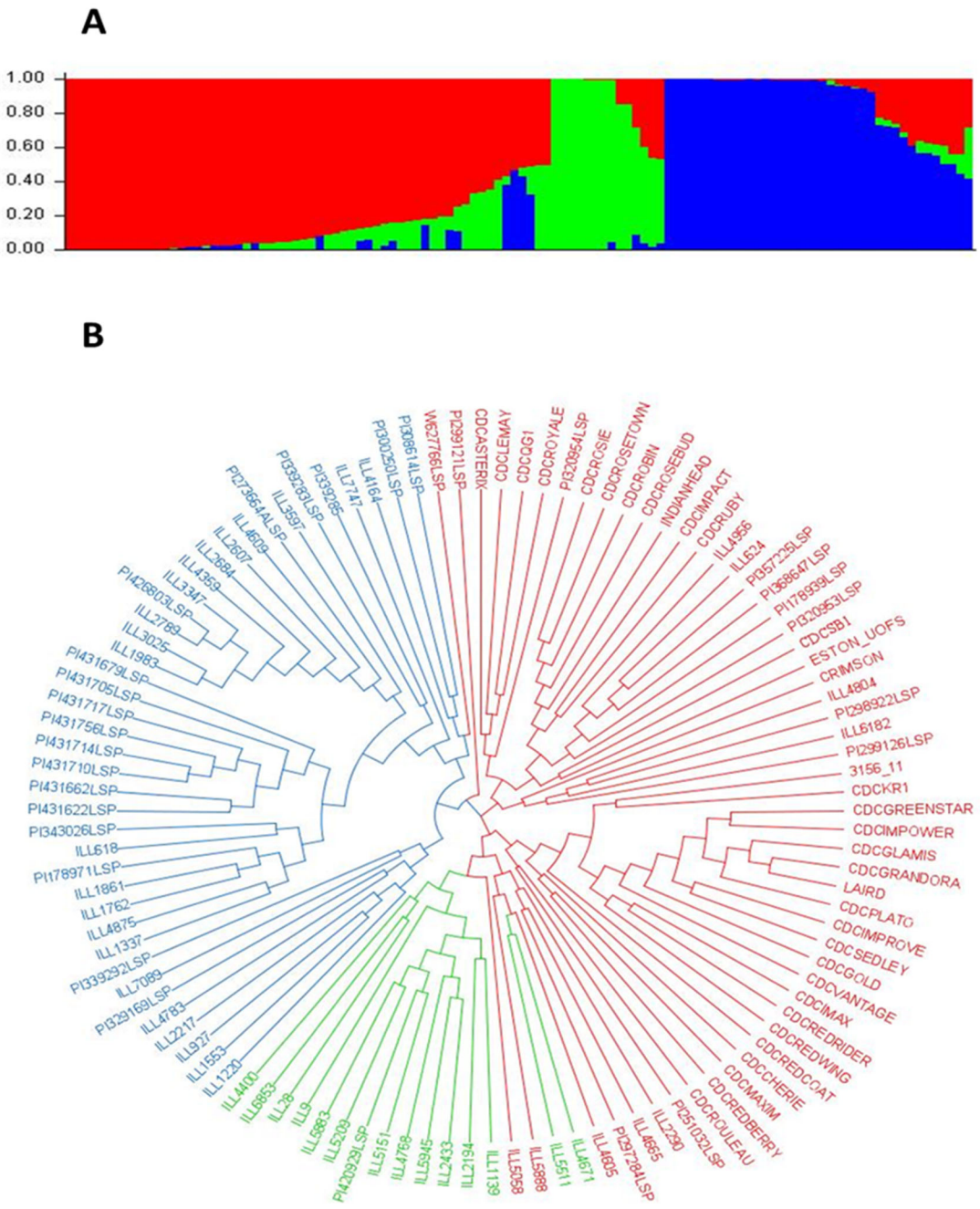



Figure 4.1. Relationship among 110 lentil accessions selected to represent global diversity of lentil germplasm. (A) STRUCTURE plot of the accessions with K = 3 clusters based on SNP data set; color composition reflects the probability of belonging to each of the three subpopulations defined by STRUCTURE. (B) Neighbor-joining tree (NJ tree), based on Nei (1972) standard genetic distance. Genotypes are coloured to match the STRUCTURE sub-groups of A.

### 4.3.2 Phenotypic Data

The mixed model analysis of variance showed that the effects of genotype was highly significant ($p>0.001$) for both injury ratings (Table 4.3). The year and location were set as fixed effect due to facet that only two years were analysed, same was in the case of locations. The mixed model usually requires five or even more levels for random intercept to achieve robust estimate of variance (Harrison et al. 2018). Year as a source of variance was significant ($p>0.001$) for both injury ratings. Location as sources of variance was significant ($p>0.001$) for injury rating collected 7 DAT, but not for injury rating collected 3 DAT. All of the tested interactions were also found to be significant ($p<0.01$) for both injury ratings.

Table 4.3 *F-values from the analysis of variance of the lentil injury ratings resulting from application of 16 g.a.i ha$^{-1}$ fluthiacet methyl at 4-6 leaf stage. Ratings were collected 3 and 7 day after treatment (DAT) at the Saskatoon and Elrose locations in 2015 and 2016.*

| Source | 3 DAT injury rating | 7 DAT injury rating |
|---|---|---|
| ***Year (Y)*** | 236.71*** | 1146.92*** |
| ***Location (L)*** | 0.16$^{NS}$ | 295.1*** |
| ***Genotype (G)*** | 9.69*** | 15.4*** |
| ***Y x L*** | 183.49*** | 58.86*** |
| ***Y x G*** | 2.43*** | 2.29*** |
| ***G x T*** | 1.83*** | 1.57*** |
| ***Y x L x G*** | 2.92*** | 1.4** |

*** $p<0.001$; ** $p<0.01$; $^{NS}$ not significant.

Injury ratings values across all genotypes can be seen in Figure 4.2. The highest mean injury ratings following fluthiacet methyl treatment were observed at the Elrose location in 2015. The overwhelming number of accessions had same injury rating of 7, on the scale of 0 to 10, in 2016 at Saskatoon location, leading to a flattened shape of the distribution (Figure 4.2).

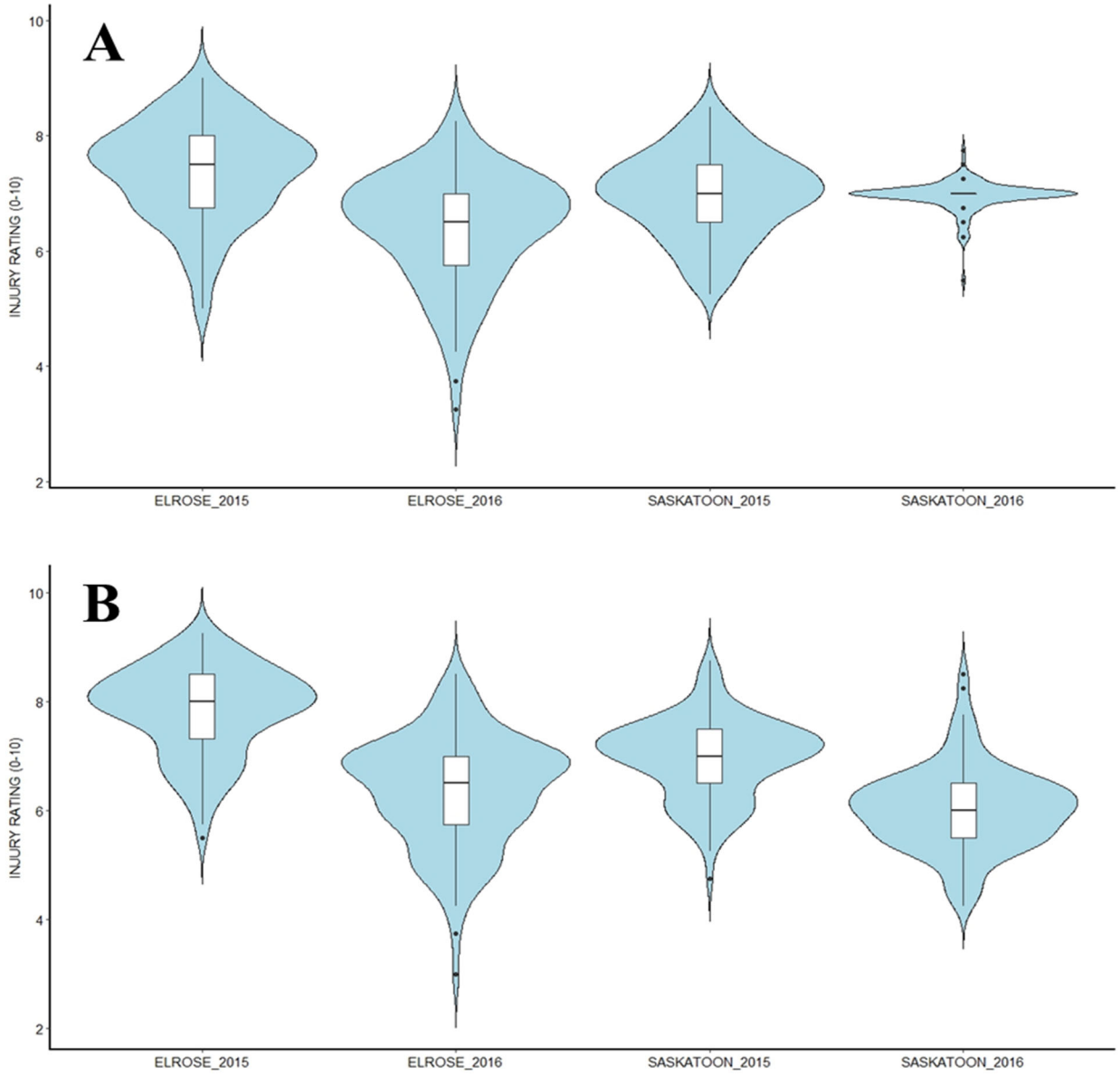



Figure 4.2. Distributions of injury ratings (on a scale of 1-no injury to 10-plant death) following fluthiacet methyl (16 g.a.i $ha^{-1}$) treatment across 110 lentil accessions, in 2015 and 2016 at Saskatoon and Elrose location A) 3 days and B) 7 days after treatment.

Analysis of variance of all four NDVI measurements showed that genotype, environment (both location and year), treatment, and all interactions between genotype, environment, and treatment,

had highly significant effects ( $p<0.01$), with a single exception of the interaction between location and treatment for NDVI at 14 DAT, which was not significant (Table 4.4).

Table 4.4 *F-values from the analysis of variance of NDVI data collected 7 days after treatment (DAT), 14 DAT, 21 DAT, and 28 DAT, for both control and 16 g.a.i ha$^{-1}$ fluthiacet methyl treatment in 2015 and 2016 at Saskatoon and Elrose*

| Source | NDVI 7 DAT | NDVI 14 DAT | NDVI 21 DAT | NDVI 28 DAT |
|---|---|---|---|---|
| ***Year (Y)*** | 3834.41*** | 3444.63*** | 2884.95*** | 904.7*** |
| ***Location (L)*** | 2356.8*** | 2976.33*** | 3349.08*** | 182.78*** |
| ***Treatment (T)*** | 255.08*** | 667.38*** | 411.25*** | 122.65** |
| ***Genotype (G)*** | 6.08*** | 7.68*** | 10.65*** | 10.27*** |
| ***Y x T*** | 6.8** | 16.22*** | 122.06*** | 177.38*** |
| ***L x T*** | 36.65*** | $0.61^{NS}$ | 40.92*** | 20.09*** |
| ***G x T*** | 1.52*** | 2.15*** | 2.53*** | 1.62*** |
| ***Y x L x T x G*** | 1.23*** | 1.48*** | 1.68*** | 1.36*** |

***p <0.001; **p <0.01; $^{NS}$, not significant

Comparisons of the distributions of NDVI values between control and fluthiacet methyl treatments across all accessions are provided in Figures 4.3 and 4.4. All fluthiacet methyl treatments displayed lower mean NDVI value than their corresponding controls. The range of NDVI values increased between the first and fourth measurement across both treatments, with the exception of Saskatoon in 2016 where the last NDVI measurement decreased in comparison to previous NDVI measurements.

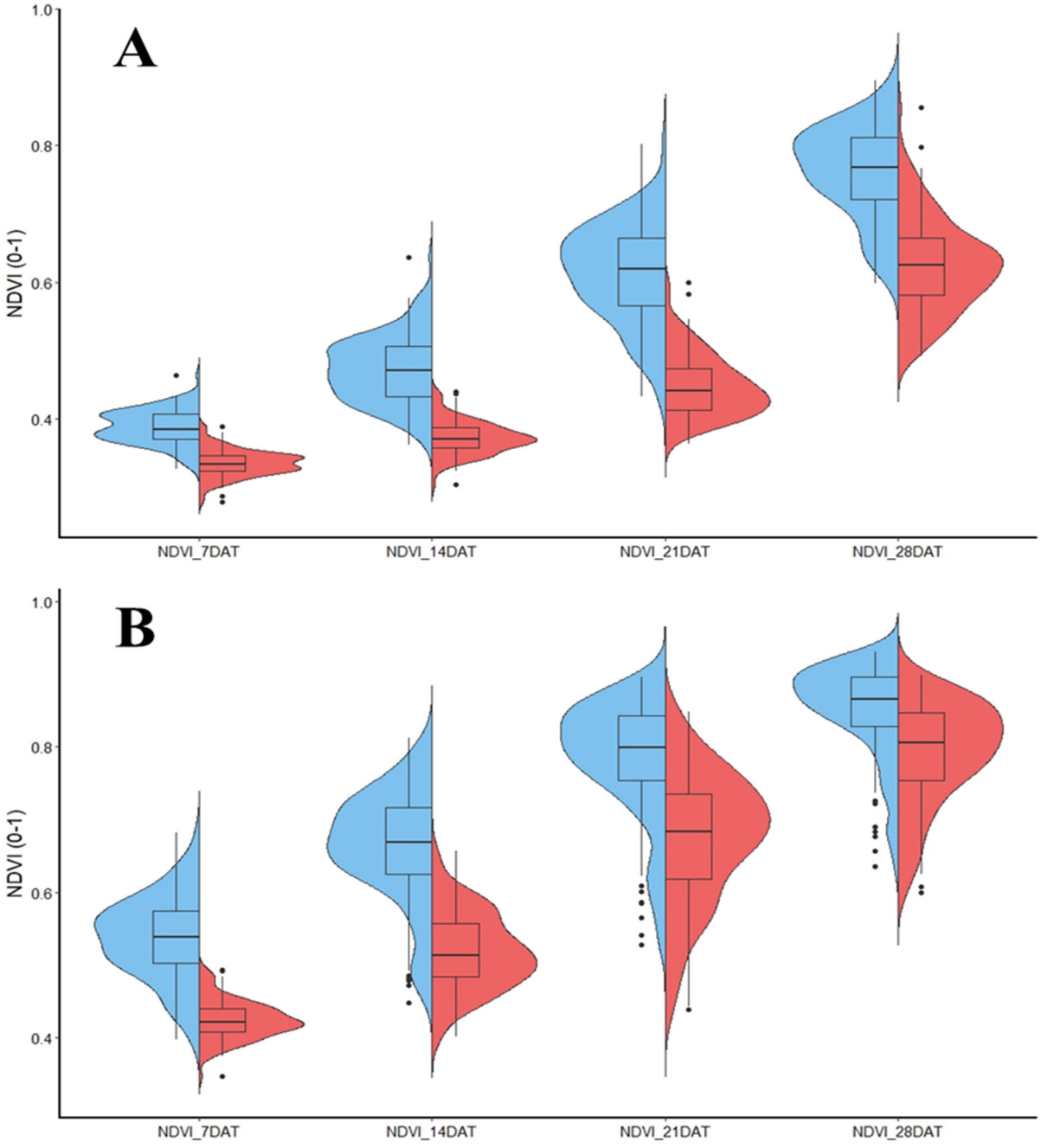



Figure 4.3 Distribution of NDVI values for control treatment (blue, left side) and fluthiacet methyl (16 g.a.i $ha^{-1}$) treatment (red, right side) at Elrose, A) in 2015 and B) in 2016, collected on 7 days intervals after treatment (7 DAT, 14 DAT, 21 DAT and 28 DAT).

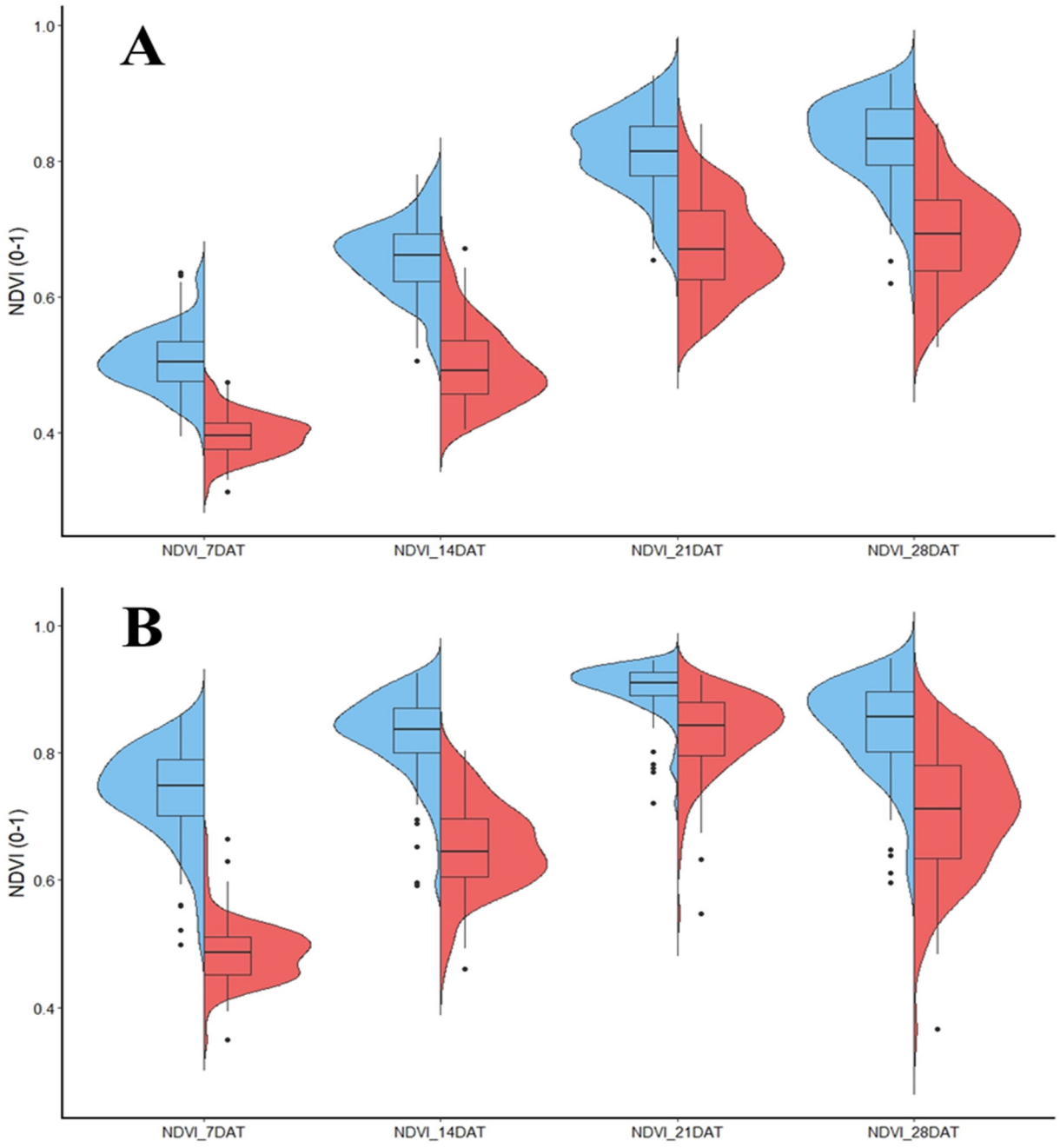



Figure 4.4 Distribution of NDVI values for control treatment (blue, left side) and fluthiacet methyl (16 g.a.i $ha^{-1}$) treatment (red, right side) at Saskatoon, A) in 2015 and B) in 2016, collected on 7 days intervals after treatment (7 DAT, 14 DAT, 21 DAT and 28 DAT).

Most hypothesized sources of variance were highly significant (p>0.001) based on the dry biomass data from controlled environment chambers, with the single exception of the interaction between experiment, treatment, and genotype (Table 4.5). The repetition of the experiment was highly significant (p>0.001), indicating that the data from the two iterations could not be merged and needed to be analyzed separately. The effect of the fluthiacet methyl treatment is visible in Figure 4.5, where the distribution of dry biomass data among all 110 accessions is given for both control and fluthiacet methyl treatment.

Table 4.5 *F-values from the analysis of variance (ANOVA) of dry biomass resulting from control and fluthiacet methyl treatments in a controlled environment collected 14 days after treatment*

| Source | F Value |
|---|---|
| ***Repeat (R)*** | 13.95*** |
| ***Treatment (T)*** | 2939.21*** |
| ***Genotype (G)*** | 21.04*** |
| ***R x T*** | 21.92*** |
| ***R x G*** | 1.84*** |
| ***T x G*** | 2.83*** |
| ***R x T x G*** | $1.18^{NS}$ |

*** p <0.001; $^{NS}$ not significant.

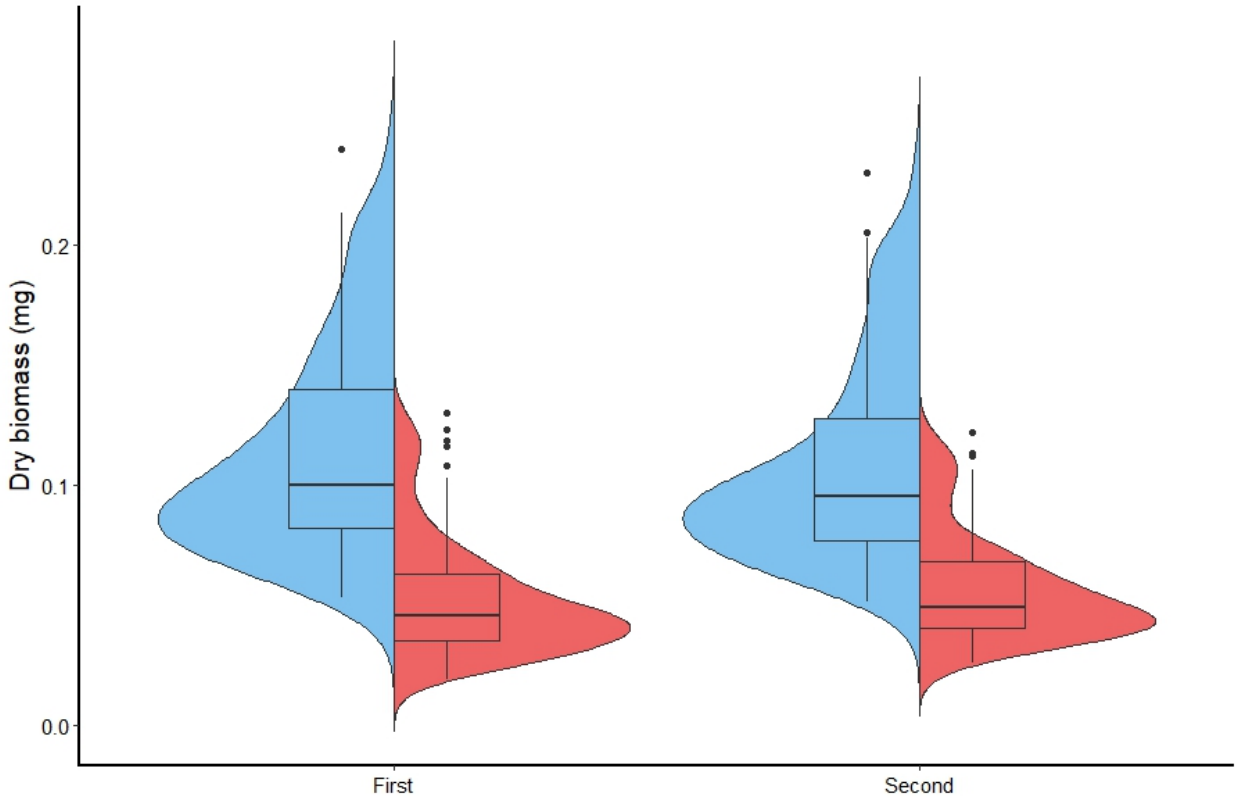



Figure 4.5. Distribution of dry biomass (mg) across 110 lentil accessions in the first run of the experiment and in the repetition under the same conditions. Control treatment results are shown on left side of the violin plots (blue), while fluthiacet methyl results occupy the right side (red).

Intraclass coefficient (ICC), which measures the proportion of variance explained by sub-population structure, ranged from 0.02 to 0.26 for all traits (Table 4.6). Among injury ratings, ICC values varied more for injury 3 DAT than for 7 DAT across all site years. NDVI values following fluthiacet methyl treatment had relatively low ICC, ranging between 0.01 and 0.18. Similar results were observed for the control treatment. ICC for dry biomass for both treatment was lower in the first repetition in comparison to the second repetition.

Table 4.6 *Intraclass coefficient calculated for injury 3 and 7 days after treatment (DAT); NDVI 7, 14, 21 and 28 days after treatment (DAT) and dry biomass following a fluthiacet methyl (16 g.a.i ha$^{-1}$) treatment or a control (untreated) at Saskatoon and Elrose in 2015 and 2016 or under controlled environment conditions.*

| Treatment | Trait | Saskatoon | | Elrose | | Controlled environment | |
|---|---|---|---|---|---|---|---|
| | | 2015 | 2016 | 2015 | 2016 | First repetition | Second repetition |
| **Fluthiacet methyl** | Injury 3 DAT | 0.22 | 0.02 | 0.07 | 0.16 | N/A | N/A |
| | Injury 7 DAT | 0.16 | 0.10 | 0.13 | 0.12 | N/A | N/A |
| | NDVI 7 DAT | 0.12 | 0.08 | 0.01 | 0.07 | N/A | N/A |
| | NDVI 14 DAT | 0.15 | 0.09 | 0.01 | 0.09 | N/A | N/A |
| | NDVI 21 DAT | 0.18 | 0.05 | 0.10 | 0.09 | N/A | N/A |
| | NDVI 28 DAT | 0.07 | 0.14 | 0.13 | 0.13 | N/A | N/A |
| | Dry Biomass | N/A | N/A | N/A | N/A | 0.10 | 0.20 |
| **Control** | NDVI 7 DAT | 0.15 | 0.13 | 0.01 | 0.09 | N/A | N/A |
| | NDVI 14 DAT | 0.08 | 0.11 | 0.03 | 0.09 | N/A | N/A |
| | NDVI 21 DAT | 0.15 | 0.07 | 0.04 | 0.09 | N/A | N/A |
| | NDVI 28 DAT | 0.10 | 0.22 | 0.09 | 0.06 | N/A | N/A |
| | Dry Biomass | N/A | N/A | N/A | N/A | 0.13 | 0.26 |

### 4.3.3 Genotype-Phenotype Associations

A total of five genotype to phenotype associations were detected across all injury rating data. One of these was significant at p <7.38 e$^{-5}$, while the remaining four were significant at p value <1.48 e$^{-4}$. At Elrose, only one association was detected in 2015, and at the Saskatoon (SPG) location four significant associations were detected, but only in 2016. None of the significant associations (p <1.48 e$^{-4}$) were detected across multiple site years. All significant associations were only between injury rating 7 DAT and the SNP markers. Most associations were localized on chromosome 7,

and only one was found on chromosome 6. The physical positions of all significant associations on the lentil genome are shown in Table 4.7. Percentage variation explained by the significant markers ranged between 3.7 and 4.5%. A list of candidate genes for each of the significant association is given in Table 4.7.

Table 4.7 *Name and position of single-nucleotide polymorphism markers found to be associated with injury rating data of fluthiacet methyl (16 gai/ha) treatment collected at 3 DAT and 7 DAT at Saskatoon and Elrose in 2015 and 2016), marker information include significance level (p value) and R² of MLM model; and name with position of candidate gene identified in proximity of marker*

| Site Year | Trait | Marker | Chromosome | Position | p value | Marker R² | Candidate gene | Position of candidate gene |
|---|---|---|---|---|---|---|---|---|
| Saskatoon 2016 | Injury (7 DAT) | SContig29662_147774 | 6 | 727282 | 9.36 $e^{-5}$ | 0.037 | *Glutathione S-transferase* | Chr6:446909..447923 |
| Saskatoon 2016 | Injury (7 DAT) | SContig582110_30365 | 7 | 21507364 | 1.38 $e^{-4}$ | 0.042 | *Hydroxyproline-rich glycoprotein family protein* | Chr7:21651719..21654192 |
| Saskatoon 2016 | Injury (7 DAT) | SContig350063_4336 | 7 | 147401005 | 8.70 $e^{-5}$ | 0.045 | *NADH-ubiquinone oxidoreductase 18 kDa subunit* | Chr7:147224470..147228183 |
| Saskatoon 2016 | Injury (7 DAT) | SContig350063_4350 | 7 | 147401019 | 1.27 $e^{-4}$ | 0.043 | *NADH-ubiquinone oxidoreductase 18 kDa subunit* | Chr7:147224470..147228183 |
| Elrose 2015 | Injury (7 DAT) | SContig86402_15872 | 7 | 192688078 | 1.04 $e^{-4}$ | 0.042 | *DUF1645 family protein* | Chr7:192809235..192809593 |

The total number of associations across all NDVI data sets for fluthiacet methyl treatment was 42. Of those, only one association was significant at p value < 7.38 $e^{-6}$; 18 were significant at p value <7.38 $e^{-5}$, and the remaining 23 associations were significant at p value <1.48 $e^{-4}$. The list of all significant associations is shown in Tables 4.8-4.11. The significant associations were detected across all lentil chromosomes, with chromosome 6 and 4 having the largest number of associations (Tables 4.8-4.11). The highest number of significant associations were detected at Saskatoon in 2016, a total of 33. The least number of significant associations were found at the Elrose location in 2015, with only one significant association. Among all detected associations, only two were found significant in different site years (i.e. under different environments). The most significant associations were detected 21 days after treatment (total of 30 associations), followed by 14 days after treatment (6 associations) and 28 days after treatment (4 associations), and least were found 7 days after treatment (only 2 associations). The percentage of variation explained by significant variation at a given locus ranged between 3.3 and 5.7% (Tables 4.8-4.11).

Analysis of NDVI data of control treatment yielded a larger number of significant associations in comparison to fluthiacet methyl. Of the 161 significant associations detected, 43 were significant at p value < 7.38 $e^{-6}$; 76 at p value < 7.38 $e^{-5}$; and the remaining 42 at p value <1.48 $e^{-4}$ across all site-years and all four time points (Appendix C). The significant associations are distributed over all lentil chromosomes, with the highest number of associations located on chromosomes 2 and 6. Distribution of significant associations across site years followed a similar trend as the phenotypic data of fluthiacet methyl treatment where most of association were found at Saskatoon in 2016 and least or in this case none were detected for the Elrose location in 2015. The number of associations found to be significant in more than one environment was 14 for all time points. Among time points, most associations were found 21 days after treatment, followed by 28 DAT, 14 DAT, and the least number of significant associations were detected 7 days after treatment. Among all significant associations (with p value <1.48 $e^{-4}$) there was only one case in which associations from fluthiacet methyl NDVI data were found in close proximity to control NDVI data (i.e. within the calculated LD for a given chromosome). The position of those associations is on the chromosome 4 with distance between them being around 100 Kbp.

