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SELECTION AND BASIS FOR 2,4-D (2,4-Dichlorophenoxyacetic acid) TOLERANCE
IN RED CLOVER (*Trifolium pratense*)

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Agriculture, Food, and Environment
at the University of Kentucky

By
Tara Leigh Burke Lewis

Lexington, Kentucky

Director: Dr. Michael Barrett,

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Lexington, Kentucky

2014

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ABSTRACT OF THESIS

SELECTION AND BASIS FOR 2,4-D (2,4-DICHLOROPHENOXYACETIC ACID) TOLERANCE IN RED CLOVER (*Trifolium pratense*)

A red clover (*Trifolium pratense*) population (UK), from a cross between the cultivar Kenland and a 2,4-D tolerant population (Florida), was recurrently selected for 2,4-D tolerance with evaluations after the 6th, 7th, and 8th selection cycles. All UK populations were more 2,4-D tolerant than Kenland. The 2,4-D tolerance following the 6th selection cycle was similar to the Florida population and tolerance was increased following 7 and 8 cycles of selection by removing plants showing 2,4-D injury and doubling the rate of 2,4-D used for selection.

Yield and forage quality were evaluated in UK and Kenland. Forage quality measurements included acid detergent fiber (ADF), neutral detergent fiber (NDF), and crude protein (CP). The UK population had improved yield and NDF.

The potential role of 2,4-D uptake, translocation, and metabolism in the red clover tolerance to the herbicide was evaluated by following behavior of ¹⁴C 2,4-D in UK and Kenland 8, 24, 48 and 72 hours after treatment. Plants were partitioned into the treated leaf, untreated shoot, and roots. There was less parent 2,4-D and more 2,4-D metabolites in all sections of UK at all sample times, indicating that enhanced 2,4-D metabolism in UK is likely the basis for tolerance in this population.

Keywords: red clover, *Trifolium pratense*, 2,4-D, metabolism, herbicide tolerance

Tara Leigh Burke Lewis

May 9, 2015

SELECTION AND BASIS FOR 2,4-D TOLERANCE IN RED CLOVER
(*Trifolium pratense*)

By

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May 9, 2015

Dedication

I would like to dedicate my thesis to the memory of my grandfather, Warren Grosch, who has always been a steadying force in my life. Both he and my grandmother, Doshia Grosch, have been like second parents to me, always pushing me to achieve, and setting me on the long path to higher education at a young age by showing me the value of hard work and perseverance.

Acknowledgements

First, I would like to thank my main advisor, Dr. Michael Barrett, for taking me on as a student. Under his guidance, I have changed the way I look at not just my Masters research, but at my career overall, and I have tried to take advantage of every one of the many opportunities he has given me for scholarship, leadership, community involvement, and of course scientific advancement and learning. I could not have asked for a better advisor. I would additionally like to thank the other members of my graduate committee, Dr. Tim Phillips and Dr. J.D. Green. Dr. Phillips has been an amazing mentor and source of advice throughout my work at the University of Kentucky; I have yet to meet another person with such a broad and deep knowledge base. Dr. Green has been a constant support and I must thank him for all of the help he has provided, and for answering my never-ending plant identification queries. Dr. Ben Goff is another faculty member at UK who I must thank for his interest in my project and help with my forage quality testing and statistical analysis. Additionally, the work of the late Dr. Norman Taylor from which this project stemmed is directly responsible for the existence of this work, so I would be remiss if I did not acknowledge Dr. Taylor. I would also like to thank Dr. David Williams for teaching and supporting me along the way, as well as Linda Williams, Charlie Slack, Sara Carter, James Roberts, Ricky King, Rich Mundell, Kristen McQuerry, and Karen Meekins for their support and experience.

I would especially like to thank Dr. Alexandra Williams and Whitney Churchman for working with me almost every day as we all progressed towards our

respective degrees; I have met two life-long friends and colleagues in the both of you. I would also like to thank my fellow graduate students whom I have worked with for their varied assistance and collaboration; thank you Chad Brabham, Bret Sparks, Kenneth Cropper, Lindsey Slaughter, Dr. Jessique Gezzie, Dan Weber, Shuang Zhou, and many others for your valuable assistance and friendship.

I would also like to thank my amazing husband Ricky Woodrow Lewis for always pushing me to think harder about my work and all of science; I would not be the scientist I am today if not for him. I also cannot thank him enough for sharing equally in our careers and lives, supporting each other in times of stress and celebrating with each other in times of joy. I would be remiss not to thank my parents, Lawrence and Linda Burke for always supporting me in my various endeavors and pushing me from a young age to achieve and explore, inspiring me to be the confident and strong scientist I am today. My twin brother Ryan must also be thanked, for always having faith in my abilities, even as he was making fun of them. Lastly, I would like to thank my grandmother, Doshia Grosch, for supporting me and my education, always pushing me to work hard, stay positive, and learn as much as I can. Thank you all.

Table of Contents

Acknowledgements	iv
Table of Contents	vi
List of Tables	viii
List of Figures.....	x
Chapter One: <i>Trifolium pratense</i> (L.) and 2,4-Dichlorophenoxyacetic Acid: A Review	1
Historical Significant of Red Clover	1
Early History	1
Modern History	4
Biology of Red Clover	6
Agriculturally Significant Traits of Red Clover	7
Crop Ecosystem Gains	7
High Quality Animal Feed	10
Implications for Farmers	10
Management Issues	11
Weed Control	12
Red Clover Genetics	13
Breeding Red Clover	14
History	14

Breeding Techniques	15
History of 2,4-D	17
Discovery and Early History	17
Wartime Use	18
Agricultural Use	18
Biochemical Action of 2,4-D	19
2,4-D in the Environment	21
Non-Target Toxicity	21
Environmental Effects	22
Weed Resistance to 2,4-D	22
Breeding for 2,4-D Tolerance	23
Breeding for 2,4-D Tolerance in Red Clover	24
Chapter Two: Polycross Recurrent and Second Year Polycross Selection for 2,4-D Tolerance in Red Clover	26
Materials and Methods.....	29
Field Based Polycross Recurrent Selection	29
Field Based Second Year Polycross Selection	32
Greenhouse Evaluation of 2,4-D Tolerance	33
Results	35
Evaluation of 2,4-D Tolerance Resulting from the 2011 Polycross Recurrent Selection.....	35

Evaluation of 2,4-D Tolerance Resulting from the 2012 Polycross Recurrent Selection	36
Evaluation of 2,4-D Tolerance Resulting from the 2013 Polycross Recurrent and Second Year Polycross Selections	37
Discussion	37
Chapter Three: Forage Quality Comparisons	56
Materials and Methods	58
Results and Discussion	60
Neutral Detergent Fiber	60
Acid Detergent Fiber and Crude Protein	60
Yield	60
Chapter Four: 2,4-D Uptake, Translocation, and Metabolism in Sensitive and Tolerant Red Clover	67
Materials and Methods.....	70
Results and Discussion	73
Uptake and Translocation	73
Metabolism of ¹⁴ C 2,4-D	76
Chapter Five: Conclusions	120
References	124
Vita	141

List of Tables

Table 2.1	P-values for each greenhouse study	44
Table 3.1	P-values for forage quality and yield experiments	62
Table 4.1	HPLC solvent gradient used for 2,4-D analysis	82
Table 4.2	P-values for each data type: the model was significant at the $p < 0.05$ level unless otherwise noted	83

List of Figures

Figure 2.1	Depiction of herbicide application equipment and representative red clover plants at time of 2,4-D application	45
Figure 2.2	Representative red clover plants at time of 2,4-D application	46
Figure 2.3	Red clover injury two weeks after 2,4-D application to Kenland, Florida 2,4-D tolerant clover (Florida), and plants grown from seed harvested from the 2010 and 2011 polycross recurrent selections (UK2010 and UK2011, respectively). Visual injury rating on a scale from 0= no damage to 10= dead. * denotes significant difference between Kenland and all other clover varieties, and ** denotes significant difference between UK2011 and Florida ($p < 0.05$)	47
Figure 2.4	Red clover fresh weights two weeks after 2,4-D application to Kenland, Florida 2,4-D tolerant clover (Florida), and plants grown from seed harvested from the 2010 and 2011 polycross recurrent selections (UK2010 and UK2011, respectively). There was no significant interaction between 2,4-D rate and clover type. Over clover types, a trend of decreasing fresh weight with increasing 2,4-D rate occurs, with 0 kg ha ⁻¹ heavier than all other 2,4-D rates. Over 2,4-D rates, Kenland is lighter than all other clover types, while Florida, UK2010, and UK2011 are all similar in weight to each other ($p < 0.05$)	48
Figure 2.5	Red clover dry weights two weeks after 2,4-D application to Kenland, Florida 2,4-D tolerant clover (Florida), and plants grown from seed harvested from the 2010 and 2011 polycross recurrent selections (UK2010 and UK2011, respectively). There is no significant interaction between 2,4-D rate and clover type, but over clover types, a trend of decreasing dry weights over increasing 2,4-D rate occurs ($p < 0.05$)	49
Figure 2.6	Red clover injury two weeks after 2,4-D application to Kenland and plants grown from seed harvested from the 2011 and 2012 polycross recurrent selections (UK2011 and UK2012, respectively). Injury rating on a scale from 0= no damage to 10= dead. * denotes significant difference between Kenland and all other clover varieties, and ** denotes significant difference between UK2011 and UK2012 ($p < 0.05$)	50

Figure 2.7	Red clover fresh weights two weeks after 2,4-D application to Kenland and plants grown from seed harvested from the 2011 and 2012 polycross recurrent selections (UK2011 and UK2012, respectively). There was no significant interaction, but over clover types there is a trend of decreasing fresh weight as rate of 2,4-D increases, and over 2,4-D rates, Kenland has a significantly lower fresh weight compared to UK2011 and UK2012 ($p < 0.05$)	51
Figure 2.8	Red clover dry weights two weeks after 2,4-D application to Kenland and plants grown from seed harvested from the 2011 and 2012 polycross recurrent selections (UK2011 and UK2012, respectively). There was no significant interaction between clover type and 2,4-D rate, but over clover types a trend of decreasing dry weights over increasing 2,4-D rate occurs, and over 2,4-D rates Kenland is significantly lower in dry weight compared to UK2011 but not UK2012 ($p < 0.05$)	52
Figure 2.9	Red clover injury two weeks after 2,4-D application to Kenland, and plants grown from seed harvested from the 2012 and 2013 polycross recurrent selections (UK2012 and UK2013, respectively) as well as the 2013 second year polycross selection (UK2013-P). Injury rating on a scale from 0= no damage to 10= dead. * denotes significant difference between Kenland and all other clover varieties, ** denotes significant difference between UK2013 and both UK2012 and UK2013-P, and *** denotes significant difference between UK2012 and both UK2013 and UK2013-P ($p < 0.05$)	53
Figure 2.10	Red clover fresh weights two weeks after 2,4-D application to Kenland, and plants grown from seed harvested from the 2012 and 2013 polycross recurrent selections (UK2012 and UK2013, respectively) as well as the 2013 second year polycross selection (UK2013-P). There is no significant interaction between clover types and 2,4-D rates, but over clover types there is a trend of decreasing fresh weight with increasing 2,4-D rate, and over 2,4-D rates Kenland has a significantly lower fresh weight compared to the other clover types while UK2013-P has a significantly higher fresh weight compared to the other clover types ($p < 0.05$)	54
Figure 2.11	Red clover dry weights two weeks after 2,4-D application to Kenland, and plants grown from seed harvested from the 2012 and 2013 polycross recurrent selections (UK2012 and UK2013, respectively) as well as the 2013 second year polycross selection	

	(UK2013-P). There was no significant interaction between clover type and 2,4-D rate, but over clover types there is a trend of decreasing dry weight with increasing 2,4-D rate, and over herbicide rates Kenland has a significantly lower fresh weight compared to the other clover types, while UK2013-P has a significantly higher fresh weight compared to the other clover types ($p < 0.05$)	55
Figure 3.1	Neutral detergent fiber contents for Kenland and UK2013. There was no significant interaction between clover types and 2,4-D rates, but Kenland has a significantly higher NDF content compared to UK2013 over 2,4-D rates ($p < 0.05$)	63
Figure 3.2	Acid detergent fiber contents for Kenland and UK2013. Bars with the same letter are not statistically different ($p < 0.05$)	64
Figure 3.3	Crude protein contents for Kenland and UK2013. Bars with the same letter are not statistically different ($p < 0.05$)	65
Figure 3.4	Individual plant yield of Kenland and UK2013 at 0 and 1.12 kg ha ⁻¹ 2,4-D. There is no significant interaction between clover type and 2,4-D rate, but over 2,4-D rates Kenland has a lower yield than UK2013 ($p < 0.05$)	66
Figure 4.1	Treatment area for studies	88
Figure 4.2	¹⁴ C 2,4-D droplets of treatment solution on red clover leaf	89
Figure 4.3	Sample radiochromatograph showing ¹⁴ C 2,4-D peak at 23 minutes and metabolite retention times	90
Figure 4.4	Radioactivity recovered from Kenland and UK2013 plants over time. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. Points with the same letter are not statistically different ($p < 0.05$)	91
Figure 4.5	Uptake of ¹⁴ C from ¹⁴ C 2,4-D by Kenland and UK2013 plants over time. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. Points with the same letter are not statistically different ($p < 0.05$)	92
Figure 4.6	Total radioactivity in the treated leaf of Kenland and UK2013. Points with the same letter are not statistically different ($p < 0.05$)	93
Figure 4.7	Total radioactivity in the untreated shoot of Kenland and UK2013. There is no significant interaction between clover type and harvest	

	interval, but over clover types recovered radioactivity increased with time ($p < 0.05$)	94
Figure 4.8	Total radioactivity in the root section of Kenland and UK2013. There is no interaction between clover types and harvest intervals, but over clover types, recovered radioactivity increased with time, and over harvest intervals Kenland has significantly more radioactivity compared to UK2013 ($p < 0.05$)	95
Figure 4.9	Translocation of ^{14}C from ^{14}C 2,4-D to the untreated shoots across 8 to 72 HAT. Data analysis was performed using the arcsine of the square root transformation in SAS. The values in here are back-transformed. There was no significant interaction between clover type and harvest interval but over clover types, translocation to the untreated shoots increased with time ($p < 0.05$)	96
Figure 4.10	Translocation of ^{14}C from ^{14}C 2,4-D to the roots from 8 to 72 HAT. Data analysis was performed using the arcsine of the square root transformation in SAS. The values in here are back-transformed. There was no significant interaction between clover type and harvest interval, but over clover types there is a trend of increasing translocation over increasing time, and over harvest intervals Kenland has significantly more translocation to the roots than UK2013 ($p < 0.05$)	97
Figure 4.11	Radioactivity extracted from the treated leaf (TL), untreated shoots (US), and roots (R), over clover types and harvest intervals. Bars with the same letter are not significantly different ($p < 0.05$)	98
Figure 4.12	Radioactivity unextracted from Kenland and UK2013 at 8, 24, 48, and 72 HAT, in the treated leaf section. There was no significant interaction between clover type and harvest interval, but over harvest intervals Kenland has a lower percentage of unextracted radioactivity compared to UK2013, and over clover types 8 HAT has a lower percentage of radioactivity unextracted compared to all other harvest intervals ($p < 0.05$)	99
Figure 4.13	Radioactivity extracted from Kenland and UK2013 at 8, 24, 48, and 72 HAT, in the treated leaf section. There was no significant interaction between clover type and harvest interval, and the main effects of clover type and harvest interval are also not significant ($p < 0.05$)	100