Table 4.*8 Name and position in lentil genome of single-nucleotide polymorphism markers found to be associated with NDVI data of fluthiacet methyl (16 gai/ha) treatment collected 7 DAT at Saskatoon and Elrose in 2015 and 2016; marker information include significance level (p value) and R² of MLM model; and name with positon of candidate gene identified in proximity of marker*

| Site Years | Marker | Chromosome | Position | p value | Marker R² | Candidate gene | Position of candidate gene |
|---|---|---|---|---|---|---|---|
| Saskatoon 2016 | SContig378614_102129 | 3 | 109666586 | 9.39 $e^{-5}$ | 0.042 | *Glutathione S-transferase* | Chr3:109371484..109372069 |
| Saskatoon 2016 | SContig95764_36324 | 7 | 210759859 | 5.87 $e^{-5}$ | 0.044 | *Respiratory burst oxidase-like protein* | Chr7:210953830..210959492 |

Table 4.9 *Name and position in lentil genome of single-nucleotide polymorphism markers found to be associated with NDVI data of fluthiacet methyl (16 gai/ha) treatment collected 14 DAT at Saskatoon and Elrose in 2015 and 2016; marker information include significance level (p value) and R² of MLM model; and name with positon of candidate gene identified in proximity of marker*

| Site Years | Marker | Chromosome | Position | p value | Marker R² | Candidate gene | Position of candidate gene |
|---|---|---|---|---|---|---|---|
| Elrose 2016 | SContig631184_11980 | 1 | 299588979 | 1.23 $e^{-4}$ | 0.042 | *Auxin response factor* | Chr1:299671582..299675319 |
| Elrose 2016 | SContig92844_47071 | 2 | 292184876 | 1.46 $e^{-4}$ | 0.043 | *Heavy metal transport/detoxification superfamily protein* | Chr2:292357550..292358626 |
| Saskatoon 2016 | SContig490393_22580 | 6 | 3346772 | 1.34 $e^{-4}$ | 0.039 | *Cytokinin oxidase/dehydrogenase-like protein* | Chr6:3300065..3303925 |
| Elrose 2016 | SContig68057_42474 | 6 | 146245581 | 8.55 $e^{-5}$ | 0.036 | *Peptidoglycan-binding LysM domain protein* | Chr6:146233175..146233874 |
| Saskatoon 2016 | SContig68057_42474 | 6 | 146245581 | 8.71 $e^{-5}$ | 0.033 | *UDP-N-acetylmuramyl-tripeptide synthetase* | Chr6:146218849..146220344 |
| SPG 2015 | SContig680789_14381 | 7 | 43941616 | 1.47 $e^{-4}$ | 0.037 | *Peroxidase* | Chr7:43980254..43980532 |

Table 4.10 *Name and position in lentil genome of single-nucleotide polymorphism markers found to be associated with NDVI data of fluthiacet methyl (16 gai/ha) treatment collected 21 DAT at Saskatoon and Elrose in 2015 and 2016; marker information include significance level (p value) and R² of MLM model; and name with positon of candidate gene identified in proximity of marker*

| Site Years | Marker | Chromosome | Position | p value | Marker R² | Candidate gene | Position of candidate gene |
|---|---|---|---|---|---|---|---|
| Saskatoon 2016 | SContig46133_43627 | 1 | 183266109 | 9.44 $e^{-5}$ | 0.045 | *RPAP1-like* | Chr1:183256013..183258403 |
| Saskatoon 2016 | SContig12452_8414 | 1 | 210520781 | 3.65 $e^{-5}$ | 0.049 | *Membrane-associated kinase regulator-like protein* | Chr1:210526307..21052850 |
| Saskatoon 2016 | SContig244638_22167 | 1 | 217545632 | 5.27 $e^{-5}$ | 0.048 | *Cytochrome P450 family 88 protein* | Chr1:217334417..217337422 |
| Saskatoon 2016 | SContig106193_75109 | 2 | 119986397 | 8.09 $e^{-5}$ | 0.047 | *Calmodulin-domain kinase CDPK protein* | Chr2:120158146..120158900 |
| Saskatoon 2016 | SContig53877_147480 | 2 | 240826722 | 9.05 $e^{-5}$ | 0.046 | *Cysteine-rich TM module stress tolerance protein* | Chr2:241039134..241039800 |
| Saskatoon 2016 | SContig296184_20763 | 3 | 133833217 | 2.35 $e^{-5}$ | 0.044 | *Transcription factor* | Chr3:133832844..133835398 |
| Saskatoon 2016 | SContig5647_56536 | 3 | 156459987 | 7.55 $e^{-5}$ | 0.047 | *Cyclin-dependent kinase C* | Chr3:156489336..156493647 |
| Saskatoon 2016 | SContig367400_13394 | 4 | 51799058 | 6.67 $e^{-5}$ | 0.048 | *Cytochrome P450 71B37* | Chr4:51708147..51710470 |
| Saskatoon 2016 | SContig367400_13405 | 4 | 51799069 | 6.67 $e^{-5}$ | 0.048 | *Cytochrome P450 71B37* | Chr4:51708147..51710470 |
| Saskatoon 2016 | SContig114750_18556 | 4 | 99552593 | 7.85 $e^{-5}$ | 0.048 | *Cytochrome P450 family 94 protein* | Chr4:99976168..99977486 |
| Saskatoon 2016 | SContig248898_2191 | 4 | 120214589 | 8.61 $e^{-5}$ | 0.046 | *Drug resistance transporter-like ABC domain protein* | Chr4:120187767..120195135 |
| Saskatoon 2016 | SContig248898_2192 | 4 | 120214590 | 1.34 $e^{-4}$ | 0.044 | *Cytochrome P450 family protein* | Chr4:120160774..120163591 |
| Saskatoon 2016 | SContig39430_73910 | 4 | 140558414 | 4.56 $e^{-5}$ | 0.049 | *Transducin/WD40 domain protein* | Chr4:140560806..140571102 |
| Saskatoon 2016 | SContig99188_53097 | 4 | 193985126 | 6.98 $e^{-5}$ | 0.047 | *Squamosa promoter-binding-like protein* | Chr4:193967582..193973198 |
| Saskatoon 2016 | SContig3058_128932 | 4 | 195921613 | 2.84 $e^{-5}$ | 0.052 | *Glycosyltransferase* | Chr4:196021949..196023400 |
| Saskatoon 2016 | SContig785617_23951 | 5 | 30061738 | 5.10 $e^{-5}$ | 0.039 | *Transcription initiation factor TFIID protein* | Chr5:30057166..30076786 |
| Saskatoon 2016 | SContig78006_13867 | 5 | 70658872 | 1.85 $e^{-6}$ | 0.057 | *Glycosyltransferase family protein* | Chr5:70669546..70671272 |
| Saskatoon 2016 | SContig504595_69465 | 5 | 213416469 | 8.64 $e^{-5}$ | 0.045 | *Terpene cyclase/mutase family member* | Chr5:213394384..213396166 |
| Elrose 2016 | SContig568407_14973 | 5 | 231562023 | 2.29 $e^{-5}$ | 0.049 | *52 kDa repressor of the inhibitor of the protein kinase* | Chr5:231535936..231536683 |
| Saskatoon 2016 | SContig125888_50268 | 6 | 23440078 | 3.13 $e^{-5}$ | 0.051 | *Malic enzyme* | Chr6:23555676..23558363 |
| Saskatoon 2016 | SContig103254_43291 | 6 | 63812430 | 8.23 $e^{-5}$ | 0.045 | *Cytochrome P450 family protein* | Chr6:64216655..64222008 |
| Saskatoon 2016 | SContig22835_44865 | 6 | 82558956 | 4.06 $e^{-5}$ | 0.050 | *Cytokinin receptor histidine kinase* | Chr6:82459237..82461073 |
| Elrose 2016 | SContig309707_65640 | 6 | 83940163 | 9.03 $e^{-5}$ | 0.044 | *Cell division FtsZ-like protein* | Chr6:83896535..83907594 |
| Saskatoon 2016 | SContig309707_65640 | 6 | 83940163 | 4.59 $e^{-5}$ | 0.049 | *Cell division FtsZ-like protein* | Chr6:83896535..83907594 |
| Saskatoon 2016 | SContig278796_18084 | 6 | 101612487 | 2.99 $e^{-5}$ | 0.050 | *DUF674 family protein* | Chr6:101610794..101612230 |
| Saskatoon 2016 | SContig61069_171944 | 6 | 106619748 | 1.15 $e^{-4}$ | 0.045 | *Glutathione S-transferase* | Chr6:106701098..106702289 |
| Saskatoon 2016 | SContig61069_171952 | 6 | 106619756 | 1.44 $e^{-5}$ | 0.043 | *Glutathione S-transferase* | Chr6:106701098..106702289 |
| Saskatoon 2016 | SContig118855_18037 | 6 | 110550476 | 3.50 $e^{-5}$ | 0.051 | *Glutathione S-transferase* | Chr6:110973236..110974237 |
| Elrose 2016 | SContig68057_42474 | 6 | 146245581 | 2.01 $e^{-5}$ | 0.042 | *Peptidoglycan-binding LysM domain protein* | Chr6:146233175..146233874 |
| SPG 2015 | SContig32030_10818 | 7 | 5941348 | 1.36 $e^{-4}$ | 0.040 | *Cytochrome P450 family 71 protein* | Chr7:5995932..6001177 |

Table 4.11 *Name and position in lentil genome of single-nucleotide polymorphism markers found to be associated with NDVI data of fluthiacet methyl (16 gai/ha) treatment collected 28 DAT at Saskatoon and Elrose in 2015 and 2016; marker information include significance level (p value) and R² of MLM model; and name with positon of candidate gene identified in proximity of marker*

| Site Years | Marker | Chromosome | Position | p value | Marker R² | Candidate gene | Position of candidate gene |
|---|---|---|---|---|---|---|---|
| Saskatoon 2016 | SContig99188_53097 | 4 | 193985126 | 1.07 $e^{-4}$ | 0.042 | *Squamosa promoter-binding-like protein* | Chr4:193967582..193973198 |
| Saskatoon 2016 | SContig3058_128932 | 4 | 195921613 | 1.10 $e^{-4}$ | 0.042 | *Glycosyltransferase* | Chr4:196021949..196023400 |
| Saskatoon 2016 | SContig78006_13867 | 5 | 70658872 | 1.18 $e^{-4}$ | 0.034 | *Glycosyltransferase family protein* | Chr5:70669546..70671272 |
| Elrose 2015 | SContig54517_17097 | 6 | 115629026 | 1.11 $e^{-4}$ | 0.043 | *Peroxidase* | Chr6:115158172..115162233 |



Table 4.12 *Name and position in lentil genome of single-nucleotide polymorphism markers found to be associated with dry biomass of fluthiacet methyl (16 gai/ha); experiment conducted in controlled environment (repeated two times); marker information include significance level (p value) and R² of MLM model; and name with positon of candidate gene identified in proximity of marker*

| Experiment | Marker | Chromosome | Position | p value | Marker R² | Candidate gene | Position of candidate gene |
|---|---|---|---|---|---|---|---|
| 1 | SContig122234_6436 | 1 | 246255562 | 1.93 $e^{-5}$ | 0.041 | *Phototropic-responsive NPH3 family protein* | Chr1:246570444..246574703 |
| 1 | SContig32652_78967 | 1 | 316765660 | 1.20 $e^{-4}$ | 0.034 | *Ovate transcriptional repressor* | Chr1:316750792..316751750 |
| 2 | SContig227048_31201 | 3 | 17958250 | 4.86 $e^{-5}$ | 0.040 | *Cyclin-like F-box protein* | Chr3:17985361..17987292 |
| 1 | SContig345038_77605 | 5 | 17904038 | 1.44 $e^{-4}$ | 0.040 | *Brassinazole-resistant 1 protein* | Chr5:17902157..17904334 |
| 1 | SContig397347_7251 | 5 | 97543767 | 4.10 $e^{-5}$ | 0.038 | *Malic enzyme* | Chr5:97759118..97761036 |
| 1 | SContig174547_199092 | 5 | 117183569 | 2.76 $e^{-5}$ | 0.041 | *Helix loop helix DNA-binding domain protein* | Chr5:117178205..117180640 |
| 1 | SContig28743_17194 | 5 | 224669057 | 1.06 $e^{-4}$ | 0.035 | *CCT motif protein* | Chr5:224666451..224667698 |
| 1 | SContig28743_17207 | 5 | 224669070 | 1.05 $e^{-4}$ | 0.035 | *CCT motif protein* | Chr5:224666451..224667698 |
| 1 | SContig161544_29360 | 7 | 32934697 | 2.12 $e^{-5}$ | 0.041 | *Glutathione S-transferase* | Chr7:32889503..32890778 |

In the indoor study, dry biomass data of fluthiacet methyl was analyzed using control data as a covariate in the model, but it did not yield significant associations. The analysis was repeated without using dry biomass of control as a covariate. In that case, a total of eight significant associations were found in the first run of the experiment and only one significant association in the second run. Five associations had significance at p value $<7.38\ e^{-5}$ and four associations were significant at p value $<1.48\ e^{-4}$. Most of these associations were located on chromosome 5, and fewer on chromosomes 1, 7 and 3. The number of associations detected for dry biomass of control treatment was 3 (p value $<1.48\ e^{-4}$), with all them being detected on chromosomes 1, 2 and 4 in the second run of the experiment (Appendix C). The overlap between the marker associations with the control and fluthiacet methyl treatment occurred only for one marker.

### 4.4 Discussion

The goal of association mapping was to reveal the genetic control of tolerance to fluthiacet methyl in a diverse population of lentil. Dayan and Duke (1997) proposed several possible mechanisms of differential levels of herbicide tolerance, including inhibition of uptake or sequestration of the herbicide, rapid metabolic degradation of the herbicide, herbicide-resistant PPOX enzyme, quenching of singlet oxygen and other toxic oxygen species, etc. The presence of more than one mechanism is possible, especially among diverse germplasm, which could influence the ability to precisely detect genetic causes underlining different levels of tolerance.

The population used for association analyses consisted of 110 accessions from different regions where lentil is grown. Analysis of population structure determined that there are three distinct subpopulations among the accessions, echoing findings of Khazaei et al. (2016), who also detected the existence of three distinct groups within a larger lentil panel, which included most of the accessions used in this study. The three sub-populations defined by Khazaei et al. (2016) were South Asian, Mediterranean, and northern temperate. The findings of population structure correspond to these groups with exception of the South Asian sub-population, which in this case included Indian as well as Iranian accessions, so it would be better named as an Asian sub-population. The results of the dendrogram generally correspond to the STRUCTURE results. The

accessions with high levels of admixture could be the consequence of breeding efforts as they have a very similar proportions of two sub-populations.

In association mapping studies, it is important to assess the effect of the population structure in order to account for this effect on the results. Intraclass coefficient (ICC) determines the amount of phenotypic variation explained by population structure. For the injury rating data, ICC varied across herbicide treatments and site-years. Overall, population structure accounted for less than one-fifth of phenotypic variation in almost all cases, suggesting that phenotypic variation for reaction to fluthiacet methyl is not predominantly controlled by population structure. Indicating that differences in tolerance level are not the solely product of population subdivisions, rather that there the factors causing differences in tolerance level are scattered across the sub-populations.

In the study, both control (unsprayed) and fluthiacet methyl treatments were used, which is not the case in some other herbicide association studies where only herbicide treatments were used in the analysis (Wang et al., 2016). The primary reason for including a control treatment is natural differences in growth (i.e. NDVI value or biomass) among tested accessions. The control data was used in two ways in this study; first as a covariate in analyzing fluthiacet methyl treatment data, and secondly, it was used to generate associations based on control data and SNPs. Having results of association analysis for control treatment serves as a reference point to the results of fluthiacet methyl treatment. Significant association found in the same region for both control and fluthiacet methyl suggest that a region plays role in general growth and development, and in principle it is not connected to fluthiacet methyl tolerance.

Estimated linkage disequilibrium (LD) decay across the seven lentil chromosomes ranged from 500 to 1700 kbp. These are high LD values, but still in the range consistent with other legume species (Saxena et al., 2014). Mating system, historical bottlenecks, and selection have likely played an important role in keeping LD high in lentil. Selfing species typically have higher LD relative to outcrossing species. Therefore, it is more appropriate to compare lentil to other selfing legumes than to outcrossing species. In the case of chickpea, LD was estimated to be in the 1.5 - 2 Mbp range (Diapari et al., 2014), slightly above this lentil estimate. The rate of LD dictates the number of markers needed for sufficient mapping resolution (Zhao et al., 2017). In this study, the number of SNP markers and their coverage of the genome provided adequate resolution for an association study. Increased LD values make identification of candidate genes more difficult as a

larger genomic area surrounding markers needs to be considered increasing the number of potential candidates.

The two separate types of experiment, field and indoor-based, were conducted to better understand the reactions of a diverse set of lentil germplasm to fluthiacet methyl. In both cases, different types of data were used to describe the phenotype. In the field experiment, injury ratings and NDVI data were used across all environments. Injury rating is cost-effective, fast, and widely used, but it tends to be subjective measurement and it is best used in conjunction with a more objective technique, such as biomass or imaging. Injury ratings as a result of fluthiacet methyl treatment were collected at two time points at each site-year: 3 and 7 d after fluthiacet methyl treatment. In 2016 at Saskatoon, overrepresentation of injury rating of 7 (on a 0 to 10 injury rating scale) at 3 DAT was observed, likely due to the delay in application of fluthiacet methyl for that site year. The delay was caused by a prolonged rainy period in early June, which postponed herbicide application until lentil plants reached 8-10 leaf stage, instead of the recommended 6 leaf stage. The increased amount of foliage meant more contact with fluthiacet methyl, resulting in more uniform appearance of damage across all accessions. At other site years, distribution of injury 3 DAT rating was more diverse. This suggests that spraying at later stages when lentil plants have more biomass leads to more uniform appearance of damage. In the case of injury rating 7 DAT, values are similarly distributed through all site-years. A rating time of 7 d after herbicide application was enough period for some plants to exhibit first signs of recovery. Several accessions with low injury ratings were large-seeded lines (i.e. CDC QG1, CDC Glamis, CDC Greenstar, CDC Grandora), which produce more biomass in early stages of development. Large-seeded accessions seem to have an innate advantage in comparison to small-seeded ones, but this advantage is not directly connected to a specific herbicide tolerance mechanism. This suggests that the development of herbicide-resistant lentil material could be more easily achieved among large-seeded material. The development of herbicide resistant small-seeded lines is not impossible, but requires a better understanding of tolerance mechanisms and preferably the application molecular markers in the screening process.

NDVI is a widely used vegetation index, often deployed in high throughput phenotyping, and can be used as an indirect measure of plant biomass and plant health (White et al., 2012). In a number of weed science studies, NDVI has been used to directly measureof herbicide damage. For

example, it was successfully used to assess damage caused by saflufenacil (group 14 herbicide) on rice cultivars (Montgomery et al., 2014); to measure the effects of lactofen (group 14 herbicide) and imazethapyr (group 2 herbicide) on soybean lines (Thelen et al., 2004); and to assess the impact of multiple herbicides on bermudagrass (Bell et al., 2000).

An NDVI curve consists of an increasing phase, or onset of greenness, followed by the maximum NDVI, and a decreasing phase, or end of greenness (Reed et al., 1994). NDVI values collected for plots exposed to fluthiacet methyl all stayed longer in the first phase, implying that treated plants had a prolonged vegetation period. Focusing on the genotypes in the extremes of the distribution, several accessions, like CDC Glamis, CDC Greenstar, and ILL 5058 were found frequently in the top ten genotypes in terms of highest NDVI values following treatment with fluthiacet methyl across all site-years. Genotypes like PI 300250, ILL 3347, ILL 3597, and W6 27766 tended to be on the lower end of NDVI values across site-years. Some of the genotypes found in the tails of NDVI distribution after fluthiacet methyl treatment are the same as the ones scoring extremely high or low under control treatment. Specifically, W6 27766 produced very low NDVI values regardless of the treatment, while CDC Glamis ranked among the genotypes with the highest NDVI values in both control and fluthiacet methyl treatment. Most of the lentil accessions did not follow this trend, however, their position in the distribution of NDVI values was significantly affected by the application of fluthiacet methyl. Statistical analysis of the NDVI data in this study showed that genotypes reacted differently to the herbicide immediately after the application, as well as during the recovery period. The environment played an important role in the experiment, as it had a great influence on plant development and the efficacy of the herbicide. Recovery of the plants following application of fluthiacet methyl was highly influenced by the environmental factors, as seen at Elrose in 2015, where frost occurred just 10 d after seeding. The minimum temperature fell to -8.2°C (Environment Canada, 2018), causing stress to the plants leading to slower growth among control plots, and at the same time affecting recovery of fluthiacet methyl plots (Figure 4.3).

The controlled nature of the indoor study prevented any major abiotic or biotic stress, and therefore reduced environmental variability. In a controlled environment, light quality plays an important role in lentil growth and development (Yuan et al., 2017). In addition to quality, light intensity plays role in determining the level of injury caused by fluthiacet methyl, as higher intensity means

higher injury at same herbicide rate (Fausey & Renner, 2001). This is important for the interpretation of the results as the level of damage is very depended on light, which is why it is difficult to reproduce results under different light systems. Phenotypic data acquired in the indoor experiment consisted of dry biomass collected 14 d after herbicide application. Fluthiacet methyl caused a reduction of biomass among most accessions compared to the results of matching control treatment (Figure 4.5). The results of the indoor experiment have an only modest match with the field results, which emphasizes the role of the environment in these experiments. The indoor experiment, although providing excellent control of some environmental factors, does not provide an adequate amount of light relative to field conditions. Environment plays a significant role in influencing not just the plants but also it is crucial to realizing the full effect of fluthiacet methyl.

The goal of association mapping analysis was to determine which genetic factors played a role in controlling tolerance in this population. The phenotypic data used in the analysis showed both significant effects of herbicide and significant differences among tested lentil accessions. Injury ratings yielded only five associations with a low significance level of $\alpha=1$, NDVI for fluthiacet methyl-treated plants yielded more significant associations than injury rating or dry biomass, although only one association had a high significance level of $\alpha=0.05$. It is important to mention that in the analysis of NDVI data, control NDVI data served as a covariate to manage the effect of differences in plant growth which naturally exist in the tested population. The same was done for dry biomass of fluthiacet methyl but it did not yield significant associations so a simpler model without control as a covariate was used, producing several significant associations. The complexity of genetic control is also reflected in the proportion of phenotypic variation explained by each individual SNP range between 3 and 5.7 %.

The total number of associations between traits under control treatment and SNP markers is considerable, indicating that these traits are under complex genetic control. Traits (i.e. NDVI values and dry biomass) measured under control treatment are generally related to growth or development of biomass. As such, these results are consistent with findings in other studies, such as genetic mapping of biomass production in alfalfa (Robins et al. 2007) and association mapping of biomass in maize (Lu et al., 2011) - in both cases they detected over 40 significant associations. The associations detected under the control treatment were not in studied in detail, rather they served as a comparison with the associations found under fluthiacet methyl treatment.

In cases where LD value is high, identification of candidate genes becomes difficult as larger genomic regions need to be taken into consideration, and this especially becomes an issue in gene-rich regions. In more complex situations, the determination of candidate genes requires a certain degree of interpretation, which is done based on the gene functions and anticipated influence of the researched trait. Among significant associations between injury rating data and SNPs, four candidate genes were identified. Two significant associations were located close to *NADH-ubiquinone oxidoreductase 18 kDa subunit*. This gene codes for an enzyme that is a part of respiratory complex I which is located in the mitochondria and consists of nearly 50 subunits (Braun et al., 2014). Respiratory complex I has high metabolic significance in generating proton gradients and production of ATP, but there is also some indication that it plays role in generating reactive oxygen species (ROS; Vinogradov & Grivennikova, 2016). There is no evidence for the direct connection between the effect of fluthiacet methyl and respiratory complex I, but if respiratory complex I can create ROS (i.e. superoxide and/(or) hydrogen peroxide) through oxygen reduction (Vinogradov & Grivennikova, 2016) it could increase the effect of fluthiacet methyl.

Among the other candidate genes identified for injury rating and SNP association are *DUF1645 family protein*, *hydroxyproline-rich glycoprotein family protein* and *glutathione-S-transferase. DUF1645 family protein* was found to play an important role in drought tolerance in rice. One of the functions of DUF1645 is to alter transcript levels of stress-responsive genes (Cui et al., 2016). Fluthiacet methyl, as a Group 14 herbicide, causes accumulation of ROS, which activate stress response in the plant cell. *Hydroxyproline-rich glycoprotein family protein* (*HRGP*) has a function in strengthening the cell walls and that contributes to plant defense reactions (Deepak et al., 2010). Knowing that fluthiacet methyl causes degradation of cell membranes and cell walls, it could be speculated that *HRGP* plays a role in mitigating some of the damage to a cell wall. *Glutathione-S-transferase* genes are known to play an important role in non-target site herbicide resistance by detoxification of plant cells (Yuan, Tranel & Stewart, 2007), so finding this gene associated with injury rating data confirms a significant role of non-target site resistance mechanisms in the response of lentil to fluthiacet methyl. A large number of associations between SNPs and NDVI data were detected for fluthiacet methyl treatments. Among candidate genes most prevalent were *cytochrome P450*s, which were identified at three different locations on chromosome 4, and at one location each on chromosomes 1, 6 and 7. *Cytochrome P450*s have been associated with metabolizing herbicide molecules in the cell, leading to less toxic forms (Werck-Reichhart, Hehn

& Didierjean, 2000). *Glutathione-S-transferases* were found at two different positions on chromosome 6 and at one on chromosome 3and as stated in the previous paragraph, have an important roles in detoxifying cells from the effect of herbicides. Both cytochrome P450s and glutathione-S-transferases are key enzymes in degradation of herbicide molecules, through oxidation and conjugation respectively, and may have the same role in the case of fluthiacet methyl and lentil. Among other candidate genes that were found multiple times were *peroxidase* and *glycosyltransferase*. *Peroxidases* code for antioxidant enzymes, which remove $H_2O_2$ from the cell (Sewelam, Kazan, & Schenk, 2016). Fluthiacet methyl, as any group 14 herbicide, leads to accumulation of ROS (i.e. $H_2O_2$), so the presence of peroxidases might alleviate some of the effects of herbicide. *Glycosyltransferases* are known to play a role in non-target site herbicide resistance through conjugation of herbicide molecules (Yuan, Tranel & Stewart, 2007). They are also involved in the modification of many small molecules and thereby influence many aspects of plant development (Li et al., 2017). In Arabidopsis thaliana, *glycosyltransferase* was found to play an important role in stress response, reducing levels of $H_2O_2$ and superoxide in the case where the gene was overexpressed (Li et al., 2017).

Several genes involved in plant growth and development were identified as candidate genes, such as *RNA polymerase II-associated protein 1 (RPAP1)-like*, which initiate stem cell differentiation in plants (Lynch et al., 2017), *cell division FtsZ-like protein* which plays a role in plastid division (Schmitz et al., 2009), *squamosa promoter-binding-like protein*, a transcription factors involved in leaf development and many others aspects of plant architecture (Chen et al., 2010), and both *peptidoglycan-binding LysM* domain protein and *UDP-N-acetylmuramyl-tripeptide synthetase,* both involved in peptidoglycan biosynthesis (i.e. cell wall formation). Several phytohormonal related genes, two cytokinins and one auxin-related gene, were also identified as candidate genes. Potential involvmentthese genes implies that the regrowth or a recovery phase following fluthiacet methyl application is controlled with multiple genes and these are essential for developing resistant varieties. Among other interesting candidate genes were different stress-related genes, including *cysteine-rich TM module stress tolerance protein* which is located in the plasma membrane and responds to various types of stresses (Venancio, & Aravind, 2009), *calmodulin-domain kinase CDPK protein,* which coordinates plant responses to environmental stresses (Zeng et al., 2015), *respiratory burst oxidase-like protein,* which in can cause increased production of ROS as a response to stress conditions (Wang et al., 2018). A *transducin/WD40* gene was also identified as

a candidate gene. These genes interact with other proteins and serve as key regulators in plant development and stress signaling (Gachomo et al., 2014). Although *ABC transporters* (or *drug resistance transporter-like ABC domain proteins*) are generally considered to play a very important role in non-target site herbicide resistance, only one significant association was found near one of these genes.

The analysis of dry biomass of fluthiacet methyl-treated plots showed certain problems, for example, the data of the control treatment served as a covariate in the model, but no significant association was detected even at α=1. Therefore, control data as a covariate was removed from the model, and several significant associations were detected. Among identified candidate genes only two could logically be related to the effect of fluthiacet methyl: *glutathione-S-transferase* and *ovate transcriptional repressor*, which play roles in cell elongation (Wang et al., 2007), so could be connected to plant recovery.

There were a large number of associations between SNPs and all traits under the control treatment. Candidate gene identification was not performed, and these results served only as a check for the results under fluthiacet methyl treatment. The associations under control conditions are related to normal plant growth, and if the same associations had been detected for the phenotypic data of herbicide treatment, it would mean that those loci do not have real significance for fluthiacet methyl tolerance. Among injury ratings and NDVI data, only one case was found where associations under the two treatments were in close proximity to each other: on chromosome 4 where several uncharacterized and *squamosa promoter-binding-like protein* gene are located. *Squamosa promoter-binding-like protein* is a transcription factor that plays a significant role in plant development (Chen et al., 2010). For dry biomass results, again only one significant association was shared between the two treatments. Candidate genes in that region include *exostosin family protein*, *disease resistance protein* (*TIR-NBS-LRR class*) and *phototropic-responsive NPH3 family protein*. The latter plays the role in photo signaling or response to blue light (Zhao et al., 2018), perhaps blue light or lack of same had a strong effect on plants growing in control environments.

The results of the association study suggest that tolerance to fluthiacet methyl in lentil is an elaborate mechanism involving a large number of genes. Drawing a definitive conclusion as to which specific genes are involved remains problematic as only two candidate genes were

significant in multiple environments. The lack of reproducibility of specific candidate genes over multiple environments is buffered by the results which repeatedly suggest involvement of the same gene families, and thereby it gives leverage in creating a broad picture of the genetic control of tolerance. It seems that fluthiacet methyl tolerance in lentil plants involves non-target site resistance mechanisms (mainly cytochrome P450 and glutathione-S-transferase) combined with general stress response, followed by a recovery phase which is controlled by phytohormones and several other genes influencing growth and development.

## Prologue to Chapter 5

Based on the results reported in chapter 4, the role of cytochrome P450 and glutathione-S-transferase in the tolerance to sulfentrazone and fluthiacet methyl was studied further. A combination of herbicide and inhibitors of those two enzyme families was used in an electrolyte leakage assay to assess the role of those enzymes. The four lentil varieties that were used in chapter 5 were selected based on the results of the electrolyte leakage assay in chapter 3.

# 5. IMPACT OF CYTOCHROME P450s AND GLUTATHIONE-S-TRANSFERASES ON THE TOLERANCE OF LENTIL TO SULFENTRAZONE AND FLUTHIACET METHYL

## 5.1 Introduction and Objectives

The ability of plant cells to metabolize herbicides, or mitigate their negative effects, is a common source of herbicide tolerance or resistance. All physiological processes involved in the reduction of herbicide effects are classified as non-target site resistance (NTSR). NTSR can be deconstructed into four phases of detoxification: the first and second phase involve chemical degradation of herbicide molecules, while the latter phases encompass transport to, and further degradation in, vacuolar and extracellular spaces (Yuan, Tranel & Stewart, 2007). In the first phase of detoxification, herbicide degradation starts with oxidation mediated by cytochrome P450 monooxygenases. In the second phase, the oxidized herbicide is conjugated with thiols or sugars (Yuan, Tranel & Stewart, 2007; Werck-Reichhart, Hehn & Didierjean, 2000). Glutathione-S-transferases are a well-known gene family involved in the second phase of detoxification. They catalyze conjugation of glutathione or homoglutathione with the herbicide molecule (Yuan, Tranel & Stewart, 2007; Edwards, Dixon & Walbot, 2000).

Délye et al. (2011) established that non-target site resistance to multiple herbicides in *Alopecurus myosuroides* (black-grass) is a result of complex genetic control. Several genes were linked to NTSR through a transcriptome study in *Alopecurus myosuroides*, including three cytochromes P450 genes—*CYP71A*, *CYP71B* and *CYP81D*, one *peroxidase*, and one disease-resistance gene (Gardin et al., 2015). Building on this knowledge, it is hypothesised that *cytochrome P450 monooxygenases* and *glutathione-S-transferases* play a role in differential response of lentil to sulfentrazone and fluthiacet methyl. Using a combination of inhibitors and herbicides and an electrolyte leakage assay (ELA) should help discern the role of these two gene families in the response of lentil to herbicides. 1-aminobenzotriazole (Sigma-Aldrich, USA) was selected as an inhibitor of *cytochrome P-450s* (Preston et al., 1996). Tridiphane (LGC Standards, USA) and

ellagic acid (Sigma-Aldrich, USA) serve as inhibitors of *glutathione-S-transferases* (Letouzé & Gasquez, 2003; Das et al., 1984).

### 5.2 Materials and Methods

Four lentil varieties were selected for testing: CDC Redberry and CDC Improve are considered to be highly tolerant material, while CDC Impala and CDC Rosetown represent susceptible lentil varieties (Chapter 3). The experiment included nine treatments:

a) control (base buffer);
b) sulfentrazone (200 µM);
c) fluthiacet methyl (25 µM);
d) control + 1-aminobenzotriazole (base buffer + 200 µM);
e) sulfentrazone + 1-aminobenzotriazole (200 µM + 200 µM);
f) fluthiacet methyl + 1-aminobenzotriazole (25 µM + 200 µM);
g) control+ tridiphane + ellagic acid (base buffer + 25 µM + 100 µM);
h) sulfentrazone+ tridiphane + ellagic acid (200 µM + 25 µM + 100 µM);
i) fluthiacet methyl + tridiphane + ellagic acid (25 µM +25 µM + 100 µM).

The base buffer solution used included 1% sucrose and 1mM of MES (4-Morpholineethanesulfonic acid), with the pH value adjusted to 6.5 using NaOH. The experiment was set up in a complete randomized design with four replicates and was conducted two times. All plants were grown in a growth chamber at University of Saskatchewan (Conviron, Controlled Environments Limited, Winnipeg, MB) in 10 $cm^2$ square pots with soilless mix (2:1 Sunshine #3: Perlite, Sun Gro Horticulture, Canada). The growth chamber conditions were as follows: day/night temperatures set at 21°C/15°C; day length of 18 h; with light intensity of 350 µmol/$m^2$s. Leaflets were cut two weeks after seeding (at about 5-6 leaf stage). Leaflets were carefully cut and weighed to ensure a sample size of approximately 250 mg. Only leaflets with no injured tissues (no physical damage or insect injury) were selected for the experiment. Preparation of the leaflets for the experiment also included washing them with a base buffer solution for one minute, to ensure that any dirt particles are removed from the surface. Washed leaflets were placed in 30 ml of the treatment solution before the first electro-conductivity value ($EC_{start}$) was recorded. After the initial measurement, all samples were placed in a dark growth chamber at room temperature (22°C) for

24 h. Following this "dark phase," the samples were placed in the growth chamber under continuous light conditions (500 μmol/$m^2$s at 22°C) and electro-conductivity was measured in 12-hour intervals for the next 72 h. After the 7$^{th}$ measurement, all samples were placed in a 95°C water bath for 1.5 h to cause complete tissue disintegration, and then cooled, before the final electro-conductivity measurements were collected. Formula 3.2 (Chapter 3.2.5.) was used to calculate percent leakage at each time point.

### 5.3 Data Analysis

The experiment had two repetitions, and since a homogeneity of variance test did not show significant differences in residual variance between the repetitions, the data were merged and analyzed in entirety. A non-linear regression method was used to fit the experimental data using the drc package (suite of dose-response curves analyses) of R statistical software (R Development Core Team, 2014; Ritz & Streibig, 2005). Based on the previous experiences with ELA data (chapter 3), log logistic and Weibull type 2 models were tested using the "mselect" function (part of the drc R package). Data for treatments without herbicide did not show any increase of leakage over time, so they could not be fitted to any of the non-linear regression models. The herbicide treatment data set was fitted to a three-parameter Weibull type 2 model (Morgan & Smith, 1992):

$$Y = D * (1 - \exp(-\exp(B(\log(X) - \log(E))))) \quad \text{.....(5.1)}$$

where $Y$ is the response (i.e. % leakage), $D$ is the upper limit (fixed at 100), $B$ is the relative slope around the inflection point E, and $X$ is time (in hours). Time until 50 % total leakage ($T_{50}$) was used as a measure of herbicide damage (described in detail in chapter 3) across different treatments, and it was calculated using the Weibull type 2 model.

### 5.4 Results

Results for every genotype are presented separately, so that potential effect of inhibitors can be assessed and compared among and across selected genotypes. The three-parameter Weibull type 2 model provided an acceptable description of the data, as evidenced by the lack of fit test p-values of 0.4807 for CDC Redberry, 0.7032 for CDC Improve, 0.8598 for CDC Impala, and 0.2265 for CDC Rosetown. Model parameters for every treatment across each genotype are provided in

Appendix D. The increase in percent of electrolyte leakage associated with each treatment is presented as a sigmoid curve resulting from the fitted model, and is displayed for each genotype tested in Figure 5.1.

$T_{50}$ values for all genotypes and treatments are presented in Table 5.1. $T_{50}$ values for herbicide alone correspond to the previously reported results (Chapter 3.3), where CDC Redberry was shown to be the most tolerant, and CDC Rosetown the most susceptible genotype to both herbicides.