Figure 4.14	Radioactivity extracted from Kenland and UK2013 at 8, 24, 48, and 72 HAT, in the treated leaf section. There was no significant interaction between clover type and harvest interval, and the main effects of clover type and harvest interval are also not significant ($p < 0.05$)	101
Figure 4.15	Metabolism of ^{14}C from ^{14}C 2,4-D: 2,4-D remaining in Kenland and UK2013 treated leaf sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. There is no significant interaction between clover type and harvest interval, but over harvest intervals the treated leaf of UK2013 contains less 2,4-D compared to Kenland, and over clover types the treated leaf at 8 HAT has more 2,4-D compared to all other harvest intervals, and the treated leaf at 24 HAT contains more 2,4-D than at 72 HAT ($p < 0.05$)	102
Figure 4.16	Metabolism of ^{14}C from ^{14}C 2,4-D: 2,4-D remaining in Kenland and UK2013 untreated shoot sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. The model for this comparison is not significant ($p < 0.05$)	103
Figure 4.17	Metabolism of ^{14}C from ^{14}C 2,4-D: 2,4-D remaining in Kenland and UK2013 root sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. There is no significant interaction between clover type and harvest interval, but over harvest intervals the roots of UK2013 contain less 2,4-D than the roots of Kenland ($p < 0.05$)	104
Figure 4.18	Metabolism of ^{14}C from ^{14}C 2,4-D: Metabolite 1 in Kenland and UK2013 treated leaf sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. There is no significant interaction between clover type and harvest interval, but over harvest intervals the treated leaf of Kenland contains less M1 than the treated leaf of UK2013, and over clover types the treated leaf at 8 HAT has more 2,4-D compared to all other harvest intervals, and the treated leaf at 24 HAT contains more 2,4-D than at 72 HAT ($p < 0.05$)	105
Figure 4.19	Metabolism of ^{14}C from ^{14}C 2,4-D: Metabolite 1 in Kenland and UK2013 untreated shoot sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The	

	values here are back-transformed. The model for this comparison is not significant ($p < 0.05$)	106
Figure 4.20	Metabolism of ^{14}C from ^{14}C 2,4-D: Metabolite 1 in Kenland and UK2013 root sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. The model for this comparison is not significant ($p < 0.05$)	107
Figure 4.21	Metabolism of ^{14}C from ^{14}C 2,4-D: Metabolite 3 in Kenland and UK2013 treated leaf sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. There is no significant interaction between clover type and harvest interval, but over clover types the treated leaf at 48 HAT contains more M3 than at both 8 and 72 HAT ($p < 0.05$)	108
Figure 4.22	Metabolism of ^{14}C from ^{14}C 2,4-D: Metabolite 3 in Kenland and UK2013 untreated shoot sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. There is no significant interaction between clover type and harvest interval, but over harvest intervals the untreated shoot of Kenland contains less M3 compared to the untreated shoot of UK2013, and over clover types the untreated shoot at 72 HAT contains more M3 compared to all other harvest intervals ($p < 0.05$)	109
Figure 4.23	Metabolism of ^{14}C from ^{14}C 2,4-D: Metabolite 3 in Kenland and UK2013 root sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. The model for this comparison is not significant ($p < 0.05$)	110
Figure 4.24	Metabolism of ^{14}C from ^{14}C 2,4-D: Metabolite 4 in Kenland and UK2013 treated leaf sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. There is no significant interaction between clover type and harvest interval, but over clover types, the treated leaf at 8 HAT contains less M4 compared with at 24 and 72 HAT, and the treated leaf at 72 HAT also contains more M4 compared with at 48 HAT ($p < 0.05$)	111
Figure 4.25	Metabolism of ^{14}C from ^{14}C 2,4-D: Metabolite 4 in Kenland and UK2013 untreated shoot sections. Data analysis was performed	

	using the arcsine of the square root transformation in SAS. The values here are back-transformed. There is no significant interaction between clover type and harvest interval, but over clover types, the untreated shoot at 8 HAT contains less M4 compared with at 24 and 72 HAT, the untreated shoot at 24 and 72 HAT also contain more M4 compared with at 48 HAT ($p < 0.05$)	112
Figure 4.26	Metabolism of ^{14}C from ^{14}C 2,4-D: Metabolite 4 in Kenland and UK2013 root sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. Points with the same letter are not statistically different ($p < 0.05$)	113
Figure 4.27	Metabolism of ^{14}C from ^{14}C 2,4-D: Metabolite 5 in Kenland and UK2013 treated leaf sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. There is no significant interaction between clover type and harvest interval, but over harvest intervals the treated leaf section of Kenland contains less M5 compared to the treated leaf section of UK2013, and over clover types the treated leaf at 8 HAT contains less M5 than at all other harvest intervals ($p < 0.05$)	114
Figure 4.28	Metabolism of ^{14}C from ^{14}C 2,4-D: Metabolite 5 in Kenland and UK2013 untreated shoot sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. The model for this comparison is not significant ($p < 0.05$)	115
Figure 4.29	Metabolism of ^{14}C from ^{14}C 2,4-D: Metabolite 5 in Kenland and UK2013 root sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. The model for this comparison is not significant ($p < 0.05$)	116
Figure 4.30	Metabolism of ^{14}C from ^{14}C 2,4-D: Metabolite 6 in Kenland and UK2013 treated leaf sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. The model for this comparison is not significant ($p < 0.05$)	117
Figure 4.31	Metabolism of ^{14}C from ^{14}C 2,4-D: Metabolite 6 in Kenland and UK2013 untreated shoot sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The	

values here are back-transformed. There is no significant interaction between clover type and harvest interval, but over clover types the untreated shoot section at 72 HAT contains more M6 than at all other harvest intervals ($p < 0.05$) 118

Figure 4.32 Metabolism of ^{14}C from ^{14}C 2,4-D: Metabolite 6 in Kenland and UK2013 root sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. The model for this comparison is not significant ($p < 0.05$) 119

Chapter One

Trifolium pratense (L.) and 2,4-Dichlorophenoxyacetic Acid: A Review

Historical Significance of Red Clover

Early History

Red clover (*Trifolium pratense* L.) is a forage legume that has been heavily utilized throughout history. As a soil fertility-building crop, red clover has been an important asset in animal production, rotational cropping, and nitrogen (N) cycling over the past thousand years (Cuttle et al. 2003; Frame et al. 1998; Isobe et al. 2014). Its beneficial traits include shade tolerance, winter hardiness, quick establishment, and the ability to grow well in areas of low soil pH, low soil fertility, drought, and poor soil drainage (Cuttle et al. 2003; Frame et al. 1998; Isobe et al. 2014). *Trifolium*, the genus to which red clover belongs, originated in the Mediterranean region, most likely in the Early Miocene, 16-23 million years ago (Ellison et al. 2006). Red clover domestication first occurred in southeastern Europe and Asia Minor, from there spreading across Europe to southern Spain by the year 1,000 (Kjaergaard 2003; Taylor 1985). It was quickly recognized for its ability to thrive in less than ideal environments.

Red clover use was recorded in Italy, France, England, and the United States as early as 1550, 1585, 1645, and 1663, respectively (Taylor and Quesenberry 1996). The classical agricultural authorities, including Theophrastus, Philny, and the patron saint of “all who cultivate the natural sciences”, Albert Magnus, recognized that legumes, such as clover, were good for the soil and animal feed, “the best crop for meadowland” and a

solution for N deficiency (Bostock and Riley 1900; Encyclopaedia Britannica 2015; Isobe et al. 2014; Kjaergaard 2003). Because of its effect on soil, clover contributed to the rise of cereal-driven agriculture (Chorley 1981). The agricultural practices of the 13th and 14th centuries generated a serious N deficit, with some scholars pointing to the resulting protracted malnourishment of the population as the cause for the bubonic plague's unparalleled severity in the 14th century (Kjaergaard 2003). Interplanting clovers with cereals, or growing clover and cereals in rotation, creates a more N stable system. The N fixing clover provides as much as two thirds of the total N annually accumulated in its tissues to adjacent plants or future rotational crops in the same field (Chorley 1981). With clover supplied N fueling the growth of cereals, these crops were able to feed the ever expanding European population.

The benefits of clover use in Europe spread beyond rotational crop nutrition. Cattle (*Bos* spp.) thrive particularly well on clovers, and this discovery helped fuel a dramatic increase in European cattle production (Cai et al. 2014; Kjaergaard 2003). This led to an impressive increase in meat and dairy production, supporting further population growth. Kjaergaard (2003) claimed that this, in turn, led to the decline of malaria in northern Europe. The most common carrier of the malaria virus is the mosquito *Anopheles atroparvus*, which prefers cattle over humans as its blood source. With more and more cattle being produced in northern Europe, *A. atroparvus* was able to choose its favored food source over a human food source. This caused less and less humans to be bitten and subsequently become infected by the malaria virus carried by the mosquito. As

cattle are unaffected by malaria, this precipitated a truncation of the virus's life cycle and led to its decline.

Another consequence of the rise of clover in Europe was a transformation of the landscape into a flower garden. This had far reaching implications. Honey production was greatly increased, not just due to the abundant pollen source, but also due to the late season mass flowering of red clover and its positive effect on bee reproduction (Rundlöf et al. 2014; Taylor 2008). Additionally, the vast fields of red clover colored the landscape and inspired the romantic art depicted by poets, musicians, and painters of the time period (Taylor 2008, Kjaergaard 2003). So far reaching are the implications of red clover cultivation, that its influence on civilization likely exceeds that of any other forage plant. Clover even contributed to the wide-spread adoption of potatoes (*Solanum tuberosum* L.), which may have remained an exotic luxury crop rather than the staple food it is today, if not for clover's impact on the N cycle (Chorley 1981; Kjaergaard 2003; Piper 1914; Taylor 1985).

Red clover was widespread throughout northern Europe, the Americas, and Eurasia by the end of the 17th century, and its range was still expanding (Taylor 2008). Red clover was an integral part of crop rotation, soil improvement, and fodder production in almost all temperate agricultural regions in the world by the 1800's (Isobe et al. 2014). Now recognized as one of the most important legumes worldwide and the most widely grown clover overall, red clover fixes more than double the N of other legumes, such as

peas, sainfoins, and lupines (Chorley 1981; Kjaergaard 2003; McKersie and Brown 1997; Piper 1914; Smith et al. 1985).

Modern History

It is no surprise that, over the past century, red clover continued to be an important forage crop in the United States as well as elsewhere in the world (Frame et al. 1998; Smith et al. 1985; Taylor 2008). Red clover is also a common cover crop and the preferred legume for use as a green manure, often frost sown into winter wheat. It is even used as a novelty plant, as a treatment for menopause, and as trap crop for certain pests (Beck et al 2005; Hudson et al. 2011; Frame et al. 1998; Isobe et al. 2014; McKersie and Brown 1997; Robinson et al. 2014). The noted forage crop specialist of the early 1900s, Charles V. Piper, remarked that red clover was “by far the most important leguminous crop grown in America” and that red clover cultivation covered five times the amount of land as alfalfa (*Medicago sativa*) (Piper 1914). Red clover acreage in the United States peaked between 1909-1927 at 12-14 million hectares (Taylor and Quesenberry 1996).

However, as the times have changed, red clover use decreased, reflected in the rapid decline of red clover seed production, starting in the 1950s and stabilizing around 1990 (Isobe et al. 2014; Taylor and Quesenberry 1996). In the decades since 1950, in particular, red clover was transformed from a highly sought after forage species to a secondary crop (Taylor 2008). One reason for this was the decline in need for biologically fixed N. In 1909, Fritz Haber developed his process of synthetic NH_3 production, which was quickly scaled up the following year by Carl Bosch. By the end of

the 1940s, widespread use of the inexpensive ammonia generated via the Haber-Bosch process resulted in lower demand for red clover and other legumes as an N source, thus red clover use declined steadily over the following decades (Frame et al. 1998; Isobe et al. 2014). Synthetic N fertilizer, however, is not without its drawbacks. Wittwer (1979) called the energy dependency of synthetic N “one of the most flagrant violations of good economics”. The recent increased interest in red clover is tied to concerns regarding the ever increasing energy costs and environmental consequences from the use and production of synthetic N fertilizers (Frame et al. 1998; Taylor 2008; Taylor 2011). In countries where synthetic N fertilizer is too costly, the use of forage legumes, like red clover, has increased (Frame et al. 1998).

Another factor contributing to the decline in red clover use was a shift in livestock production practices. Increasing amounts of livestock are raised in concentrated animal feeding operations (CAFOs) and other non-pasture, large scale, intensive systems where their diet is composed of grains like cereals, corn and soybean more so than the traditional pasture crops (Naylor et al. 2005). This apparent “de-linking of livestock from the supporting natural resource base” resulted in less demand for forage crops like red clover (Naylor et al. 2005).

A third factor in the decline of red clover is the use of herbicides in modern agriculture. Starting with the discovery of the first selective herbicides in the 1940’s, an increasing variety of chemical weed control options have become available for agricultural use. This has proved to be highly beneficial for crops which show tolerance

towards these selective herbicides, but for red clover it has meant difficult decisions for land managers who want to cultivate it, as red clover shows little tolerance to herbicides (Ferrell and Sellers 2004; Gianessi and Reigner 2007; Hudson et al. 2011; Robinson et al. 2014). Thus, land managers must give up any advantage they would gain from using these modern chemical solutions to weed control if they are inclined to take advantage of red clover's beneficial traits.

Despite the development of low cost synthetic N fertilizer, shifting livestock production practices, and chemical solutions to weed control, red clover is still grown today on approximately 4 million hectares worldwide, predominantly in the United States and several European countries (Frame et al. 1998; Isobe et al. 2014). Red clover is the most important forage legume in Scandinavia and the second most important forage legume in both the United States and the United Kingdom (Frame et al. 1998; Ohlsson and Wedin 1989; Rhodes and Ortega 1996). In the future, the use of red clover and other forage legumes is widely predicted to increase due to shifting priorities and advances in our understanding of this crop (Frame et al. 1998).

Biology of Red Clover

Red clover is a cool season legume of the Fabaceae family included in the 10% of Fabaceae species which are utilized as forage plants in commercial agriculture (Williams and Nichols 2011). Red clover is a small, herbaceous, short lived perennial, with a deep taproot about 1 meter long. Plants possess dark green trifoliate leaves, which commonly show a delta shaped, light colored leaf mark, and have varying amounts of pubescence on

their leaves and stems (Ball et al. 2002; Isobe et al. 2014; Taylor and Quesenberry 1996). Once the plant reaches maturity, capitate flower heads, composed of around 85 reddish pink zygomorphic florets, form from spring to fall. Each flower contains 2 ovaries and each flower head will produce, on average, 25 seeds, with the optimum time for seed set occurring when each flower is half open (Cope and Taylor 1985; Piper 1914; Taylor and Quesenberry 1996). With stands established in spring or autumn, cuttings or seed harvest can occur up to three times a year (Isobe et al. 2014). Two main types of red clover are used for agriculture: Medium and Mammoth. These types of red clover differ mainly in the time of flowering, density of stems, and persistence, with Medium red clover the primary type grown in the United States (Piper 1941). There is some disconnect between the germplasm of North America and elsewhere in the world in that the North American germplasm persists poorly elsewhere and germplasm from elsewhere persists poorly in North America (Isobe et al. 2014, Piper 1914).

Naturalized in most temperate regions across the world, red clover is adapted to a wide range of soil types, pH levels, and environmental conditions. It can thrive in N deficient conditions due to its ability to form a symbiotic association with the soil bacteria, *Rhizobium leguminosarum* biovar *trifolii* (Smith et al. 1985, Williams and Nichols 2011). The symbiotic relationship allows red clover to utilize the ammonia produced from atmospheric N assimilated by the microbe, increasing its fitness when this growth limiting plant nutrient is in short supply. Nodules which house the bacteria begin to form on the roots of red clover as early as seven days post emergence. The N fixation

which occurs within these nodules provides up to 460 kg N/ha/year to the above ground red clover tissue (Cuttle et al. 2003; Piper 1914).

Agriculturally Significant Traits of Red Clover

Crop Ecosystem Gains

The reason behind red clover's continued prominence is the multitude of benefits it provides to crop ecosystems, livestock, and farmers. As an N fixing legume, it can obtain >95% of its own N requirements from the atmosphere (Cuttle et al. 2003). This has the added benefit of enriching its rhizosphere and surrounding soil with N, which increases the amount of N available to nearby plants. Two thirds of the total N fixed by a legume crop becomes available in the next growing season (Berg et al. 1987; Chorley 1981). Legumes, like red clover, have a high turnover of roots and nodules; when interplanted with grasses, legumes can deliver up to 36% of the N needs of neighboring plants through below ground decomposition (Cuttle et al. 2003; Heichel and Henjum 1991). Just as the grass is benefited by the N output of the red clover, so is the red clover benefited by the competition for soil N by the associated grasses, which stimulates more N fixation via the red clover-rhizobium symbiosis (Chorley 1981; Cuttle et al. 2003; Nyfeler et al. 2011). Interplanting red clover with grasses gives a higher yield per acre than when a grass-only pasture is fertilized with synthetic N (Cuttle et al. 2003). The N provided by red clover is also less susceptible to losses due to leaching and volatilization than synthetic N applications, although N from both these sources is utilized with equal effectiveness by the plant (Chorley 1981). Red clover harvest residues can contain up to

118 kg N ha⁻¹, providing this essential plant nutrient for future plants grown in the same field through residue decomposition and mineralization. These harvest residues contain more than 5000 kg ha⁻¹ (dry mass) of organic matter, outperforming even the more popular alfalfa in enriching the soil for future crops (Bowley et al. 1988).

Red clover benefits the soil ecosystem in other ways as well, increasing soil organic matter, decreasing erosion, improving soil water holding capacity, decreasing soil pH, improving soil microbial diversity, enhancing soil structure, and improving soil porosity (Berg et al. 1987; Biederbeck et al. 2005; Frame et al. 1998; Nyatsanga and Pierre 1973). The same taproot that allows red clover to improve soil porosity and soil structure extends deep into the soil horizons. Essential nutrients are transported from deep in the soil, where they can be utilized by plants with shallow roots (Berg et al. 1987, Chorley 1981). This lessens leaching of these important nutrients and prevents them from leaving the rooting zone (Cuttle et al. 2003). The leading expert on crop nutrition for Great Britain in the mid 1900's, E. M. Crowther, postulated that this ability of red clover “may well be an essential feature of the recovery of soil fertility [in pastures and hay fields]” (Chorley 1981; Cooke and Gething 1980). Overall, red clover is an asset to the ecosystem in which it is grown, increasing nutrient availability to neighboring plants, enhancing the soil, and improving soil quality and N availability for the future. This is why the use of red clover and other legumes has been cited as one of the lessons to be drawn from historic agriculture as we move forward with sustainability in mind (Frossard et al. 2009).

High Quality Animal Feed

In addition to benefiting the crop ecosystem where it is grown, red clover is a highly nutritious forage for livestock, both grazed in pastures and fed as hay, haylage, or silage (Ball et al. 2002; Cai et al. 2014; Isobe et al. 2014). Red clover exceeds grass species in nutritive value, voluntary intake, and animal performance, showing an overall superior feeding value (Frame et al. 1998). When compared to other forage legumes, not only does red clover surpass alfalfa in terms of its environmental range and energy density, dairy cows fed on it also outperform those fed on alfalfa (Ball et al. 2002; Bowren et al. 1969; Broderick et al. 2000; Broderick et al. 2001). Cattle weight gains and reproductive rates also increase when clover is added to the diet (Taylor 2008). Overall, red clover provides excellent nutrition for livestock, even when compared to other forage legumes.

Implications for Farmers

All of the benefits that red clover provides to crop ecosystems where it is grown, and to the livestock that consume it, form a complex of advantages to the farmers that choose to cultivate red clover. Nutrients made available to the red clover, as well as adjacent plants, surrounding soil, and future crops, allow lower fertilizer costs and healthier fields (Chorley 1981). The increased health of the land is also due to the soil quality improvements made by red clover. Finally, farmers benefit from increases in the value of their livestock, which are more productive when their diet includes red clover. This allows farmers to obtain a greater income per animal, thus creating higher returns on

their investments while decreasing their input costs (Broderick et al. 2000; Broderick et al. 2001; Frame et al. 1998).

Management Issues

Unfortunately, red clover also has several drawbacks from a farmer's perspective. For example, when red clover is grown as a monoculture, it can cause a condition called "bloat" in ruminant species that graze it. Interplanting red clover with grass species will prevent this condition (Taylor and Quesenberry 1996; Undersander 1993). Reproductive issues in sheep (*Ovis* spp.) can also occur with diets high in red clover, due to the presence of certain isoflavones, but this can also be managed by planting in a mixture with grass species (Taylor and Quesenberry 1996). Red clover is susceptible to a number of diseases and insect pests (Fame et al. 1998; Smith et al. 1985; Taylor and Quesenberry 1996). Clover infected with black spot (causative agent *Rhisoctonia leguminicola* Gough & Elliot) can cause excessive salivation syndrome, which can be remedied by removing the contaminated hay or planting red clover and grass mixtures (Taylor and Quesenberry 1996).

Red clover, like other forage legumes, can be unpredictable in terms of yield, particularly in the spring, when compared to grass forage species. This is exacerbated by the tendency of farmers to apply N to increase yield before the first grazing, which decreases red clover growth (Sprent and Mannetje 1996). Although red clover persists for 2-3 years, and up to 4 years with certain cultivars, it can be maintained in pasture for longer time periods using pasture renovation techniques (Isobe et al. 2014; Piper 1914;

Smith et al. 1985; Taylor and Anderson 1973). Short persistence is a major limitation of red clover, especially compared to the persistence of alfalfa, the top forage legume in the United States (Ortega et al. 2014; Piper 1914). Alfalfa also outperforms red clover in terms of yield and growth rate (Bowley et al. 1988; Heichel and Henjum 1991).

Weed Control

Land managers have relied on herbicides for easier, cheaper, and more effective weed control for more than half a century. Chemical weed control is used on 90% of the area of most crops in the United States, totaling 87 million hectares (Gianessi and Reigner 2007). Herbicides have helped farmers to reduce crop losses due to weeds, which in the pre-herbicide era could be 50% or greater (Gianessi and Reigner 2007). The adoption of herbicides increases crop yields by 24-167%, depending on the crop and herbicide used (Gianessi and Reigner 2007). The benefits of herbicide use pose an issue for red clover, as it is sensitive to the majority of herbicides available for weed control in pastures, and many that are labeled for use on red clover have problematic limitations (Ferrell and Sellers 2004; Robinson et al. 2014; Undersander et al. 1990). For example, “In grass pastures interseeded with clover or other forage legumes, selective herbicide options are not available for use as broadcast treatments” (Green et al. 2006). As a result, many land managers have phased out red clover in favor of other herbicide tolerant forage crops. Outside of a forage or animal feed situation, when red clover is frost seeded into winter wheat as a cover crop, spring weed control can be problematic, as few herbicide options for winter wheat can be tolerated by red clover (Hudson et al. 2011;

Robinson et al. 2014). Farmers that do choose to grow red clover have been forced to ignore the valuable resource of chemical weed control in order to reap the benefits of this excellent forage legume.

Red Clover Genetics

Red clover is a gametic self-incompatible cross pollinated species and, like other cross breeding species, is susceptible to inbreeding depression. Although there have been several methods proposed to allow for inbred line maintenance, none are considered successful (Duncan et al. 1973; McKersie and Brown 1997; Taylor 2011; Taylor 2008; Taylor and Quesenberry 1996). The two main pollinators for this legume are honeybees (*Apis mellifera* L.) and bumblebees (*Bombus* spp.). Red clover is ordinarily a diploid with chromosome number $2n=14$ and an estimated genome size of 435Mb. Tetraploid varieties ($2n=28$) have been developed which often have superior yield and disease resistance; however, these are not typically cultivated in North America (Isobe et al. 2014; Smith et al. 1985). All red clover cultivars are heterogeneous populations with numerous heterozygous individuals. This creates an excellent source of genetic variability for further breeding efforts but also can lead to genetic shifts during seed production (Piper 1914; Smith et al. 1985; Taylor et al. 1979).

Breeding Red Clover

History

Before 1940, most of the red clover grown was landraces or ecotypes, developed by natural selection or farmer-driven artificial selection rather than concentrated breeding efforts (Fergus and Hollowell 1960). Red clover was one of the first forage plants focused on by plant breeders at the dawn of modern plant breeding, around turn of the 20th century in the United States, Sweden, Denmark, and Switzerland (Isobe et al. 2014; Piper 1914; Taylor 2011). In that period, most breeders focused on developing red clover cultivars with adaptation to a wide environmental range. Mass selection was the method by which most of these early cultivars were developed (Hollowell 1951; Piper 1914).

In the latter half of the 19th century, the focus turned towards breeding red clover for increased adaptability and persistence. Some programs also focused on regrowth potential and winter hardiness (Smith et al. 1985; Taylor 2008). The end goal of all such programs was yield, which in red clover is highly linked to persistence, and which is strongly influenced by disease susceptibility (Isobe et al. 2014). It follows then that pest resistance became a major objective of most breeding programs (Cope and Taylor 1985). The first breeding efforts for disease resistance in red clover were performed at the Tennessee Experimental Station in 1905 by Samuel M. Bain, who was attempting to develop an anthracnose (causal agent *Kabatella caulivora* Kirchn.) resistant red clover (Allen 1919). His, and others, efforts were so successful that southern anthracnose has not been economically important in the United States since the early 1950s. Kenland is a

notable anthracnose resistant cultivar. Kenland was developed at the Kentucky Agricultural Experiment Station in cooperation with the USDA and was released in 1947. Developed through screening for southern anthracnose resistance followed by several generations of recurrent mass selections, Kenland is prized for its regional adaptability and disease resistance and for many years was the top-selling red clover cultivar in the United States (Taylor 2011; Taylor 2008; Taylor and Quesenberry 1996).

Resistance to other microbial diseases has been effectively bred into red clover through numerous breeding programs. Breeding viral disease resistance into red clover was, at one point, considered such an important goal in the southeastern United States that “Regional Project S-228, Forage Legume Viruses: Identification and Genetic Resistance for Improved Productivity” was formed. Resistance to the root knot nematode has also been a goal of red clover breeding and resistant cultivars have been released. Insect pests, however, have not been considered significant enough to warrant breeding resistance to them (Taylor 2008). Breeding for increased persistence also yielded cultivars like the novel Australian cultivar Astred, which has enhanced vegetative reproductive ability and disease resistance, and Milvus, a novel Swiss cultivar which exhibits good regrowth capacity and disease resistance (Rhodes and Ortega 1996).

Breeding Techniques

Most red clover breeding has been carried out by the public sector and, since a focus on improving red clover through intensive breeding began, many improved cultivars have been developed (McKersie and Brown 1997). Currently, the OECD lists

252 cultivars still eligible for seed certification, up from 153 in 1993 (Isobe et al. 2014; Taylor and Quesenberry 1996). Breeding practices for red clover must allow for cross pollination to avoid inbreeding depression thus breeding populations must be large (Taylor and Quesenberry 1996). Mass selection is still the preferred method, with plants selected for their superior traits allowed to either cross pollinate with a set of elite individual plants, as in the polycross method, or to be cross pollinated with the entire population, as in the open pollinated method (Smith et al. 1985). Phenotypic recurrent selection, mass selection carried out as consecutive cycles over several generations, has been very effective, particularly for developing pest resistant varieties. Although less common, backcrossing has received some attention, as it was used to incorporate disease resistance into the ‘Kenstar’ cultivar developed at the University of Kentucky (UK) (Smith et al. 1985; Taylor 2008).

There is significant interest in interspecific hybridization as a means for increasing genetic diversity and as a way to import desirable agronomic traits (Cope and Taylor 1985). Although efforts in the past have proved unsuccessful, there is evidence that, with new methods, there are gains to be made using interspecific hybridization. In particular, *Trifolium medium* and *Trifolium sarosiense* are promising wild relatives, each containing genes which may improve the perenniality of red clover (Williams and Nichols 2011). Hybridization has also received some interest but has been largely ineffective, mostly due to problems with maintaining the parental lines (Smith et al. 1985). To this end, embryo rescue is a method used for overcoming post fertilization barriers preventing hybridization in red clover (McKersie and Brown 1997). Polyploid

breeding is also done to a limited extent but mostly outside the United States. This type of breeding has resulted in tetraploid red clover with increased disease tolerance, yield, and winter hardiness at the expense of low seed yield and dry matter content (Cope and Taylor 1985; McKersie and Brown 1997; Rhodes and Ortega 1996). Finally, there are several genetic linkage and QTL maps which have been developed as molecular breeding tools for red clover, and transgenic red clover has been produced experimentally although no transgenic varieties have been commercially released (Isobe et al. 2014).

History of 2,4-D

Discovery and Early History

Of all the currently available herbicides, 2,4-dichlorophenoxyacetic acid (2,4-D) is one of the most commonly used in pastures and hay fields (Anderson 1996). 2,4-D is also used extensively in wheat and other row crops as well as in turfgrass systems (Robinson et al. 2014; Anderson 1996). Use of 2,4-D in row crops is expected to increase in the wake of Enlist™ crops, genetically engineered to withstand both glyphosate and 2,4-D (Joseph 2014). Between 1940 to 1943, four separate research groups independently discovered the herbicide 2,4-D. It and MCPA (monochlorophenoxyacetic acid) were the first selective herbicides to be developed (Mithila et al. 2011; Troyer 2001). 2,4-D was first marketed in 1945 by the American Chemical Paint Company as a chemical solution for the control of broadleaf weeds in grass systems and soon enjoyed wide-spread use (Anderson 1996; Peterson 1967). As an inexpensive product, 2,4-D serves as a perfect example of how the use of herbicides lowered weed control costs for farmers. 2,4-D in

1945 sold for less than \$3.00 a pound, \$39.12 in today's money, and by 1950 the price had dropped to \$0.50, with prices continuing to decline thereafter (BLS 2015; Mithila et al. 2011; Peterson 1967).

Wartime Use

This herbicide was also used as a weapon in the Vietnam War (1955-1975). Over the course of the war, the United States applied 56 million pounds of 2,4-D, 53% of all the herbicide used during this conflict. 2,4-D was used as a defoliator and was effective on both the mangroves forests and crop-lands of Vietnam as a means to prevent ambush. The most well-known formulation used for this purpose was Agent Orange, a mixture of equal parts 2,4-D and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), which accounted for 10.7 million of the 17.7 million gallons of herbicides using during the war (Munro et al. 1992). It was eventually discovered that the human health effects of this mixture were not due to 2,4-D but, rather, dioxin contamination of 2,4,5-T (Schechter et al. 2001).

Agricultural Use

The use of 2,4-D in cereal crops has been said to have “revolutionized agricultural production throughout the world” and for 70 years it has been one of the most popular and widely used herbicides worldwide (Mithila et al. 2011; Troyer 2001). The 2006-2007 EPA survey of pesticide sales and usage in the United States placed 2,4-D as the 7th most commonly used herbicide in the agricultural market sector, ranking as the single most commonly used herbicide in the other two market sectors – home/garden and

industry/commercial/government (Gube et al. 2011). According to 1995 federal and state figures, despite fewer pounds being sold annually compared to other herbicides, 2,4-D remained the most widespread used herbicide, covering a larger acreage than any other herbicide on the market (Colborn 1999). Today, 2,4-D is the third most commonly used herbicide in the United States and ranks as the number one most commonly used herbicide worldwide, providing excellent control of broadleaf weeds in monocotyledonous crops (Joseph 2014).