Table 5.1 *$T_{50}$ values (in hours) of ELA with standard error (SE) for sulfentrazone and fluthiacet methyl treatment with and without inhibitors of cytochrome P450s (1-aminobenzotriazole) and glutathione-S-transferases (tridiphane or ellagic acid) among four lentil varieties*

| **Treatment** | **CDC Redberry** | | **CDC Improve** | | **CDC Impala** | | **CDC Rosetown** | |
|---|---|---|---|---|---|---|---|---|
| | $T_{50}$ | SE | $T_{50}$ | SE | $T_{50}$ | SE | $T_{50}$ | SE |
| *FM25* | 73.1 | 0.82 | 64.3 | 1.03 | 62.0 | 0.98 | 58.2 | 0.90 |
| *GSH_FM25* | 69.6 | 0.81 | 62.7 | 0.93 | 56.8 | 0.89 | 58.6 | 0.76 |
| *P450_FM25* | 61.3 | 0.74 | 59.0 | 0.93 | 56.1 | 0.92 | 54.0 | 0.89 |
| *SF200* | 83.6 | 0.85 | 75.7 | 1.08 | 72.6 | 0.98 | 68.3 | 0.95 |
| *GSH_SF200* | 81.8 | 0.67 | 76.6 | 0.86 | 68.9 | 0.86 | 68.3 | 0.84 |
| *P450_SF200* | 75.6 | 0.74 | 70.8 | 0.94 | 66.8 | 0.89 | 66.5 | 0.88 |

*FM25-fluthiacet methyl 25µM; GSH_FM25-fluthiacet methyl 25µM+ inhibitors glutathione-S-transferase (tridiphane or ellagic acid); P450_FM25-fluthiacet methyl 25µM+ inhibitor cytochrome P450 (1-aminobenzotriazole); SF200-sulfentrazone 200µM; GSH_SF200-sulfentrazone 200µM+ inhibitors glutathione-S-transferase (tridiphane or ellagic acid); P450_SF200-sulfentrazone 200µM+ inhibitor cytochrome P450 (1-aminobenzotriazole).*

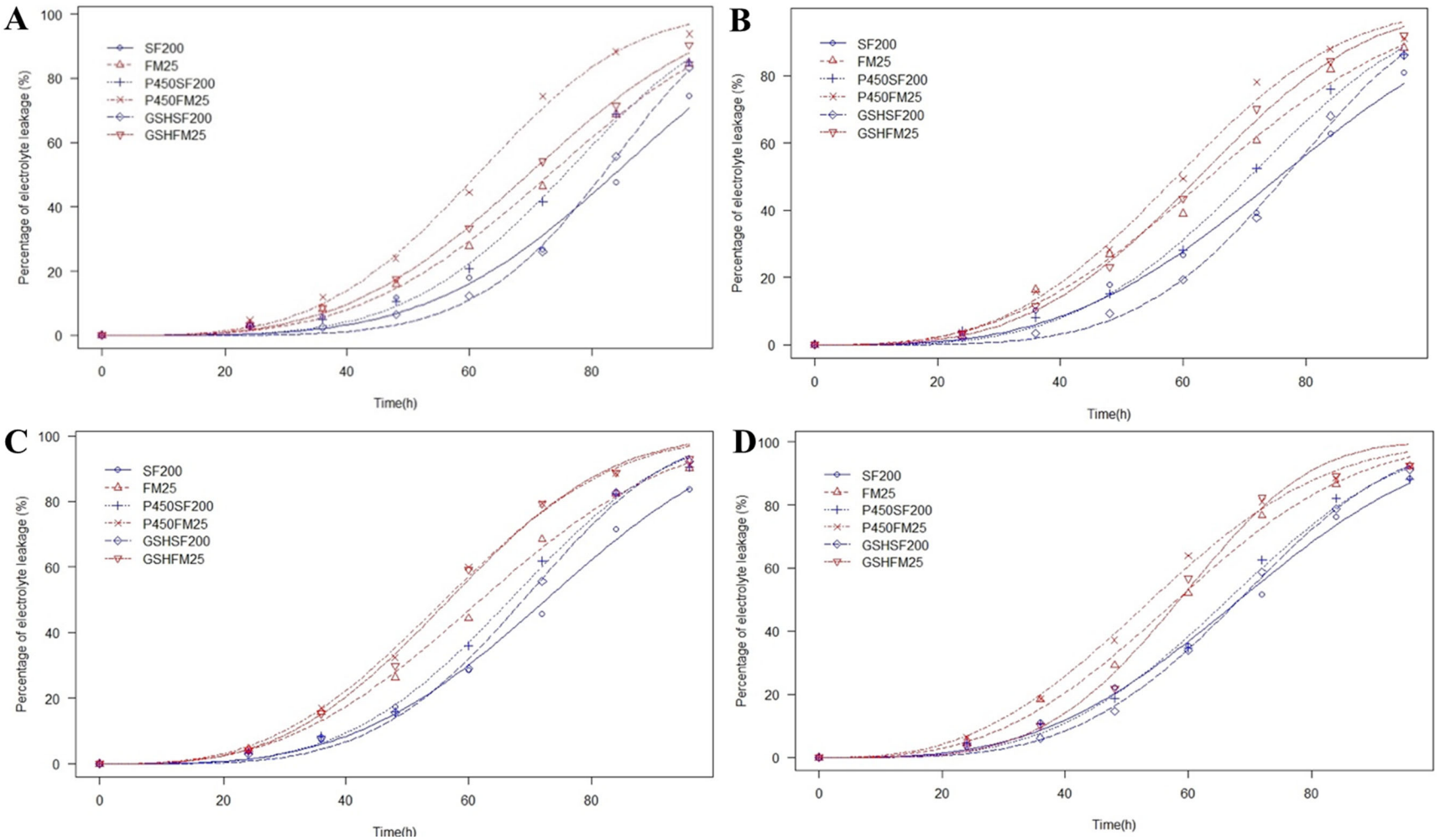



Figure 5.1. Non-linear curves representing ELA results for SF200 (sulfentrazone 200µM), FM25 (fluthiacet methyl 25µM), P450SF200 (sulfentrazone 200µM + inhibitor cytochrome P450), P450FM25 (fluthiacet methyl 25µM + inhibitor cytochrome P450), GSHSF200 (sulfentrazone 200µM + inhibitors glutathione-S-transferase), and GSHFM25 (fluthiacet methyl 25µM + inhibitors glutathione-S-transferase) treatments, A) CDC Redberry, B) CDC Improve, C) CDC Impala, D) CDC Rosetown. Blue denotes sulfentrazone treatments and red marks fluthiacet methyl treatments.

Inhibitor treatments caused a lowering of $T_{50}$ values, but they did not change the relative order of genotypes in terms of tolerance and susceptibility. A comparison of $T_{50}$ values between herbicide treatments with and without inhibitors for every genotype is given in Table 5.2.

Table 5.2 *T-values stemming from the comparison of $T_{50}$ values through the t-test of herbicide treatments without inhibitors (sulfentrazone 200 µM and fluthiacet methyl 25 µM) and herbicide treatments with inhibitors of cytochrome P450s (1-aminobenzotriazole) and glutathione-S-transferases (tridiphane or ellagic acid)*

| Varieties | Herbicide treatment | Inhibitor of cytochrome P450s | Inhibitors of glutathione-S-transferase |
|---|---|---|---|
| **CDC Redberry** | Sulfentrazone 200 µM | -7.54*** | $-1.73^{NS}$ |
| | Fluthiacet methyl 25 µM | 9.76*** | 2.95** |
| **CDC Improve** | Sulfentrazone 200 µM | -3.54*** | $6.77^{NS}$ |
| | Fluthiacet methyl 25 µM | 1.09*** | $1.16^{NS}$ |
| **CDC Impala** | Sulfentrazone 200 µM | -4.59*** | -2.87** |
| | Fluthiacet methyl 25 µM | 4.19*** | 3.76*** |
| **CDC Rosetown** | Sulfentrazone 200 µM | $-1.48^{NS}$ | $-4.68^{NS}$ |
| | Fluthiacet methyl 25 µM | 3.19** | $-3.10^{NS}$ |

*** $p<0.001$; ** $p<0.01$; * $p< 0.05$; $^{NS}$, not significant

Inhibitor of cytochrome P450 (1-aminobenzotriazole) in combination with both herbicides caused a statistically significant ($p<0.001$) decrease in $T_{50}$ values across almost all genotypes, with CDC Rosetown being the exception. Glutathione-S-transferases inhibitors (tridiphane and ellagic acid) caused a significant reduction in the $T_{50}$ value of CDC Impala when combined with either herbicide ($p<0.01$). In the case of CDC Redberry, glutathione-S-transferases inhibitors caused a significant decrease in the $T_{50}$ value in the fluthiacet methyl treatment only.

CDC Rosetown, considered the most susceptible genotype, experienced a significant reduction in $T_{50}$ only when exposed to the cytochrome P450 inhibitor (1-aminobenzotriazole) in combination with fluthiacet methyl ($p<0.01$). For CDC Redberry, all but one inhibitor treatment caused a significant ($p<0.01$) reduction in the $T_{50}$ value, while for CDC Improve, only the inhibitor of cytochrome P450 caused a significant ($p<0.001$) decrease. CDC Impala was the only genotype in which all inhibitor treatments caused a significant ($p<0.01$) reduction in $T_{50}$ values.

## 5.5 Discussion

Cytochrome P450 monooxidases and glutathione-S-transferases are two enzyme families that play a major role in detoxification of herbicides (Kawahigashi, 2009). The effect of these two enzyme families on the tolerance to group 14 herbicides was assessed by inhibiting them and observing the impact on damage to four lentil genotypes through an electrolyte leakage assay (ELA). This approach provides a simple technique to test the *in vivo* role of cytochrome P450s and glutathione-S-transferases in herbicide tolerance. In contrast to some other approaches such as spraying or drenching and measuring whole plant response, an ELA provides a reliable method for inhibitors to reach their targets in plant cells, as leaf tissue are in constant contact with solution of inhibitors. In an earlier study assessing the role of cytochrome P450s in herbicide resistance of *Lolium rigidum* (ryegrass) (Preston et al., 1996), the concentration of 1-aminobenzotriazole applied was 70 µM. In a study sourcing herbicide resistance in *Alopecurus myosuroides* (Letouzé & Gasquez, 2003), tridiphane was used at a concentration of 15 µM. The concentration of the ellagic acid of 83 µM was determined to match half maximal inhibitory concentration ($I_{50}$) of glutathione-S-transferase (Das et al., 1984). At this time, no credible information exists regarding the potency of these inhibitors in lentil. To ensure inhibition of enzymes in the experiments, the concentration of the inhibitors applied was higher than the values used in recent literature.

The results of these series of ELAs suggest that *cytochrome P450*s do play a role in detoxifying plant cells from the herbicides in almost all genotypes, while *glutathione-S-transferases* have a more variable impact on the effect of herbicides, depending on the genotype. Inhibition of cytochrome P450s resulted in more damage exerted in a shorter period of time relative to samples exposed only to the herbicides, regardless of the genotype. Cytochrome P450s and glutathione-S-transferases are known to play role in chemical degradation of herbicides, but glutathione-S-transferases are also known to play an important role in protecting cells from oxidative stress (Didierjean et al., 2002; Veal et al., 2002). It is not possible to determine specific differences in the mechanisms without deeper biochemical study. Inhibiting cytochrome P450s increased damage over time across all genotypes tested, with CDC Redberry exhibiting the largest reduction of $T_{50}$ value for both herbicides implying faster destruction of leaf tissue. Based on these results, it is possible that cytochrome P450s plays an important role in tolerance, presumably through

metabolizing of herbicide molecules as reported in other research (Yuan et al., 2007). The only genotype where inhibitors of cytochrome P450s caused significant change was CDC Rosetown, which is among more susceptible genotypes (Chapter 3). The higher level of susceptibility in CDC Rosetown could be attributed to the low efficacy of cytochrome P450s in this genotype in metabolizing herbicide. Additional studies are needed to provide more definitive answers.

The effects of the inhibitors of glutathione-S-transferases also varied across the four genotypes, suggesting there may be a role for this enzyme in tolerance to sulfentrazone and fluthiacet methyl. CDC Rosetown, a very susceptible genotype, did not show any effect of inhibitors of glutathione. The same effect was noted for CDC Improve, which has a higher tolerance level than CDC Rosetown. On the other hand, in CDC Impala and to a lesser degree CDC Redberry, inhibiting glutathione-S-transferases did produce a measurable decrease in tolerance. This mixed result provides evidence that the role of glutathione-S-transferases varies among lentil genotypes and that a combination of genetic variants in both of these genes play a role in regulating the response of a genotype to herbicide exposure.

Application of inhibitors, along with herbicides, in an ELA revealed a role played by cytochrome P450s and glutathione-S-transferase in tolerance of lentil to group 14 herbicides. Across the four genotypes tested, cytochrome P450s appear to play an important role in reducing the effect of both sulfentrazone and fluthiacet methyl. Glutathione-S-transferases seem to play a greater role in tolerance to fluthiacet methyl, while the role of cytochrome P450s it is less prominent.

## Prologue to Chapter 6

In chapter 6, the focus is on investigating genes encoding target sites of the sulfentrazone and fluthiacet methyl. Plants have two different protoporphyrinogen IX oxidase enzymes - one is localized in chloroplasts and other is localized in mitochondria. The DNA sequences for both of the protoporphyrinogen IX oxidases are identified and localized in the lentil genome. The genomic data of 80 lentil genotypes was used to discover several polymorphisms in the DNA sequences of target genes. A combination of phenotypic data from chapter 4 with newly discovered genetic polymorphisms were tested to determine the effect of genotype on the response to sulfentrazone and fluthiacet methyl.

# 6. SEQUENCE DIVERSITY OF PROTOPORPHYRINOGEN IX OXIDASE GENES, TARGET SITES OF GROUP 14 HERBICIDES, AMONG LENTIL GENOTYPES

## 6.1 Introduction and Objectives

The change of the herbicide target site sequence in plants is a common source of herbicide tolerance or resistance. Alterations, through amino acid insertion, deletion, or substitution, can lead to diminished effects of an herbicide within plant cells, thereby increasing the tolerance of the plant. In plant breeding, this approach has been widely used for the development of most commercially available herbicide resistant crops. Simple genetic control is the biggest breeding advantage of this mechanism. In some cases, however, alteration of a target site leads to a fitness penalty as the physiological functionality of those enzyme changes, e.g. triazine resistant canola which has lower yield and seedling vigor than susceptible varieties (Beversdorf, Hume, & Daonnelly-Vanderloo 1988).

The target sites for group 14 herbicides are protoporphyrinogen IX oxidase (PPOX) enzymes in plant cells (Matringe et al., 1989). Plants have two PPOX enzymes, one is localized in the chloroplasts, and another in the mitochondria. These enzymes are encoded by two different nuclear genes: *chloroplast PPOX* (*Lcu_Ch_PPOX*) encodes the chloroplast enzyme, while *mitochondria PPOX* (*Lcu_Mh_PPOX*) encodes the mitochondria one. Examples of target site tolerance to group 14 herbicides have been found and confirmed in three weed species: common waterhemp, common ragweed, and palmer amaranth (Dayan et al., 2014; Salas-Perez et al., 2017). None of the past studies of the source of tolerance in cultivated species has focused on target site exclusively, so there is limited data available for crop species. Several amino acid changes have been identified as critical to acquiring resistance to some group 14 herbicides (Dayan, Barker & Tranel 2018).
Only limited information is available regarding *PPOX* genes in the lentil genome. Thus, the objectives of this chapter were to: identify *Lcu_Ch_PPOX* and *Lcu_Mh_PPOX* genes in the lentil genome and identify single nucleotide polymorphisms (SNPs) in these genes, focusing on the ones

causing amino acid changes. Secondly, we wanted totest their effect on tolerance to fluthiacet methyl and sulfentrazone via phenotypic data. The research objective also included investigating diversity of these genes among three wild *Lens* species (*L. odemensis*, *L. lamottei* and *L. ervoides*).

## 6.2 Materials and Methods

### 6.2.1 Identification of PPOX genes in the lentil genome

The *PPOX* genes from *Medigaco truncatula* and *Glycine max* were identified using a word search of the Phytozome v.12.1.5 database (Goodstein et al., 2011). In these databases, genes annotated as *PPOX* are not further defined as chloroplast- or mitochondria-specific, so the presence of chloroplast or mitochondria transit peptides was used to confirm proper identification of *PPOX* genes. Transit peptides were identified through TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/ from Emanuelsson et al., 2000). Exon and intron sequences of lentil *Lcu_Ch_PPOX* and *Lcu_Mh_PPOX* genes were identified using BLASTn searches of the lentil genome assembly available through the KnowPulse web portal (http://knowpulse.usask.ca/portal/blast/nucleotide/nucleotide). The most recent lentil genome assembly (LcV1.2) was used to identify the chromosome positions of the genes as well. Coding DNA sequence (CDS) of the two lentil *PPOX* genes were used in a BLASTn against existing *L. ervoides*, *L. lamottei* and *L. odemensis* genome assemblies available through the same site. CDSs of the *PPOX* genes in the wild lentil relatives were translated to protein sequences and aligned with the protein sequences of those from cultivated lentil using the CLUSTAL O (1.2.4) online service (https://www.ebi.ac.uk/Tools/msa/clustalo/ ; McWilliam et al., 2013).

### 6.2.2 Plant material, genomic and phenotypic data

SNP data for PPOX genes from 86 diverse lentil cultivars (Appendices J and K) were retrieved from the KnowPulse database (http://knowpulse.usask.ca/portal/project/AGILE%3A-Application-of-Genomic-Innovation-in-the-Lentil-Economy). These SNPs were derived from a lentil exome capture assay (details available in Ogucten et al., 2018). The genotypes were selected as they have exome capture data, and were also part of the lentil association mapping panel that was phenotyped for response to sulfentrazone (280 g.a.i $ha^{-1}$) and fluthiacet methyl (16 g.a.i $ha^{-1}$)

treatments collected in 2016 from two locations, Saskatoon and Scott (Chapter 4). The details of the experimental design and NDVI data are provided in Chapter 4. Normalized difference vegetation index (NDVI) results collected four times in weekly interval after herbicide applications (7 days, 14 days, 21 days and 28 days) were used in this analysis. NDVI data were transformed using a Box-Cox transformation as they exhibit some deviation from normal distribution, transformation was performed with PROC TRANSREG function of SAS v9.4 (SAS Institute, Cary, NC). Single marker analysis (single-point analysis), using analysis of variance (ANOVA), was performed to detect associations between molecular marker and NDVI data for all treatments.

### 6.3 Results

The *Ch_PPOX* gene is present in a single copy in the Medicago genome, on chromosome 1 (Medtr1g085730.1), whereas the soybean genome contains two copies— on chromosomes 2 and 10 (Glyma.10G138600.1 and Glyma.02G007200.1). The presence of two copies in soybean are an aftereffect of ancient polyploidy event in soybean (Schmutz et al., 2010). A single copy of the *Ch_PPOX* gene was identified in the lentil genome located on chromosome 1 (Lc04510), (http://knowpulse.usask.ca/portal/jbrowse/Lentil/?loc=LcChr1:255090777..255097785&tracks=Lc1.2genes,Mt4CDS,Gmax275Wm82a2v1). *Lcu_Ch_PPOX* gene is 6074 bp long and consists of 9 exons (Figure 6.1). Exon lengths range from 81 to 563 bp.

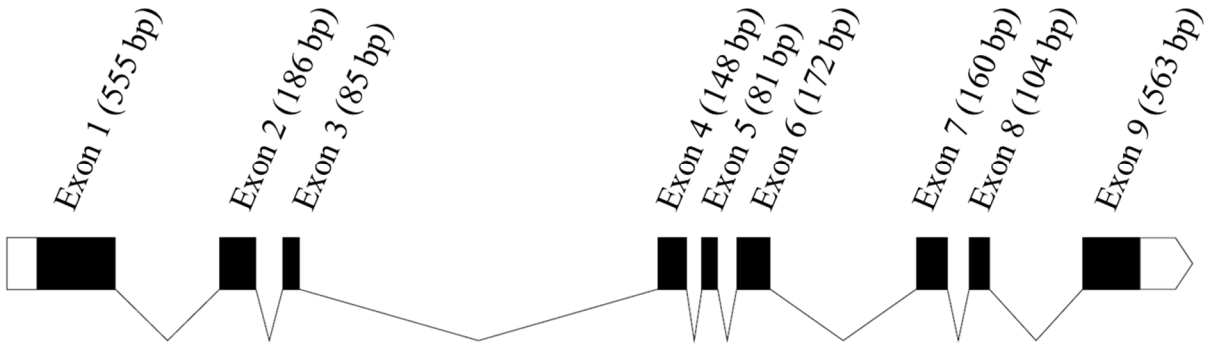



Figure 6.1 The structure of lentil *Lcu_Ch_PPOX* (Lc04510, lentil genome v 1.2) with exons length given in base pairs (bp).

Both Medicago and soybean have a single copy of *Lcu_Mh_PPOX*. In Medicago, it is located on chromosome 7 (Medtr7g031310); in soybean it is found on chromosome 19

(Glyma.19G087600.1). In lentil, the *Lcu_Mh_PPOX* gene is found in a single copy on chromosome_6(Lc26711), (http://knowpulse.usask.ca/portal/jbrowse/Lentil/?loc=LcChr6:40640299..40663388&tracks=Lc1.2genes,Mt4CDS,Gmax275Wm82a2v1). *Lcu_Mh_PPOX* gene is 17,869 bp long, and consists of 17 exons (Figure 6.2). Exons vary in length from 37 to 309 bp, with large intron spaces between them. Peptide sequence alignment of *Lcu_Mh_PPOX* and *Lcu_Ch_PPOX* showed only 29.7% overlap in amino acid residues, which were scattered across the whole alignment

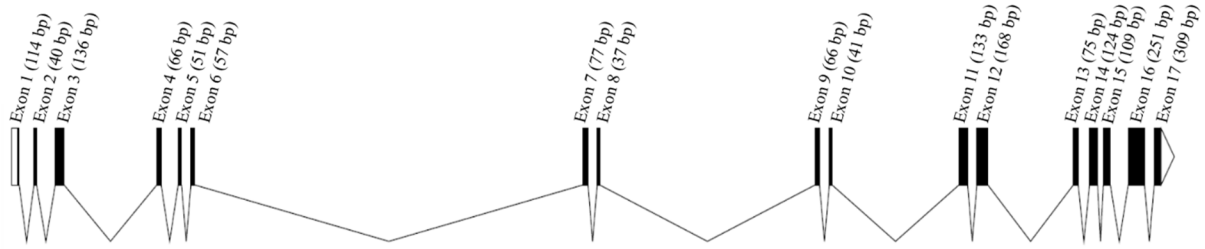



Figure 6.2 The structure of lentil *Lcu_Mh_PPOX* (Lc25711, lentil genome v 1.2) with exon length given in base pairs (bp).

A total of nine SNPs relative to the CDC Redberry reference were detected within the exons of *Lcu_Ch_PPOX* across the 86 cultivars for which sequence data were available (Appendix J). Only two SNPs would lead to amino acid substitutions in the protein: SLCCHR1_255094897 (further this SNP is labeled as Lcu_Ch_PPOX1) and SLCCHR1_255094997 (further this SNP is labeled as Lcu_Ch_PPOX2). The distribution of those two SNPs, in each of the 86 genotypes is given in Appendix E. In the case of Lcu_Ch_PPOX1, the reference (CDC Redberry) allele (G) is present in 86% of genotypes and the alternative (A) is present in 14% of genotypes. The second SNP, Lcu_Ch_PPOX2, is present as a reference allele (A) in 80% of genotypes and the alternative (G) is present in 20% of the genotypes. The amino acid changes to the chloroplast PPOX enzyme caused by Lcu_Ch_PPOX1 and Lcu_Ch_PPOX2 SNPs are $Asp^{304}$→Asn and $Gln^{337}$→Arg, respectively.

For *Lcu_Mh_PPOX*, five SNPs were observed relative to the reference (Appendix K), only two of which would cause an amino acid substitution: SLCCHR6_40659440 (further this SNP is labeled as Lcu_Mh_PPOX1) and SLCCHR6_40659466 (further this SNP is labeled as Lcu_Mh_PPOX2).

Lcu_Mh_PPOX1 is present in only one genotype as an alternative allele (T) and in the rest of genotypes have the reference allele (C). The Lcu_Mh_PPOX2 SNP is more diversely distributed through the 86 genotypes, where the reference allele (A) is present among 58% of genotypes and the alternative allele (G) is present in 42% of the genotypes (Appendix E). The detected amino acid changes in mitochondria PPOX enzymes are $Val^{334}\rightarrow$ Ala (for Lcu_Mh_PPOX1) and $Lys^{346}\rightarrow$Arg (for Lcu_Mh_PPOX2).

The results of the single-marker analysis (ANOVA) for all allele pairs and NDVI data is given in Tables 6.1 to 6.4. NDVI data differed significantly between individuals carrying the two different alleles at Lcu_Ch_PPOX1 in all treatments, including the control. The allele state at Lcu_Mh_PPOX2 also had a strong association with fluthiacet methyl treatment results ($p<0.05$) in all site years. NDVI data did not differ between alleles for most sulfentrazone treatments. There is no evidence that the allele state at Lcu_Ch_PPOX2 and Lcu_Mh_PPOX1 resulted in differing NDVI data for individuals treated with either herbicide.

Table 6.1 *Single-marker analysis (ANOVA) of Lcu_Ch_PPOX1 allele state association with NDVI data for three herbicide treatments (Control (unsprayed), Sulfentrazone (280 g.a.i ha$^{-1}$), Fluthiacet methyl (16 g.a.i ha$^{-1}$)) collected at Saskatoon and Elrose locations in 2016 over four weekly time points post herbicide application*

| | **Treatment** | **Elrose** | | **Saskatoon** | |
|---|---|---|---|---|---|
| | | F value | $R^2$ | F value | $R^2$ |
| **7 DAT** | Control | 11.40*** | 11.8 | 16.47*** | 16.2 |
| | Sulfentrazone (280 g.a.i ha$^{-1}$) | 16.98*** | 16.7 | 8.87** | 9.5 |
| | Fluthiacet methyl (16 g.a.i ha$^{-1}$) | 37.31*** | 30.4 | 26.33*** | 23.7 |
| **14 DAT** | Control | 9.96** | 10.5 | 9.75** | 10.3 |
| | Sulfentrazone (280 g.a.i ha$^{-1}$) | 15.90*** | 17.6 | 7.24** | 7.9 |
| | Fluthiacet methyl (16 g.a.i ha$^{-1}$) | 41.57*** | 32.8 | 22.50*** | 20.9 |
| **21 DAT** | Control | 9.08** | 9.6 | 12.82*** | 13.1 |
| | Sulfentrazone (280 g.a.i ha$^{-1}$) | 15.89*** | 15.8 | 6.29* | 6.9 |
| | Fluthiacet methyl (16 g.a.i ha$^{-1}$) | 32.18*** | 27.5 | 15.47*** | 15.4 |
| **28 DAT** | Control | 6.15* | 6.8 | 15.90*** | 15.8 |
| | Sulfentrazone (280 g.a.i ha$^{-1}$) | 7.94** | 8.5 | 10.18** | 10.7 |
| | Fluthiacet methyl (16 g.a.i ha$^{-1}$) | 30.27*** | 26.3 | 17.53*** | 17.1 |

*** $p<0.001$; ** $p<0.01$; * $p< 0.05$.

Table 6.2 *Single-marker analysis (ANOVA) of Lcu_Ch_PPOX2 allele state association with NDVI data for three herbicide treatments (Control (unsprayed), Sulfentrazone (280 g.a.i ha$^{-1}$), Fluthiacet methyl (16 g.a.i ha$^{-1}$)) collected at Saskatoon and Elrose locations in 2016 over four weekly time points post herbicide application*

| | Treatment | Elrose | | Saskatoon | |
|---|---|---|---|---|---|
| | | F value | $R^2$ | F value | $R^2$ |
| **7 DAT** | Control | $3.71^{NS}$ | N/A | 4.22* | 4.7 |
| | Sulfentrazone (280 g.a.i ha$^{-1}$ ) | $2.14^{NS}$ | N/A | $1.80^{NS}$ | N/A |
| | Fluthiacet methyl (16 g.a.i ha$^{-1}$) | $0.74^{NS}$ | N/A | $0.57^{NS}$ | N/A |
| **14 DAT** | Control | $1.94^{NS}$ | N/A | 4.41* | 4.9 |
| | Sulfentrazone (280 g.a.i ha$^{-1}$) | $2.27^{NS}$ | N/A | $2.57^{NS}$ | N/A |
| | Fluthiacet methyl (16 g.a.i ha$^{-1}$) | $1.89^{NS}$ | N/A | $0.52^{NS}$ | N/A |
| **21 DAT** | Control | $0.97^{NS}$ | N/A | $2.64^{NS}$ | N/A |
| | Sulfentrazone (280 g.a.i ha$^{-1}$) | $0.08^{NS}$ | N/A | $1.56^{NS}$ | N/A |
| | Fluthiacet methyl (16 g.a.i ha$^{-1}$) | $1.93^{NS}$ | N/A | $0.03^{NS}$ | N/A |
| **28 DAT** | Control | $0.10^{NS}$ | N/A | $1.98^{NS}$ | N/A |
| | Sulfentrazone (280 g.a.i ha$^{-1}$) | $0.80^{NS}$ | N/A | $1.09^{NS}$ | N/A |
| | Fluthiacet methyl (16 g.a.i ha$^{-1}$) | $1.74^{NS}$ | N/A | $0^{NS}$ | N/A |

* $p< 0.05$; $^{NS}$, not significant.

Table 6.3 *Single-marker analysis (ANOVA) of Lcu_Mh_PPOX1 allele state association with NDVI data for three herbicide treatments (Control (unsprayed), Sulfentrazone (280 g.a.i ha$^{-1}$), Fluthiacet methyl (16 g.a.i ha$^{-1}$)) collected at Saskatoon and Elrose locations in 2016 over four weekly time points post herbicide application*

| | Treatment | Elrose | | Saskatoon | |
|---|---|---|---|---|---|
| | | F value | $R^2$ | F value | $R^2$ |
| **7 DAT** | Control | $0.33^{NS}$ | N/A | $0.36^{NS}$ | N/A |
| | Sulfentrazone (280 g.a.i ha$^{-1}$) | $0.07^{NS}$ | N/A | $0.22^{NS}$ | N/A |
| | Fluthiacet methyl (16 g.a.i ha$^{-1}$) | $2.99^{NS}$ | N/A | $0.28^{NS}$ | N/A |
| **14 DAT** | Control | $0.10^{NS}$ | N/A | $0.11^{NS}$ | N/A |
| | Sulfentrazone (280 g.a.i ha$^{-1}$) | $0.68^{NS}$ | N/A | $0.25^{NS}$ | N/A |
| | Fluthiacet methyl (16 g.a.i ha$^{-1}$) | $0.45^{NS}$ | N/A | $0.02^{NS}$ | N/A |
| **21 DAT** | Control | $0.18^{NS}$ | N/A | $0.21^{NS}$ | N/A |
| | Sulfentrazone (280 g.a.i ha$^{-1}$) | $0.12^{NS}$ | N/A | $0.06^{NS}$ | N/A |
| | Fluthiacet methyl (16 g.a.i ha$^{-1}$) | $0.80^{NS}$ | N/A | $0.01^{NS}$ | N/A |
| **28 DAT** | Control | $0.26^{NS}$ | N/A | $0.31^{NS}$ | N/A |
| | Sulfentrazone (280 g.a.i ha$^{-1}$) | $3.15^{NS}$ | N/A | $0.02^{NS}$ | N/A |
| | Fluthiacet methyl (16 g.a.i ha$^{-1}$) | $0^{NS}$ | N/A | $0.02^{NS}$ | N/A |

$^{NS}$, not significant

Table 6.4 *Single-marker analysis (ANOVA) of Lcu_Mh_PPOX2 allele state association with NDVI data for three herbicide treatments (Control (unsprayed), Sulfentrazone (280 g.a.i ha$^{-1}$), Fluthiacet methyl (16 g.a.i ha$^{-1}$)) collected at Saskatoon and Elrose locations in 2016 over four weekly time points post herbicide application*

| | Treatment | Elrose | | Saskatoon | |
|---|---|---|---|---|---|
| | | F value | $R^2$ | F value | $R^2$ |
| **7 DAT** | Control | $0.09^{NS}$ | N/A | $0.61^{NS}$ | N/A |
| | Sulfentrazone (280 g.a.i ha$^{-1}$) | $0^{NS}$ | N/A | $1.31^{NS}$ | N/A |
| | Fluthiacet methyl (16 g.a.i ha$^{-1}$) | 8.79** | 9.4 | 13.52*** | 13.7 |
| **14 DAT** | Control | $0.10^{NS}$ | N/A | $2.17^{NS}$ | N/A |
| | Sulfentrazone (280 g.a.i ha$^{-1}$) | $0.82^{NS}$ | N/A | $1.88^{NS}$ | N/A |
| | Fluthiacet methyl (16 g.a.i ha$^{-1}$) | 12.08*** | 12.5 | 7.7** | 8.3 |
| **21 DAT** | Control | $0.7^{NS}$ | N/A | $0.11^{NS}$ | N/A |
| | Sulfentrazone (280 g.a.i ha$^{-1}$) | $1.03^{NS}$ | N/A | $3.42^{NS}$ | N/A |
| | Fluthiacet methyl (16 g.a.i ha$^{-1}$) | 6.61* | 7.2 | 11.08** | 11.5 |
| **28 DAT** | Control | $0.04^{NS}$ | N/A | $1.76^{NS}$ | N/A |
| | Sulfentrazone (280 g.a.i ha$^{-1}$) | $0.75^{NS}$ | N/A | 6.64* | 7.2 |
| | Fluthiacet methyl (16 g.a.i ha$^{-1}$) | $3.49^{NS}$ | N/A | 7.76** | 8.4 |

*** $p<0.001$; ** $p<0.01$; * $p< 0.05$; $^{NS}$, not significant

Both *Lcu_Ch_PPOX* and *Lcu_Mh_PPOX* gene sequences were present in three wild lentil assemblies for which sequences were available. The alignment of the peptide sequences of chloroplast PPOX revealed eight polymorphisms ($Arg^{11}$→Ser, $Arg^{39}$→His, $Ser^{63}$→Phe, $Thr^{292}$→Ala, $Asp^{304}$→Asn, $Gly^{321}$→Glu, $Gln^{457}$→Arg and $Val^{488}$→Ile), in which amino acids differ between *L. culinaris* and one or more wild relative (Figure 6.3). *Lens lamottei* has the highest number of polymorphisms in comparison to *L. culinaris*, followed by *L. ervoides* and *L. odemensis*. The $Val^{488}$ allele was specific only to *L. culinaris*, while the rest of the wild species had $Ile^{488}$. The $Asp^{304}$→Asn polymorphism was found in all wild sequences and is the same as the Lcu_Ch_PPOX1 variant found among some of the *L. culinaris* genotypes surveyed. The other seven polymorphism were not observed among *L. culinaris* genotypes, suggesting they may be unique to the wild lentil species. The peptide sequences of mitochondria PPOXs from *L. culinaris* and wild relatives were very similar, with only five polymorphisms (Figure 6.4). One is a codon deletion, which means that $Gln^{10}$ is missing, in *L. ervoides*. The other four polymorphism are $Asn^{140}$→Lys, $Lys^{346}$→Arg, $Thr^{404}$→Ile, $Asp^{519}$→Glu. *L. odemensis* had the highest number of

number of polymorphisms, followed by *L. lamottei* and *L. ervoides*. The Lys$^{346}$→Arg found in all the wild species sequences corresponds to the Lcu_Mh_PPOX2 found among the *L. culinaris* genotypes surveyed.

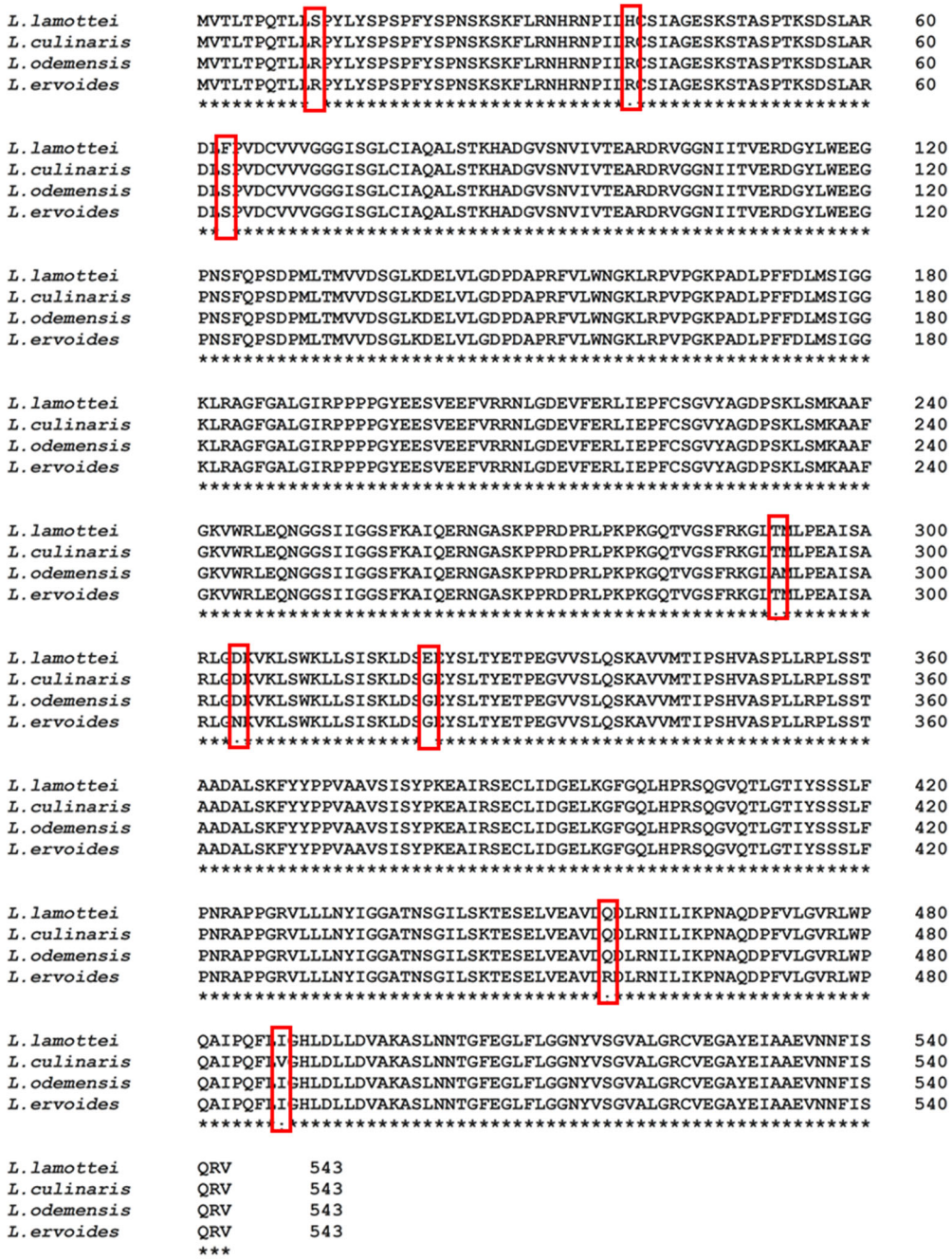



Figure 6.3 Alignment of the amino acid sequences of chloroplast PPOX enzymes among *L. culinaris* (CDC Redberry); *L. ervoides*; *L. lamottei* and *L.odemensis*. Polymorphisms are highlighted with a red box.

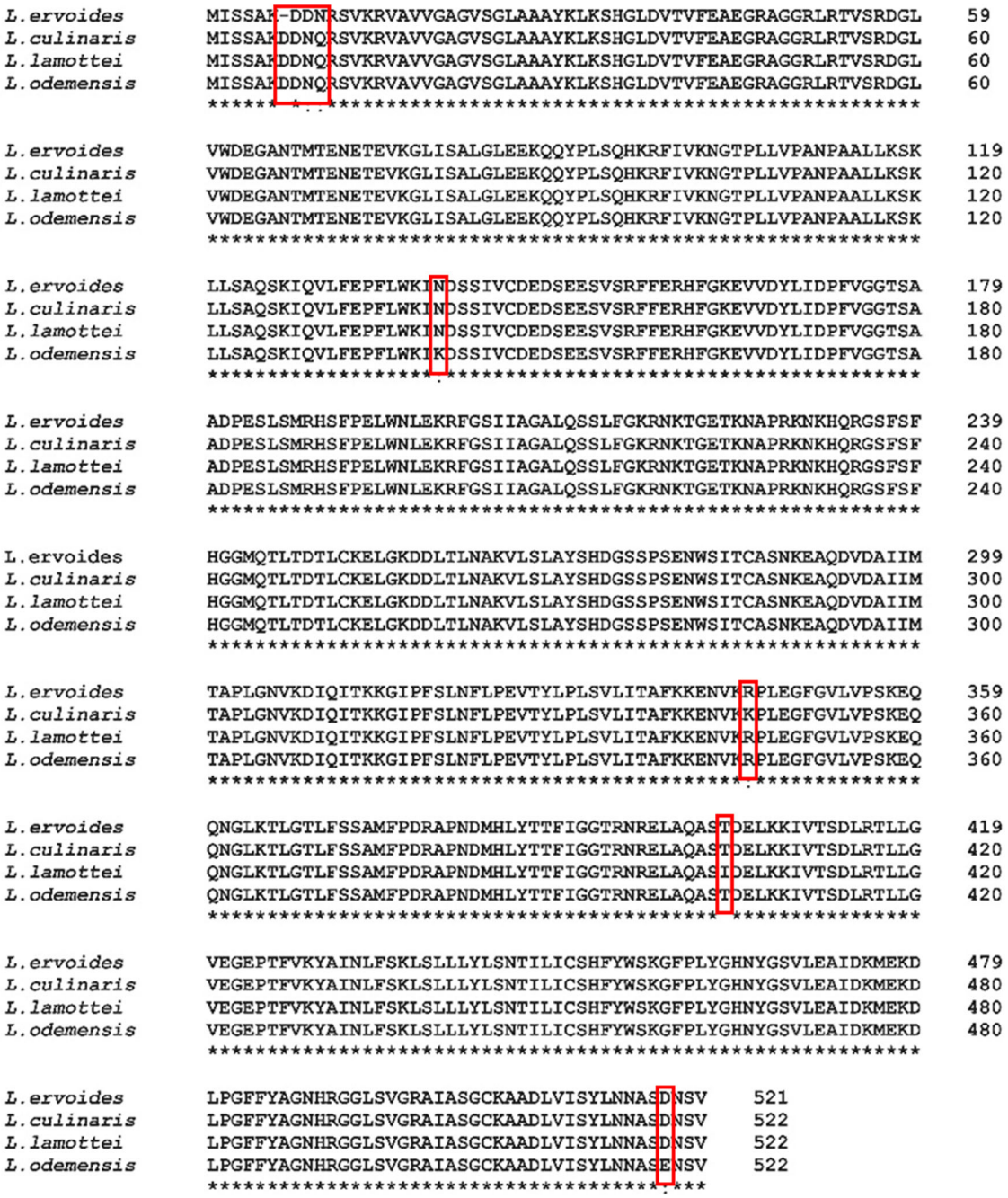



Figure 6.4 Alignment of the amino acid sequences of mitochondria PPOX enzymes among *L. culinaris* (CDC Redberry), *L. ervoides, L. lamottei* and *L.odemensis*. Polymorphisms are highlighted with a red box.

## 6.4 Discussion

Modification of the herbicide target site is the most common mechanism for developing herbicide resistance in plants. The target sites of group 14 herbicides are two PPOX enzymes, one targeted in the chloroplast, and the other in the mitochondria (Lermontova et al., 1997). In the lentil genome a single copy each of these was found on two different chromosomes.

In recent years, research efforts have focused on herbicide resistant weeds, so sources of target site resistance to group 14 herbicides are better understood among weed species. For example, in the case of *Amaranthus tuberculatus*, a codon deletion at the $Gly^{210}$ position of mitochondria PPOX is documented as the primary reason for resistance to group 14 herbicides (Patzoldt et al., 2006). This deletion has significant architectural consequences for the enzyme, and leads to increased cavity size of the enzyme activity site by almost 50%, without impacting the physiological function of the enzyme (Dayan et al., 2010). In common ragweed, a single point mutation caused an amino acid substitution of $Arg^{98}$→Leu which has been shown to lead to an increase in herbicide tolerance (Rousonelos et al., 2012). In palmer amaranth populations, both $Arg^{98}$, and $Gly^{210}$, along with $Arg^{128}$, were identified as causes of herbicide resistance (Giacomini et al., 2017; Salas-Perez et al., 2017). A structural study of the PPOX enzyme in tobacco predicted that $Arg^{98}$ would be one of the critical amino acids for substrate binding, and therefore important for herbicide resistance. In addition to $Arg^{98}$, amino acids $Leu^{356}$, $Leu^{372}$, and $Phe^{392}$ are involved in interaction substrate and active site of mitochondria PPOX enzyme, and therefore, considered important amino acids for herbicide resistance (Heinemann et al., 2007).

In cultivated lentil, amino acid polymorphisms were observed at the $Val^{334}$ and $Lys^{346}$ positions in the mitochondria PPOX. These substitutions have not previously been mentioned as important amino acids for herbicide target site interaction. In the single marker analysis with Lcu_Mh_PPOX1, no statistically significant differences in response to herbicide treatments were observed between the two groups with different alleles. This leads to the conclusion that the $Val^{334}$→ Ala change likely does not play a role in herbicide tolerance. The single marker analysis of Lcu_Mh_PPOX2 revealed a different pattern, where the $Lys^{346}$→Arg change resulted in different responses only to fluthiacet methyl, and not for control or sulfentrazone treatment. Lentil genotypes with $Lys^{346}$ in mitochondria PPOX had higher NDVI values under fluthiacet methyl

treatment compared to ones that have $Arg^{346}$ suggesting this is a good candidate for increasing herbicide tolerance in lentil. The percentage of variation explained by Lcu_Mh_PPOX2 across all site years is relatively low, so should not be considered the sole source of resistance in the breeding program.

Unlike mitochondria PPOX, the impact of amino acid substitutions in the chloroplast PPOX enzyme on herbicide tolerance has not generally been the focus of much research. A comparison of amino acid sequences between mitochondria and chloroplast PPOX enzymes in lentil showed only 29.8% similarity, corresponding to findings from other plant species (Lermontova et al., 1997; Watanabe et al., 2000). Overlaps between the two enzymes are randomly distributed across the length of the enzymes, so it is hard to pinpoint which amino acids in the chloroplast PPOX could play a crucial role in increasing herbicide tolerance. Two SNPs were found in the lentil chloroplast *PPOX* gene which lead to two different amino acid substitutions. The results of single marker analysis of Lcu_Ch_PPOX1 ($Asp^{304}$→Asn) do not provide enough evidence to form a definitive answer on the possible role of this gene in resistance to sulfentrazone and fluthiacet methyl. Differences existed between individuals with different Lcu_Ch_PPOX1 alleles in both herbicide treatments but also the control. Although differences in herbicide treatment lead to the conclusion that the marker plays a role in tolerance, at the same time those differences also exist in the control treatment meaning that, independently of treatment, differences exist among groups having different loci. No significant differences were observed between the two groups of genotypes that differed for allele state at Ch_PPOX2 for any treatment, leading to the conclusion that the variation at Ch_PPOX2 does not play a role in tolerance to sulfentrazone and fluthiacet methyl.

In addition to cultivated lentil germplasm, wild lentil species were investigated as a source of additional polymorphisms in both the mitochondria and chloroplast PPOX enzymes. Three amino acid substitutions, and one amino acid deletion, were found only among wild lentil mitochondria PPOX. In addition, eight additional polymorphisms were discovered for chloroplast PPOX enzyme, confirming the notion that wild species can serve as a source of new variation for cultivated plants. In addition to these polymorphisms, $Lys^{346}$→Arg in mitochondria PPOX and $Asp^{304}$→Asn substitution in chloroplast PPOX are present both among wild lentil species and in cultivated lentil germplasm, meaning that this substitution is commonly found among *Lens* species. The genus *Lens*, in addition to four species used in this study, also includes *L. orientalis*

and *L. tomentosus* (in the primary gene pool) and *L. nigricans* (in the quaternary gene pool) (Wong et al., 2015). Mining of existing genomic databases is a relatively inexpensive approach for assessing genetic diversity in a specific genomic region or gene. The amount of genetic information generated through new sequencing techniques exceeds the needs of the original project and becomes a valuable resource for additional research. Using existing data from the lentil database led to the identification of six polymorphisms in mitochondria PPOX and nine polymorphisms in chloroplast PPOX, among cultivated and wild lentil species. Based on these findings, a more comprehensive study is needed to assess the usability of these amino acid substitutions in herbicide breeding programs. In lentil breeding, wild species are actively used to bring new genetic variability. The same can be done for *PPOX* genes where marker assisted selection can be essential for success.

## Prologue to Chapter 7

The focal point of chapter 7 is the application of chemically induced mutagenesis to increasing tolerance to sulfentrazone and fluthiacet methyl in lentil. An existing mutant lentil population was screened in the field with both sulfentrazone and fluthiacet methyl. Individual plants and M2-derived lines were selected for further testing. In the case of sulfentrazone, mutant lines were re-tested in the field and selected mutant plants were re-tested using an electrolyte leakage assay. In the case of fluthiacet methyl, mutant lines were re-tested using a dose response study and electrolyte leakage assay.

# 7. INCREASING TOLERANCE OF LENTIL TO SULFENTRAZONE AND FLUTHIACET METHYL THROUGH MUTATION BREEDING

## 7.1 Introduction

Chemical mutagenesis is widely used in crop research and breeding programs and the most common agents are ethyl methanesulfonate (EMS) and sodium azide. These two chemicals cause different kinds of DNA alterations (Shu et al., 2012). A good example of commonly used herbicide resistance achieved through mutation breeding is resistance to imidazolinone herbicides. Many crops, including corn, wheat, rice, and soybean, have imidazolinone resistance developed through successful application of mutagenesis (Green & Owen, 2011). Mutation breeding was also used to develop imidazolinone resistance in lentil, through an alteration in the lentil acetohydroxyacid synthase (*AHAS*) gene (Slinkard, Vandenberg & Holm, 2007; Thompson, 2013). However, examples of successful mutagenesis applications leading to increased tolerance to group 14 herbicides are rare, and so far, non-existent among major crops. The rare cases include Arabidopsis and green algae *Chlamydomonas reinhardtii*, whose increased tolerance was achieved through target site mutation, i.e. *protoporphyrinogen IX oxidase* (Li & Nicholl, 2005). In this case, mutations led to a ten-fold (or higher) increase in the level of resistance to different group 14 herbicides (Li & Nicholl, 2005). Although chemical mutations attempts were focused on target sites of herbicide, a large pool of non-target site resistance genes are not typically being exploited. These non-target site genes could serve as additional sources of herbicide resistance.

The objective of this experiment was to screen, identify, and validate mutant lines of CDC Redberry for resistance to sulfentrazone and fluthiacet methyl. Initial screening was performed under field conditions, while subsequent experiments included smaller subsets of selected mutagenized lines. In the validation studies, performance of selected lines was compared to that of non-mutagenized CDC Redberry.

## 7.2 Material and Methods

### 7.2.1 Plant Material

The mutant lentil population was developed in cooperation with Dr. Victor Raboy, USDA Idaho, who developed and conducted the protocol for mutagenizing lentil seeds using sodium azide. CDC Redberry seeds were used in the process that began with a four-hour exposure to 1 mM concentration of sodium azide solution. Seeds were then washed with water and dried. A total of 1,171 M3 lines were derived from M1 seeds, and provided for this study. The sets of five different lentil varieties: CDC Improve, CDC Impala, CDC KR1, CDC Redberry, and CDC Rosetown, were used as checks during initial screening. In the follow up experiments, CDC Redberry was used as a check.

### 7.2.2 Initial Field Screening of M3 Lines with High Rates of Sulfentrazone and Fluthiacet Methyl

The initial screening of mutagenized lines with sulfentrazone was performed under field conditions in 2014 at one location: Saskatoon (52°03'48.9"N 106°26'22.1"W). M3 lines were seeded in 1 $m^2$ microplots at a seeding rate of 100 seeds per plot. The experiment was sown in an augmented design, where mutagenized lines were un-replicated, and a set of five checks (CDC Improve, CDC Impala, CDC KR-1, CDC Redberry, and CDC Rosetown) was replicated five times across the trial. A faba bean plot, as a tolerant crop, was seeded after every ten microplots to ensure proper identification of the microplots. Sulfentrazone (Authority™ 480g.a.i $l^{-1}$, FMC Canada) was applied post seeding but pre-emergence, using a tractor sprayer. The product was mixed with water to produce twice the label-recommended concentration, and then sprayed in two passes in an overlapping spraying pattern. As a result, the final rate of sulfentrazone corresponded to 560 g.a.i $ha^{-1}$, or four times recommended product rate.

The number of germinated seeds per plot was counted two weeks after seeding. Because of high levels of damage, microplots with 20 or more plants were tagged. In subsequent weeks, injury ratings were recorded in the tagged plots. All plants from the tagged plots were harvested at maturity and seeds were bulked on a line basis after harvest. In addition to the selected whole plots,

several individual plants, particularly those located in areas with heavy injuries but showing low damage symptoms, were harvested.

The initial screening of mutagenized lines with fluthiacet methyl was conducted at the same location, using the same plot size, seeding rate, and experimental design. The rate of fluthiacet methyl used for screening mutants was 32 g.a.i $ha^{-1}$, which is eight times recommended rate for this product. Fluthiacet methyl (Cadet™ 10.8%, FMC Canada) was mixed with adjuvant Agral 90 (Syngenta, Canada) at 0.25 vol %. The herbicide was applied post emergence at the 4-6 leaf stage, using a tractor sprayer in one pass. Three days after spraying all plots were rated using an injury scale of 1 to 5, where 1 stood for no or minimal injury among plants in the plot, and 5 represented complete plant death through whole plot. Mutagenized lines with the lowest injury ratings were tagged and selected for further rating. After the initial injury most plots showed recovery, and symptomology of herbicide injury was not present in later ratings. Therefore, later ratings were based on general appearance. All plants in selected plots were harvested at maturity and the seed bulked on a plot basis. No additional individual plants were selected for harvest in the fluthiacet methyl experiment, since plants within microplots showed uniform levels of injury. Following the initial screening, a set of 13 selected M4 lines was tested in a controlled environment under same protocol as in Chapter 3.2.6. The rate of fluthiacet methyl used in this part of the experiment was 8 g.a.i $ha^{-1}$, and it was applied using a single 8001 nozzle delivering 100 L/ha at 240 kPa in spray cabinet. Above ground, biomass was collected fourteen days after herbicide treatment (Appendix F). Based on these results set of four M4 lines was chosen for further study.

### 7.2.3 Field Testing of Selected M4 Lines with Sulfentrazone

Twenty-two mutagenized lines were validated through field tests to assess their levels of tolerance to sulfentrazone relative to CDC Redberry. Mutagenized lines and checks were seeded in individual 2.4 m long rows, at a seeding rate of 100 seeds per row. The trial was conducted near Saskatoon (Sutherland 52°10'19.9"N 106°30'21.2"W) during the summer of 2015. The experimental design was a split-plot with herbicide treatments (control and 280 g.a.i $ha^{-1}$ sulfentrazone) representing main plots, and genotypes (i.e. mutagenized lines and checks) representing sub-plots, with four replications. The application of 280 g.a.i $ha^{-1}$ sulfentrazone was

performed the previous fall. The experiment was repeated, with the second seeding date one week after the first.

Dry biomass data were collected from above ground biomass samples harvested 21 days after seeding, and dried in an oven at 80°C for 48 h before weighing. NDVI data was collected using a hand-held GreenSeeker™ RT-100 (Trimble Navigation Limited, Sunnyvale, CA) the day before the biomass was harvested from the field. The GreenSeeker™ sensor was held approximately 30 cm above the top of the lentil canopy. This device records reflectance at 660 nm and 780 nm which correspond to red and infrared part of light spectra, respectively, which is referred to as "red NDVI" in some literature. GreenSeeker Capture™ software, part of RT100 system, collects, displays current reading, and stores measurements for further use (Trimble Navigation Limited, 2010).

### 7.2.4 Dose Response of Selected M4 Lines to Fluthiacet Methyl

Based on the results of the initial, 2014 field screening, and the additional indoor testing, a set of 4 mutagenized lines (Lot2-11; Lot2-418; Lot2-457; Lot2-498) were selected for a dose response study with CDC Redberry as the check. Plants were grown in a controlled environment under the following conditions: day length was set to 18 h, at 21°C temperature, and light intensity of 350 $\mu mol/m^2s$, while the night phase was set to 6 h at 15°C. Planting medium was a mix of Sunshine #3 and Perlite (Sun Gro Horticulture, Canada) at a 2:1 ratio in 10 $cm^2$ square pots. Twelve rates of fluthiacet methyl were used: 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, and 256 g.a.i $ha^{-1}$. Non-ionic surfactant Agral-90 (Syngenta, Canada) was applied with the herbicide at 0.25% v/v. Plants were sprayed at the 4-5 leaf stage with a single 8001 nozzle delivering 100 L/ha at 240 kPa in the spray cabinet. Above soil biomass was harvested 14 d after spraying and dried for 48 h at 80°C before weighing. The experiment was repeated a second time.

### 7.2.5 Electrolyte Leakage Assay of Selected Individual Mutant Plants and Selected M4 Lines with Sulfentrazone and Fluthiacet Methyl

An ELA was used to test mutant plants selected for possible resistance to either sulfentrazone or fluthiacet methyl. CDC Redberry served as a check. The protocol involved growing plants and conducting ELA was the same as in Chapter 3.3. Sulfentrazone screening of selected mutant lines

was done with sulfentrazone at a concentration of 150 µM, while the fluthiacet methyl screen used a concentration of 25 µM. Detailed description of sample preparation, measurement of electro-conductivity and calculation of the percentage of leakage is provided in chapter 3.3.

### 7.2.6 Statistical Analysis

Dry biomass and NDVI data were tested for normality and homogeneity of variance using Shapiro–Wilk test and Levene's test, respectively, in SAS 9.4 software package (SAS Institute, Cary, NC). Dry biomass data failed both tests, and were thus transformed using Box-Cox transformation, which successfully corrected both normality and homogeneity of variance issues. NDVI data did not fail either the Shapiro–Wilk or the Levene's test. Analysis of variance of both dry biomass and NDVI data was performed using SAS 9.4 software, by means of a general linear model (PROC GLM). Tukey's studentized range test (HSD) was conducted using the means of genotypes for both dry biomass and NDVI data. Pearson coefficient of correlation between dry biomass and NDVI score was calculated using PROC CORR of SAS 9.4.

Statistical analysis of the ELA data was conducted using the drc package of R software (R Development Core Team, 2014). This analysis is predicated on the selection of an appropriate model for accurate data description. Tested models included three, four and five-parameter log-logistic, and three and four-parameter Weibull type 1 and type 2 models. All of the models tested had the upper limit fixed at 100, since the percentage of leakage could not exceed 100%. Akaike information criterion (AIC) was used to asses and compare fitted models. The model with the lowest AIC score was selected for further analysis. Data from the sulfentrazone and fluthiacet methyl ELAs were separately fitted to a three-parameter Weibull type II model (Equation 5.1)

$T_{50}$ values (described in detail in chapter 3.3.3) were calculated and pairwise comparison of $T_{50}$ values was performed using the EDcomp function of the drc package of R. Control treatments were not used in this analysis, since they represent linear functions, and are thus not suitable for this non-linear analysis.

Dose responses of selected mutant lines and CDC Redberry were tested for homogeneity of variance of the two replications of the experiments. This did not reveal significant differences, so the data were combined for further analyses. A set of different dose response models was tested

and three-parameter Weibull type 1 model was used to regress dry biomass data over herbicide rates (Wild & Seber 1989). The formula for the Weibull type 1 model is as follows:

$$Y = D * exp\big(-exp\big(B(log(X) - log(E))\big)\big) \quad (7.1)$$

where $Y$ is the response (dry biomass), $D$ is the upper limit, $B$ is the relative slope around the inflection point $E$, and $X$ is the herbicide dose. The drc package of the R software (R Development Core Team, 2014) was used to perform statistical analyses. $ED_{50}$ values from the dose response curves were calculated for the all genotypes and a comparison of $ED_{50}$ values was conducted using the EDcomp function of the drc package.

## 7.3 Results

### 7.3.1 Results of the Initial Screening of M3 Lines with Sulfentrazone and Fluthiacet Methyl

Among the 1,171 mutagenized lines in the initial sulfentrazone screening, almost five hundred did not show any signs of germination after two weeks (Figure 7.1A). The highest number of plants per microplot was 49, and only four lines had more than 40 germinating plants out of the 100 seeded. Only 41 mutagenized lines had more than twenty germinating plants (Figure 7.1B). Selection among those 41 mutagenized lines was based on the appearance and lack of injury (i.e., yellowing and necrosis). The final selection included 22 lines that were then validated. Among the checks, the number of germinating plants ranged from 2, for CDC KR-1, to 6.8 for CDC Improve (Figure 7.1C).


| | Number of germinated plants per plot | Standard error |
|---|---|---|
| CDC Impala | 3.4 | 1.99 |
| CDC Improve | 6.8 | 2.83 |
| CDC KR-1 | 2 | 0.94 |
| CDC Redberry | 2.8 | 1.68 |
| CDC Rosetown | 4 | 2.92 |

Figure 7.1. Results of initial field screening in 2014 where mutagenized lentil lines and the set of five checks were exposed to 560 g.a.i $ha^{-1}$ sulfentrazone. (A) Representative subset of plots, picture taken seven weeks after seeding. (B) Distribution of the number of germinated plants per plot for mutagenized line two weeks after seeding (seeding rate was 100 seeds per microplot). (C) Number of germinated plants per plot for each check, collected two weeks after seeding.

In addition to the selection of entire plots, individual surviving plants were also selected. Those individual plants were, in most cases, the only healthy plants in a plot, or across even larger areas (Figure 7.2). Ten individual plants were caged for protection and later harvested. Due to a low number of harvested seeds, those plants were only tested using the ELA.

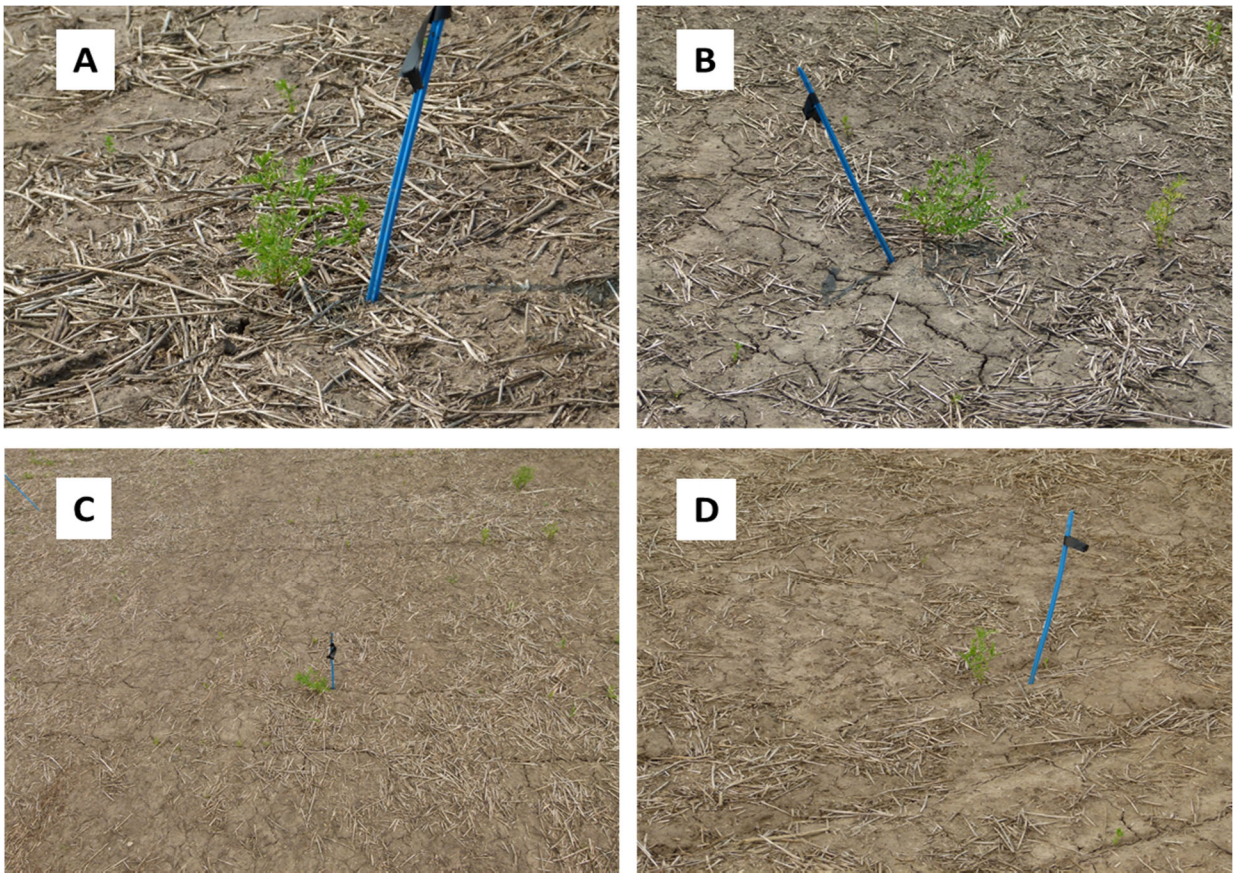
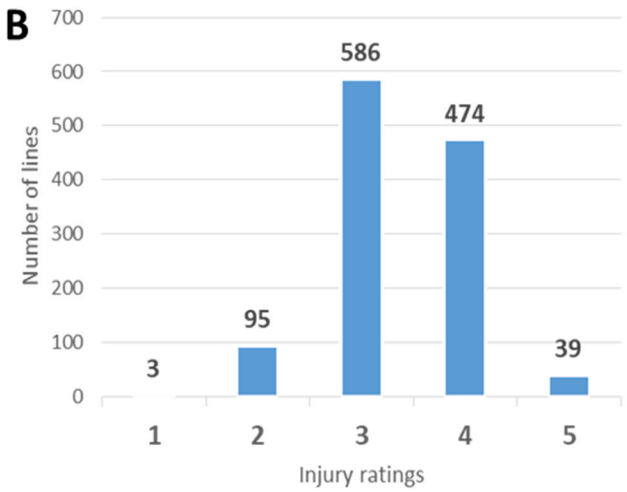



Figure 7.2. Examples of selected individual mutants from the 2014 sulfentrazone (560 g.a.i $ha^{-1}$) field screening at Saskatoon (SPG) location. (A) Selected plant MLA282. (B) Selected plant MLA382. (C) Selected plant MLA591. (D) Selected plant MLA707. All images were taken seven weeks after seeding.

In the fluthiacet methyl field screening, most injury scores ranged between 3 and 4, and only a few produced extreme results. The lowest injury rating was observed in only three lines (Figure 7.3B). Among the checks, injury ratings ranged from 4.8 for CDC Rosetown to 3.0 for CDC KR-1 (Figure 7.3C). No individual plants were selected, since plants within microplots recorded uniform levels of injury.


B




C

| | Injury rating | Standard Error |
|---|---|---|
| CDC Impala | 3.4 | 0.36 |
| CDC Improve | 3.6 | 0.22 |
| CDC KR-1 | 3 | 0.28 |
| CDC Redberry | 3.6 | 0.22 |
| CDC Rosetown | 4.8 | 0.18 |

Figure 7.3. Results of 2014 field trial where mutagenized lentil lines and set of five checks were exposed to 32 g.a.i $ha^{-1}$ of fluthiacet methyl. (A) Representative subset of plots, picture taken seven weeks after seeding. (B) Distribution of injury ratings across all microplots three days after fluthiacet methyl spraying. (C) Distribution of injury ratings across five checks three days after fluthiacet methyl spraying.

### 7.3.2 Results of Field Testing of Selected M4 Lines with Sulfentrazone

Analysis of variance of the dry biomass data showed that both treatment and seeding date were highly significant ($p<0.001$) sources of variance. In contrast, genotypes, interaction between genotype and seeding date, interaction between genotypes and herbicide treatment, interaction between seeding date and herbicide treatment, and interaction between genotype, seeding date, and herbicide treatment were not significant sources of variance (Table 7.1).

The same overarching effects were observed in the NDVI data, where seeding date and treatment were found to be significant ($p<0.001$), and genotype, interaction of genotype and seeding date, interaction of genotypes and herbicide treatment, interaction of seeding date and herbicide treatment, and interaction of genotype, seeding date and herbicide treatment were not significant to the variability observed.

Table 7.1 *F-values from the analysis of variance (ANOVA) of dry biomass and NDVI data across 22 selected mutagenized lines and CDC Redberry tested with 280 g.a.i ha$^{-1}$ sulfentrazone in the field at Saskatoon in 2015*

| Source | Dry biomass | NDVI |
|---|---|---|
| *Genotype (G)* | $0.69^{NS}$ | $0.64^{NS}$ |
| *Seeding date (S)* | $62.09^{***}$ | $58.01^{***}$ |
| *Herbicide treatment (HT)* | $403.88^{***}$ | $365.31^{***}$ |
| *GxS* | $1.01^{NS}$ | $1.05^{NS}$ |
| *GxHT* | $1.03^{NS}$ | $0.97^{NS}$ |
| *SxHT* | $1.40^{NS}$ | $5.47^{NS}$ |
| *GxSxHT* | $0.48^{NS}$ | $0.60^{NS}$ |

*** $p<0.001$; ** $p<0.01$; $^{NS}$ not significant.

The mean and standard deviation of dry biomass for all tested mutant lines and CDC Redberry are given in Appendix G. The mean NDVI values and standard deviations for all mutagenized lines and the check (CDC Redberry) are provided in Appendix G. The largest NDVI value in the control treatment was observed for Lot2-1207, and the smallest for Lot2-494. Under the sulfentrazone treatment, the largest NDVI values were associated with Lot2-418, while the smallest value was

observed in Lot2-297. Tukey's studentized range test (HSD) found no difference in dry biomass among genotypes tested under either treatment.

NDVI and dry biomass data combined across the treatments and seeding dates had a positive correlation ($r^2 = 0.845$; $p<0.0001$). Data were also analyzed for each seeding date individually. Pearson correlation coefficient between NDVI and dry biomass data from the first seeding date was 0.923 ($p<0.0001$), while the coefficient for the data from the second seeding date was $r^2= 0.834$ ($p<0.0001$) (Appendix H).