Biochemical Action of 2,4-D

An herbicide of the phenoxy family, 2,4-D is called an auxin mimic or synthetic auxin due to its chemical structure and mechanism of action, which is similar to the plant growth hormone auxin. The toxic effects of 2,4-D on a plant manifest in such a way that the plant appears to be “growing itself to death” (Devine et al. 1993; Grossmann 2000; Mithila et al. 2011). Epinasty, stem fasciation, leaf strapping, leaf cupping, and abnormal callus tissue growth are all symptoms of 2,4-D toxicity in plants (Anderson 1996).

The site and mechanism of action of 2,4-D have long been poorly understood. In 1953, six years after it entered the agricultural marketplace, the mechanism of action was hypothesized to be competitive activity with endogenous auxin, which presumably caused 2,4-D to preferentially bind to substrates normally bound to auxin. As the activities of endogenous auxins were themselves poorly understood at the time, it followed that the activity of an auxin mimic was also poorly understood; although it

progressed from the previous hypothesis that 2,4-D was simply stronger than endogenous auxins (Weintraub 1953). By the 1960's, the metabolism of 2,4-D in microbes, known since the late 1940's to be responsible for the degradation of 2,4-D in soil, began to be understood (Audus, 1949; Duxbury et al. 1970). In plants, the black box of auxin molecular action contributed to the limited understanding of the mechanism of action for 2,4-D, but nucleic acid metabolism was thought to be involved (Key et al. 1966). By the 1970's, a considerable body of work had accumulated concerning the metabolism of sublethal doses of 2,4-D in sensitive plants, although the exact mechanism of action was still unknown. At this point, the metabolism of 2,4-D in resistant species attracted significant interest from the scientific community and the conversion of 2,4-D to herbicidally inactive 3-(2,4-DP) was discussed as a possible mechanism of resistance (Hagin et al. 1970).

In the 1990's, Klaus Grossmann and others began to develop a mechanism of action for the synthetic auxin herbicides (Grossmann 2000, 2003, 2010; Grossmann et al. 1996; Grossman et al. 2004). This was made possible by recent discoveries in auxin metabolism, which shed light on a previously unknown family of nuclear AFB proteins involved in the regulation of plant response to auxin (Dharmasiri et al. 2005; Schenck et al. 2010). As a result of this discovery, in 2010, Grossmann proposed the TIR1/AFB auxin receptors as the site of action for synthetic auxin herbicides. Perception by these receptors leads to the targeting of transcriptional regulator proteins for degradation through the ubiquitin-proteasome pathway. The loss of these transcriptional regulator proteins leads to the de-repression of transcriptional activator proteins, called auxin

response factors (ARFs), which activate and subsequently overexpress the auxin-responsive genes, including those involved in ethylene and 1-aminocyclopropane-1-carboxylic acid (ACC) biosynthesis. The result of this overexpression yields the biochemical and physiological events associated with the toxicity of these herbicides; excessive stimulation of ACC paralleled by an increase in ethylene production, which is followed by an overproduction of abscisic acid (ABA), creating the symptomology of synthetic auxins (Grossmann 2010).

2,4-D in the Environment

Non-Target Toxicity

2,4-D is considered low in toxicity for non-target organisms, with minimal toxicity to mammals and practical non-toxicity in bees (Gervais et al. 2008; Williams et al. 2012). Numerous studies have been performed to evaluate the safety of 2,4-D, revealing no toxicity to grazing animals and minimal residues in agricultural products (milk, meat), even when consumed in excess of what would reasonably occur on any farm (Gervais et al. 2008; Klingman et al. 1966; Munro et al. 1992; Peterson 1967). It has also been evaluated for cancer risk in humans and was, at one point, thought to cause non-Hodgkin's lymphoma. However, the comprehensive integrated review and evaluation by Munro et al. in 1992 proved the link between 2,4-D and cancer to be tenuous, at best. Due to 2,4-D's high water solubility, it does not accumulate in tissues, but is instead rapidly excreted which contributes to its low toxicity (Munro et al. 1992). It still has a level of toxicity, and can cause various effects in non-target organisms, from

eye irritation to kidney damage. Neurotoxicity, as well as reproductive and developmental toxicities, have also been shown (Gervais et al. 2008).

Environmental Effects

2,4-D is degraded quickly by microbes in the soil, with a half-life of 1-15 days, depending on the formulation and site of application. (Audus 1949; Walker and Newman 1956; Gervais et al. 2008). This results in a low residual activity for the herbicide, allowing for short pre-harvest intervals and few problems when switching to sensitive crops (Mithila et al. 2011). 2,4-D has been detected in surface and ground water in both rural and urban areas. It has also been detected in finished drinking water, although at concentrations well below the maximum contaminant level set by the EPA. Its half-life in aquatic systems is equal to that in soil. 2,4-D degrades slowly on foliage and leaf litter, detectable for up to 3 years post-application in one study on forest leaf litter (Gervais et al. 2008; Torstensson et al. 1989). Drift and volatilization are also a concern for this herbicide, particularly for the ester formulation. Applications must occur in low-wind situations to avoid off-target movement and improper application can often result in damage to nearby broadleaf plants (Gervais et al. 2008).

Weed Resistance to 2,4-D

In the case of 2,4-D, there is a relatively low incidence of weed resistance. Only 16 species of weeds have evolved resistance to 2,4-D worldwide. This, as compared to glyphosate, with 31 separate weed species showing resistance, is a relatively low number,

particularly when considering 2,4-D has been in use for 70 years (Heap 2015). The number of 2,4-D resistant weed species has not increased since 2009 (Heap 2015). This low frequency of resistance has been attributed to the low residual activity of 2,4-D as well as the complex nature of the mechanism of action, naturally low levels of 2,4-D resistance alleles in wild weed populations, and fitness tradeoffs associated with 2,4-D resistance (Mithila et al. 2011). Management to prevent further development of 2,4-D resistant weeds should still be a priority especially as 2,4-D is set to see increased use with the release of Enlist™ crops. Despite the complexity of the 2,4-D mechanism of action, there are some cases of 2,4-D resistance being conferred by a single dominant allele (Egan et al. 2011). This indicates that 2,4-D resistance has the potential to develop and spread rapidly making the evolution of 2,4-D resistant weeds of particular concern (Egan et al. 2011).

Breeding for 2,4-D Tolerance

In recent years, red clover has joined numerous other crops in being bred for tolerance to herbicides, in particular, the herbicide 2,4-D. Over the years, several crops have been bred for tolerance to this herbicide. The first successful use of recurrent selection to develop herbicide tolerance was reported in 1975 with birdsfoot trefoil (*Lotus corniculatus* L.), with the goal of developing it as a 2,4-D tolerant forage crop (Devine et al. 1975). Genetic engineering has also been employed to develop 2,4-D tolerance in target species; the major success being the Enlist™ soybean (*Glycine max* L.), cotton

(*Gossypium hirsutum* L.), and corn (*Zea mays* L.) varieties (Bayley et al. 1992; Joseph 2014; Lyon et al. 1989; McLean and Charest 2000).

Breeding for 2,4-D Tolerance in Red Clover

There have been three major breeding efforts aimed at improving 2,4-D tolerance in red clover. A southern adapted red clover with enhanced 2,4-D tolerance was developed at the University of Florida (UF) in 1989 using recurrent half-sib selection (Taylor et al. 1989a). The parent material for this line, an equal mixture of the cultivars Kenstar and Nolins, as well as the Florida breeding population QC5, was treated with 2,4-D. Individual plants with the highest 2,4-D tolerance were then selected for intercrossing into half-sib families that were individually evaluated and intercrossed for further selection (Taylor et al. 1989a). Efforts are currently ongoing towards the release of a cultivar derived from this work (Quesenberry et al. 2015; Munoz et al. 2015). The 2,4-D tolerant, southern adapted, red clover developed in Florida fostered two separate efforts, in Wisconsin and Kentucky, to develop a northern adapted red clover with 2,4-D tolerance. In Wisconsin, using seed from the population developed at UF, work was initiated at the USDA-ARS by Heathcliffe Riday resulting in a potentially northern adapted red clover with enhanced 2,4-D tolerance. Dr. Riday's selections relied on an initial polycross, in which 50% of the plants were from the Florida population and the remaining 50% were elite northern adapted red clover germplasm (Riday 2014). In Kentucky, progress towards the development of a northern adapted 2,4-D tolerant red clover began with an initial cross of two populations, followed by polycross recurrent

selection. The initial cross was performed in 2005 when Dr. Norman Taylor crossed the population developed at UF with the northern adapted red clover cultivar Kenland (Taylor 2011; M Barrett, personal communication). Selections are ongoing towards the improvement of this population and are the basis for the work outlined in this thesis.

Chapter Two

Polycross Recurrent and Second Year Polycross Selection for 2,4-D Tolerance in Red Clover

Red clover is a beneficial crop for hay fields and pastures, where farmers can take advantage of the benefits offered by this underutilized forage crop. This legume confers many benefits to the soil ecosystem where it is grown, as well as to adjacent plants, decreasing erosion, decreasing soil pH, improving soil water holding capacity, improving soil porosity, improving soil microbial diversity, enhancing soil structure, and increasing soil organic matter (Berg et al. 1987; Biederbeck et al. 2005; Bowren et al. 1969; Frame et al. 1998; Nyatsanga and Pierre 1973). Nitrogen fixation by rhizobia associated with red clover roots supplies it with >95% of its N requirements while the rapid belowground decomposition of red clover tissues provides up to 36% of the N requirements of nearby plants (Cuttle et al. 2003; Heichel and Henjum 1991). Red clover also has a long taproot which can access mineral nutrients deep in the soil. This reduces the leaching of these nutrients and brings them to shallower soil depths where nearby plants with shallower roots can acquire them (Berg et al. 1987, Chorley 1981). Animals fed clover have higher weight gains and reproductive rates compared to grass or alfalfa fed animals due to the greater nutritive value of this forage and the greater intake rates it triggers in livestock (Frame et al. 1998; Taylor 2008). Due to the decrease in N fertilizer costs and increase in income per animal with red clover, farm profits are increased. Red clover was historically heavily used but that has declined over the past century. This decline is associated with factors such as availability of inorganic N, lessened use of

grazing in animal production, and the development and widespread use of herbicides for pasture weed control (Ferrell and Sellers 2004; Frame et al. 1998; Gianessi and Reigner 2007; Hudson et al. 2011; Isobe et al. 2014; Naylor et al. 2005; Robinson et al. 2014). Pasture weed control is problematic when growing red clover. Many herbicide options are available for broadleaf weed control in pastures; however, red clover is sensitive to these herbicides, precluding their use where it is grown (Green et al. 2006). If red clover tolerated more pasture herbicides, it would expand weed management options in mixed red clover-grass pastures. A candidate for this purpose is 2,4-D, one of the oldest selective herbicides and one of the most commonly used for weed control in pastures and forage fields (Mithila et al. 2011; Troyer 2001).

Genetic variability in 2,4-D tolerance was demonstrated in red clover, suggesting the possibility of increasing 2,4-D tolerance in red clover through breeding (Taylor et al. 1989b). A red clover with increased 2,4-D tolerance and adapted to the southern U.S. was developed at the University of Florida (UF) (Taylor 1989a). However, the Florida red clover is not adapted to the south central clover belt, including Kentucky, Tennessee, North Carolina, Virginia, West Virginia, Missouri, and the southern parts of Indiana, Ohio, and Illinois, which precludes its use in this region (M Barrett, personal communication). Others have used the Florida red clover germplasm to develop a northern adapted red clover with elevated 2,4-D tolerance (Riday 2014). It was used in this study for the same purpose.

The studies in this thesis originated with the work of Dr. Norman Taylor at the University of Kentucky (UK). In 2005, he made a cross between the popular red clover cultivar Kenland, selected because of its excellent agronomic traits and regional adaptation, and the 2,4-D tolerant red clover germplasm developed at UF (M Barrett, personal communication). Recurrent, phenotypic, field based selection was used by Dr. Taylor in the early stages of this work; from 2006 until 2011. We continued this field based polycross recurrent selection. Polycross recurrent selection is the most effective and efficient breeding approach for the clover genus when performed annually and utilizing phenotypic selection methods (Taylor 2011). Recurrent selection was successfully used in selecting for herbicide tolerance in multiple instances (Devine 1975; Taylor 1989a, 1989b). The Florida 2,4-D tolerant red clover was also developed using phenotypic recurrent selection (Taylor 1989a).

The objective of the current research was to continue with, and improve upon, the recurrent, phenotypic, field based polycross recurrent selection of the established breeding population and to quantify the progress made towards developing increased 2,4-D tolerance in the red clover population. The selection methodology previously in use was an effective means of developing 2,4-D tolerance in the red clover population but additional steps were required before considering this breeding population ready for release as a novel, 2,4-D tolerant, northern adapted cultivar of red clover. We considered that, in order for the tolerance level to be useful for farmers, a 2,4-D rate double the recommended use rate of 1.12 kg/ha should cause minimal damage to the red clover. This would provide a two-fold safety factor, insurance against application problems such as

spray overlap. This level of tolerance will be very useful to farmers who wish to add red clover to their forage or pasture fields while still being able to use 2,4-D for weed management.

Materials and Methods

Field Based Polycross Recurrent Selection

Breeding for a northern adapted 2,4-D tolerant red clover was started in 2005 by Dr. Norman Taylor at UK with an initial cross between the 2,4-D sensitive red clover cultivar Kenland (Victory Seed Company, PO box 192-Molalla, Oregon 97038) and the 2,4-D tolerant red clover germplasm developed at UF (Florida) (seed provided by Dr. Ken Quesenberry, University of Florida)(Taylor et al. 1989a). To allow for the selection of hybrids between these two lines, the parents used in this cross were of two phenotypes; Florida plants that displayed a V-shaped, light colored, water mark on each leaflet of their trifoliolate leaves and Kenland plants that lacked this mark. After crossing, F₁ offspring were grown from seed collected from the Kenland plants and selected based on the presence of the leaf mark indicating plants resulting from a Florida-Kenland cross. The selected plants were allowed to cross-pollinate and the population resulting from this selection was recurrently selected from 2006-2011 in the field by Dr. Taylor, using 0.56 kg ha⁻¹ of 2,4-D application as selection pressure, and harvesting seed from those plants which survived this application.

Seed harvested from the 6th cycle of selection, conducted in 2011, were mechanically scarified, treated with the fungicide thiram (dimethylcarbamothioylsulfanyl

N,N-dimethylcarbamodithioate) (Arasan Fungicide E.I. du Pont de Nemours and Company, 1007 Market Street Wilmington, DE 19898), and planted in Styrofoam float trays in a mixture of 2:1 of PRO-MIX® (Premier Tech Horticulture, 127 South Fifth Street, #300 Quakertown, PA18951 USA) and Maury silt loam soil (fine, mixed, mesic typic Paleudalfs) in a glass greenhouse in January of 2012. In early spring of 2012, the plants were trimmed to 18 cm and transplanted into a 0.40 ha field on UK's Spindletop research farm in central Kentucky, using a mechanical transplanter (Rain-Flo Irrigation, LLC 929 Reading Rd East Earl, Pennsylvania 17519, Model 1600). The field soil was a Maury silt loam with 2.6% organic matter and a pH of 6.5-7. No supplemental fertility was applied to the field. The location was scouted prior to planting to ensure the absence of existing red clover plants in order to limit the risk of outcrossing with naturalized populations. Weeds were controlled with a combination of hand hoeing and an application of 47 ml ai ha⁻¹ imazethapyr (Pursuit® Herbicide, BASF Corporation 26 Davis Drive Research Triangle Park, NC 27709) and 11.7 ml ai ha⁻¹ clethodim (Select® 2EC Herbicide Valent U.S.A. Corporation P.O. Box 8025 Walnut Creek, CA 94946) with 1% v/v crop oil concentrate (Maximizer, Loveland Products, 3005 Rocky Mountain Ave. Loveland, CO 80538) applied in early May of 2012, as well as 11.7 ml ai ha⁻¹ of clethodim with 1% v/v crop oil concentrate applied in late June of 2012. In late May of 2012, 1.12 kg ai ha⁻¹ of 2,4-D amine (Weedar 64® Herbicide Nufarm Americas Inc., 11901 South Austin Avenue Alsip, IL. 60803) was applied using an all-terrain-vehicle sprayer. Red clover plants with epinasty, twisting, or leaf-cupping symptoms of 2,4-D injury were manually removed from the field two weeks after treatment (WAT).

Plants infected with powdery mildew were also removed at this time. In mid-August of 2012, 2.34 L ha⁻¹ of paraquat (Gramoxone Inteon® Herbicide, Syngenta Crop Protection, LLC, Schwarzwaldallee 215, 4002 Basel, Switzerland) was applied as a pre-harvest drying agent. Red clover seed was harvested 24 hours after paraquat treatment (HAT) using a Hege Model 140 seed combine (Wintersteiger Ag, 4910 Ried, Austria, Dimmelstrasse 9). Harvested seed was dried in cloth bags in a crop dryer with no heat used and the dried seed was cleaned using a Clipper seed cleaner (A.T. Ferrell Company Inc. 1440 South Adams Street, Bluffton, IN 46714). Cleaned seed was stored at -20C until used in greenhouse studies.

This selection process was repeated in 2013 and 2014. Weeds were controlled in 2013 through a combination of hand hoeing and chemical control using 47 ml ai ha⁻¹ imazethapyr (Pursuit® Herbicide, BASF Corporation 26 Davis Drive Research Triangle Park, NC 27709), 5.9 ml ai ha⁻¹ clethodim, and 0.4 L ai ha⁻¹ bentazon (Basagran® Herbicide Arysta Life Science North America, LLC 15401 Weston Parkway Suite 150 Cary NC 27513) with 1% v/v crop oil concentrate applied in late May of 2013. A second treatment of bentazon (0.4 L ai ha⁻¹) plus 1% v/v crop oil concentrate was applied in mid-August of 2013. In 2014, weed control was maintained through a combination of hand hoeing and chemical control using of 0.4 L ai ha⁻¹ bentazon, 47 ml ai ha⁻¹ imazethapyr, and 5.9 ml ai ha⁻¹ clethodim with 1% v/v crop oil concentrate applied in early June. The 2,4-D (Formula 40® Herbicide Nufarm Americas Inc., 11901 South Austin Avenue Alsip, IL. 60803) rate was increased to 2.24 kg ai ha⁻¹ in 2013 to increase selection pressure. The 2,4-D was applied in late June. The same 2,4-D rate was used in

2014 and was also applied in late June. Plants with 2,4-D or with powdery mildew symptoms were removed from the field 2 WAT. Paraquat (2.34 L ai ha⁻¹) was used as a desiccant in late August of 2013 and mid-November of 2014 with seed harvested soon thereafter. Seed harvest occurred later in 2014 than in previous years to allow for maximum regrowth following damage from a late summer hailstorm. The harvested seed was dried, cleaned, and stored at -20C until used in greenhouse studies.

Field Based Second Year Polycross Selection

In 2013, a second year polycross plot was established using red clover plants overwintered from the 7th cycle of polycross recurrent selection, conducted in 2012. In late April of 2013, 2.24 kg ai ha⁻¹ of 2,4-D amine (Weedar 64® Herbicide Nufarm Americas Inc, 11901 South Austin Avenue Alsip, IL. 60803) was applied using a hand held CO₂ pressurized sprayer. Red clover plants completely free of 2,4-D and powdery mildew symptoms were flagged 2 WAT. Seventy flagged plants were transplanted into a new area in late May of 2013. This area was approximately 1.6 miles away from the polycross recurrent selection field. Irrigation was provided as needed for 2 weeks following transplantation and weed control was maintained by hand hoeing. The plants in the second year polycross plot were allowed to cross pollinate and seed was manually harvested in August and again in September of 2013. Harvested seed was dried in cloth bags in a crop dryer, cleaned by hand, and stored at -20C until used in greenhouse studies.

In 2014, the plot was mowed to a height of 12 cm in late June, when the plants were in the early bloom stage, and 2,4-D (2.24 kg ai ha⁻¹) was applied in early July. Seed harvest was performed by hand in late October to allow for maximum regrowth following damage from a late summer hailstorm. Harvested seed was dried in a crop dryer, cleaned by hand, and stored at -20C until used in greenhouse studies.

Greenhouse Evaluation of 2,4-D Tolerance

Three separate evaluations of the progress made towards the development of a 2,4-D tolerant red clover were performed. The first evaluation was of the progress made during the 5th and 6th cycles of field based polycross recurrent selection, performed in 2010 and 2011, respectively. Plants of the red clover cultivar Kenland (Kenland), the Florida 2,4-D tolerant germplasm (Florida), and plants grown from seed collected after the 5th and 6th cycle of field based polycross recurrent selections (UK2010 and UK2011, respectively) were established and grown to the 5-8 leaf stage in the following manner. Approximately 100 seeds of each were scarified with 011K Crystal Bay medium emery cloth (3M, 3M Center St. Paul, MN 55144) and coated with thiram before being placed on 9.0 cm Whatman filter paper circles (Fisher Scientific, 300 Industry Drive Pittsburgh, PA 15275) in a Petri plate (Fisher Scientific, 300 Industry Drive Pittsburgh, PA 15275) containing 8ml of distilled water. Three Petri plates were used for each seed source. The Petri plates were sealed with Parafilm® (Fisher Scientific, 300 Industry Drive Pittsburgh, PA 15275) prior to their transfer into a growth room. The growth room had a 24 h photoperiod provided by fluorescent bulbs (0.56 $\mu\text{mol m}^{-2} \text{sec}^{-1}$) and it was maintained at

24C. Just after cotyledon emergence, after about one week in the growth room, seedlings were transplanted and grown individually in Cone-tainers™ (Stuewe & Sons, Inc. 31933 Rolland Drive Tangent, Oregon 97389 USA) containing a 3:1 mixture of PRO-MIX® and Maury silt loam. After transplanting, the plants were placed in a greenhouse. Moisture was maintained by subirrigation. The temperature in the greenhouse ranged from 21-30 C and a 16 h photoperiod was maintained with supplemental lighting when necessary. Once the 5-8 leaf stage was reached, a set of homogeneous, healthy plants from each clover type was selected (Figures 2.1 and 2.2). The plants were arranged in a split plot design, with 2,4-D application rate as the main plot and red clover type as the subplot. There were five replications (individual plants) per treatment and the entire experiment was repeated seven times. The treatments were 0, 0.56, 1.12, 1.68, and 2.24 kg/ha⁻¹ 2,4-D amine (Weedar 64® Herbicide Nufarm Americas Inc., 11901 South Austin Avenue Alsip, IL. 60803). All 2,4-D treatments were applied in a CO² pressurized spray chamber with a carrier rate volume of 280 L/ha-1 at 207 kPa spray pressure (Figure 2.1). The 2,4-D treated plants and the untreated control plants were harvested 2 WAT. At harvest, 2,4-D injury was rated on a scale from zero to ten, with zero indicating a plant with no 2,4-D injury and ten being a dead plant. Fresh and dry weights were determined for harvested plants.

The same experimental procedure was used to evaluate the 2,4-D tolerance of plants grown from seed from the 7th cycle of field based polycross recurrent selection, performed in 2012 (UK2012), except UK2010 and Florida were not included. This

experiment was repeated three times, and experiments were pooled for statistical analysis.

The same experimental procedure was also used to evaluate the 2,4-D tolerance in plants grown from seed from the 8th cycle of field based polycross recurrent selection, performed in 2013 (UK2013), and plants grown from seed from the field based second year polycross selection (UK2013-P). Tolerance of these plants was compared to Kenland and UK2012. This experiment was repeated five times, and experiments were pooled for statistical analysis.

Data from all the studies was subjected to an analysis of variance using ProcGLM in SAS® statistical software. Mean separation was performed using LSmeans at $\alpha=0.05$.

Results

Evaluation of 2,4-D Tolerance Resulting from the 2011 Polycross Recurrent Selection

There was a significant interaction between clover type and herbicide rate for the visual injury data from the evaluation of the 2011 polycross recurrent selection (Table 2.1). Kenland was less tolerant to 2,4-D compared to all the other clover varieties, Florida, UK2010, and UK2011, at all 2,4-D rates (Figure 2.3). This confirms the lack of 2,4-D tolerance in Kenland compared to the populations developed at UK and UF. The clover other than Kenland were still injured by 2,4-D but much less than Kenland. There was little difference in 2,4-D injury between Florida, UK2010, and UK2011, except at 1.68 kg ai ha⁻¹ 2,4-D, where UK2011 was injured more than Florida (Figure 2.3). There

were no significant interactions between clover and 2,4-D rate for the fresh and dry weight. However, overall, the fresh weights of Kenland plants were less than those of the other three clover varieties. Conversely, there was no overall difference in dry weights between the clovers. Both fresh and dry weights were decreased in a rate dependent manner for all clover varieties (Table 2.1). Visual injury was a better measure of 2,4-D tolerance differences between the varieties than weights (Figures 2.3, 2.4, and 2.5).

Evaluation of 2,4-D Tolerance Resulting from the 2012 Polycross Recurrent Selection

There was a significant interaction between clover type and herbicide rate for the visual injury data from the evaluation of the 2012 polycross recurrent selection (Table 2.1). Kenland was less 2,4-D tolerant than all the other clovers (UK2011, and UK2012) at all 2,4-D rates (Figure 2.6).

While UK2011 and UK2012 are more 2,4-D tolerant than Kenland, UK2012 was only injured less by 2,4-D than UK2011 at 1.12 kg ai ha⁻¹ (Figure 2.6). In our initial evaluation of progress towards a 2,4-D tolerant red clover, UK2011 had similar tolerance to that of Florida (Figure 2.3). Thus, while the difference is small, the difference in injury at 1.12 kg/ha 2,4-D suggests some improvement in tolerance between 2011 and 2012 and from Florida. As for the evaluation of the 2011 polycross recurrent selection, there were no interactions between 2,4-D rate and clover type for fresh or dry weight data, although there were differences for both dry and fresh weight between clovers; the overall Kenland fresh weights are significantly lower than the other clovers and dry weights are lower

than UK2012, and both fresh and dry weights decrease in a 2,4-D rate dependent manner for all clover types (Table 2.1, Figures 2.7 and 2.8).

Evaluation of 2,4-D Tolerance Resulting from the 2013 Polycross Recurrent and Second Year Polycross Selections

There was a significant interaction between 2,4-D rate and clover type for the visual injury data (Table 2.1). Kenland once again had the lowest tolerance to 2,4-D this time in comparison to UK2012, UK2013, and UK2013-P (from the second year polycross plot) at all 2,4-D rates (Figure 2.9). The 2,4-D tolerance of UK2013 was greater than that of UK2012 at the two highest rates of 2,4-D, 1.68 and 2.24 kg ai ha⁻¹. Also, UK2013-P was injured less than UK2012 at 2.24 kg ai ha⁻¹ 2,4-D. However, this difference was not seen at 1.68 kg ai ha⁻¹ 2,4-D. As in the other studies, there were no interactions between clover type and 2,4-D rate for fresh and dry weights, but there are differences, in both fresh and dry weights, between clovers across 2,4-D rates, as Kenland and UK2013-P had lower fresh and dry weights compared to UK2012 and UK2013, across all clovers plant fresh and dry weights were greater at 0 kg ai ha⁻¹ 2,4-D compared to all other rates (Table 2.1, Figures 2.10 and 2.11).

Discussion

Since the initial cross for this breeding population, polycross recurrent selections were conducted from 2006 to 2011, using 0.56 kg ha⁻¹ 2,4-D as selection pressure, and allowing all surviving plants to contribute to the genetics of the progeny. The initial

cross and subsequent selection cycles were effective in introducing the 2,4-D tolerance trait from the Florida germplasm into the Kenland cultivar. However, we felt that changes in the selection process were required in order to increase the level of 2,4-D tolerance above that of the Florida parent. Two changes were instituted. The first change, began in 2012, was the removal of any red clover plants from the breeding population that showed 2,4-D injury. The objective was to prevent any of the injured plants from crossing with uninjured plants in the polycross recurrent selection field and contributing to the genetics of the progeny from that year. We did not see improvement in 2,4-D tolerance between 2010 and 2011 (Figure 2.3) and this might be because 2,4-D injured plants were not removed. In fact, UK2011 was more injured than UK2010 at 1.68 kg/ha 2,4-D. After the removal of 2,4-D affected plants, which was initiated in 2012, 2,4-D tolerance was increased in 2012 and 2013 (Figures 2.6 and 2.9).

The second change to the breeding methodology was increasing the rate of 2,4-D applied to the breeding population. Prior to 2012, 0.56 kg ha⁻¹ was the rate of 2,4-D applied. This was increased to 1.12 kg ha⁻¹ in 2012, and to 2.24 kg ha⁻¹ in 2013, which was maintained in 2014. The increase in tolerance observed from the 2012 and 2013 selection cycles may be partially due to this change, in addition to removing 2,4-D affected plants.

The third change to the breeding methodology was the establishment of a second year polycross plot utilizing 70 uninjured individual red clover plants from the overwintered 2012 polycross recurrent selection field that was treated with 2.24 kg/ha

2,4-D in the spring. Although these plants showed high levels of 2,4-D tolerance when selected, the second year polycross plot did not yield more improvement in 2,4-D tolerance compared to the polycross recurrent selection field. The failure to improve more quickly on 2,4-D tolerance using the polycross plot method highlights the need for a very large population when breeding red clover. Had a larger population been utilized in the polycross plot, increases in 2,4-D tolerance could have been more substantial. For alfalfa, a minimum population size of 75 is recommended to prevent inbreeding depression, a condition which can be severe in red clover (Bowley 1997; Taylor and Quesenberry 1996). Increases in 2,4-D tolerance from the second year polycross plot could be greater over time, if the seed from this plot is planted and selected upon recurrently. However, after one year, the gains in 2,4-D tolerance from the second year polycross method were insufficient to support this more labor-intensive selection method as being superior to the polycross recurrent selection method.

Future work towards the goal of developing a 2,4-D tolerant red clover cultivar should continue with the polycross recurrent selection method, maintaining the current method of 2,4-D sensitive plant removal. Increasing the 2,4-D rate, perhaps to 4.48 kg ha⁻¹, could accelerate improvement in 2,4-D tolerance. Additionally, a larger second year polycross plot could yield faster gains in tolerance. The initial polycross plot used in the development of a northern adapted 2,4-D tolerant red clover in Wisconsin contained 192 individuals, which is more than double the number of plants used in the second year polycross plot in our study (Riday 2014).