### 7.3.3. Results of Electrolyte Leakage Assay of Individually Selected M4 Plants Treated with Sulfentrazone

The leakage data obtained from the ten individually selected mutant plants and CDC Redberry were fitted to a three-parameter Weibull type 2 model (Figure 7.4). A lack of fit test (p value = 1) proved that the model accurately represented the data. $T_{50}$ values show a slight grouping of tested genotypes, where MLA397, MLA418, MLA561, and MLA835 all have $T_{50}$ values close to 65, while the rest of the mutants and CDC Redberry ranged between 79 and 92 (Table 7.2). The lowest $T_{50}$ value (64.7) was observed for MLA397. Pairwise comparisons of $T_{50}$ values between CDC Redberry and the mutants showed that MLA397, MLA418, MLA561 and MLA835 had significantly ($p<0.001$) lower $T_{50}$ values than CDC Redberry. The $T_{50}$ value of MLA194 was not significantly different from that of CDC Redberry. $T_{50}$ values of MLA282 and MLA707 were found to be significantly ($p<0.05$) higher than that of CDC Redberry; MLA382 and MLA893 were even higher ($p<0.01$); and MLA591 had the highest $T_{50}$ value of 92.3.

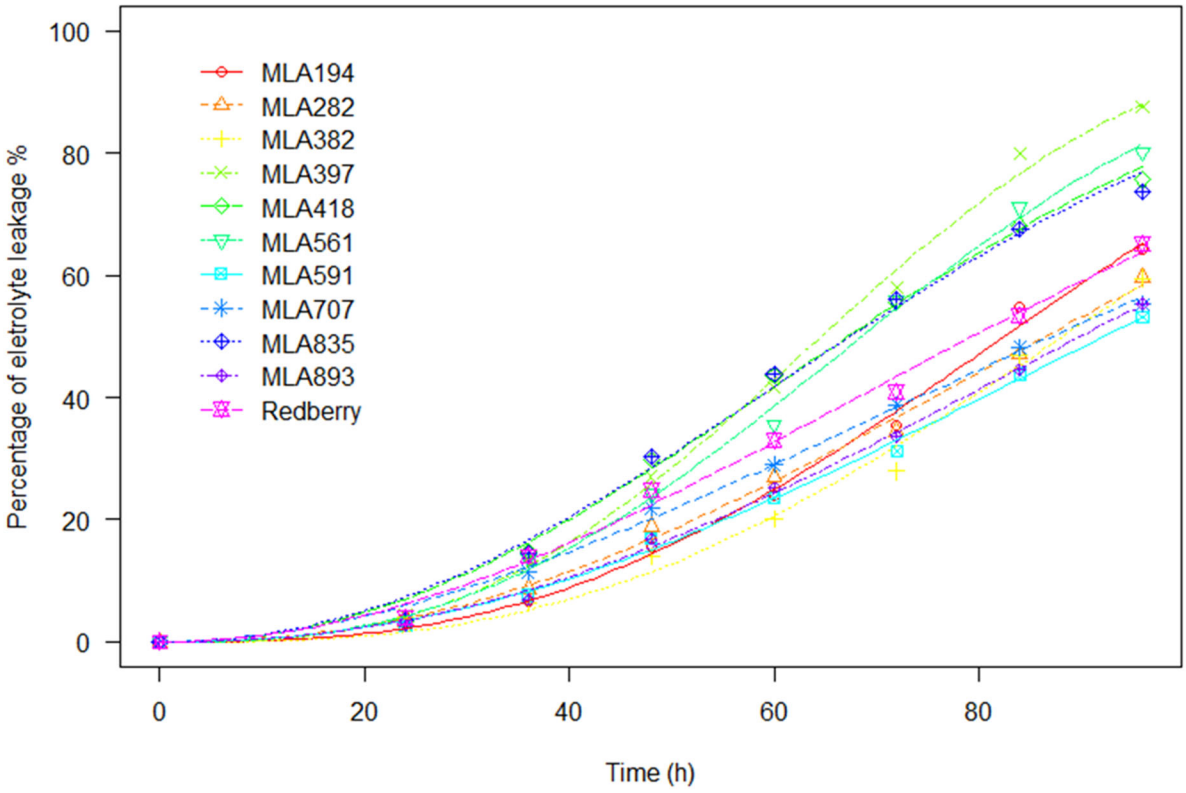



Figure 7.4. Response curves of electrolyte leakage assays fitted with three-parameter Weibull type 2 model for 10 selected mutant plants and CDC Redberry, all treated with 150 µM of sulfentrazone.

Table 7.2 *$T_{50}$ values of electrolyte leakage assay and* t *values with significance of paired t-tests between $T_{50}$ values of CDC Redberry and ten selected mutants treated with 150 µM of sulfentrazone*

| | CDC Reddbery | MLA194 | MLA282 | MLA382 | MLA397 | MLA418 | MLA561 | MLA591 | MLA707 | MLA835 | MLA893 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| T50 | 79.4 | 82.5 | 86.5 | 88.2 | 64.7 | 67.2 | 68.6 | 92.3 | 87.2 | 67.43 | 89.9 |
| SE | 2.37 | 1.93 | 2.69 | 2.31 | 1.42 | 1.72 | 1.53 | 3.45 | 3.25 | 1.75 | 3.08 |
| CDC Reddbery | | -1.03$^{NS}$ | -2.06* | -2.88** | 5.00*** | 3.89*** | 3.64*** | 3.40*** | -2.05* | 3.80*** | -2.93** |
| MLA194 | | | -1.26$^{NS}$ | -1.98* | 6.74*** | 5.36*** | 5.22*** | -2.69** | -1.29$^{NS}$ | 5.23*** | -2.69* |
| MLA282 | | | | -4.82$^{NS}$ | 6.64*** | 5.36*** | 5.22*** | -1.37$^{NS}$ | 1.54$^{NS}$ | 5.44*** | -8.53$^{NS}$ |
| MLA382 | | | | | 7.81*** | 6.51*** | 6.47*** | -1.01$^{NS}$ | 2.60$^{NS}$ | 6.39*** | -4.52$^{NS}$ |
| MLA397 | | | | | | -1.18$^{NS}$ | -1.94$^{NS}$ | -9.86*** | -8.04*** | -1.26$^{NS}$ | -9.61*** |
| MLA418 | | | | | | | -6.00$^{NS}$ | -8.24*** | -6.57*** | 8.83$^{NS}$ | 7.92*** |
| MLA561 | | | | | | | | -7.94*** | -6.23*** | 0.48$^{NS}$ | -7.62*** |
| MLA591 | | | | | | | | | 1.05$^{NS}$ | 5.92*** | 0.50$^{NS}$ |
| MLA707 | | | | | | | | | | 4.98*** | -6.26$^{NS}$ |
| MLA835 | | | | | | | | | | | -7.76*** |
| MLA893 | | | | | | | | | | | |

*** $p < 0.001$; ** $p < 0.01$, * $p < 0.05$; $^{NS}$ not significant.

### 7.3.4 Results of Electrolyte Leakage Assay Performed on Selected M4 Lines Treated with Fluthiacet Methyl

The ELA for the four mutant lines and CDC Redberry exposed to 25 µM fluthiacet methyl also fitted a three-parameter Weibull type 2 model as confirmed by a lack of fit test ($p = 0.9469$). ELA curves for the mutagenized lines showed higher rates of leakage over time than CDC Redberry (Figure 7.5). All mutant lines had significantly ($p<0.001$) lower $T_{50}$ values than did CDC Redberry ($T_{50} = 74.4$). Lot2-418 had the lowest $T_{50}$ value of 55.2, while Lot2-11 had the highest value, 68.1. All mutant lines had significantly ($p<0.01$) lower $T_{50}$ values than CDC Redberry (Table 7.3).

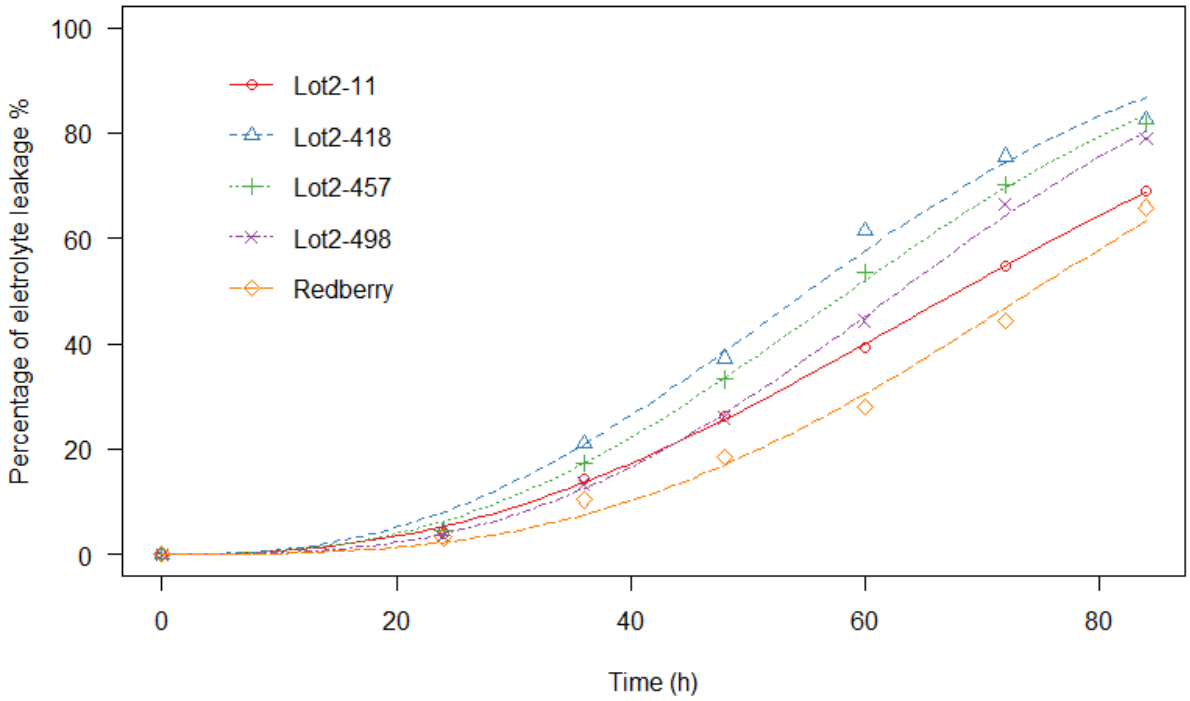



Figure 7.5. Response curves of electrolyte leakage assay for four mutagenized lines (Lot2-11; Lot2-418; Lot2-457; Lot2-498) and CDC Redberry, treated with 25 µM of fluthiacet methyl.

Table 7.3 *$T_{50}$ values of electrolyte leakage assay and standard error for selected mutagenized lines (Lot2-11, Lot2-418, Lot2-457, Lot2-498) and CDC Redberry, and t values with significance of paired t-tests between tested genotypes treated with electrolyte leakage assay at 25 µM concentration of fluthiacet methyl*

| **Line** | **Lot2-11** | **Lot2-418** | **Lot2-457** | **Lot2-498** | **CDC Redberry** |
|---|---|---|---|---|---|
| **$T_{50}$** | 68.1 | 55.2 | 58.7 | 63.0 | 74.4 |
| **SE** | 1.09 | 0.86 | 0.88 | 0.86 | 1.1 |
| **Lot2-11** | | 7.55*** | 5.61*** | 3.15*** | -3.79*** |
| **Lot2-418** | | | -2.62** | -6.08*** | -1.44*** |
| **Lot2-457** | | | | -3.23** | -1.13*** |
| **Lot2-498** | | | | | -8.00*** |
| **CDC Redberry** | | | | | |

***p <0.001; **p <0.01; $^{NS}$, not significant.

### 7.3.5 Fluthiacet Methyl Dose Response of Selected M4 Lines

In the dose response study, a three-parameter Weibull type 1 model was used to analyze the data. A lack of fit test indicated that this model provides an accurate description of the data (p = 0.19). Dose response curves for the mutant lines and CDC Redberry are shown in Figure 7.6. The $ED_{50}$ values among mutant lines ranged from 15.67 (for Lot2-418) to 34.59 (for Lot2-457), while the $ED_{50}$ for CDC Redberry was 9.75 (Table 7.4). Comparison of $ED_{50}$ values showed that Lot2-11 and Lot2-418 were not significantly different from CDC Redberry, but Lot2-457 and Lot2-498 had significantly ($p<0.001$) higher $ED_{50}$ values than CDC Redberry (Table 7.4).

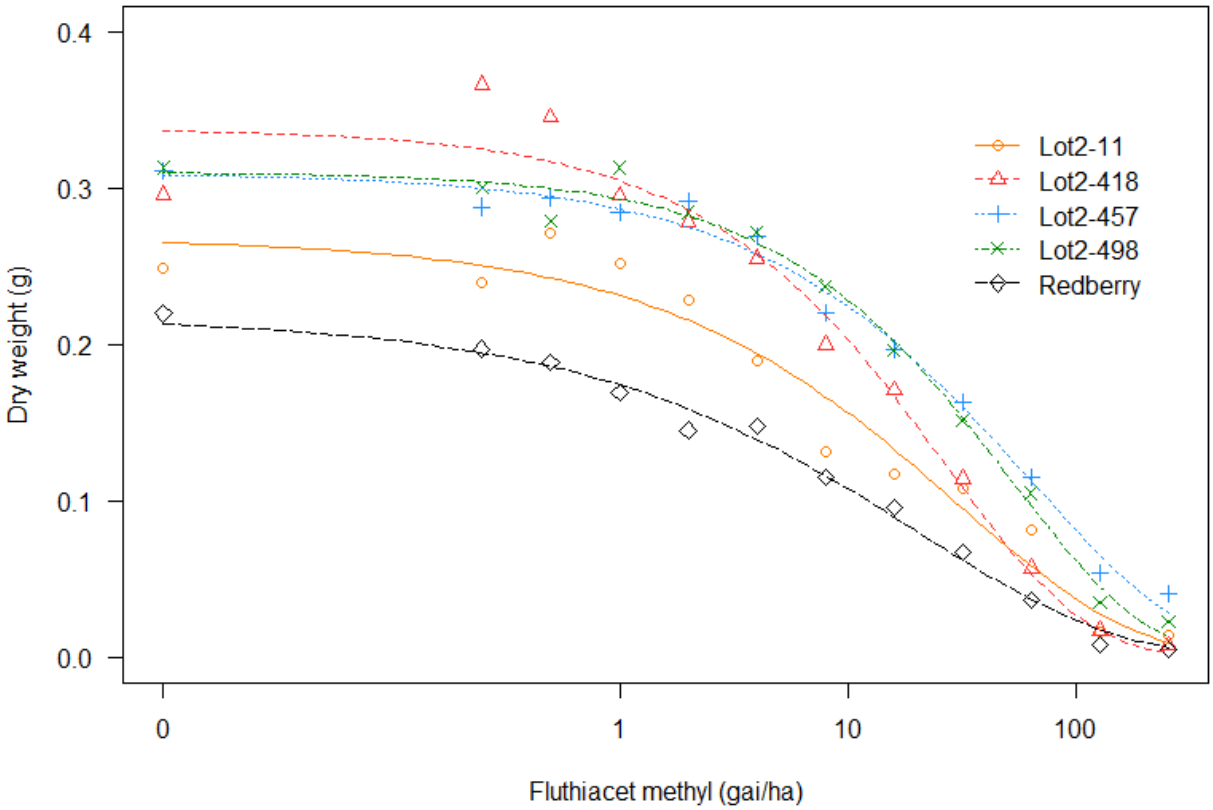



Figure 7.6. Response of selected mutagenized lines (Lot2-11; Lot2-418; Lot2-457; Lot2-498) and the CDC Redberry check to the application of fluthiacet methyl, expressed with dry biomass. Each point represents an average of four replications.

Table 7.4 *Effective dose 50 ($ED_{50}$) calculated for selected mutagenized lines (Lot2-11; Lot2-418; Lot2-457; Lot2-498) and CDC Redberry, and multiple comparison of the $ED_{50}$ between examined material*

| **Line** | **CDC Redberry** | **Lot2-11** | **Lot2-418** | **Lot2-457** | **Lot2-498** |
|---|---|---|---|---|---|
| **$ED_{50}$** | 9.75 | 15.69 | 15.67 | 34.59 | 34.20 |
| **SE** | 2.88 | 2.99 | 1.97 | 5.21 | 4.08 |
| **CDC Redberry** | | $-1.74^{NS}$ | $1.89^{NS}$ | -7.69*** | -6.81*** |
| **Lot2-11** | | | $0.008^{NS}$ | -4.96*** | -4.28*** |
| **Lot2-418** | | | | -6.15*** | -5.46*** |
| **Lot2-457** | | | | | $0.49^{NS}$ |
| **Lot2-498** | | | | | |

***p <0.001; **p <0.01; $^{NS}$, not significant.

## 7.4 Discussion

Mutation breeding has been successfully used to generate lentil germplasm with resistance to imidazoline and sulfonylureas herbicides (both Group 2) (Slinkard, Vandenberg & Holm, 2007; Rizwan et al., 2017). Experiments conducted in this study sought to find resistance to herbicides group 14.

Initial screening of M3 lines with sulfentrazone caused extensive damage to all mutant lines and the checks. Selected M4 lines were subsequently screened under field conditions at a lower rate of 280 g.a.i $ha^{-1}$ sulfentrazone. Injuries ranged from reduced plant growth to necrosis. The rate of sulfentrazone used was four times higher than the rate recommended for weed control, and as such would not typically be applied in production in Western Canada. The strategy of applying a high rate of sulfentrazone was executed in anticipation of finding large genetic gains in herbicide resistance. When genetic material is highly susceptible, a different strategy is clearly needed. Application of lower rates of herbicide can also lead to the development of herbicide resistance, as evidenced by rigid ryegrass and diclofop. Exposing a herbicide-susceptible population of rigid ryegrass (*Lolium rigidum*) to low rates of diclofop, led to the rapid development of herbicide resistance in the screened population (Manalil et al., 2011). The lentil genotypes exhibited different levels of herbicide tolerance, and screening at a low rate would only exclude the most susceptible lines from the selection process, leaving a large number of plants for further testing.

Dry biomass and NDVI values were the main focus of the field data collection. Correlation between dry biomass and NDVI was very high, suggesting that NDVI could be used as an estimator of biomass. Among the advantages of using NDVI data are lower costs and the ability to collect data multiple times without disturbing (i.e. cutting) the plants. Reported problems with NDVI include saturation due to a high volume of biomass in the field, which can lead to an overestimation of NDVI values (Prabhakara, Hively & McCarty, 2015). However, saturation does not present a serious problem to exploration of herbicide injury in lentil since the plant size is relatively small, and the maximal biomass is not reached by the time of data collection. Lentil plants generally reach maximum biomass in early to late bud formation stages, corresponding to 42–56 days after emergence (Malhi et al., 2007). Statistical analysis showed that in terms of both dry biomass and NDVI, M4 lines under sulfentrazone treatment did not perform any better than CDC Redberry.

In addition to screening M4 lines, 10 individual M4 plants were tested with an ELA to assess the sensitivity of the material to sulfentrazone (Li, Wehtje, & Hancock, 2000). Leaf samples of M4 plants were used in the ELA, meaning that observed differences stem from the ability of leaf cells to cope with, and delay the effects of an herbicide. This also implies that the assay did not take into account the entire plant. Other potential mechanisms of herbicide tolerance, including root absorbance or herbicide translocation, are not evaluated with this assay. Results of the ELA for the selected M4 individuals showed that five of the ten plants selected had higher $T_{50}$ values than the check (CDC Redberry). Those five selected M4 plants—MLA282, MLA382, MLA593, MLA707, and MLA893—are all potential candidates for inclusion in the breeding program, although additional testing should be conducted in a variety of environments to confirm the presence of stable and elevated herbicide tolerance.

Initial screening of M3 lines with fluthiacet methyl at a rate of 32 g.a.i $ha^{-1}$, caused high initial injury across all lines. Only a small number of mutagenized lines showed limited herbicide damage at this rate. Injury rating scores for the M3 lines were highest in the first week after herbicide application. Almost all M3 lines showed rapid recovery in subsequent weeks. Selection of M3 lines for further study was, therefore, focused on the initial injury and early recovery results.

A dose response experiment showed that two selected M4 lines; Lot2-457 and Lot2-498, had significantly higher $ED_{50}$ values than did CDC Redberry, implying that they should possess higher levels of tolerance than CDC Redberry. The results of the ELA, however, indicated that all four mutagenized lines tested had lower $T_{50}$ than CDC Redberry, suggesting that they are less tolerant. These contradictory results could be explained by considering the two stages of herbicide effect. The first stage is the initial injury caused by the herbicide, and the second stage is the recovery from the initial effects of the herbicide. Initial injury and recovery from herbicide in this case should be treated independently. ELA with fluthiacet methyl is a short-term experiment based on exposing the leaf tissue to a solution of herbicide and measuring tissue decay over time (Shimizu et al., 1995). In contrast, dose response is a longer lasting experiment, and in the case of fluthiacet methyl it can take up to 14 d, giving the plants sufficient time to recover from damaged tissue. Selected M4 lines did not gain the ability to metabolize or mitigate effects of herbicide in the cell, rather they had the ability to regrow faster than CDC Redberry after injury. Selecting material based on initial injury does not automatically lead to improved recovery, and vice versa, as they

are controlled by different genetic loci. Selecting lentil material based on both initial injury and recovery would require proper phenotyping of both traits, which could present a significant issue as it is not clear how to distinguish when initial injury ends and when recovery starts. Developing low cost and high throughput phenotypic techniques, able to distinguish between these two traits is essential for further progress. New developments in phenomics, such as imaging technologies, could be used to bridge this gap. A successful combination of improvements in these traits could result in higher levels of tolerance to the herbicide.

All currently available, commercial, non-transgenic, herbicide tolerant crops were created through mutation of the gene that encodes an herbicide target site (Tan & Bowe, 2009). In previous years, three target sites were used the most for development of herbicide tolerant crops: acetyl-CoA carboxylase (ACCase) target of herbicides group 1, acetohydroxyacid synthase (AHAS) target site of herbicides group 2, and D1 protein of photosynthesis II target of herbicide group 5 (Tan & Bowe, 2009). Herbicide tolerant mutants with altered target sites tend to exhibit very high levels of herbicide resistance (Tan et al., 2005). Mutant selection for resistance to sulfentrazone in this study did yield five potential candidates but the results did not show the anticipated extremely high levels of resistance. The increase in fluthiacet methyl tolerance was found more in the biomass regrowth than in the actual response to the initial herbicide application. Therefore, alteration of target site was not the underlying cause of the improved results relative to the check.

If the target site is not altered, resistance can be a result of a mutation of genes controlling metabolic (non-target site) resistance. However, a major issue with non-target site resistance is pinpointing the exact gene responsible, since some of the candidate genes belong to diverse gene families, such as *cytochrome P450s*, *glutathione-S-transferases* and *ABC transporters*. Mutation can also lead to increased susceptibility, as shown in the case of two rice mutants whose mutation on cytochrome P450 (*CYP81A6*) caused 60-fold more susceptibility to bentazon than wild-type (Pan et al., 2006).

In the light of experience and knowledge gathered through this set of experiments, new strategies need to be proposed for selecting mutants with increased levels of tolerance. Particularly, a two-step selection process followed by validation studies is recommended. The first step would involve screening a very large number of M2 seeds in a controlled environment. Seeds could be placed on germination paper in large tubs, soaked with a high concertation of an herbicide, and left to

germinate. The concentration of an herbicide should high enough to cause plant death among the most tolerant available genotypes. Germinating plants with low or no injury would be rescued and transferred to growing media in pots for seed increase. In the case of group 14 herbicides, light conditions play a crucial role, so it would be critical to provide uniform light conditions throughout the selection process. The advantage provided by this step is that it could be repeated in a uniform fashion as many times as needed, and thereby allow for screening of much larger numbers of mutant seed material, without the added stress of the field environment. The disadvantage of this step higher cost in comparison to mass field screening. Adding costs for this initial step is justified in case of sulfentrazone, as large variations in efficacy of this herbicide were observed in field conditions which could lead to numerous false positives and there for jeopardizing the whole selection process.

Following the initial step of selection, mutants should then be tested under field conditions against a set of checks. Depending on the seed availability, field experiments should have at least four replications for any selected mutant. Unreplicated trials should be avoided because of possible variability in herbicide efficacy across fields. Implementation of this two-step strategy would also deal with the issue of initial injury and recovery. The first step focuses on the level of initial injury, and the second step of selection deals with the long-term effect of an herbicide. Any mutant that successfully meets both should be included in a breeding program and/or used for further studies. This two-step approach is more complex in comparison to previous strategies which heavily depend on initial mass field selection, but it provides a much higher level of certainty when selection for herbicide resistance.

# 8. GENERAL DISCUSSION

The function of herbicides in lentil production is to bridge the gap between the weak weed competitiveness and stable high yield. The ongoing spread of herbicide-resistant weeds across Western Canada reduces the effectiveness of several key herbicides in lentil production. The strategies proposed to mitigate the pressure of herbicide-resistant weeds in Western Canada, such as rotation of herbicides with different mode-of-action, herbicide tank mixes, and promoting more competitive crops (Beckie & Harker, 2017), could potentially put lentil in a precarious position. Lentil has few registered herbicides, which limits the possible tank mixes or herbicide rotations. It is also one of the weakest competitors among major crops in Western Canada and could easily be marginalized among producers. Increasing the number of registered herbicides could ensure lentil remains a viable part of crop rotation.

Sulfentrazone and fluthiacet methyl, group 14 herbicides, have never been used or registered in lentil for weed control. Testing the effects of sulfentrazone and fluthiacet methyl on seven selected lentil varieties showed that different levels of herbicide tolerance to both herbicides exist in lentil germplasm. The differences among tested lentil varieties were more evident in some environments as efficacy of herbicide is highly influenced by environment. The experiments revealed different effects on lentil growth and development by these herbicides. Sulfentrazone is a soil-applied pre-emergent herbicide, so the maximum effects occur as early as the germination stage, and persist throughout the vegetative period due to a long half-life in the soil (Mueller et al., 2014). In contrast, fluthiacet methyl is a contact herbicide applied when lentil is at the 5-6 leaf stage. The effects of fluthiacet methyl reach a pinnacle just days after the application and are followed by recovery, even at rates several times higher than the recommended rate. The prolonged effects of sulfentrazone and fast recovery after fluthiacet methyl are evident in the yield data presented in chapter 3, where in most cases sulfentrazone did cause a reduction in yield, and fluthiacet did not. The environment has a very important role in facilitating the effects of these two herbicides, especially sulfentrazone. Inconsistency in the efficacy of sulfentrazone was observed among testing locations. The experiments at the Scott location had lower injury in comparison to the

Saskatoon location. The two locations differ in content of organic matter and soil composition, where soil at Scott location is more composed of sand and silt with less organic matter, while the soil at Saskatoon (Kernen Farm) includes more clay and organic matter. The lower organic content, coarser soil structure, and higher pH value enable greater soil mobility of sulfentrazone (Ohmes & Mueller, 2007). Sulfentrazone in Scott soil was more mobile and leached in the deeper layers reducing the concentation at the root zone. The activation of sulfentrazone depends heavily on precipitation, so late precipitation or the lack thereof leads to less injury on lentil. The focus of ELA was on the leaf tissue and its ability to deal with the effect of herbicide. The results of ELA point out that tolerance at the tissue and cellular level is different among tested varieties. The range of diversity of fluthiacet methyl tolerance could also be found in the dose-response experiment, where the most susceptible variety had an almost four times smaller median effective dose ($ED_{50}$) than the most tolerant variety. The experiments conducted and presented in chapter 3 laid the groundwork for further work, as they show that lentil genotypes do vary in the level of tolerance to sulfentrazone and fluthiacet methyl.

A larger set of lentil genotypes was gathered to answer what is the genetic background of fluthiacet methyl tolerance in lentil. The study did originally include sulfentrazone as a treatment, but due to lack of herbicide activity at a majority of site years, data were excluded from further analyses. Future sulfentrazone studies of this type should be performed in areas where irrigation is available to ensure timely activation of sulfentrazone. Associations between traits that describe damage of fluthiacet methyl and SNPs revealed an intricate mechanism which influenced tolerance levels. Lentils, depending on seed size, vigor, adaptability etc., vary in plant biomass at the same stages of development, so to accommodate for that, additional elements were added to the model. Control data served as a covariate in the model to interact the natural differences in plant size.
The most abundant among candidate genes were *cytochrome P450s* and *glutathione-S-transferases*, which corresponds with one predicted mechanism of non-target site resistance. Non-target site herbicide resistance involves herbicide metabolism, which can be divided into three phases: first being activation, second conjugation, and third compartmentalization of the herbicide molecule (Ghanizadeh & Harrington, 2017, Yuan, Tranel & Stewart, 2007). *Cytochrome P450* belongs to large superfamily of plant enzymes, whose function is to insert an oxygen molecule into targeted molecules making them more reactive. In the case of herbicides, insertion of oxygen leads to faster metabolism of the herbicide molecule in the plant cell (Werck-Reichhart &

Feyereisen 2000). One of the characteristics of this cytochrome P450 mechanism is a wide array of the herbicides that could be affected by it, e.g., bentazon and sulfonylurea tolerance in rice (Zhang et al., 2007), nicosulfuron, foramsulfuron and mesotrione tolerance in corn (Pataky et al., 2008), diclofop-methyl and chlorsulfuron tolerance in *Lolium rigidum* (Busi et al., 2011). The second phase of metabolism of a herbicide is dominated by glutathione-S-transferases, which catalyze the conjugation of glutathione or homoglutathione to the target molecule (Yuan et al., 2007). The glutathione S-transferase enzymes in addition to the metabolism of herbicide could also play a role in controlling effects of reactive oxygen species in a plant cell (Gechev et al., 2006). Many cases of glutathione conjugates of herbicides, such as atrazine, metolachlor, flurodifen, sulfonylureas, were found in plants and as such conjugates, they are not toxic to the target enzyme (McGonigle et al., 2000).

An additional study was performed on a smaller set of lentils to confirm the role of cytochrome P450s and glutathione-S-transferases in tolerance to herbicides of group 14. Inhibition of cytochrome P450s lead to increase in damage due to exposure to either sulfentrazone or fluthiacet methyl. The situation with glutathione-S-transferases was more complicated as in some cases inhibition did lead to increased damage, and in others it did not. Inhibitors used in this study are non-selective meaning that they inhibit all members of those enzyme families, so gathered information cannot be used to confirm the specific role of candidate genes. Rather it serves as additional proof of involvement of cytochrome P450s and glutathione-S-transferases in tolerance to sulfentrazone and fluthiacet methyl. Confirmation of candidate genes identified in the association study will require additional research, such as measuring the concentration of different metabolites and measuring production of ROS combined with gene expression studies.

The current model of non-target site resistance is over simplified, and the results of an association study show that plant response to these two herbicides can also involve different stress response genes, transcription factors, and a variety of other genes. The more complex model is more appropriate for group 14 herbicides, as they cause an accumulation of reactive oxygen species which triggers different stress responses in plant. Genes involved in cell wall formation, regulation of phytohormones, and plastid multiplication are also found to be important for tolerance to fluthiacet methyl. All of these genes can act independently or in combination with each other, providing diverse levels of tolerance and potentially making every case of elevated tolerance

distinct. Diverse mechanisms also provide opportunity for the breeder to combine them in new and unique ways, producing even higher level of herbicide resistance. This is an unexplored area which has high potential for the development herbicide resistant crops.

In recorded cases of herbicide resistance among weed species, protoporphyrinogen IX oxidase (*PPOX*) genes were determined to be sources of the resistance. Either through codon deletion or amino acid substitution, some plants were able to survive several folds higher rates of herbicide. Among the cultivated lentils examined, polymorphisms exist in both the chloroplast and mitochondria PPOX enzymes. One amino acid substitution in the mitochondria PPOX enzyme seems to play a role in fluthiacet methyl tolerance. Additional biochemical studies are needed to understand what effect this substitution has specifically on herbicide-enzyme interactions. Interestingly, this amino acid substitution was only significant for fluthiacet methyl and not for sulfentrazone, so it would be compelling to test it with a range of other group 14 herbicides. In the chloroplast PPOX enzyme, one substitution was significant for both herbicide treatments and control. In this case it is hard to determine the utility of this variability based only on these results, so additional research is needed. The studies of PPOX activity are commonly used to assess inhibitory properties of herbicides (Matringe et al., 1989), and in this case two isoforms of PPOX enzyme can be used to asses where herbicides have higher inhibitory properties.

The improvement of the specific trait depends on the variability of that trait in the population, and in some cases levels of variation are not adequate for breeding, so induced mutations are used to generate new genetic variability (Novak & Brunner, 1992). Mutation breeding is the major non-transgenic approach for developing herbicide tolerance (Green & Owen, 2011). Seed and pollen mutagenesis are used in corn, wheat, rice and soybean for developing commercially available herbicide-tolerant crops (Green & Owen, 2011). Mutation breeding is a proven method in developing herbicide tolerance among different crops, especially in the case of groups 2 and 5 herbicides (Rizwan & Akhtar, 2015). This success could be attributed to the relative ease with which mutations to the target sites of these two herbicide groups lead to herbicide tolerance. In herbicide screenings of mutants, selecting the appropriate rate is critical, as it needs to enable easy identification of potentially resistant candidates. Sulfentrazone is a difficult herbicide for mass selection under field conditions as its efficacy is heavily influenced by environment, which produces number of false positives due to the inconsistency of herbicide efficacy across the field.

Screening of the effect of fluthiacet methyl proved to be less complex than sulfentrazone, although, after initial injury most lentil plants started recovering. Mutant lines selected under fluthiacet methyl did surpass the check in a dose-response trial, but not in an ELA; which means that the level of tolerance to fluthiacet methyl at tissue level is the same among mutants and check, but mutants have the ability to recover faster. Selecting these fast-recovering mutants is a consequence of putting emphasis on the recovery phase, where the increase of biomass was used as a predictor of recovery. In both cases, new strategies should be developed to mitigate the drawbacks of previous screening efforts.

### 8.1 Future work

This work was able to answer several critical questions, but at the same time, it raised a number of other questions. Diversity in the effects of sulfentrazone and fluthiacet methyl were observed both in smaller set of seven genotypes and, later, in a much larger set of over a hundred genotypes. This shows that the potential for breeding for higher tolerance exists in within available lentil germplasm. At the same, it time raises a very important question: What is the highest level of tolerance to sulfentrazone and fluthiacet methyl among lentil populations? Screening of additional diverse germplasm with both of these herbicides should reveal the limits of herbicide tolerance. The screening should be performed both under field conditions and in controlled environments, following some of the recommendations made in this thesis. This would lead to the identification of genotypes with elevated tolerance and thereby generating a pool of donors for the breeding program.

The second goal of this research was to study and understand the underlying mechanisms which conveys herbicide tolerance. That goal was achieved, but only in a broad sense as the generalized picture of tolerance mechanisms was created with some of the important factors identified. The genes encoding target sites of sulfentrazone and fluthiacet methyl have certain diversity among lentil germplasm. In addition to studying the target site mechanisms of the effect of group 14 herbicides in lentils, non-target site mechanisms deserves in-depth research. The result suggests that a number of cytochrome P450s and glutathione-S-transferases play an important role in protecting lentil from effects of group 14 herbicides. The identity of individual genes involved in non-target site mechanisms need to be confirmed with additional study, proposed combination of

metabolic and biochemical studies. The phenotyping of herbicide damage should be improved with usage of new technologies, such imaging technology, and measurement of biochemical markers.

The future use of mutation breeding in the development of tolerance to group 14 herbicides depends on the strategy of the breeding program. If naturally occurring diversity does not match the need of the program, induced mutation could generate increased variability. According to the results of this work, multiple genetic factors play role in herbicide tolerance, which underlines that the number of potential targets for induced mutation is much higher than previously anticipated. Mutation breeding should be a long-term effort, which implies that generating and screening of mutagenized seed be conducted continuously until desired results are achieved. Depending on future trends in food legumes, gene editing could be considered as a substitute to chemically induced mutation breeding. Genome editing is a process of making specific changes (deletion, insertion or modification) to the genome using engineered nucleases (zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) or clustered regularly interspaced short palindromic repeats (CRISPR/Cas)) (Shah et al., 2018). In comparison to induced mutation, which randomly occurs across the whole genome, genome editing is a technique which would enable modification of only the specific genes involved in herbicide tolerance and leaves the rest of genome intact. Genome editing, i.e. CRISPR/Cas9 may cause unexpected additional mutations across the genome (Kosicki, Tomberg & Bradley, 2018), however, so certain degree of caution is recommended.

The development of tolerance to sulfentrazone and fluthiacet methyl for lentils would benefit both producers and consumers. Additional herbicide options will avert some of the yield losses caused by weeds, keeping lentil production sustainable in long run. A new paradigm in the development of herbicide resistance is needed, as previous solutions are effective for only short period of times. The time of simple, “silver bullet” type of solutions is behind us, and the time of complex solutions is upon us.

## LITERATURE CITED


Ainley, W. M., Sastry-Dent, L., Welter, M. E., Murray, M. G., Zeitler, M. G., Amora, R., … Petolino, J. F. (2013). Trait stacking via targeted genome editing. *Plant Biotechnology Journal, 11*(9), 1126-1134.

Aizawa, H., & Brown, H. M. (1999). Metabolism and degradation of porphyrin biosynthesis inhibitor herbicides. In Böger, P., & Wakabayashi, K. (Eds.), *Peroxidizing Herbicides* (347-381). Springer.

Alo, F., Furman, B. J., Akhunov, E., Dvorak, J., & Gepts, P. (2011). Leveraging genomic resources of model species for the assessment of diversity and phylogeny in wild and domesticated lentil. *Journal of Heredity*, *102*(3), 315-329.

Asami, T., & Yoshida, S. (1999). Strategy for peroxidizing herbicide-resistant crops. In Böger, P., & Wakabayashi, K. (Eds.), *Peroxidizing Herbicides* (329-345). Springer.

Ashton, F. M., & Crafts, A. S. (1981). *Mode of Action of Herbicides*. John Wiley & Sons, Inc.

Baird, J. M., Shirtliffe, S. J., & Walley, F. L. (2009). Optimal seeding rate for organic production of lentil in the northern Great Plains. *Canadian Journal of Plant Science*, *89*(6), 1089-1097.

Ball, D. A. (1992). Weed seedbank response to tillage, herbicides, and crop rotation sequence. *Weed Science*, *40*(4), 654-659.

Beckie, H. J. (2013). Herbicide-resistant (HR) crops in Canada: HR gene effects on yield performance. *Prairie Soils Crops Journal*, *6*, 33-39.

Beckie, H. J., & Harker, K. N. (2017). Our top 10 herbicide-resistant weed management practices. *Pest Management Science*, *73*(6), 1045-1052.

Bell, G. E., Martin, D. L., Kuzmic, R. M., Stone, M. L., & Solie, J. B. (2000). Herbicide tolerance of two cold-resistant bermudagrass (*Cynodon spp*.) cultivars determined by visual assessment and vehicle-mounted optical sensing. *Weed Technology*, *14*(3), 635-641

Beversdorf, W. D., Hume, D. J., & Daonnelly-Vanderloo, M. J. (1988). Agronomic performance of trianzine-resistant and susceptible reciprocal spring canola hybrids. *Crop Science*, *28*(6), 932-934.

Blackshaw, R. E., O'Donovan, J. T., Harker, K. N., & Li, X. (2002). Beyond herbicides: new approaches to managing weeds. In *Proceedings of the International Conference on Environmentally Sustainable Agriculture for Dry Areas,* 305-312.

Bradbury, P. J., Zhang, Z., Kroon, D. E., Casstevens, T. M., Ramdoss, Y., & Buckler, E. S. (2007). TASSEL: software for association mapping of complex traits in diverse samples. *Bioinformatics*, *23*(19), 2633-2635.

Braun, H. P., Binder, S., Brennicke, A., Eubel, H., Fernie, A. R., Finkemeier, I., ... & Obata, T. (2014). The life of plant mitochondrial complex I. *Mitochondrion*, *19*, 295-313.

Breseghello, F., & Sorrells, M. E. (2005). Association Mapping of Kernel Size and Milling Quality in Wheat (Triticum aestivum L.) Cultivars. *Genetics, 172*(2), 1165-1177.

Busi, R., Vila-Aiub, M. M., & Powles, S. B. (2011). Genetic control of a cytochrome P450 metabolism-based herbicide resistance mechanism in Lolium rigidum. *Heredity*, *106*(5), 817.

Camadro, J. M., Arnould, S., Le Guen, L., Santos, R., Matringe, M., & Mornet, R. (1999). Characteristics of protoporphyrinogen oxidase. In Böger, P., & Wakabayashi, K. (Eds.), *Peroxidizing Herbicides* (245-277). Springer.

Camadro, J. M., Matringe, M., Scalla, R., & Labbe, P. (1991). Kinetic studies on protoporphyrinogen oxidase inhibition by diphenyl ether herbicides. *Biochemical Journal*, *277*(1), 17-21.

Cardina, J., & Smith, A. E. (1995). Biological weed management. In A. Smith (Ed), *Handbook of Weed Management Systems* (279-341), Marcel Dekker Inc.

Chen, X., Zhang, Z., Liu, D., Zhang, K., Li, A., & Mao, L. (2010). SQUAMOSA promoter-binding protein-like transcription factors: Star players for plant growth and development. *Journal of Integrative Plant Biology*, *52*(11), 946-951.

Cornah, J. E., Terry, M. J., & Smith, A. G. (2003). Green or red: what stops the traffic in the tetrapyrrole pathway? *Trends in Plant Science*, *8*(5), 224-230.

Cubero, J. I., Pérez, D. L. V., & Fratini, R. (2009). Origin, phylogeny, domestication and spread. In W. Erskine, F. Muehlebauer, A. Sarker and B. Sharma (Eds.), *The Lentil: Botany, Production and Uses* (13-33). CABI Wallingford.

Cui, Y., Wang, M., Zhou, H., Li, M., Huang, L., Yin, X., ... & Xu, G. (2016). OsSGL, a novel DUF1645 domain-containing protein, confers enhanced drought tolerance in transgenic rice and Arabidopsis. *Frontiers in Plant Science*, *7*, 2001.

Czarnecki, O., & Grimm, B. (2012). Post-translational control of tetrapyrrole biosynthesis in plants, algae, and cyanobacteria. *Journal of Experimental Botany*, *63*(4), 1675-1687.

Das, M., Bickers, D. R., & Mukhtar, H. (1984). Plant phenols as invitro inhibitors of glutathione S-transferase (s). *Biochemical and Biophysical Research Communications*, *120*(2), 427-433.

Davies, P. A., Lülsdorf, M. M., & Ahmad, M. (2007). Wild relatives and biotechnological approaches. In Yadav, S., McNeil, D, Stevenson, P. (Eds.), *Lentil* (225-240). Springer.

Dayan, F. E., & Duke, S. O. (1997). Phytotoxicity of protoporphyrinogen oxidase inhibitors: Phenomenology, mode of action and mechanisms of resistance. In Roe, R. M., Burton, J. D., & Kuhr, R. J. (Eds.), *Herbicide Activity: Toxicology, Biochemistry and Molecular Biology* (11-36). IOS Press.

Dayan, F. E., Armstrong, B. M., & Weete, J. D. (1998). Inhibitory activity of sulfentrazone and its metabolic derivatives on soybean (Glycine max) protoporphyrinogen oxidase. *Journal of Agricultural and Food Chemistry*, *46*(5), 2024-2029.

Dayan, F. E., Barker, A., & Tranel, P. J. (2018). Origins and structure of chloroplastic and mitochondrial plant protoporphyrinogen oxidases: implications for the evolution of herbicide resistance. *Pest Management Science*, *74*(10), 2226-2234.

Dayan, F. E., Daga, P. R., Duke, S. O., Lee, R. M., Tranel, P. J., & Doerksen, R. J. (2010). Biochemical and structural consequences of a glycine deletion in the α-8 helix of

protoporphyrinogen oxidase. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, *1804*(7), 1548-1556.

Dayan, F. E., Green, H. M., Weete, J. D., & Hancock, H. G. (1996). Postemergence activity of sulfentrazone: effects of surfactants and leaf surfaces. *Weed Science*, 797-803.

Dayan, F. E., Owens, D. K., Tranel, P. J., Preston, C., & Duke, S. O. (2014). Evolution of resistance to phytoene desaturase and protoporphyrinogen oxidase inhibitors–state of knowledge. *Pest Management Science*, *70*(9), 1358-1366.

Dayan, F. E., Weete, J. D., Duke, S. O., & Hancock, H. G. (1997). Soybean (Glycine max) cultivar differences in response to sulfentrazone. *Weed Science*, 634-641.

Deepak, S., Shailasree, S., Kini, R. K., Muck, A., Mithöfer, A., & Shetty, S. H. (2010). Hydroxyproline-rich glycoproteins and plant defence. *Journal of Phytopathology*, *158*(9), 585-593.

Délye, C. (2012). Unravelling the genetic bases of non-target-site-based resistance (NTSR to herbicides: a major challenge for weed science in the forthcoming decade. *Pest Management Science*, *69*(2), 176-187.

Délye, C., Gardin, J. A., Boucansaud, K., Chauvel, B., & Petit, C. (2011). Non-target-site-based resistance should be the centre of attention for herbicide resistance research: Alopecurus myosuroides as an illustration. *Weed Research, 51*(5), 433-437.

DePristo, M. A., Banks, E., Poplin, R., Garimella, K. V., Maguire, J. R., Hartl, C., . . . Daly, M. J. (2011). A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nature Genetics, 43*(5), 491-498.

Devine, M. D., Duke, S. O., & Fedtke, C. (1993). *Physiology of Herbicide Action*. PTR Prentice-Hall. Inc.

Diapari, M., Sindhu, A., Bett, K., Deokar, A., Warkentin, T. D., & Tar'An, B. (2014). Genetic diversity and association mapping of iron and zinc concentrations in chickpea (Cicer arietinum L.). *Genome, 57*(8), 459-468.

Didierjean, L., Perkins, R., Lau, S. M., Schaller, H., O'Keefe, D. P., & Werck-Reichhart, D. (2002). Engineering herbicide metabolism in tobacco and arabidopsis with CYP76B1, a cytochrome P450 enzyme from Jerusalem artichoke. *Plant Physiology, 130*(1), 179-189.

Dröge, W., Broer, I., & Pühler, A. (1992). Transgenic plants containing the phosphinothricin-N-acetyltransferase gene metabolize the herbicide L-phosphinothricin (glufosinate) differently from untransformed plants. *Planta*, *187*(1), 142-151.

Duke, S. O. (2005). Taking stock of herbicide-resistant crops ten years after introduction. *Pest Management Science, 61*(3), 211-218.

Duke, S. O., Lydon, J., Becerril, J. M., Sherman, T. D., Lehnen, L. P., & Matsumoto, H. (1991). Protoporphyrinogen oxidase-inhibiting herbicides. *Weed Science*, *39*(3), 465-473.

Edwards, R., Dixon, D. P., & Walbot, V. (2000). Plant glutathione S -transferases: Enzymes with multiple functions in sickness and in health. *Trends in Plant Science, 5*(5), 193-198.

Emanuelsson, O., Nielsen, H., Brunak, S., & Von Heijne, G. (2000). Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *Journal of Molecular Biology*, *300*(4), 1005-1016.

Environment Canada. Daily Data Report for May 2015. http://climate.weather.gc.ca/climate_data/daily_data_e.html?hlyRange=1994-02-01|2018-04-23&dlyRange=1992-12-01|2018-04-21&mlyRange=1996-01-01|2007-11-01&StationID=10784&Prov=SK&urlExtension=_e.html&searchType=stnName&optLimit=yearRange&StartYear=2014&EndYear=2016&selRowPerPage=25&Line=0&searchMethod=contains&Month=5&Day=23&txtStationName=rosetown&timeframe=2&Year=2015 (Retrieved April 24, 2018).

Erskine, W., & Muehlbauer, F. J. (1991). Allozyme and morphological variability, outcrossing rate and core collection formation in lentil germplasm. *Theoretical and Applied Genetics*, *83*(1), 119-125.

Evanno, G., Regnaut, S., & Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology*, *14*(8), 2611-2620.

Fausey, J. C., & Renner, K. A. (2001). Environmental effects on CGA-248757 and flumiclorac efficacy/soybean tolerance. *Weed Science*, *49*(5), 668-674.

Fausey, J. C., Penner, D., & Renner, K. A. (2000). Physiological basis for CGA-248757 and flumiclorac selectivity in five plant species. *Weed Science*, *48*(4), 405-411.

Fedoruk, L. K., Johnson, E. N., & Shirtliffe, S. J. (2011). The Critical Period of Weed Control for Lentil in Western Canada. *Weed Science, 59*(04), 517-526.

Ferrell, J. A., Witt, W. W., & Vencill, W. K. (2003). Sulfentrazone absorption by plant roots increases as soil or solution pH decreases. *Weed Science*, *51*(5), 826-830.

Fisher, L. R., Smith, W. D., & Wilcut, J. W. (2003). Effect of sulfentrazone rate and application method on weed control and stunting in flue-cured tobacco. *Tobacco Science*, 12-16.

Finney, D. J. (1971). *Probit Analysis*, Cambridge University Press.

FMC Agricultural Solutions (2017). Cadet. http://www.fmccrop.com/grower/Products/Herbicides/Cadet.aspx (Retrieved April 12, 2017).

FMC Canada (2017). Authority. http://fmccrop.ca/products/authority-480-west/ (Retrieved April 12, 2017).

FMC Corporation (1989). *Technical Bulletin of Sulfentrazone (F6285).* Philadelphia, PA: Agricultural Chemical Group.

Food and Agriculture Organization of the United Nations (2019). FAOSTAT Database. Rome, Italy: FAO. http://www.fao.org/faostat/en/#data (Retrieved January 30, 2019).

Gachomo, E. W., Jimenez-Lopez, J. C., Baptiste, L. J., & Kotchoni, S. O. (2014). GIGANTUS1 (GTS1), a member of Transducin/WD40 protein superfamily, controls seed germination, growth and biomass accumulation through ribosome-biogenesis protein interactions in Arabidopsis thaliana. *BMC Plant Biology*, *14*(1), 37.

Gardin, J. A., Gouzy, J., Carrère, S., & Délye, C. (2015). ALOMYbase, a resource to investigate non-target-site-based resistance to herbicides inhibiting acetolactate-synthase (ALS) in the major grass weed Alopecurus myosuroides (black-grass). *BMC Genomics, 16*(1).

Gechev, T. S., Van Breusegem, F., Stone, J. M., Denev, I., & Laloi, C. (2006). Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. *Bioessays*, *28*(11), 1091-1101.

Ghanizadeh, H., & Harrington, K. C. (2017). Non-target site mechanisms of resistance to herbicides. *Critical Reviews in Plant Sciences*, *36*(1), 24-34.

Giacomini, D. A., Umphres, A. M., Nie, H., Mueller, T. C., Steckel, L. E., Young, B. G., ... & Tranel, P. J. (2017). Two new PPX2 mutations associated with resistance to PPO-inhibiting herbicides in Amaranthus palmeri. *Pest Management Science*, *73*(8), 1559-1563.

Goodstein, D. M., Shu, S., Howson, R., Neupane, R., Hayes, R. D., Fazo, J., ... & Rokhsar, D. S. (2011). Phytozome: a comparative platform for green plant genomics. *Nucleic Acids Research*, *40*(D1), 1178-1186.

Government of Saskatchewan. (2017). 2017 Guide To Crop Protection. - http://www.publications.gov.sk.ca/details.cfm?p=77706 (Retrieved April 7, 2017).

Government of Saskatchewan (2018). Varieties of Grain Crops 2018. http://publications.gov.sk.ca/documents/20/96889-Varieties%20of%20Grain%20Crops_2018.pdf (Retrieved May 15, 2018).

Green, J. M. (2007). Review of glyphosate and ALS-inhibiting herbicide crop resistance and resistant weed management. *Weed Technology*, *21*(2), 547-558.

Green, J. M., & Owen, M. D. (2011). Herbicide-Resistant Crops: Utilities and Limitations for Herbicide-Resistant Weed Management. *Journal of Agricultural and Food Chemistry, 59*(11), 5819-5829.

Grey, T. L., Walker, R. H., & Hancock, H. G. (1997). Sulfentrazone adsorption and mobility as affected by soil and pH. *Weed Science*, 733-738.

Groenewegen J., & S. Thompson (2016). Economic Impact of Plant Breeding in The Crop Development Centre Final Report. Retrieved from: https://agbio.usask.ca/documents/centres-and-facilities/CDC_FINAL_REPORT_November2016.pdf (Retrieved May 18, 2017).

Gruszka, D., Szarejko, I., & Maluszynski, M. (2012). Sodium azide as a mutagen. *Plant Mutation Breeding and Biotechnology*. CABI Wallingford, 159-166.

Hager A (2009). *Weed Management*. Illinois Agronomy Handbook, 153-177.

Hagood, E. S., Williams, J. L., & Bauman, T. T. (1980). Influence of herbicide injury on the yield potential of soybeans (Glycine max). *Weed Science*, *28*(1), 40-45.

Hardy, O. J., & Vekemans, X. (2002). SPAGeDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. *Molecular Ecology Notes*, *2*(4), 618-620.

Harker, K. N., O'donovan, J. T., Blackshaw, R. E., Beckie, H. J., Mallory-Smith, C., & Maxwell, B. D. (2012). Our view. *Weed Science*, *60*(2), 143-144.

Harrington, R. B., & Powles, S. B. (2012). Harrington seed destructor: a new nonchemical weed control tool for global grain crops. *Crop Science*, *52*(3), 1343-1347.

Harrison, X. A., Donaldson, L., Correa-Cano, M. E., Evans, J., Fisher, D. N., Goodwin, C. E., ... & Inger, R. (2018). A brief introduction to mixed effects modelling and multi-model inference in ecology. *PeerJ*, *6*, e4794.

Heap, I. (2018). International Survey of Herbicide Resistant Weeds. http://weedscience.org/ (Retrieved April 18, 2018).

Heinemann, I. U., Diekmann, N., Masoumi, A., Koch, M., Messerschmidt, A., Jahn, M., & Jahn, D. (2007). Functional definition of the tobacco protoporphyrinogen IX oxidase substrate-binding site. *Biochemical Journal*, *402*(3), 575-580.Hirai, K. (1999). Structural evolution and synthesis of diphenyl ethers, cyclic imides and related compounds. In Böger, P., & Wakabayashi, K. (Eds.), *Peroxidizing Herbicides* (15-71). Springer.

Holm, F. A., Vandenberg, A., Sapsford, K. L., & Johnson, E. (2012). *Developing Sulfentrazone Tolerance in Lentil* (Vol. BRE0901, Rep.). Saskatchewan Pulse Growers.

Jhala, A. J., Sandell, L. D., Sarangi, D., Kruger, G. R., & Knezevic, S. Z. (2017). Control of glyphosate-resistant common waterhemp (Amaranthus rudis) in glufosinate-resistant soybean. *Weed Technology*, *31*(1), 32-45.

Kauss, D., Bischof, S., Steiner, S., Apel, K., & Meskauskiene, R. (2012). FLU, a negative feedback regulator of tetrapyrrole biosynthesis, is physically linked to the final steps of the Mg++-branch of this pathway. *FEBS Letters, 586*(3), 211-216.

Kawahigashi, H. (2009). Transgenic plants for phytoremediation of herbicides. *Current Opinion in Biotechnology, 20*(2), 225-230.

Khazaei, H., Caron, C. T., Fedoruk, M., Diapari, M., Vandenberg, A., Coyne, C. J., . . . Bett, K. E. (2016). Genetic Diversity of Cultivated Lentil (Lens culinaris Medik.) and Its Relation to the Worlds Agro-ecological Zones. *Frontiers in Plant Science, 7*, 1093.

Khazaei, H., Podder, R., Caron, C. T., Kundu, S. S., Diapari, M., Vandenberg, A., & Bett, K. E. (2017). Marker–Trait Association Analysis of Iron and Zinc Concentration in Lentil (Medik.) Seeds. *The Plant Genome, 10*(2).

Kosicki, M., Tomberg, K., & Bradley, A. (2018). Repair of double-strand breaks induced by CRISPR–Cas9 leads to large deletions and complex rearrangements. *Nature Biotechnology*, *36*(8), 765.

Ladizinsky, G. (1979). The origin of lentil and its wild genepool. *Euphytica*, *28*(1), 179-187.

Ladizinsky, G., & Abbo, S. (1993). Cryptic speciation in Lens culinaris. *Genetic Resources and Crop Evolution, 40*(1), 1-5.

Lee, H. J., & Duke, S. O. (1994). Protoporphyrinogen IX-oxidizing activities involved in the mode of action of peroxidizing herbicides. *Journal of Agricultural and Food Chemistry*, *42*(11), 2610-2618.

Lee, R. M., Hager, A. G., & Tranel, P. J. (2008). Prevalence of a novel resistance mechanism to PPO-inhibiting herbicides in waterhemp (Amaranthus tuberculatus). *Weed Science*, *56*(3), 371-375.

Lermontova, I., Kruse, E., Mock, H. P., & Grimm, B. (1997). Cloning and characterization of a plastidal and a mitochondrial isoform of tobacco protoporphyrinogen IX oxidase. *Proceedings of the National Academy of Sciences*, *94*(16), 8895-8900.

Letouzé, A., & Gasquez, J. (2003). Enhanced activity of several herbicide-degrading enzymes: A suggested mechanism responsible for multiple resistance in blackgrass (Alopecurus myosuroides Huds.). *Agronomie, 23*(7), 601-608.

Li, H., & Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics, 25*(14), 1754-1760.

Li, P., Li, Y. J., Wang, B., Yu, H. M., Li, Q., & Hou, B. K. (2017). The Arabidopsis UGT87A2, a stress-inducible family 1 glycosyltransferase, is involved in the plant adaptation to abiotic stresses. *Physiologia Plantarum*, *159*(4), 416-432.

Li, X., & Nicholl, D. (2005). Development of PPO inhibitor-resistant cultures and crops. *Pest Management Science*, *61*(3), 277-285.

Li, X., Volrath, S. L., Nicholl, D. B., Chilcott, C. E., Johnson, M. A., Ward, E. R., & Law, M. D. (2003). Development of protoporphyrinogen oxidase as an efficient selection marker for Agrobacterium tumefaciens-mediated transformation of maize. *Plant Physiology*, *133*(2), 736-747.

Li, Z., Walker, R. H., Wehtje, G., & Hancock, H. G. (2000). Using electrolyte leakage to detect soybean (Glycine max) cultivars sensitive to sulfentrazone. *Weed Technology*, *14*(4), 699-704.

Li, Z., Wehtje, G. R., & Walker, R. H. (2000). Physiological basis for the differential tolerance of Glycine max to sulfentrazone during seed germination. *Weed Science*, *48*(3), 281-285.

Liebman, M., & Dyck, E. (1993). Crop rotation and intercropping strategies for weed management. *Ecological Applications*, *3*(1), 92-122.

Lindén, L., Palonen, P., & Lindén, M. (2000). Relating freeze-induced electrolyte leakage measurements to lethal temperature in red raspberry. *Journal of the American Society for Horticultural Science*, *125*(4), 429-435.

Loiselle, B. A., Sork, V. L., Nason, J., & Graham, C. (1995). Spatial genetic structure of a tropical understory shrub, Psychotria officinalis (Rubiaceae). *American Journal of Botany*, *82*(11), 1420-1425.

Lu, Y., Xu, J., Yuan, Z., Hao, Z., Xie, C., Li, X., . . . Xu, Y. (2011). Comparative LD mapping using single SNPs and haplotypes identifies QTL for plant height and biomass as secondary traits of drought tolerance in maize. *Molecular Breeding, 30*(1), 407-418.

Lynch, C. J., Bernad, R., Calvo, I., Nóbrega-Pereira, S., Ruiz, S., Ibarz, N., ... & Angarica, V. E. (2018). The RNA Polymerase II Factor RPAP1 Is Critical for Mediator-Driven Transcription and Cell Identity. *Cell Reports*, *22*(2), 396-410.

Malhi, S. S., Johnston, A. M., Schoenau, J. J., Wang, Z. H., & Vera, C. L. (2007). Seasonal biomass accumulation and nutrient uptake of pea and lentil on a black chernozem soil in Saskatchewan. *Journal of Plant Nutrition*, *30*(5), 721-737.

Manalil, S., Busi, R., Renton, M., & Powles, S. B. (2011). Rapid evolution of herbicide resistance by low herbicide dosages. *Weed Science*, *59*(2), 210-217.

Matringe, M., Camadro, J. M., Block, M. A., Joyard, J., Scalla, R., Labbe, P., & Douce, R. (1992). Localization within chloroplasts of protoporphyrinogen oxidase, the target enzyme for diphenylether-like herbicides. *Journal of Biological Chemistry*, *267*(7), 4646-4651.

Matringe, M., Camadro, J. M., Labbe, P., & Scalla, R. (1989). Protoporphyrinogen oxidase as a molecular target for diphenyl ether herbicides. *Biochemical Journal*, *260*(1), 231-235.

Matsunaka, S. (1969). Acceptor of light energy in photoactivation of diphenyl ether herbicides. *Journal of Agricultural and Food Chemistry*, *17*(2), 171-175.

Matsunaka, S. (1999). History and early investigation of mode of action of peroxidizing herbicides. In Böger, P., & Wakabayashi, K. (Eds.), *Peroxidizing Herbicides* (1-14). Springer.

Mba, C. (2013). Induced mutations unleash the potentials of plant genetic resources for food and agriculture. *Agronomy*, *3*(1), 200-231.

McGonigle, B., Keeler, S. J., Lau, S. M. C., Koeppe, M. K., & O'Keefe, D. P. (2000). A genomics approach to the comprehensive analysis of the glutathione S-transferase gene family in soybean and maize. *Plant Physiology*, *124*(3), 1105-1120.

Mckenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., . . . Depristo, M. A. (2010). The Genome Analysis Toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Research, 20*(9), 1297-1303.

McWilliam, H., Li, W., Uludag, M., Squizzato, S., Park, Y. M., Buso, N., ... & Lopez, R. (2013). Analysis tool web services from the EMBL-EBI. *Nucleic Acids Research*, *41*(W1), W597-W600.

Meskauskiene, R., Nater, M., Goslings, D., Kessler, F., op den Camp, R., & Apel, K. (2001). FLU: a negative regulator of chlorophyll biosynthesis in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences*, *98*(22), 12826-12831.

Miyazawa, T., Kawano, K., Shigematsu, S., Yamaguchi, M., Matsunari, K., Porpiglia, P., & Gutbrod, K. G. (1993). KIH-9201, a new low-rate post-emergence herbicide for maize (Zea mays) and soybeans (Glycine max) Brighton crop protection conference, weeds Proceedings of an International Conference, Brighton, UK, 22-25 November 1993 (Vol. I): 23-28.

Mohd-Yusoff, N. F., Ruperao, P., Tomoyoshi, N. E., Edwards, D., Gresshoff, P. M., Biswas, B., & Batley, J. (2015). Scanning the effects of ethyl methanesulfonate on the whole genome of Lotus japonicus using second-generation sequencing analysis. *G3: Genes, Genomes, Genetics*, *5*(4), 559-567.

Montgomery, G. B., Bond, J. A., Golden, B. R., Gore, J., Edwards, H. M., Eubank, T. W., & Walker, T. W. (2014). Response of Commercial Rice Cultivars to Postemergence Applications of Saflufenacil. *Weed Technology, 28*(04), 679-684.

Morgan, B. J., & Smith, D. M. (1992). A Note on Wadley's Problem with Overdispersion. *Applied Statistics, 41*(2), 349.

Morrall, R. A. A. (1997). Evolution of lentil diseases over 25 years in western Canada. *Canadian Journal of Plant Pathology*, *19*(2), 197-207.

Moulin, M., & Smith, A. G. (2005). Regulation of tetrapyrrole biosynthesis in higher plants. *Biochemical Society Transactions*, *33*(4), 737-742.

Mueller, T. C., Boswell, B. W., Mueller, S. S., & Steckel, L. E. (2014). Dissipation of fomesafen, saflufenacil, sulfentrazone, and flumioxazin from a Tennessee soil under field conditions. *Weed Science*, *62*(4), 664-671.

Myles, S., Peiffer, J., Brown, P. J., Ersoz, E. S., Zhang, Z., Costich, D. E., & Buckler, E. S. (2009). Association Mapping: Critical Considerations Shift from Genotyping to Experimental Design. *The Plant Cell Online, 21*(8), 2194-2202.

Nagano, E. (1999). Herbicidal efficacy of protoporphyrinogen oxidase inhibitors. In Böger, P., & Wakabayashi, K. (Eds.), *Peroxidizing Herbicides* (293-302). Springer.

Nei, M. (1972). Genetic distance between populations. *The American Naturalist*, 106(949), 283-292.

Novak, F. J., & Brunner, H. (1992). *Plant Breeding: Induced Mutation Technology for Crop Improvement*. IAEA Bull, 4, 25-33.

Ohmes, G. A., & Mueller, T. C. (2007). Sulfentrazone adsorption and mobility in surface soil of the southern United States. *Weed Technology*, *21*(3), 796-800.

Oladosu, Y., Rafii, M. Y., Abdullah, N., Hussin, G., Ramli, A., Rahim, H. A., ... & Usman, M. (2016). Principle and application of plant mutagenesis in crop improvement: a review. *Biotechnology & Biotechnological Equipment*, *30*(1), 1-16.

Owen, M. D., & Zelaya, I. A. (2005). Herbicide-resistant crops and weed resistance to herbicides. *Pest Management Science, 61*(3), 301-311.

Padgette, S. R., Kolacz, K. H., Delannay, X., Re, D. B., LaVallee, B. J., Tinius, C. N., ... & Peschke, V. M. (1995). Development, identification, and characterization of a glyphosate-tolerant soybean line. *Crop Science*, *35*(5), 1451-1461.

Pan, G., Zhang, X., Liu, K., Zhang, J., Wu, X., Zhu, J., & Tu, J. (2006). Map-based cloning of a novel rice cytochrome P450 gene CYP81A6 that confers resistance to two different classes of herbicides. *Plant Molecular Biology*, *61*(6), 933-943.

Pataky, J. K., Meyer, M. D., Bollman, J. D., Boerboom, C. M., & Williams, M. M. (2008). Genetic basis for varied levels of injury to sweet corn hybrids from three cytochrome P450-

metabolized herbicides. *Journal of the American Society for Horticultural Science*, *133*(3), 438-447.

Patzoldt, W. L., Hager, A. G., McCormick, J. S., & Tranel, P. J. (2006). A codon deletion confers resistance to herbicides inhibiting protoporphyrinogen oxidase. *Proceedings of the National Academy of Sciences*, *103*(33), 12329-12334.

Poulson, R., & Polglase, W. J. (1975). The enzymic conversion of protoporphyrinogen IX to protoporphyrin IX. Protoporphyrinogen oxidase activity in mitochondrial extracts of Saccharomyces cerevisiae. *Journal of Biological Chemistry*, *250*(4), 1269-1274.

Powles, S. B., & Yu, Q. (2010). Evolution in Action: Plants Resistant to Herbicides. *Annual Review of Plant Biology, 61*(1), 317-347.

Pozniak, C. J., Birk, I. T., O'Donoughue, L. S., Ménard, C., Hucl, P. J., & Singh, B. K. (2004). Physiological and molecular characterization of mutation-derived imidazolinone resistance in spring wheat. *Crop Science*, *44*(4), 1434-1443.

Prabhakara, K., Hively, W. D., & McCarty, G. W. (2015). Evaluating the relationship between biomass, percent groundcover and remote sensing indices across six winter cover crop fields in Maryland, United States. *International Journal of Applied Earth Observation and Geoinformation*, *39*, 88-102.

Pratt S. High prices help lentils take top spot in exports. https://www.producer.com/2016/02/high-prices-help-lentils-take-top-spot-in-exports/ (Retrieved February 25, 2016).

Preston, C., Tardif, F. J., Christopher, J. T., & Powles, S. B. (1996). Multiple Resistance to Dissimilar Herbicide Chemistries in a Biotype of Lolium rigidum Due to Enhanced Activity of Several Herbicide Degrading Enzymes. *Pesticide Biochemistry and Physiology, 54*(2), 123-134.

Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, *155*(2), 945-959.

PubChem (2018). Sulfentrazone. https://pubchem.ncbi.nlm.nih.gov/compound/86369#section=Top (Retrieved September 15, 2018).

PubChem (2018b). Fluthiacet methyl. Retrieved from: https://pubchem.ncbi.nlm.nih.gov/compound/93542#section=Top (Retrieved September 15, 2018).

R Development Core Team (2014). R: *A Language and Environment for Statistical Computing*.

Rao, V. S. (2000). *Principles of Weed Science*. CRC Press.

Reddy, S. S., Stahlman, P. W., Geier, P. W., Bean, B. W., & Dozier, T. (2014). Grain sorghum response and Palmer amaranth control with postemergence application of fluthiacet-methyl. *International Journal of Pest Management*, *60*(3), 147-152.

Reed, B. C., Brown, J. F., Vanderzee, D., Loveland, T. R., Merchant, J. W., & Ohlen, D. O. (1994). Measuring phenological variability from satellite imagery. *Journal of Vegetation Science, 5*(5), 703-714.

Ritz, C., & Streibig, J. C. (2005). Bioassay Analysis using R. *Journal of Statistical Software, 12*(5). 1-22.

Rizwan, M., & Akhtar, S. (2015). Development of herbicide resistant crops through induced mutations. *Advancements in Life Sciences*, *3*(1), 1-8.

Rizwan, M., Aslam, M., Asghar, M. J., Abbas, G., Shah, T. M., & Shimelis, H. (2017). Pre-breeding of lentil (Lens culinaris Medik.) for herbicide resistance through seed mutagenesis. *PloS One*, *12*(2), e0171846.

Robins, J. G., Luth, D., Campbell, T. A., Bauchan, G. R., He, C., Viands, D. R., . . . Brummer, E. C. (2007). Genetic Mapping of Biomass Production in Tetraploid Alfalfa. *Crop Science, 47*(1), 1.

Rousonelos, S. L., Lee, R. M., Moreira, M. S., VanGessel, M. J., & Tranel, P. J. (2012). Characterization of a common ragweed (Ambrosia artemisiifolia) population resistant to ALS-and PPO-inhibiting herbicides. *Weed Science*, *60*(3), 335-344.

Roychowdhury, R., & Tah, J. (2013). Mutagenesis—A potential approach for crop improvement. In Hakeem, K. R., Ahmad, P., Öztürk, M. (Eds.), *Crop Improvement* (149-187). Springer.