Based on visual injury ratings, the progress made towards developing a 2,4-D tolerant population of red clover has been substantial. Improvements from the initial 2,4-D tolerant parent have been made at several rates of application, and all populations evaluated showed significant gains over Kenland. Our observations are that the level of tolerance observed in the field is even higher than measured in the greenhouse studies (data not shown). In the field, recovery in the UK red clover is observed from any 2,4-D injury. The plants in the selection field are grown as individual plants, in order to observe them as individuals so that selections can be made. When grown interspersed, as they would after broadcast seeding into a pasture, almost all plants survived and even thrived after an application of 2,4-D (observed after application of 2.24 kg/ha 2,4-D to the overwintered 2012 polycross recurrent selection field). This was likely due to the overlap of the individual plant crowns providing some degree of protection to each other, and supports the observation of red clover breeders that “The performance in plant spaced nurseries *will* be different from the broadcast or drilled plantings that farmers use” (Taylor and Quesenberry 1996). It may also mean that more mature plants are generally more tolerant. In the future, field evaluations of tolerance should be done in order to quantify this observation, which may indicate that the level of tolerance currently obtained is more than sufficient to withstand 2.24 kg ha⁻¹ of 2,4-D in a field setting. It should also be noted that the plants tested for tolerance in greenhouse were much younger than those treated in the polycross recurrent selection and second year polycross plots. It is possible that a more mature plant can withstand 2,4-D treatment more than a younger one. However, the tolerance observed in UK2013 is still impressive.

The weight data, both fresh and dry, did not correlate with observed differences in 2,4-D tolerance, measured as visual injury, and were, thus, not useful measures of red clover response to 2,4-D. The absence of consistent differences in fresh and dry weights following 2,4-D treatment between Kenland, the 2,4-D sensitive parent of the initial cross, and Florida, the 2,4-D tolerant parent of the initial cross, illustrates this inadequacy. One reason for this may be the tendency in plants treated with 2,4-D to have excessive plant growth prior to the death of sensitive plants. Plants which exhibit the twisting, cupping, thickening, epinasty, and browning symptoms typical of 2,4-D injury often weigh more than plants which are unaffected by the herbicide application, although a visual assessment can easily determine which plant is being damaged by 2,4-D exposure (Anderson 1996; Cedergreen 2007; Di Meo 2012; Mithila et al. 2011) .

Another reason that plant mass does not correlate well with 2,4-D tolerance is related to how selections are made. Red clover breeding populations are maintained as a heterogeneous population with many heterozygotic individuals in order to prevent inbreeding depression (Smith et al. 1985; Taylor et al. 1979; Taylor and Quesenberry 1996). Additionally, when making selections in the field for plants with no 2,4-D injury symptoms, there has been no distinction made between 2,4-D tolerant plants which were small and those that had grown significantly after, and perhaps in response to, the 2,4-D application. The lack of selection for this particular trait, and the heterogeneous, heterozygotic nature of the breeding population and individuals therein, allows for significant variability in plant characteristics, including weight gain, in response to 2,4-D

exposure. The variability of this response precludes the use of either fresh or dry weight as an adequate measure of a red clover plant's response to 2,4-D.

Knowing that individual plant weights was not indicative of red clover response to 2,4-D in this breeding study, other metrics must be utilized which accurately measure 2,4-D tolerance. Visual injury ratings, discussed above and used in the initial breeding work for the Florida variety, are effective, but can vary significantly depending on the researcher performing them (Taylor et al. 1989). For subjective metrics, such as visual injury ratings, overall trends may be compared between different researchers, but exact values are more difficult to draw comparisons from. This highlights the need for another system for measuring plant response to 2,4-D. Plant weight, while not an acceptable metric at the individual level, may illustrate 2,4-D response more clearly at the population level. Top growth harvest measurements, a type of plant population weight measurement, have been used to effectively demonstrate differing levels of 2,4-D tolerance in *L. corniculatus*, and may be applicable to this response in red clover as well (Devine et al. 1975).

Other researchers breeding for 2,4-D tolerance utilized a percent survival metric to measure the 2,4-D response of a population (Devine et al. 1975; Riday 2014). Percent survival is not appropriate for further progress evaluations of this work, but could be utilized if a substantially larger population size were used in conjunction with much higher rates of 2,4-D application. The red clover population utilized by Riday (2014) contained 3,418 individual plants across 3 rates of 2,4-D ($\frac{1}{8}$, $\frac{1}{4}$, and $\frac{1}{2}$ the recommended

use rate) in the fifth round of selection; this is much larger than the population size employed during our progress evaluations. Unlike Riday's research, the population used for the progress evaluations discussed here does not contribute to the breeding population of the following year, and thus does not need to be large enough to prevent inbreeding depression. However, in order to provide enough individuals to get an accurate representation of survival percentages, the population sample size would need to be increased substantially. Also, the survival percentages for the progress evaluations here would be quite high, with not many plants, outside of the Kenland cultivar, being killed by 2,4-D at the time of harvest. Rather, damage was assessed as mild from most 2,4-D rates. If higher rates of 2,4-D were applied, such that the survival decreased significantly, survival could be a useful metric to employ alongside visual injury ratings.

As further information on the 2,4-D mechanism of action and well as the basis for 2,4-D tolerance in red clover becomes available, it may be possible to identify new metrics for use in developing 2,4-D tolerant crops, including red clover. The addition of such metrics to breeding programs that are selecting for 2,4-D tolerance could increase a breeder's ability to identify genotypes to include in the process and, hopefully, accelerate progress towards their objective.

Table 2.1. P-values from the greenhouse studies. ^A

Greenhouse Study	Data Type	Source	P-Value
Kenland x Florida x UK2010 x UK2011	Visual Injury	Clover	<0.0001
		Herbicide	<0.0001
		Herbicide*Clover	0.0008
Kenland x Florida x UK2010 x UK2011	Fresh Weights	Clover	<0.0001
		Herbicide	<0.0001
		Herbicide*Clover	0.1535
Kenland x Florida x UK2010 x UK2011	Dry Weights	Clover	0.0775
		Herbicide	<0.0001
		Herbicide*Clover	0.1584
Kenland x UK2011 x UK2012	Visual Injury	Clover	<0.0001
		Herbicide	0.0002
		Herbicide*Clover	0.0254
Kenland x UK2011 x UK2012	Fresh Weights	Clover	0.0127
		Herbicide	0.0002
		Herbicide*Clover	0.5239
Kenland x UK2011 x UK2012	Dry Weights	Clover	0.0278
		Herbicide	<0.0001
		Herbicide*Clover	0.1078
Kenland x UK2012 x UK2013 x UK2013-P	Visual Injury	Clover	<0.0001
		Herbicide	<0.0001
		Herbicide*Clover	<0.0001
Kenland x UK2012 x UK2013 x UK2013-P	Fresh Weights	Clover	<0.0001
		Herbicide	0.0014
		Herbicide*Clover	0.6393
Kenland x UK2012 x UK2013 x UK2013-P	Dry Weights	Clover	<0.0001
		Herbicide	0.0009
		Herbicide*Clover	0.8703

^A All data analyzed via Analysis of Variance.



Figure 2.1. Depiction of herbicide application equipment and representative red clover plants at time of 2,4-D application.

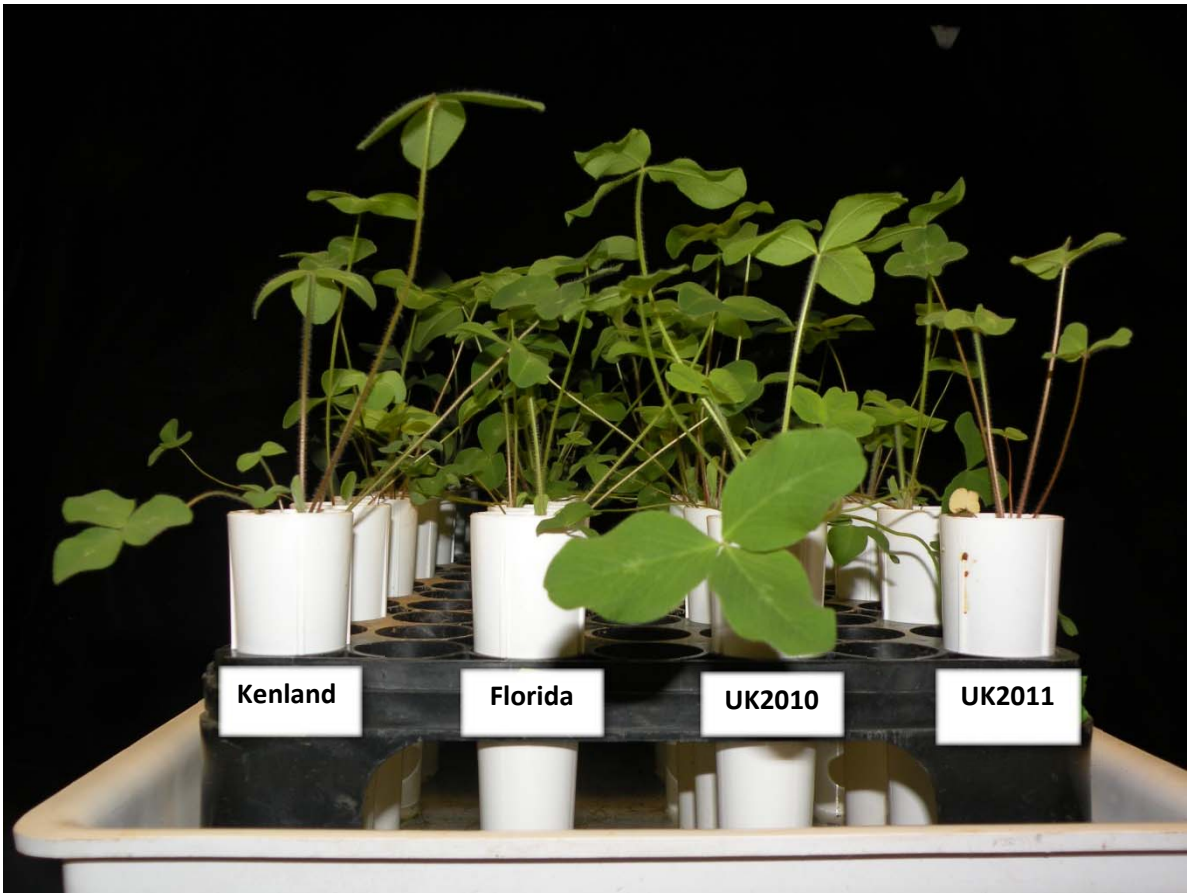


Figure 2.2. Representative red clover plants at time of 2,4-D application.

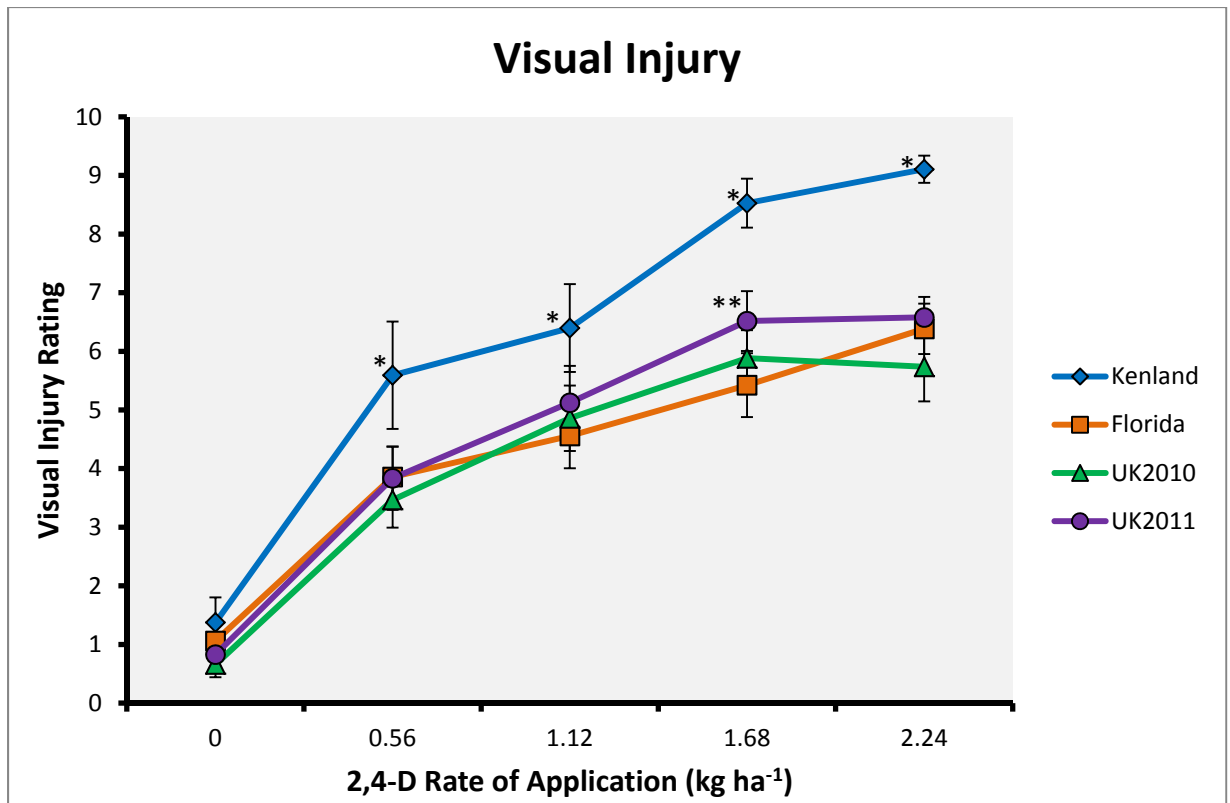


Figure 2.3. Red clover injury two weeks after 2,4-D application to Kenland, Florida 2,4-D tolerant clover (Florida), and plants grown from seed harvested from the 2010 and 2011 polycross recurrent selections (UK2010 and UK2011, respectively). Visual injury rating on a scale from 0= no damage to 10= dead. * denotes significant difference between Kenland and all other clover varieties, and ** denotes significant difference between UK2011 and Florida ($p < 0.05$).