Salas-Perez, R. A., Burgos, N. R., Rangani, G., Singh, S., Refatti, J. P., Piveta, L., ... & Scott, R. C. (2017). Frequency of Gly-210 deletion mutation among protoporphyrinogen oxidase inhibitor–resistant Palmer amaranth (Amaranthus palmeri) populations. *Weed Science*, *65*(6), 718-731.

Sandhu, J. S., & Singh, S. (2007). History and origin. In Yadav, S., McNeil, D, Stevenson, P. (Eds.), *Lentil* (1-9). Springer.

SAS Institute Inc. 2011. *SAS/STAT™ 9.3 User's Guide*. Cary, NC: SAS Institute Inc.

Saxena, M. S., Bajaj, D., Kujur, A., Das, S., Badoni, S., Kumar, V., ... & Parida, S. K. (2014). Natural allelic diversity, genetic structure and linkage disequilibrium pattern in wild chickpea. *PLoS One*, *9*(9), e107484.

Schmitz, A. J., Glynn, J. M., Olson, B. J., Stokes, K. D., & Osteryoung, K. W. (2009). Arabidopsis FtsZ2-1 and FtsZ2-2 are functionally redundant, but FtsZ-based plastid division is not essential for chloroplast partitioning or plant growth and development. *Molecular Plant*, *2*(6), 1211-1222.

Schmutz, J., Cannon, S. B., Schlueter, J., Ma, J., Mitros, T., Nelson, W., ... & Xu, D. (2010). Genome sequence of the palaeopolyploid soybean. *Nature*, *463*(7278), 178.

Seber, G., & Wild, C.(1989). *Nonlinear Regression*, Wiley, New York.

Seefeldt, S. S., Jensen, J. E., & Fuerst, E. P. (1995). Log-Logistic Analysis of Herbicide Dose-Response Relationships. *Weed Technology, 9*(02), 218-227.

Sewelam, N., Kazan, K., & Schenk, P. M. (2016). Global plant stress signaling: reactive oxygen species at the cross-road. *Frontiers in Plant Science*, *7*, 187.

Shah, T., Andleeb, T., Lateef, S., & Noor, M. A. (2018). Genome editing in plants: Advancing crop transformation and overview of tools. *Plant Physiology and Biochemistry*, *131*, 12-21.

Shaner, D. L. (2014). *Herbicide Handbook*. Weed Science Society of America.

Sherman, T. D., Becerril, J. M., Matsumoto, H., Duke, M. V., Jacobs, J. M., Jacobs, N. J., & Duke, S. O. (1991). Physiological basis for differential sensitivities of plant species to protoporphyrinogen oxidase-inhibiting herbicides. *Plant Physiology*, *97*(1), 280-287.

Shimizu, T., Hashimoto, N., Nakayama, I., Nakao, T., Mizutani, H., Unai, T., ... & Abe, H. (1995). A novel isourazole herbicide, fluthiacet-methyl, is a potent inhibitor of protoporphyrinogen oxidase after isomerization by glutathione S-transferase. *Plant and Cell Physiology*, *36*(4), 625-632.

Shu, Q. Y., Forster, B. P., Nakagawa, H., & Nakagawa, H. (Eds.). (2012). *Plant Mutation Breeding and Biotechnology*. CABI Wallingford.

Sikora, P., Chawade, A., Larsson, M., Olsson, J., & Olsson, O. (2011). Mutagenesis as a tool in plant genetics, functional genomics, and breeding. *International Journal of Plant Genomics*, 2011.

Siva, N., Johnson, C. R., Duckett, S., Jesch, E. D., & Thavarajah, P. (2017). Can lentil (Lens culinaris Medikus) reduce the risk of obesity?. *Journal of Functional Foods*, *38*, 706-715.

Skibinski, D. O. F., Rasool, D., & Erskine, W. (1984). Aspartate aminotransferase allozyme variation in a germplasm collection of the domesticated lentil (Lens culinaris). *Theoretical and Applied Genetics*, *68*(5), 441-448.

Slinkard, A. E. (1981). Eston lentil. *Canadian Journal of Plant Science*, *61*(3), 733-734.

Slinkard, A. E., & Bhatty, R. S. (1979). Laird lentil. *Canadian Journal of Plant Science*, *59*(2), 503-504.

Slinkard, A. E., Vandenberg, A., & Holm, F. A. (2007). U.S. Patent No. 7,232,942. Washington, DC: U.S. Patent and Trademark Office.

Smitchger, J. A., Burke, I. C., & Yenish, J. P. (2012). The critical period of weed control in lentil (Lens culinaris) in the Pacific Northwest. *Weed Science*, *60*(1), 81-85.

Soldatova, O., Apchelimov, A., Radukina, N., Ezhova, T., Shestakov, S., Ziemann, V., ... & Grimm, B. (2005). An Arabidopsis mutant that is resistant to the protoporphyrinogen

oxidase inhibitor acifluorfen shows regulatory changes in tetrapyrrole biosynthesis. *Molecular Genetics and Genomics*, *273*(4), 311-318.

Song, Y. (2014). Insight into the mode of action of 2, 4-dichlorophenoxyacetic acid (2, 4-D) as an herbicide. *Journal of Integrative Plant Biology*, *56*(2), 106-113.

Statistics Canada (2018). Field and special crops. http://www.statcan.gc.ca/tables-tableaux/sum-som/l01/cst01/prim11a-eng.htm (Retrieved August 2, 2018).

Swanton, C. J., Harker, K. N., & Anderson, R. L. (1993). Crop losses due to weeds in Canada. *Weed Technology*, *7*(2), 537-542.

Szmigielski, A. M., Schoenau, J. J., Johnson, E. N., Holm, F. A., Sapsford, K. L., & Liu, J. (2009). Development of a laboratory bioassay and effect of soil properties on sulfentrazone phytotoxicity in soil. *Weed Technology*, *23*(3), 486-491.

Szmigielski, A. M., Schoenau, J. J., & Johnson, E. N. (2012). Use of Sugar Beet as a Bioindicator Plant for Detection of Flucarbazone and Sulfentrazone Herbicides in Soil. *Herbicides-Environmental Impact Studies and Management Approaches*. InTech.

Tan, S., & Bowe, S. J. (2009). Developing herbicide-tolerant crops from mutations. In *Induced Plant Mutations in the Genomics Era.* Food and Agriculture Organization of the United Nations.

Tan, S., & Bowe, S. J. (2012). Herbicide-tolerant crops developed from mutations. In Shu, Q. Y., Forster, B. P., Nakagawa, H., & Nakagawa, H. (Eds.), *Plant Mutation Breeding and Biotechnology* (423-436), CABI Wallingford.

Tan, S., Evans, R. R., Dahmer, M. L., Singh, B. K., & Shaner, D. L. (2005). Imidazolinone-tolerant crops: history, current status and future. *Pest Management Science*, *61*(3), 246-257.

Tanaka, R., & Tanaka, A. (2007). Tetrapyrrole biosynthesis in higher plants. *Annual Review Plant Biology*, *58*, 321-346.

Taylor-Lovell, S., Wax, L. M., & Nelson, R. (2001). Phytotoxic response and yield of soybean (Glycine max) varieties treated with sulfentrazone or flumioxazin. *Weed Technology*, *15*(1), 95-102.

Thelen, K. D., Kravchenko, A. N., & Lee, C. D. (2004). Use of Optical Remote Sensing for Detecting Herbicide Injury in Soybean. *Weed Technology, 18*(02), 292-297.

Theodoridis, G. (2007). Protoporphyrinogen - IX - oxidase Inhibitors. In Krämer, W., Schirmer, U., Jeschke, P., & Witschel, M. (Eds.). *Modern Crop Protection Compounds* (53-186), John Wiley & Sons.

Thinglum, K. A., Riggins, C. W., Davis, A. S., Bradley, K. W., Al-Khatib, K., & Tranel, P. J. (2011). Wide distribution of the waterhemp (Amaranthus tuberculatus) ΔG210 PPX2 mutation, which confers resistance to PPO-inhibiting herbicides. *Weed Science*, *59*(1), 22-27.

Thomas, W. E., Troxler, S. C., Smith, W. D., Fisher, L. R., & Wilcut, J. W. (2005). Uptake, translocation, and metabolism of sulfentrazone in peanut, prickly sida (Sida spinosa), and pitted morningglory (Ipomoea lacunosa). *Weed Science*, *53*(4), 446-450.

Thompson, C. L. (2013). Genetic characterization of the acetohydroxyacid synthase (AHAS) gene responsible for imidazolinone tolerance (Master dissertation, College of Graduate Studies and Research In Partial Fulfillment of the Requirements For the Degree of Master of Science In the Department of Plant Sciences, University of Saskatchewan Saskatoon).

Tidemann, B. D., Hall, L. M., Johnson, E. N., Beckie, H. J., Sapsford, K. L., Willenborg, C. J., & Raatz, L. L. (2014). Additive efficacy of soil-applied pyroxasulfone and sulfentrazone combinations. *Canadian Journal of Plant Science*, *94*(7), 1245-1253.

Tranel, P.J., Wright, T.R. and Heap, I.M. (2018). ALS Mutations from Herbicide-resistant Weeds. www.weedscience.com (Retrieved September 1, 2018).

Tullu, A., Diederichsen, A., Suvorova, G., & Vandenberg, A. (2011). Genetic and genomic resources of lentil: status, use and prospects. *Plant Genetic Resources*, *9*(1), 19-29.

Van der Auwera, G. A., Carneiro, M. O., Hartl, C., Poplin, R., Angel, G. D., Levy-Moonshine, A., . . . Depristo, M. A. (2013). From FastQ Data to High-Confidence Variant Calls: The Genome Analysis Toolkit Best Practices Pipeline. *Current Protocols in Bioinformatics, 43*(11), 1-33.

Van der Weide, R. Y., Bleeker, P. O., Achten, V. T. J. M., Lotz, L. A. P., Fogelberg, F., & Melander, B. (2008). Innovation in mechanical weed control in crop rows. *Weed Research*, *48*(3), 215-224.

Veal, E. A., Toone, W. M., Jones, N., & Morgan, B. A. (2002). Distinct Roles for GlutathioneS-Transferases in the Oxidative Stress Response in Schizosaccharomyces pombe. *Journal of Biological Chemistry, 277*(38), 35523-35531.

Venancio, T. M., & Aravind, L. (2009). CYSTM, a novel cysteine-rich transmembrane module with a role in stress tolerance across eukaryotes. *Bioinformatics*, *26*(2), 149-152.

Vencill, W. K. (2002). *Herbicide Handbook* (No. Ed. 8). Weed Science Society of America.

Vinogradov, A. D., & Grivennikova, V. G. (2016). Oxidation of NADH and ROS production by respiratory complex I. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1857*(7), 863-871.

Wakabayashi, K., & Böger, P. (1999). General physiological characteristics and mode of action of peroxidizing herbicides. In Böger, P., & Wakabayashi, K. (Eds.), *Peroxidizing Herbicides* (163-190). Springer.

Walker, R. H. (1995). Preventive weed management. In *Handbook of Weed Management Systems* (35-50). Marcel Dekker Inc, New York.

Walsh, M., Newman, P., & Powles, S. (2013). Targeting weed seeds in-crop: a new weed control paradigm for global agriculture. *Weed Technology*, *27*(3), 431-436.

Wang, S., Chang, Y., Guo, J., & Chen, J. G. (2007). Arabidopsis Ovate Family Protein 1 is a transcriptional repressor that suppresses cell elongation. *The Plant Journal*, *50*(5), 858-872.

Wang, W., Chen, D., Zhang, X., Liu, D., Cheng, Y., & Shen, F. (2018). Role of plant respiratory burst oxidase homologs in stress responses. *Free Radical Research*, *52*(8), 826-839.

Wang, Y., Zhou, Z., Wang, X., Cai, X., Li, X., Wang, C., . . . Wang, K. (2016). Genome-wide association mapping of glyphosate-resistance in Gossypium hirsutum races. *Euphytica, 209*(1), 209-221.

Watanabe, N., Che, F. S., Terashima, K., Takayama, S., Yoshida, S., & Isogai, A. (2000). Purification and properties of protoporphyrinogen oxidase from spinach chloroplasts. *Plant and Cell Physiology*, *41*(7), 889-892.

Weed Science Society of America (1998) Technical Notes. *Weed Technology 12*(4), 789.

Werck-Reichhart, D., & Feyereisen, R. (2000). Cytochromes P450: a success story. *Genome Biology*, *1*(6), reviews 3003-1.A

Werck-Reichhart, D., Hehn, A., & Didierjean, L. (2000). Cytochromes P450 for engineering herbicide tolerance. *Trends in Plant Science, 5*(3), 116-123.

White, J. W., Andrade-Sanchez, P., Gore, M. A., Bronson, K. F., Coffelt, T. A., Conley, M. M., . . . Wang, G. (2012). Field-based phenomics for plant genetics research. *Field Crops Research, 133*, 101-112.

Wild, C. J., & Seber, G. A. F. (1989). *Nonlinear Regression*. Wiley.

Witkowski, D. A., & Halling, B. P. (1989). Inhibition of plant protoporphyrinogen oxidase by the herbicide acifluorfen-methyl. *Plant Physiology*, *90*(4), 1239-1242.

Wong, M. M., Gujaria-Verma, N., Ramsay, L., Yuan, H. Y., Caron, C., Diapari, M., ... & Bett, K. E. (2015). Classification and characterization of species within the genus Lens using genotyping-by-sequencing (GBS). *PLoS One*, *10*(3), e0122025.

Yang, Z., Li, Z., & Bickel, D. R. (2013). Empirical Bayes estimation of posterior probabilities of enrichment: a comparative study of five estimators of the local false discovery rate. *BMC Bioinformatics*, *14*(1), 87.

Yenish, J. P., Brand, J., Pala, M., & Haddad, A. (2009). Weed Management. In W. Erskine, F. Muehlebauer, A. Sarker and B. Sharma (Eds.), *The Lentil: Botany, Production and Uses* (326-342). CABI Wallingford.

Yuan, H. Y., Saha, S., Vandenberg, A., & Bett, K. E. (2017). Flowering and growth responses of cultivated lentil and wild Lens germplasm toward the differences in red to far-red ratio and photosynthetically active radiation. *Frontiers in Plant Science*, *8*, 386.

Yuan, J. S., Tranel, P. J., & Stewart, C. N. (2007). Non-target-site herbicide resistance: A family business. *Trends in Plant Science, 12*(1), 6-13.

Zeng, H., Xu, L., Singh, A., Wang, H., Du, L., & Poovaiah, B. W. (2015). Involvement of calmodulin and calmodulin-like proteins in plant responses to abiotic stresses. *Frontiers in Plant Science*, *6*, 600.

Zhang, L., Lu, Q., Chen, H., Pan, G., Xiao, S., Dai, Y., ... & Tu, J. (2007). Identification of a cytochrome P450 hydroxylase, CYP81A6, as the candidate for the bentazon and sulfonylurea herbicide resistance gene, Bel, in rice. *Molecular Breeding*, *19*(1), 59-68.

Zhao, J., Huang, L., Ren, X., Pandey, M. K., Wu, B., Chen, Y., . . . Jiang, H. (2017). Genetic Variation and Association Mapping of Seed-Related Traits in Cultivated Peanut (*Arachis hypogaea L.*) Using Single-Locus Simple Sequence Repeat Markers. *Frontiers in Plant Science, 8*.

Zhao, X., Zhao, Q. P., Xu, C. Y., Wang, J., Zhu, J. D., Shang, B. S., & Zhang, X. (2018). Phot2-regulated relocation of NPH3 mediates phototropic response to high-intensity blue light in Arabidopsis thaliana. *Journal of Integrative Plant Biology*, *60*(7), 562-577

Zimdahl, R. L. (2007). *Fundamentals of Weed Science*. Academic Press.

Zohary, D. (1999). Monophyletic vs. polyphyletic origin of the crops on which agriculture was founded in the Near East. *Genetic Resources and Crop Evolution*, *46*(2), 133-142.

# APPENDIX

## APPENDIX A: List of lentil accessions used in genome wide association study

*Table A.1. List of lentil accessions used in association mapping with country of origin and their STRUCTURE sub-group assignment (K = 3 clusters based on SNP data set).*

| | Accession | Origin | STRUCTURE Sub-group assignment |
|---|---|---|---|
| 1 | CDC Asterix | Canada | 2 |
| 2 | CDC Rosie | Canada | 2 |
| 3 | 3156-11 | Canada | 2 |
| 4 | CDC Greenstar | Canada | 2 |
| 5 | CDC Cherie | Canada | 2 |
| 6 | CDC Glamis | Canada | 2 |
| 7 | CDC Gold | Canada | 2 |
| 8 | CDC Grandora | Canada | 2 |
| 9 | CDC Imax | Canada | 2 |
| 10 | CDC Impact | Canada | 2 |
| 11 | CDC Impower | Canada | 2 |
| 12 | CDC Improve | Canada | 2 |
| 13 | CDC KR-1 | Canada | 2 |
| 14 | CDC LeMay | Canada | 2 |
| 15 | CDC Maxim | Canada | 2 |
| 16 | CDC Plato | Canada | 2 |
| 17 | CDC QG-1 | Canada | 2 |
| 18 | CDC Red Rider | Canada | 2 |
| 19 | CDC Redberry | Canada | 2 |
| 20 | CDC Redcoat | Canada | 2 |
| 21 | CDC Redwing | Canada | 2 |
| 22 | CDC Robin | Canada | 2 |
| 23 | CDC Rosebud | Canada | 2 |
| 24 | CDC Rosetown | Canada | 2 |
| 25 | CDC Rouleau | Canada | 2 |
| 26 | CDC Royale | Canada | 2 |
| 27 | CDC Ruby | Canada | 2 |
| 28 | CDC SB-1 | Canada | 2 |
| 29 | CDC Sedley | Canada | 2 |
| 30 | CDC Vantage | Canada | 2 |
| 31 | Crimson | USA | 2 |
| 32 | Eston | Canada | 2 |
| 33 | ILL 9 | Jordan | 1 |

| | | | |
|---|---|---|---|
| 34 | ILL 28 | Syria | 1* |
| 35 | ILL 618 | Tajikistan | 3 |
| 36 | ILL 624 | Macedonia | 2 |
| 37 | ILL 927 | Turkey | 3 |
| 38 | ILL 1139 | Lebanon | 1 |
| 39 | ILL 1220 | Iran | 3 |
| 40 | ILL 1337 | Iran | 3 |
| 41 | ILL 1553 | Iran | 3 |
| 42 | ILL 1762 | Afghanistan | 3 |
| 43 | ILL 1861 | Sudan | 3 |
| 44 | ILL 1983 | Ethiopia | 3 |
| 45 | ILL 2194 | Pakistan | 1 |
| 46 | ILL 2217 | Afghanistan | 3 |
| 47 | ILL 2290 | Chile | 2 |
| 48 | ILL 2433 | Ethiopia | 1 |
| 49 | ILL 2501 | India | 2 |
| 50 | ILL 2607 | India | 3 |
| 51 | ILL 2684 | India | 3 |
| 52 | ILL 2789 | India | 3 |
| 53 | ILL 3025 | India | 3 |
| 54 | ILL 3347 | India | 3 |
| 55 | ILL 3597 | India | 3 |
| 56 | ILL 4164 | India | 3* |
| 57 | ILL 4359 | India | 3 |
| 58 | ILL 4400 | Syria | 1 |
| 59 | ILL 4605 | Argentina | 2 |
| 60 | ILL 4609 | Pakistan | 3 |
| 61 | ILL 4665 | Hungary | 2 |
| 62 | ILL 4671 | USA | 1 |
| 63 | ILL 4768 | Yemen | 1 |
| 64 | ILL 4783 | Czech Republic | 2* |
| 65 | ILL 4804 | Libya | 2 |
| 66 | ILL 4875 | Uzbekistan | 3 |
| 67 | ILL 4956 | Portugal | 2 |
| 68 | ILL 5058 | Spain | 2 |
| 69 | ILL 5151 | India | 1 |
| 70 | ILL 5209 | Jordan | 1 |
| 71 | ILL 5511 | Syria | 1 |
| 72 | ILL 5588 | Jordan | 2* |
| 73 | ILL 5883 | Jordan | 1 |
| 74 | ILL 5945 | Ethiopia | 1 |
| 75 | ILL 6182 | Tunisia | 2 |
| 76 | ILL 6853 | Syria | 1 |
| 77 | ILL 7089 | Russia | 2* |
| 78 | ILL 7747 | Syria | 3* |

| | | | |
|---|---|---|---|
| 79 | Indianhead | Canada | 2 |
| 80 | Laird | Canada | 2 |
| 81 | PI 178939 | Turkey | 2 |
| 82 | PI 178971 | Turkey | 3 |
| 83 | PI 251032 | Iran | 2 |
| 84 | PI 273664 | Ethiopia | 3 |
| 85 | PI 297284 | Argentina | 2 |
| 86 | PI 298922 | Italy | 2 |
| 87 | PI 299121 | Mexico | 2 |
| 88 | PI 299126 | Mexico | 2 |
| 89 | PI 300250 | Syria | 3 |
| 90 | PI 308614 | Syria | 3 |
| 91 | PI320953 | Germany | 2 |
| 92 | PI 320954 | Hungary | 2 |
| 93 | PI 329169 | Iran | 3* |
| 94 | PI 339283 | Turkey | 3 |
| 95 | PI 339285 | Turkey | 3 |
| 96 | PI 339292 | Turkey | 1 |
| 97 | PI 343026 | Russia | 3 |
| 98 | PI 357225 | Serbia | 2 |
| 99 | PI 368647 | Macedonia | 2 |
| 100 | PI 420929 | Jordan | 1 |
| 101 | PI 426803 | Pakistan | 3 |
| 102 | PI431622 | Iran | 3 |
| 103 | PI 431662 | Iran | 3 |
| 104 | PI 431679 | Iran | 3 |
| 105 | PI 431705 | Iran | 3 |
| 106 | PI 431710 | Iran | 3 |
| 107 | PI 431714 | Iran | 3 |
| 108 | PI 431717 | Iran | 3 |
| 109 | PI 431756 | Iran | 3 |
| 110 | W6 27766 | USA | 2 |

*Note*. * labels genotypes with high level of admixture

## APPENDIX B: Linkage disqulibrium for each lentil chromosome

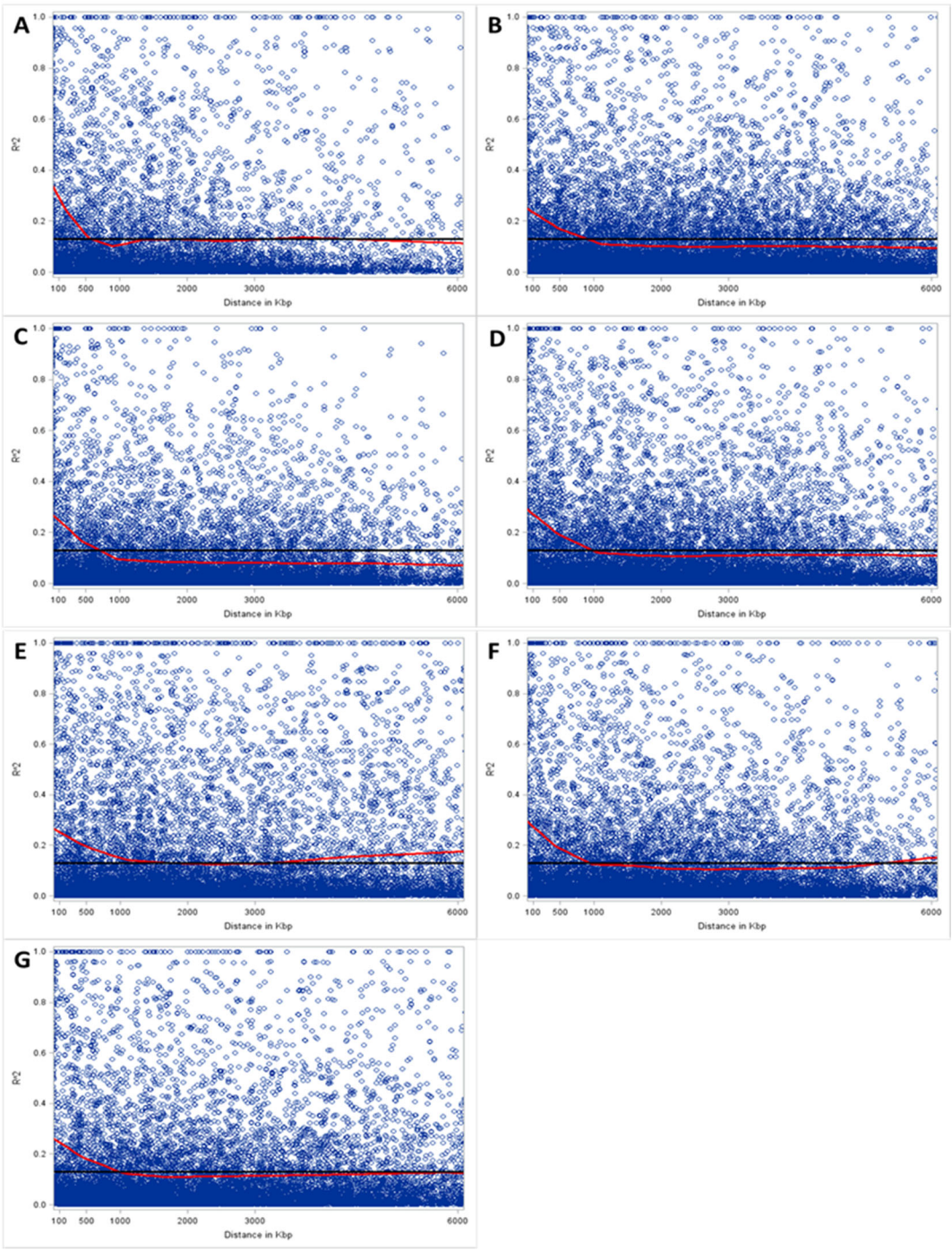



Figure B.1. Plots of LD decay based on set of 6,779 markers given by chromosomes, A) chromosome 1; B) chromosome 2; C) chromosome 3; D) chromosome 4; E) chromosome 5; F) chromosome 6; G) chromosome 7. Red line represents LOESS curve and black line represents critical $r^2$ value.

**APPENDIX C: List of significant associations in the control treatment**

*Table C.1. Name and position in lentil genome of single-nucleotide polymorphism markers found to be associated with NDVI data of control (unsprayed) treatment collected at 7 DAT; 14 DAT; 21 DAT and 28 DAT at Saskatoon and Elrose in 2015 and 2016; marker information include significance level (p value) and R² of MLM model.*