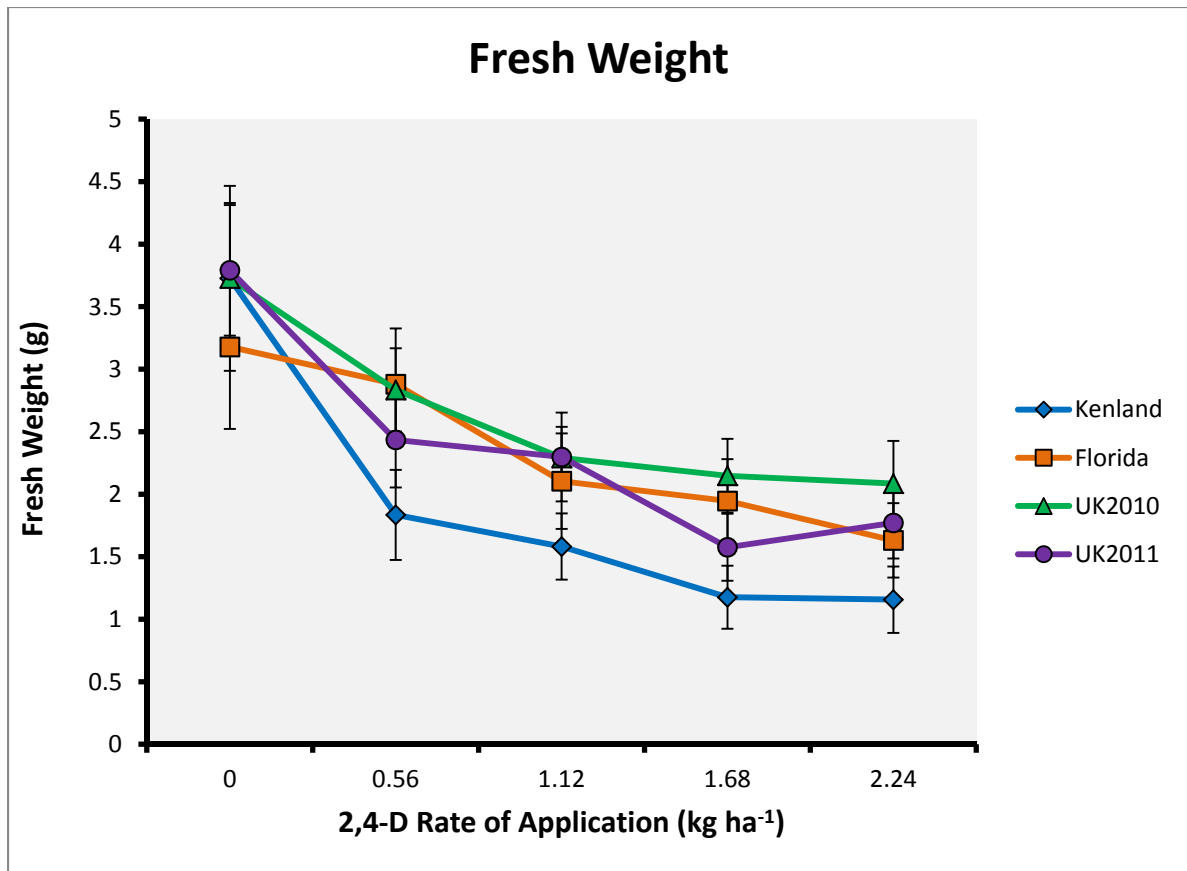


Figure 2.4. Red clover fresh weights two weeks after 2,4-D application to Kenland, Florida 2,4-D tolerant clover (Florida), and plants grown from seed harvested from the 2010 and 2011 polycross recurrent selections (UK2010 and UK2011, respectively). There was no significant interaction between 2,4-D rate and clover type. Over clover types, a trend of decreasing fresh weight with increasing 2,4-D rate occurs, with 0 kg ha⁻¹ heavier than all other 2,4-D rates. Over 2,4-D rates, Kenland is lighter than all other clover types, while Florida, UK2010, and UK2011 are all similar in weight to each other ($p < 0.05$).

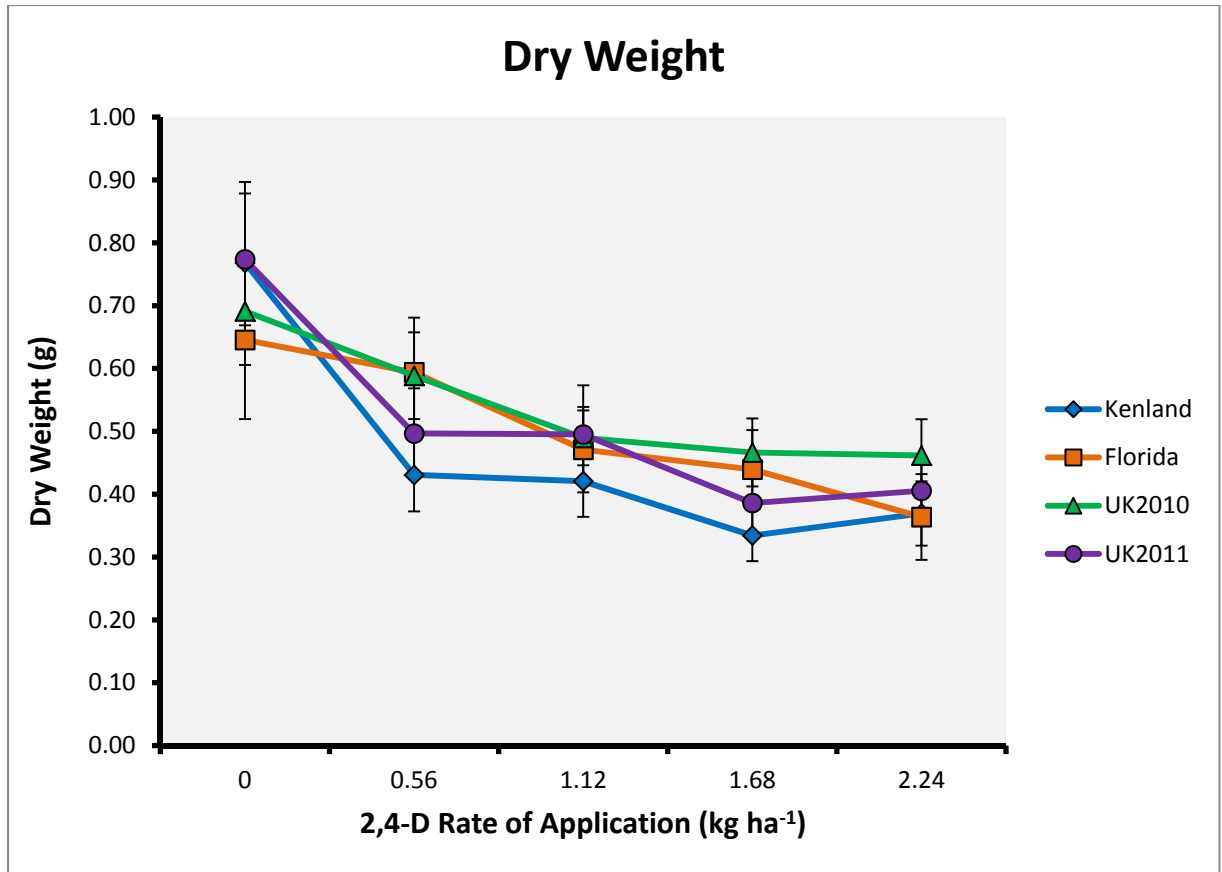


Figure 2.5. Red clover dry weights two weeks after 2,4-D application to Kenland, Florida 2,4-D tolerant clover (Florida), and plants grown from seed harvested from the 2010 and 2011 polycross recurrent selections (UK2010 and UK2011, respectively). There is no significant interaction between 2,4-D rate and clover type, but over clover types, a trend of decreasing dry weights over increasing 2,4-D rate occurs ($p < 0.05$).

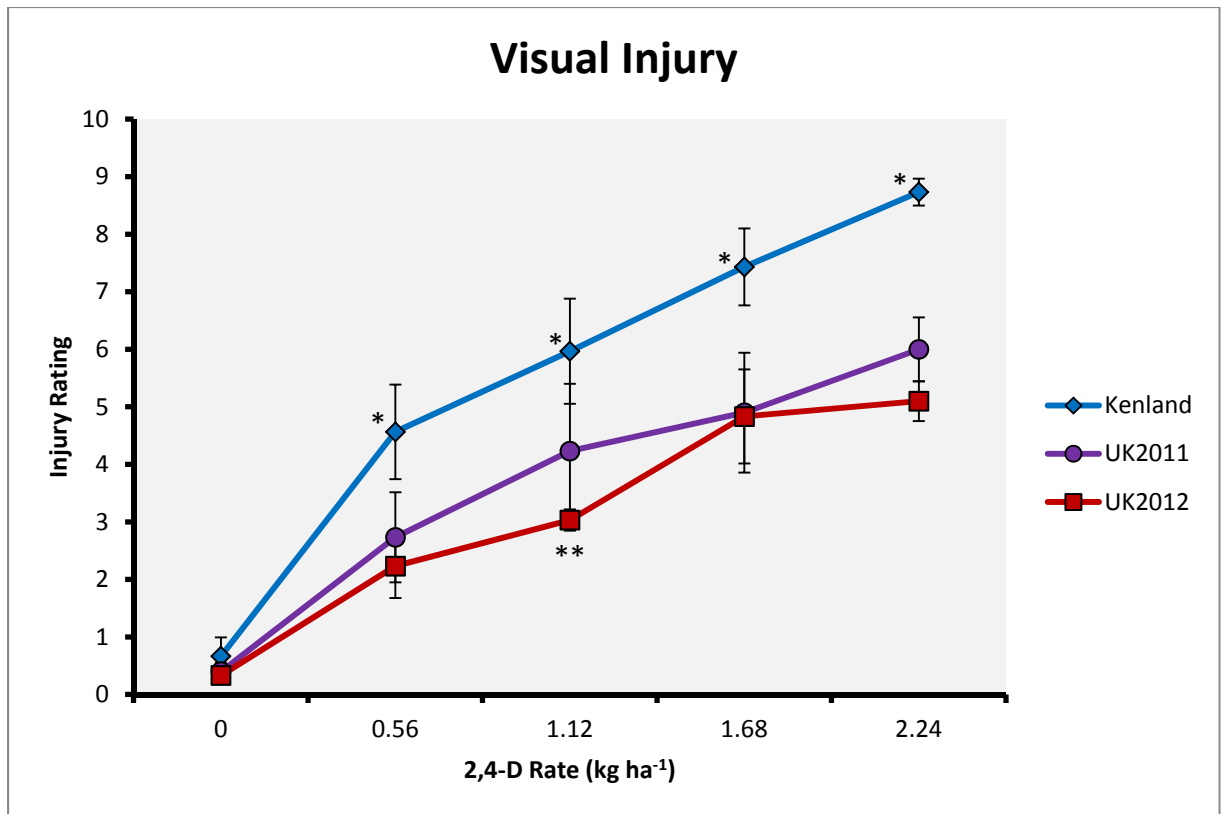


Figure 2.6. Red clover injury two weeks after 2,4-D application to Kenland and plants grown from seed harvested from the 2011 and 2012 polycross recurrent selections (UK2011 and UK2012, respectively). Injury rating on a scale from 0= no damage to 10= dead. * denotes significant difference between Kenland and all other clover varieties, and ** denotes significant difference between UK2011 and UK2012 ($p < 0.05$).

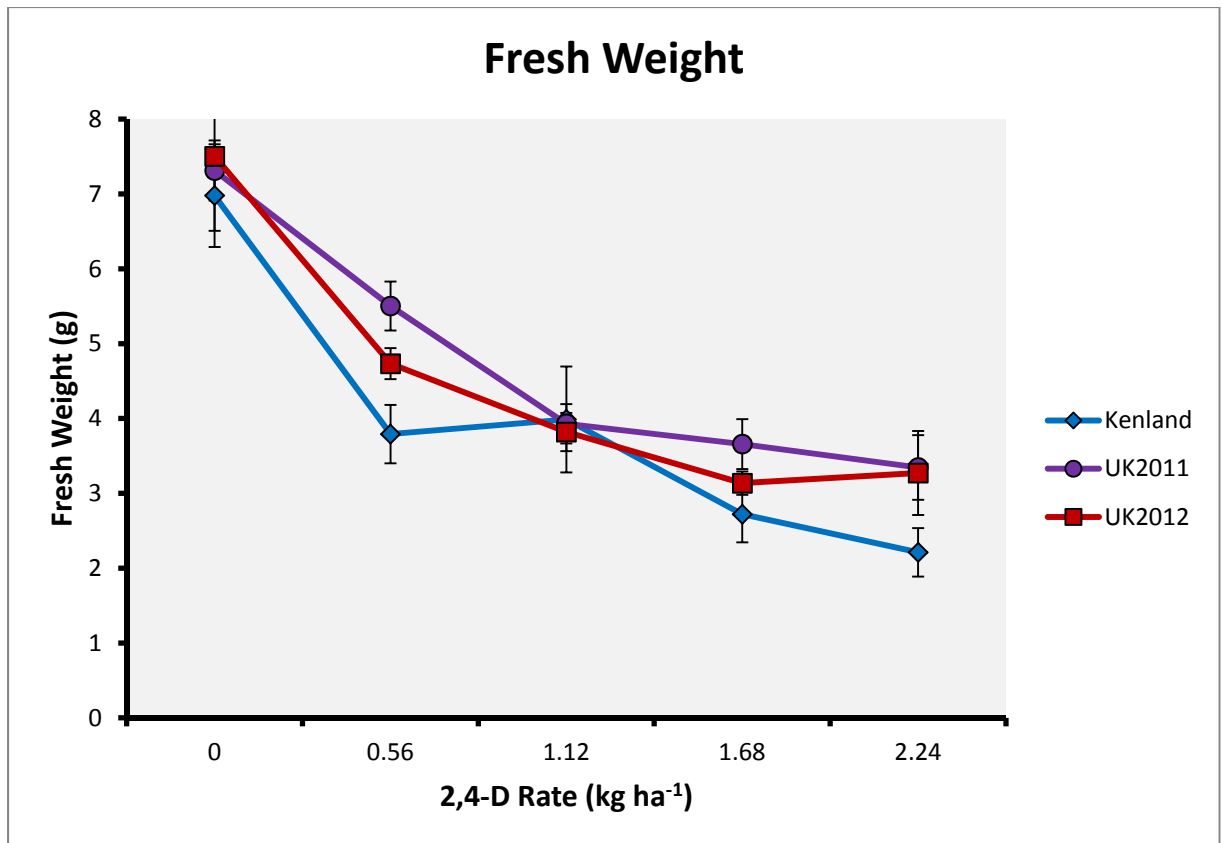


Figure 2.7. Red clover fresh weights two weeks after 2,4-D application to Kenland and plants grown from seed harvested from the 2011 and 2012 polycross recurrent selections (UK2011 and UK2012, respectively). There was no significant interaction, but over clover types there is a trend of decreasing fresh weight as rate of 2,4-D increases, and over 2,4-D rates, Kenland has a significantly lower fresh weight compared to UK2011 and UK2012 ($p < 0.05$).

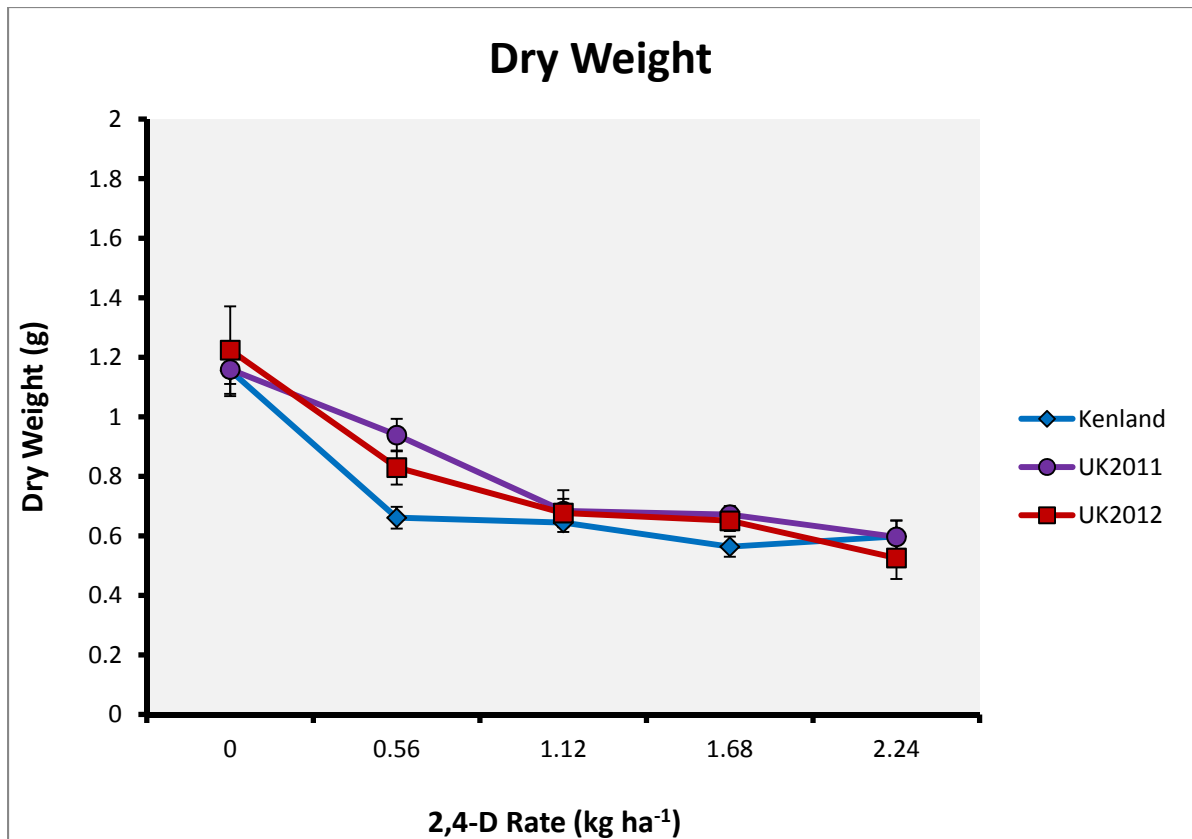


Figure 2.8. Red clover dry weights two weeks after 2,4-D application to Kenland and plants grown from seed harvested from the 2011 and 2012 polycross recurrent selections (UK2011 and UK2012, respectively). There was no significant interaction between clover type and 2,4-D rate, but over clover types a trend of decreasing dry weights over increasing 2,4-D rate occurs, and over 2,4-D rates Kenland is significantly lower in dry weight compared to UK2011 but not UK2012 ($p < 0.05$).