| Site Year | NDVI | Marker | Chromosome | Position | p value | Marker $R^2$ |
|---|---|---|---|---|---|---|
| Elrose 2016 | 28 DAT | SContig82450_22284 | 1 | 2924796 | 3.19 $e^{-5}$ | 0.048 |
| SPG 2016 | 28 DAT | SContig82450_22284 | 1 | 2924796 | 5.33 $e^{-6}$ | 0.053 |
| SPG 2016 | 14 DAT | SContig24784_43829 | 1 | 34945603 | 1.09 $e^{-5}$ | 0.055 |
| SPG 2016 | 21 DAT | SContig24784_43829 | 1 | 34945603 | 1.11 $e^{-6}$ | 0.068 |
| SPG 2016 | 14 DAT | SContig24784_43850 | 1 | 34945624 | 1.09 $e^{-5}$ | 0.055 |
| SPG 2016 | 21 DAT | SContig24784_43850 | 1 | 34945624 | 1.11 $e^{-6}$ | 0.068 |
| Elrose 2016 | 21 DAT | SContig512466_7786 | 1 | 52389929 | 6.28 $e^{-5}$ | 0.036 |
| Elrose 2016 | 28 DAT | SContig512466_7786 | 1 | 52389929 | 3.77 $e^{-5}$ | 0.040 |
| SPG 2016 | 28 DAT | SContig512466_7786 | 1 | 52389929 | 2.85 $e^{-6}$ | 0.051 |
| Elrose 2016 | 14 DAT | SContig183102_9014 | 1 | 84257992 | 6.75 $e^{-5}$ | 0.047 |
| SPG 2016 | 14 DAT | SContig183102_9014 | 1 | 84257992 | 4.15 $e^{-7}$ | 0.073 |
| Elrose 2016 | 21 DAT | SContig183102_9014 | 1 | 84257992 | 1.67 $e^{-6}$ | 0.063 |
| SPG 2016 | 21 DAT | SContig183102_9014 | 1 | 84257992 | 1.29 $e^{-8}$ | 0.094 |
| Elrose 2016 | 28 DAT | SContig183102_9014 | 1 | 84257992 | 5.38 $e^{-8}$ | 0.082 |
| SPG 2015 | 28 DAT | SContig183102_9014 | 1 | 84257992 | 3.63 $e^{-5}$ | 0.050 |
| SPG 2016 | 28 DAT | SContig183102_9014 | 1 | 84257992 | 1.07 $e^{-6}$ | 0.061 |
| SPG 2016 | 7 DAT | SContig183102_9014 | 1 | 84257992 | 3.46 $e^{-5}$ | 0.047 |
| SPG 2016 | 14 DAT | SContig499725_16683 | 1 | 114557329 | 4.28 $e^{-5}$ | 0.047 |
| SPG 2016 | 21 DAT | SContig499725_16683 | 1 | 114557329 | 1.21 $e^{-6}$ | 0.066 |
| SPG 2016 | 14 DAT | SContig314561_38356 | 1 | 131144724 | 4.78 $e^{-5}$ | 0.048 |
| SPG 2016 | 21 DAT | SContig314561_38356 | 1 | 131144724 | 1.26 $e^{-6}$ | 0.069 |
| SPG 2016 | 14 DAT | SContig52066_212556 | 1 | 140073150 | 9.16 $e^{-6}$ | 0.058 |
| Elrose 2016 | 21 DAT | SContig52066_212556 | 1 | 140073150 | 1.51 $e^{-5}$ | 0.052 |
| SPG 2016 | 21 DAT | SContig52066_212556 | 1 | 140073150 | 1.35 $e^{-6}$ | 0.075 |
| Elrose 2016 | 28 DAT | SContig52066_212556 | 1 | 140073150 | 9.48 $e^{-5}$ | 0.044 |
| SPG 2016 | 28 DAT | SContig52066_212556 | 1 | 140073150 | 2.19 $e^{-6}$ | 0.059 |
| Elrose 2016 | 21 DAT | SContig654170_9586 | 1 | 214568143 | 1.35 $e^{-4}$ | 0.040 |
| SPG 2016 | 21 DAT | SContig17789_76148 | 1 | 310634904 | 5.33 $e^{-5}$ | 0.047 |
| SPG 2016 | 28 DAT | SContig17789_76148 | 1 | 310634904 | 5.91 $e^{-6}$ | 0.052 |
| Elrose 2016 | 21 DAT | SContig219249_26951 | 1 | 332482362 | 1.21 $e^{-4}$ | 0.033 |
| SPG 2016 | 14 DAT | SContig204845_44086 | 2 | 54047612 | 2.57 $e^{-5}$ | 0.049 |
| SPG 2016 | 21 DAT | SContig204845_44086 | 2 | 54047612 | 5.94 $e^{-7}$ | 0.069 |
| SPG 2016 | 14 DAT | SContig193490_44597 | 2 | 59185677 | 3.30 $e^{-5}$ | 0.040 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Elrose 2016 | 21 DAT | SContig193490_44597 | 2 | 59185677 | 3.10 $e^{-5}$ | 0.038 |
| SPG 2016 | 21 DAT | SContig193490_44597 | 2 | 59185677 | 1.18 $e^{-5}$ | 0.046 |
| SPG 2016 | 28 DAT | SContig193490_44597 | 2 | 59185677 | 2.64 $e^{-5}$ | 0.038 |
| SPG 2016 | 14 DAT | SContig134783_45499 | 2 | 64591088 | 4.16 $e^{-5}$ | 0.047 |
| SPG 2016 | 21 DAT | SContig134783_45499 | 2 | 64591088 | 1.40 $e^{-6}$ | 0.065 |
| SPG 2016 | 14 DAT | SContig134783_45531 | 2 | 64591120 | 3.60 $e^{-5}$ | 0.047 |
| SPG 2016 | 21 DAT | SContig134783_45531 | 2 | 64591120 | 1.47 $e^{-6}$ | 0.065 |
| Elrose 2016 | 28 DAT | SContig297173_49769 | 2 | 77590428 | 7.20 $e^{-5}$ | 0.044 |
| Elrose 2016 | 21 DAT | SContig262092_8999 | 2 | 157511846 | 1.32 $e^{-4}$ | 0.033 |
| SPG 2016 | 21 DAT | SContig210047_36268 | 2 | 253012652 | 1.67 $e^{-5}$ | 0.053 |
| Elrose 2016 | 28 DAT | SContig210047_36268 | 2 | 253012652 | 8.60 $e^{-5}$ | 0.042 |
| SPG 2016 | 21 DAT | SContig210047_36301 | 2 | 253012685 | 1.67 $e^{-5}$ | 0.053 |
| Elrose 2016 | 28 DAT | SContig210047_36301 | 2 | 253012685 | 8.60 $e^{-5}$ | 0.042 |
| SPG 2016 | 14 DAT | SContig187359_107835 | 2 | 263774361 | 4.11 $e^{-5}$ | 0.048 |
| SPG 2016 | 21 DAT | SContig187359_107835 | 2 | 263774361 | 1.71 $e^{-6}$ | 0.065 |
| SPG 2016 | 14 DAT | SContig187359_107860 | 2 | 263774386 | 4.11 $e^{-5}$ | 0.048 |
| SPG 2016 | 21 DAT | SContig187359_107860 | 2 | 263774386 | 1.71 $e^{-6}$ | 0.065 |
| SPG 2016 | 14 DAT | SContig187359_107873 | 2 | 263774399 | 4.11 $e^{-5}$ | 0.048 |
| SPG 2016 | 21 DAT | SContig187359_107873 | 2 | 263774399 | 1.71 $e^{-6}$ | 0.065 |
| Elrose 2016 | 21 DAT | SContig103789_23699 | 2 | 263953718 | 2.25 $e^{-5}$ | 0.050 |
| Elrose 2016 | 28 DAT | SContig103789_23699 | 2 | 263953718 | 8.25 $e^{-6}$ | 0.056 |
| Elrose 2016 | 21 DAT | SContig103789_23713 | 2 | 263953732 | 2.25 $e^{-5}$ | 0.050 |
| Elrose 2016 | 28 DAT | SContig103789_23713 | 2 | 263953732 | 8.25 $e^{-6}$ | 0.056 |
| SPG 2016 | 28 DAT | SContig385761_16083 | 2 | 271005014 | 1.89 $e^{-5}$ | 0.047 |
| Elrose 2016 | 28 DAT | SContig385761_21136 | 2 | 271010067 | 1.46 $e^{-4}$ | 0.041 |
| SPG 2016 | 28 DAT | SContig385761_21136 | 2 | 271010067 | 1.82 $e^{-5}$ | 0.048 |
| Elrose 2016 | 21 DAT | SContig10195_112544 | 2 | 278574919 | 3.49 $e^{-6}$ | 0.050 |
| Elrose 2016 | 28 DAT | SContig10195_112544 | 2 | 278574919 | 1.47 $e^{-6}$ | 0.054 |
| Elrose 2016 | 21 DAT | SContig10195_112551 | 2 | 278574926 | 3.49 $e^{-6}$ | 0.050 |
| Elrose 2016 | 28 DAT | SContig10195_112551 | 2 | 278574926 | 1.47 $e^{-6}$ | 0.054 |
| SPG 2016 | 28 DAT | SContig64135_27046 | 2 | 294703825 | 1.37 $e^{-4}$ | 0.040 |
| SPG 2016 | 28 DAT | SContig60118_112018 | 2 | 305736671 | 1.24 $e^{-5}$ | 0.049 |
| Elrose 2016 | 21 DAT | SContig517126_46950 | 3 | 43783264 | 4.64 $e^{-6}$ | 0.048 |
| Elrose 2016 | 28 DAT | SContig517126_46950 | 3 | 43783264 | 7.06 $e^{-7}$ | 0.058 |
| SPG 2016 | 14 DAT | SContig418808_67587 | 3 | 96473057 | 8.67 $e^{-5}$ | 0.044 |
| Elrose 2016 | 21 DAT | SContig418808_67587 | 3 | 96473057 | 3.27 $e^{-5}$ | 0.046 |
| SPG 2016 | 21 DAT | SContig418808_67587 | 3 | 96473057 | 1.33 $e^{-5}$ | 0.055 |
| SPG 2016 | 28 DAT | SContig418808_67587 | 3 | 96473057 | 1.19 $e^{-4}$ | 0.039 |
| SPG 2016 | 28 DAT | SContig20558_16079 | 3 | 102919652 | 9.05 $e^{-5}$ | 0.040 |
| Elrose 2016 | 21 DAT | SContig48529_60614 | 3 | 175231642 | 8.38 $e^{-5}$ | 0.035 |
| Elrose 2016 | 21 DAT | SContig314500_120141 | 3 | 178110671 | 1.26 $e^{-4}$ | 0.033 |
| Elrose 2016 | 21 DAT | SContig47024_102709 | 3 | 198404429 | 3.20 $e^{-5}$ | 0.046 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Elrose 2016 | 28 DAT | SContig47024_102709 | 3 | 198404429 | 9.19 $e^{-6}$ | 0.052 |
| SPG 2016 | 14 DAT | SContig625136_1930 | 4 | 32881541 | 1.47 $e^{-4}$ | 0.033 |
| SPG 2016 | 21 DAT | SContig625136_1930 | 4 | 32881541 | 2.89 $e^{-5}$ | 0.042 |
| SPG 2016 | 28 DAT | SContig625136_1930 | 4 | 32881541 | 3.13 $e^{-5}$ | 0.037 |
| SPG 2016 | 21 DAT | SContig111750_39435 | 4 | 35782876 | 4.03 $e^{-6}$ | 0.063 |
| SPG 2016 | 28 DAT | SContig111750_39435 | 4 | 35782876 | 7.86 $e^{-6}$ | 0.051 |
| SPG 2016 | 28 DAT | SContig10155_54135 | 4 | 110981700 | 6.28 $e^{-5}$ | 0.041 |
| SPG 2016 | 14 DAT | SContig247223_38057 | 4 | 149038870 | 3.37 $e^{-5}$ | 0.048 |
| SPG 2016 | 21 DAT | SContig247223_38057 | 4 | 149038870 | 1.49 $e^{-6}$ | 0.065 |
| SPG 2016 | 28 DAT | SContig10132_13830 | 4 | 154828211 | 1.07 $e^{-5}$ | 0.043 |
| SPG 2016 | 7 DAT | SContig10132_13830 | 4 | 154828211 | 1.25 $e^{-4}$ | 0.033 |
| SPG 2016 | 28 DAT | SContig16640_93027 | 4 | 193871222 | 5.09 $e^{-5}$ | 0.043 |
| SPG 2016 | 7 DAT | SContig16640_93027 | 4 | 193871222 | 6.57 $e^{-5}$ | 0.043 |
| Elrose 2016 | 21 DAT | SContig351502_48852 | 4 | 236451820 | 3.82 $e^{-6}$ | 0.049 |
| Elrose 2016 | 28 DAT | SContig351502_48852 | 4 | 236451820 | 9.34 $e^{-7}$ | 0.055 |
| Elrose 2016 | 21 DAT | SContig357157_40236 | 5 | 9155195 | 1.38 $e^{-4}$ | 0.033 |
| Elrose 2016 | 21 DAT | SContig357157_40263 | 5 | 9155222 | 1.38 $e^{-4}$ | 0.033 |
| SPG 2016 | 28 DAT | SContig214927_27896 | 5 | 66517673 | 1.62 $e^{-5}$ | 0.048 |
| Elrose 2016 | 21 DAT | SContig158549_8118 | 5 | 127137818 | 3.99 $e^{-5}$ | 0.046 |
| Elrose 2016 | 21 DAT | SContig221371_11589 | 5 | 130924827 | 2.95 $e^{-5}$ | 0.047 |
| Elrose 2016 | 28 DAT | SContig221371_11589 | 5 | 130924827 | 9.99 $e^{-6}$ | 0.053 |
| SPG 2016 | 28 DAT | SContig221371_11589 | 5 | 130924827 | 1.43 $e^{-5}$ | 0.050 |
| Elrose 2016 | 21 DAT | SContig262042_35133 | 5 | 131977580 | 3.92 $e^{-5}$ | 0.038 |
| Elrose 2016 | 28 DAT | SContig262042_35133 | 5 | 131977580 | 2.18 $e^{-5}$ | 0.042 |
| SPG 2016 | 28 DAT | SContig262042_35133 | 5 | 131977580 | 5.00 $e^{-6}$ | 0.045 |
| SPG 2016 | 14 DAT | SContig188945_45691 | 5 | 139936571 | 3.16 $e^{-5}$ | 0.048 |
| SPG 2016 | 21 DAT | SContig188945_45691 | 5 | 139936571 | 2.57 $e^{-7}$ | 0.073 |
| SPG 2016 | 28 DAT | SContig188945_45691 | 5 | 139936571 | 1.49 $e^{-5}$ | 0.049 |
| SPG 2016 | 14 DAT | SContig166260_57935 | 5 | 179478904 | 7.88 $e^{-5}$ | 0.036 |
| SPG 2016 | 21 DAT | SContig166260_57935 | 5 | 179478904 | 3.95 $e^{-5}$ | 0.040 |
| SPG 2016 | 28 DAT | SContig166260_57935 | 5 | 179478904 | 1.60 $e^{-6}$ | 0.050 |
| Elrose 2016 | 28 DAT | SContig7280_72461 | 5 | 210596232 | 9.60 $e^{-5}$ | 0.042 |
| Elrose 2016 | 21 DAT | SContig19091_55595 | 5 | 217775768 | 5.51 $e^{-6}$ | 0.047 |
| Elrose 2016 | 28 DAT | SContig19091_55595 | 5 | 217775768 | 7.17 $e^{-6}$ | 0.048 |
| Elrose 2016 | 21 DAT | SContig530113_43694 | 5 | 235669831 | 1.15 $e^{-4}$ | 0.041 |
| SPG 2016 | 28 DAT | SContig530113_43694 | 5 | 235669831 | 1.43 $e^{-5}$ | 0.038 |
| SPG 2015 | 28 DAT | SContig28208_49428 | 6 | 6534090 | 9.64 $e^{-6}$ | 0.054 |
| Elrose 2016 | 21 DAT | SContig178295_46200 | 6 | 59451936 | 8.68 $e^{-5}$ | 0.035 |
| SPG 2016 | 21 DAT | SContig178295_46200 | 6 | 59451936 | 5.32 $e^{-5}$ | 0.040 |
| Elrose 2016 | 28 DAT | SContig178295_46200 | 6 | 59451936 | 3.76 $e^{-5}$ | 0.040 |
| Elrose 2016 | 28 DAT | SContig58198_40629 | 6 | 59693382 | 6.28 $e^{-5}$ | 0.045 |
| SPG 2016 | 28 DAT | SContig58198_40629 | 6 | 59693382 | 1.05 $e^{-4}$ | 0.040 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Elrose 2016 | 28 DAT | SContig3755_55676 | 6 | 60354663 | 7.75 $e^{-5}$ | 0.044 |
| Elrose 2016 | 28 DAT | SContig2295_8109 | 6 | 60479222 | 2.46 $e^{-5}$ | 0.048 |
| Elrose 2016 | 28 DAT | SContig2295_15520 | 6 | 60486633 | 3.20 $e^{-5}$ | 0.048 |
| Elrose 2016 | 21 DAT | SContig2295_93325 | 6 | 60564438 | 4.22 $e^{-5}$ | 0.046 |
| Elrose 2016 | 28 DAT | SContig2295_93325 | 6 | 60564438 | 1.64 $e^{-5}$ | 0.050 |
| Elrose 2016 | 28 DAT | SContig2295_93385 | 6 | 60564498 | 1.18 $e^{-4}$ | 0.041 |
| Elrose 2016 | 28 DAT | SContig2295_93389 | 6 | 60564502 | 1.18 $e^{-4}$ | 0.041 |
| Elrose 2016 | 28 DAT | SContig132806_22043 | 6 | 60755739 | 2.65 $e^{-5}$ | 0.048 |
| Elrose 2016 | 21 DAT | SContig132806_22070 | 6 | 60755766 | 1.17 $e^{-4}$ | 0.041 |
| Elrose 2016 | 28 DAT | SContig132806_22070 | 6 | 60755766 | 2.98 $e^{-5}$ | 0.048 |
| SPG 2016 | 14 DAT | SContig350426_25067 | 6 | 88559462 | 5.97 $e^{-5}$ | 0.047 |
| SPG 2016 | 21 DAT | SContig350426_25067 | 6 | 88559462 | 4.58 $e^{-9}$ | 0.096 |
| SPG 2016 | 28 DAT | SContig350426_25067 | 6 | 88559462 | 5.75 $e^{-6}$ | 0.054 |
| Elrose 2016 | 21 DAT | SContig18621_71438 | 6 | 90335439 | 9.25 $e^{-5}$ | 0.043 |
| Elrose 2016 | 28 DAT | SContig18621_71438 | 6 | 90335439 | 4.14 $e^{-5}$ | 0.048 |
| Elrose 2016 | 28 DAT | SContig72314_2698 | 6 | 91454880 | 6.34 $e^{-5}$ | 0.048 |
| Elrose 2016 | 28 DAT | SContig105765_27648 | 6 | 145390514 | 1.29 $e^{-4}$ | 0.033 |
| Elrose 2016 | 21 DAT | SContig53493_43414 | 6 | 159002885 | 8.69 $e^{-5}$ | 0.035 |
| SPG 2016 | 14 DAT | SContig183945_87668 | 6 | 164871241 | 2.04 $e^{-5}$ | 0.054 |
| SPG 2016 | 21 DAT | SContig183945_87668 | 6 | 164871241 | 1.08 $e^{-6}$ | 0.075 |
| SPG 2015 | 28 DAT | SContig183945_87668 | 6 | 164871241 | 6.30 $e^{-5}$ | 0.048 |
| Elrose 2016 | 21 DAT | SContig64867_15703 | 6 | 176068901 | 1.03 $e^{-4}$ | 0.042 |
| Elrose 2016 | 28 DAT | SContig64867_15703 | 6 | 176068901 | 8.28 $e^{-5}$ | 0.043 |
| SPG 2016 | 14 DAT | SContig211014_47202 | 6 | 184910036 | 4.20 $e^{-5}$ | 0.048 |
| SPG 2016 | 21 DAT | SContig211014_47202 | 6 | 184910036 | 1.34 $e^{-6}$ | 0.066 |
| SPG 2016 | 14 DAT | SContig211014_47234 | 6 | 184910068 | 4.20 $e^{-5}$ | 0.048 |
| SPG 2016 | 21 DAT | SContig211014_47234 | 6 | 184910068 | 1.34 $e^{-6}$ | 0.066 |
| Elrose 2016 | 21 DAT | SContig81226_15849 | 6 | 192758865 | 1.15 $e^{-4}$ | 0.033 |
| Elrose 2016 | 14 DAT | SContig59937_312737 | 7 | 28750169 | 1.91 $e^{-5}$ | 0.043 |
| Elrose 2016 | 21 DAT | SContig59937_312737 | 7 | 28750169 | 9.55 $e^{-7}$ | 0.055 |
| Elrose 2016 | 28 DAT | SContig59937_312737 | 7 | 28750169 | 1.44 $e^{-7}$ | 0.064 |
| SPG 2016 | 14 DAT | SContig35848_30155 | 7 | 51256667 | 4.45 $e^{-5}$ | 0.047 |
| SPG 2016 | 21 DAT | SContig35848_30155 | 7 | 51256667 | 1.33 $e^{-6}$ | 0.066 |
| Elrose 2016 | 21 DAT | SContig207818_14738 | 7 | 61249018 | 7.99 $e^{-5}$ | 0.035 |
| SPG 2016 | 28 DAT | SContig191651_3893 | 7 | 76339989 | 7.57 $e^{-6}$ | 0.044 |
| SPG 2016 | 28 DAT | SContig256375_14426 | 7 | 77342084 | 7.48 $e^{-6}$ | 0.045 |
| Elrose 2016 | 21 DAT | SContig1432_29369 | 7 | 99120487 | 1.22 $e^{-4}$ | 0.033 |
| SPG 2016 | 21 DAT | SContig700452_16671 | 7 | 120222490 | 8.18 $e^{-5}$ | 0.046 |
| Elrose 2016 | 21 DAT | SContig52916_6071 | 7 | 123632218 | 1.36 $e^{-4}$ | 0.032 |
| Elrose 2016 | 21 DAT | SContig20091_47697 | 7 | 195488860 | 1.26 $e^{-4}$ | 0.034 |
| SPG 2016 | 28 DAT | SContig121993_44454 | 7 | 195888836 | 8.92 $e^{-5}$ | 0.040 |
| Elrose 2016 | 21 DAT | SContig172094_82216 | 7 | 212463561 | 1.25 $e^{-4}$ | 0.033 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Elrose 2016 | 21 DAT | SContig172094_82258 | 7 | 212463603 | 1.25 $e^{-4}$ | 0.033 |
| Elrose 2016 | 21 DAT | SContig155293_110574 | 7 | 243230877 | 7.62 $e^{-5}$ | 0.037 |

*Table C.2. Name and position in lentil genome of single-nucleotide polymorphism markers found to be associated with dry biomass of control (unsprayed) treatment, experiment was conducted in controlled environment, marker information include significance level (p value) and $R^2$ of MLM model.*

| Marker | Chromosome | Position | p value | Marker $R^2$ |
|---|---|---|---|---|
| SContig122234_6436 | 1 | 246255562 | 8.91 $e^{-5}$ | 0.035 |
| SContig52253_142091 | 2 | 2201958 | 1.17 $e^{-4}$ | 0.034 |
| SContig144478_3859 | 4 | 70059946 | 8.59 $e^{-5}$ | 0.036 |

**APPENDIX D: Model parameters for electrolyte leakage assay of inhibitors of cytochrome P450 and glutathione-S-transferase**

*Table D.1. Model parameters of Weibull 2 model with standard error (SE) for all treatments (SF200 (sulfentrazone 200µM), FM25 (fluthiacet methyl 25µM), P450SF200 (sulfentrazone 200µM + inhibitor cytochrome P450), P450FM25 (fluthiacet methyl 25µM + inhibitor cytochrome P450), GSHSF200 (sulfentrazone 200µM + inhibitor glutathione-S-transferase), GSHFM25 (fluthiacet methyl 25µM + inhibitors glutathione-S-transferase)).*

| Genotypes | | SF200 | FM25 | P450SF200 | P450FM25 | GSHSF200 | GSHFM25 |
|---|---|---|---|---|---|---|---|
| CDC Redberry | ***b*** | 4.12 | 3.51 | 4.36 | 3.56 | 5.74 | 3.45 |
| | SE | 0.31 | 0.21 | 0.26 | 0.21 | 0.39 | 0.19 |
| | ***e*** | 91.42 | 81.12 | 82.22 | 67.96 | 87.18 | 77.40 |
| | SE | 1.10 | 0.92 | 0.81 | 0.79 | 0.73 | 0.87 |
| CDC Improve | ***b*** | 3.24 | 2.90 | 3.72 | 3.17 | 4.73 | 3.36 |
| | SE | 0.25 | 0.19 | 0.26 | 0.22 | 0.35 | 0.23 |
| | ***e*** | 84.75 | 72.94 | 78.15 | 66.23 | 82.81 | 69.88 |
| | SE | 1.29 | 1.11 | 1.03 | 1.00 | 0.93 | 1.00 |
| CDC Impala | ***b*** | 3.47 | 2.93 | 3.78 | 2.99 | 4.21 | 3.16 |
| | SE | 0.24 | 0.19 | 0.26 | 0.20 | 0.30 | 0.22 |
| | ***e*** | 80.67 | 70.22 | 73.55 | 63.37 | 75.20 | 63.72 |
| | SE | 1.10 | 1.06 | 0.96 | 1.01 | 0.90 | 0.98 |
| CDC Rosetown | ***b*** | 3.16 | 2.94 | 3.51 | 2.80 | 3.86 | 3.97 |
| | SE | 0.20 | 0.18 | 0.23 | 0.18 | 0.25 | 0.31 |
| | ***e*** | 76.73 | 65.91 | 73.77 | 61.54 | 75.09 | 64.22 |
| | SE | 1.03 | 0.97 | 0.95 | 0.97 | 0.91 | 0.88 |

**APPENDIX E: Polymorphisms found on chloroplast and mitochondria PPOX genes among lentil genotypes**

*Table E.1. List of 86 diverse lentil genotypes with allele calls of two SNPs (Lcu_Ch_PPOX1 and Lcu_Ch_PPOX2) located on chloroplast PPOX gene and two SNPs (Lcu_Mh_PPOX1 and Lcu_Mh_PPOX2) located on mitochondria PPOX gene.*

| Genotypes | Lcu_Ch_PPOX1 | Lcu_Ch_PPOX2 | Lcu_Mh_PPOX1 | Lcu_Mh_PPOX2 |
|---|---|---|---|---|
| **3156-11** | G | A | C | A |
| **CDC Asterix** | G | A | C | A |
| **CDC Cherie** | G | A | C | A |
| **CDC Glamis** | A | A | C | A |
| **CDC Greenstar** | A | A | C | A |
| **CDC Imax** | A | A | C | A |
| **CDC Impower** | A | A | C | A |
| **CDC KR-1** | G | A | C | A |
| **CDC LeMay** | A | A | C | G |
| **CDC Maxim** | G | A | C | A |
| **CDC QG-1** | A | A | C | A |
| **CDC Red Rider** | A | A | C | A |
| **CDC Redberry** | G | A | C | A |
| **CDC Redcoat** | G | A | C | A |
| **CDC Redwing** | G | A | C | G |
| **CDC Robin** | G | A | C | A |
| **CDC Rosebud** | G | A | C | A |
| **CDC Rosetown** | G | A | C | A |
| **CDC Rosie** | G | A | C | A |
| **CDC Rouleau** | A | A | C | A |
| **CDC Royale** | A | A | C | G |
| **CDC Ruby** | G | A | C | A |
| **CDC SB-1** | G | A | C | G |
| **CDC Sedley** | A | A | C | G |
| **CDC Vantage** | G | A | C | A |
| **Crimson** | G | G | C | G |
| **Eston** | G | A | C | A |
| **ILL 1762** | G | A | T | G |
| **ILL 1983** | G | A | C | A |
| **ILL 2194** | G | A | C | G |
| **ILL 28** | G | A | C | G |
| **ILL 3025** | G | A | C | A |
| **ILL 313** | G | A | C | G |
| **ILL 3347** | G | A | C | A |
| **ILL 3597** | G | A | C | A |
| **ILL 4164** | G | A | C | G |
| **ILL 4400** | G | A | C | G |
| **ILL 4605** | G | A | C | G |
| **ILL 4609** | G | A | C | A |

| | | | | |
|---|---|---|---|---|
| **ILL 4671** | G | A | C | G |
| **ILL 4768** | G | A | C | G |
| **ILL 4804** | G | A | C | G |
| **ILL 4875** | G | A | C | A |
| **ILL 4956** | G | A | C | A |
| **ILL 5058** | G | A | C | G |
| **ILL 5151** | G | A | C | G |
| **ILL 5209** | G | A | C | G |
| **ILL 5883** | G | A | C | G |
| **ILL 5888** | G | A | C | A |
| **ILL 5945** | G | A | C | G |
| **ILL 6182** | G | A | C | G |
| **ILL 618** | G | G | C | A |
| **ILL 624** | G | A | C | A |
| **ILL 6853** | G | A | C | G |
| **ILL 7089** | G | A | C | A |
| **ILL 7747** | G | A | C | G |
| **ILL 9** | G | A | C | G |
| **Indianhead** | G | A | C | A |
| **Laird** | A | A | C | A |
| **PI 178939LSP** | G | G | C | G |
| **PI 178971LSP** | G | G | C | A |
| **PI 217949LSP** | G | A | C | A |
| **PI 273664LSP** | G | A | C | A |
| **PI 298631 LSP** | G | A | C | G |
| **PI 298922LSP** | G | A | C | G |
| **PI 299121LSP** | G | G | C | G |
| **PI 299126LSP** | G | G | C | G |
| **PI 300250LSP** | G | A | C | G |
| **PI 308614LSP** | G | A | C | G |
| **PI 320953LSP** | G | G | C | A |
| **PI 320954LSP** | A | A | C | G |
| **PI 339283LSP** | G | A | C | A |
| **PI 339285** | G | A | C | A |
| **PI 339292LSP** | G | G | C | A |
| **PI 343026LSP** | G | G | C | A |
| **PI 368647LSP** | G | G | C | A |
| **PI 420929LSP** | G | A | C | G |
| **PI 431622LSP** | G | G | C | A |
| **PI 431662LSP** | G | G | C | A |
| **PI 431679LSP** | G | A | C | A |
| **PI 431705LSP** | G | G | C | A |
| **PI 431710LSP** | G | G | C | A |
| **PI 431714LSP** | G | G | C | A |
| **PI 431717LSP** | G | G | C | A |
| **PI 431756LSP** | G | G | C | A |
| **W6 27766LSP** | G | A | C | G |

**APPENDIX F: Indoor pre-screening of mutagenized lines with fluthiacet methyl**

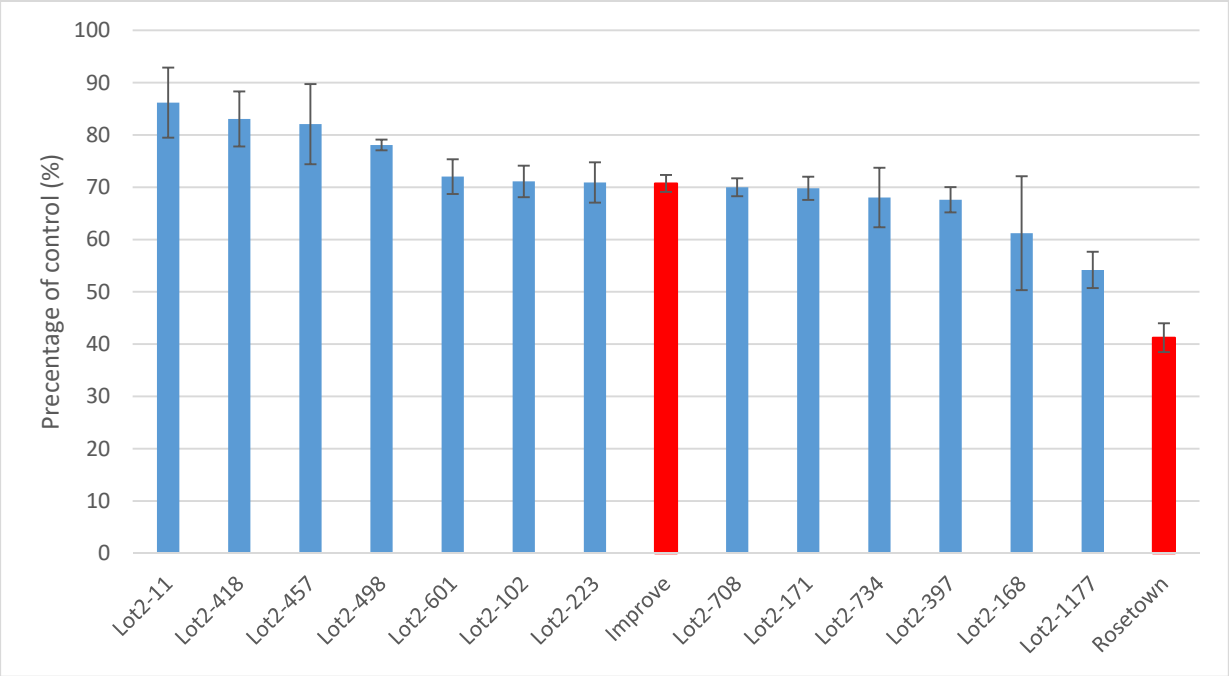



Figure F.1. Selected M4 lentil lines and two checks (CDC Improve and CDC Rosetown) tested in controlled environment with 8 g.a.i ha-1 fluthiacet methyl, above ground dry biomass presented as percentage of control.

**APPENDIX G: Field screening of selected mutagenized lines with sulfentrazone**

*Table G.1. Mean of dry biomass and standard deviation (SD) with Tukey's (HSD) grouping for 22 selected M4 lentil lines and CDC Redberry (check) for control (unsprayed) and sulfentrazone (280 g.a.i ha$^{-1}$) treatment at Saskatoon in 2015.*

| Line | CONTROL | | SULFENTRAZONE | |
|---|---|---|---|---|
| | **Dry biomass (g) (SE)** | **Tukey's HSD grouping** | **Dry biomass (g) (SE)** | **Tukey's HSD grouping** |
| **CDC Redberry** | 42.45(6.29) | ABC | 15.92(6.47) | A |
| **Lot2-1081** | 43.32(3.50) | ABC | 17.71(5.79) | A |
| **Lot2-11** | 47.15(4.53) | ABC | 17.60(7.27) | A |
| **Lot2-1102** | 47.87(6.04) | AB | 16.73(5.42) | A |
| **Lot2-1114** | 42.45(4.07) | ABC | 11.06(3.24) | A |
| **Lot2-1145** | 42.34(7.54) | ABC | 13.88(3.85) | A |
| **Lot2-1206** | 39.54(5.38) | ABC | 13.11(4.18) | A |
| **Lot2-1207** | 54.62(7.61) | A | 17.84(6.00) | A |
| **Lot2-151** | 38.84(6.07) | ABC | 21.17(5.80) | A |
| **Lot2-259** | 35.72(2.57) | BC | 13.90(4.04) | A |
| **Lot2-28** | 42.15(3.07) | ABC | 14.73(8.14) | A |
| **Lot2-282** | 38.00(4.85) | ABC | 9.74(2.95) | A |
| **Lot2-297** | 42.30(5.99) | ABC | 13.09(5.58) | A |
| **Lot2-340** | 42.63(4.35) | ABC | 14.99(5.56) | A |
| **Lot2-382** | 36.35(5.98) | BC | 9.99(3.26) | A |
| **Lot2-418** | 42.69(9.97) | ABC | 22.10(5.96) | A |
| **Lot2-427** | 52.43(4.35) | ABC | 12.66(2.75) | A |
| **Lot2-447** | 44.40(7.00) | ABC | 15.83(5.96) | A |
| **Lot2-494** | 38.20(4.87) | ABC | 13.19(3.85) | A |
| **Lot2-559** | 37.89(4.73) | ABC | 15.03(5.09) | A |
| **Lot2-75** | 41.67(6.70) | ABC | 14.25(3.86) | A |
| **Lot2-904** | 30.83(3.69) | C | 9.08(3.44) | A |
| **Lot2-962** | 42.72(2.77) | ABC | 16.83(6.25) | A |

*Table G.2. Mean of NDVI values and standard deviation (SD) with Tukey's (HSD) grouping for 22 selected M4 lentil lines and CDC Redberry (check) for control (unsprayed) and sulfentrazone (280 g.a.i ha$^{-1}$) treatment at Saskatoon in 2015.*

| Line | CONTROL | | SULFENTRAZONE | |
|---|---|---|---|---|
| | **NDVI (SE)** | **Tukey's HSD grouping** | **NDVI (SE)** | **Tukey's HSD grouping** |
| **CDC Redberry** | 0.459(0.032) | A | 0.364(0.025) | A |
| **Lot2-1081** | 0.450(0.022) | A | 0.363(0.016) | A |
| **Lot2-11** | 0.459(0.026) | A | 0.375(0.027) | A |
| **Lot2-1102** | 0.458(0.024) | A | 0.383(0.023) | A |
| **Lot2-1114** | 0.455(0.018) | A | 0.356(0.008) | A |
| **Lot2-1145** | 0.446(0.026) | A | 0.367(0.009) | A |
| **Lot2-1206** | 0.453(0.015) | A | 0.351(0.021) | A |
| **Lot2-1207** | 0.477(0.022) | A | 0.386(0.014) | A |
| **Lot2-151** | 0.434(0.026) | A | 0.367(0.022) | A |
| **Lot2-259** | 0.438(0.023) | A | 0.347(0.016) | A |
| **Lot2-28** | 0.462(0.016) | A | 0.348(0.026) | A |
| **Lot2-282** | 0.427(0.020) | A | 0.342(0.011) | A |
| **Lot2-297** | 0.453(0.028) | A | 0.339(0.015) | A |
| **Lot2-340** | 0.447(0.014) | A | 0.359(0.022) | A |
| **Lot2-382** | 0.445(0.028) | A | 0.349(0.015) | A |
| **Lot2-418** | 0.450(0.036) | A | 0.401(0.017) | A |
| **Lot2-427** | 0.477(0.010) | A | 0.365(0.008) | A |
| **Lot2-447** | 0.435(0.014) | A | 0.366(0.022) | A |
| **Lot2-494** | 0.423(0.016) | A | 0.367(0.011) | A |
| **Lot2-559** | 0.442(0.022) | A | 0.367(0.022) | A |
| **Lot2-75** | 0.447(0.026) | A | 0.382(0.013) | A |
| **Lot2-904** | 0.428(0.018) | A | 0.365(0.021) | A |
| **Lot2-962** | 0.452(0.017) | A | 0.387(0.020) | A |

## APPENDIX H: Correlation between NDVI values and dry biomass

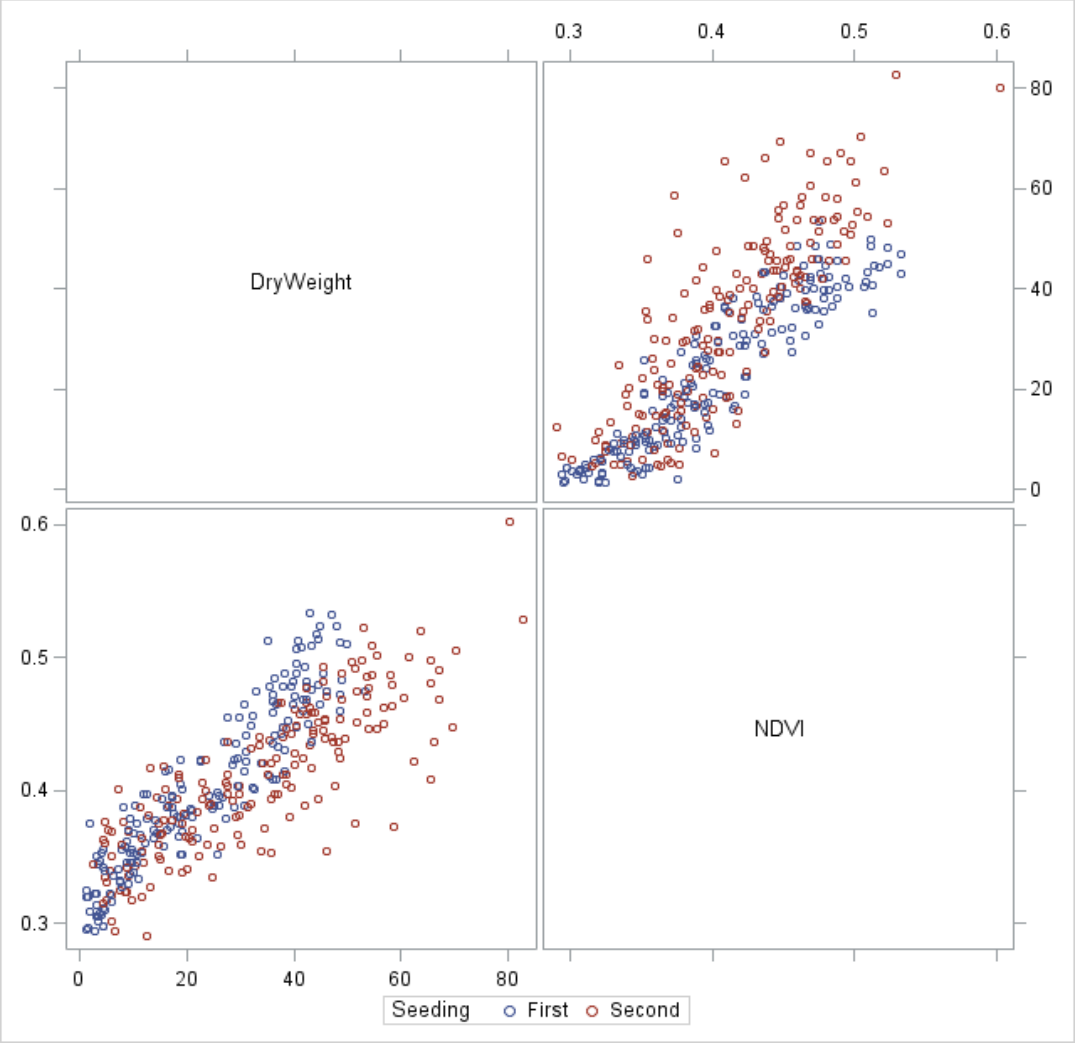



Figure H.1. Correlation between dry biomass and NDVI of 22 selected M4 lines and CDC Redberry, dry biomass and NDVI data was collected 21 days after seeding (○ first seeding; ○ second seeding) at Saskatoon during 2015.

**APPENDIX I: File containg SNP data used in genome wide association study**

Link for the file containing genotypic information of 110 diverse lentil accessions, total number of SNP marker is 6,779 with missing data threshold per each SNP set at 5%. Data is in VCF (Variant Call Format) file format.

http://knowpulse.usask.ca/portal/filter_vcf?vcf_file_id=8

## APPENDIX J: Single nucleotide polymorphisms located on the chloroplast protoporphyrinogen oxidase gene

Link for the file containing SNPs located in genomic region of lentil's chloroplast protoporphyrinogen oxidase (Lcu_Ch_PPOX) at chromosome 1, filtering SNPs based on missing data was not performed. Data is in VCF (Variant Call Format) file format.

http://knowpulse.usask.ca/portal/filter_vcf?vcf_file_id=9

## APPENDIX K: Single nucleotide polymorphisms located on the mitochondria protoporphyrinogen oxidase gene

Link for the file containing SNPs located in genomic region of lentil's mitochondria protoporphyrinogen oxidase (Lcu_Mh_PPOX) at chromosome 6, filtering SNPs based on missing data was not performed. Data is in VCF (Variant Call Format) file format.

http://knowpulse.usask.ca/portal/filter_vcf?vcf_file_id=10