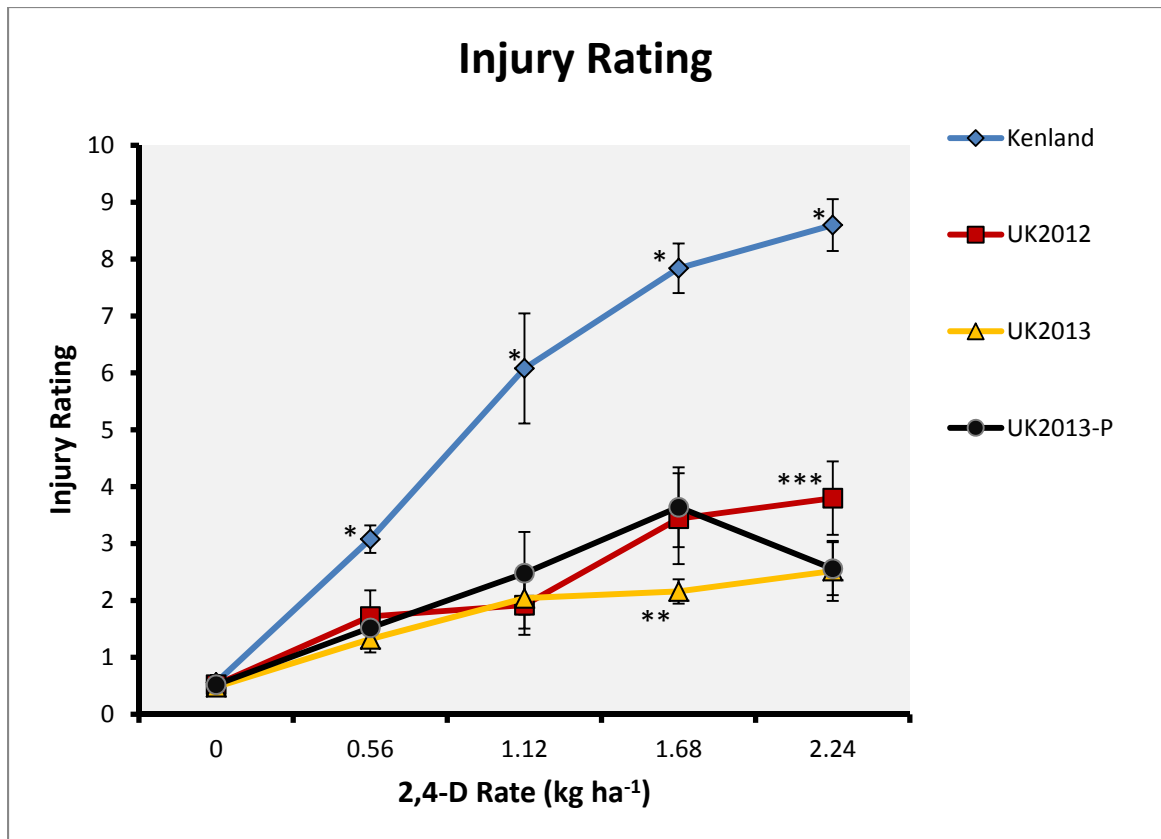


Figure 2.9. Red clover injury two weeks after 2,4-D application to Kenland, and plants grown from seed harvested from the 2012 and 2013 polycross recurrent selections (UK2012 and UK2013, respectively) as well as the 2013 second year polycross selection (UK2013-P). Injury rating on a scale from 0= no damage to 10= dead. * denotes significant difference between Kenland and all other clover varieties, ** denotes significant difference between UK2013 and both UK2012 and UK2013-P, and *** denotes significant difference between UK2012 and both UK2013 and UK2013-P ($p < 0.05$).

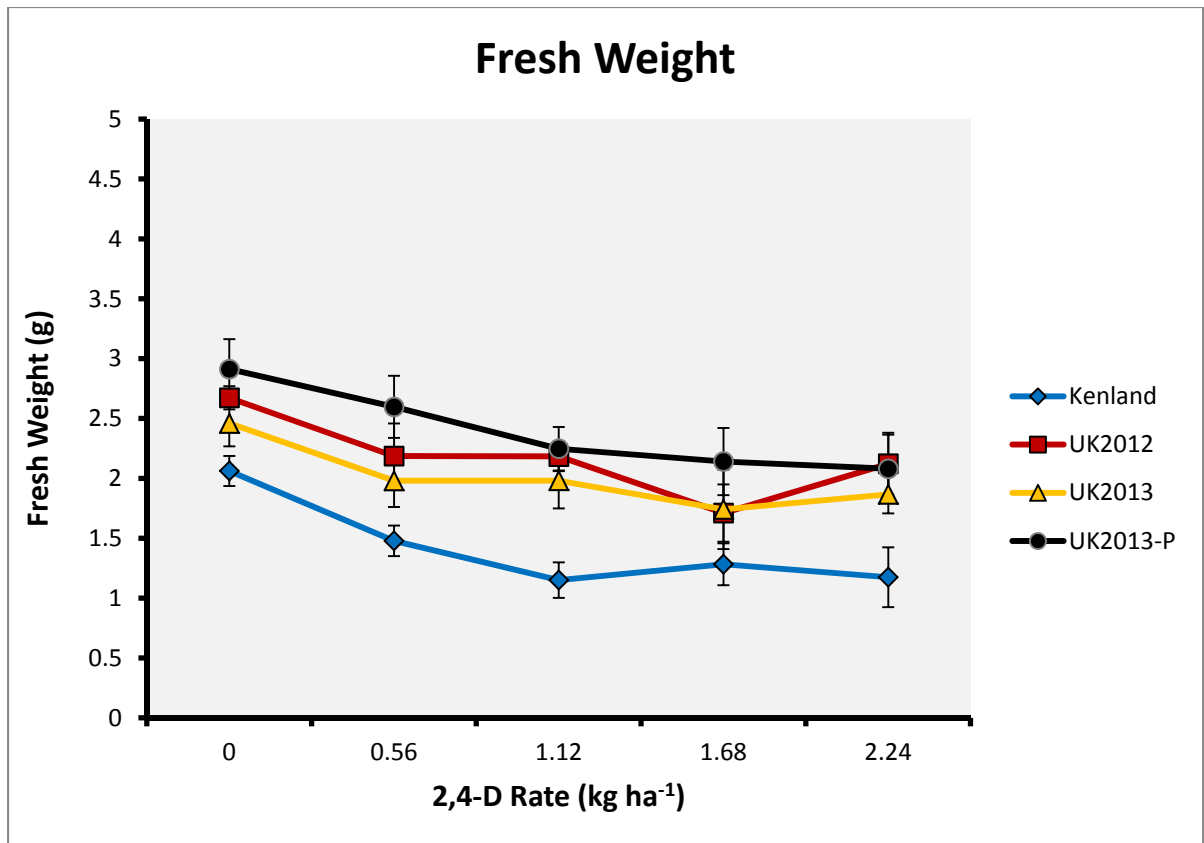


Figure 2.10. Red clover fresh weights two weeks after 2,4-D application to Kenland, and plants grown from seed harvested from the 2012 and 2013 polycross recurrent selections (UK2012 and UK2013, respectively) as well as the 2013 second year polycross selection (UK2013-P). There is no significant interaction between clover types and 2,4-D rates, but over clover types there is a trend of decreasing fresh weight with increasing 2,4-D rate, and across all 2,4-D rates Kenland has a significantly lower fresh weight compared to the other clover types while UK2013-P has a significantly higher fresh weight compared to the other clover types ($p < 0.05$).

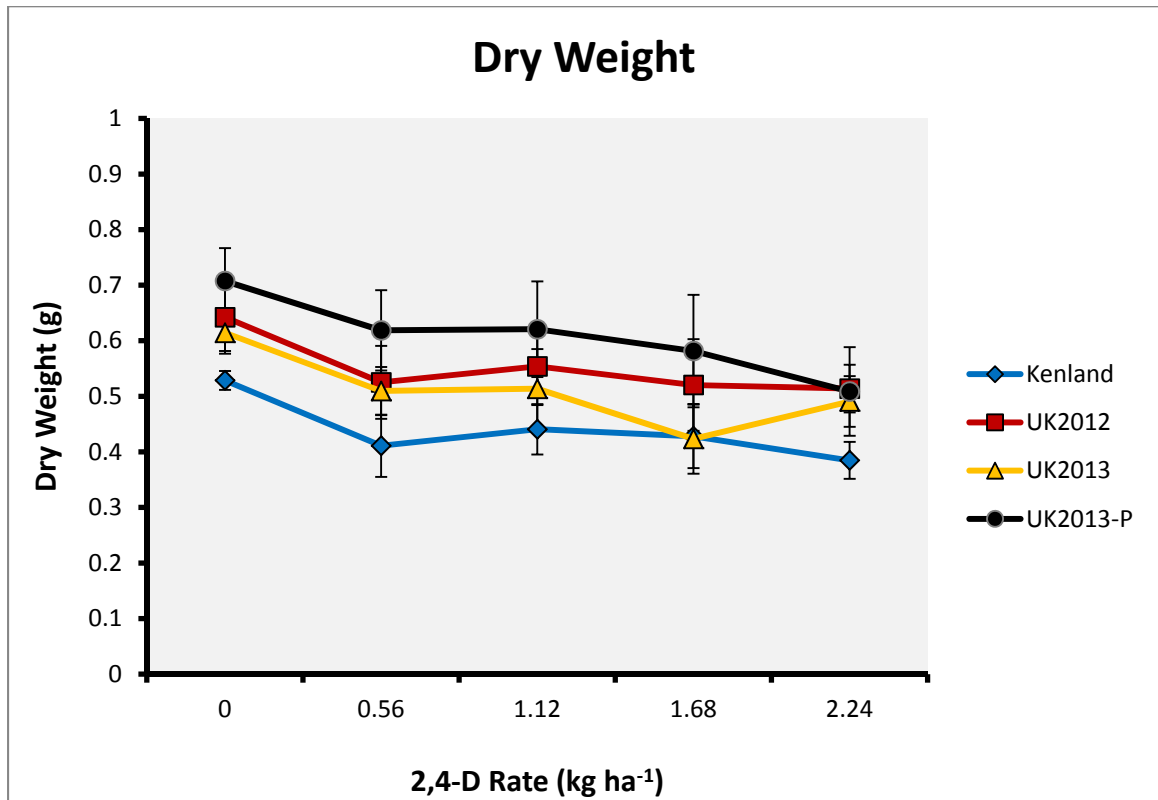


Figure 2.11. Red clover dry weights two weeks after 2,4-D application to Kenland, and plants grown from seed harvested from the 2012 and 2013 polycross recurrent selections (UK2012 and UK2013, respectively) as well as the 2013 second year polycross selection (UK2013-P). There was no significant interaction between clover type and 2,4-D rate, but over clover types there is a trend of decreasing dry weight with increasing 2,4-D rate, and over herbicide rates Kenland has a significantly lower fresh weight compared to the other clover types, while UK2013-P has a significantly higher fresh weight compared to the other clover types ($p < 0.05$).

Chapter Three

Forage Quality Comparisons

Red clover has been described as, “one of the most important legumes in the world” in the latter half of the 20th century (Smith et al. 1985). It is a common forage crop, often regarded protectively by farmers that grow it (JD Green, personal communication; Taylor and Quesenberry 1996). Red clover is usually interseeded with grasses and the mixture provides numerous benefits compared to monoculture grass pastures. These benefits include enhanced soil structure, porosity, nutrient cycling, and other pedagogical traits, increased livestock performance, and higher profit through decreased fertilizer costs and increased income per animal (Ball et al. 2002; Berg et al. 1987; Biederbeck et al. 2005; Cai et al. 2014; Chorley 1981; Frame et al. 1998; Isobe et al. 2014 Nyatsanga and Pierre 1973).

However, often these benefits cannot be fully realized due to the herbicides used in grass pastures today, to which red clover is sensitive (Ferrell and Sellers 2004; Robinson et al. 2014; Undersander et al. 1990). A red clover is currently in development at the University of Kentucky to overcome this limitation by incorporating tolerance to 2,4-D, one of the most common herbicides used in pastures. 2,4-D is a synthetic auxin in the phenoxy herbicide family and is one of the oldest selective herbicides. Discovered in the 1940’s for broadleaf control in grasses, 2,4-D has been heavily utilized worldwide since its initial marketing in 1945 (Devine et al. 1993; Grossmann 2000; Mithila et al. 2011; Troyer 2001). A 2,4-D tolerant red clover cultivar would allow farmers to use this herbicide while gaining access to the benefits provided by red clover. Currently, “In grass pastures interseeded with clover or other forage legumes, selective herbicide options are

not available for use as broadcast treatments” (Green et al. 2006). This clearly indicates a need for an herbicide tolerant red clover.

In the process of developing a 2,4-D tolerant red clover cultivar, the agronomic traits of the cultivar must be evaluated prior to its release. Agronomically important traits must not be sacrificed in favor of 2,4-D tolerance. In addition to yield, an important agronomic trait is forage quality, a measure of the nutritional quality of the crop (Ohlsson and Wedin 1989). An important component of forage quality is forage digestibility, which is measured by the neutral digestible fiber (NDF) and acid digestible fiber (ADF) contents. The protein content of the forage, which is measured by the content of crude protein (CP), is also important (Buxton 1996). NDF is a measure of the concentration of cell wall components in the forage and is inversely related to intake potential. ADF is a measure of plant components, including cellulose and lignin, with the lowest digestibility. As such, it is inversely related to digestibility. CP is the overall protein content of the forage and is important when considering the nutritional requirements of grazing animals (Buxton 1996). All three of these metrics can be useful, not only for determining the best forage crop or mixture of forage crops to feed to livestock, but also for determining the phenological scheme of the forage, which is used to relate climate and periodic biological events to determine timing of grazing or cutting at certain yield and/or nutritive values (Ohlsson and Wedin 1989). The cultivar Kenland has long been an established standard for high forage quality in red clover. It is also one of the parents of the 2,4-D tolerant cultivar being developed. Understanding relative NDF, ADF, CP, and yield levels of the 2,4-D tolerant red clover relative Kenland would help determine if the 2,4-D tolerant population has enough of the Kenland characteristics to be successful.

Therefore, the objective of this research was to evaluate the UK developed 2,4-D tolerant red clover population for forage quality traits and yield compared to those of the popular elite cultivar Kenland.

Materials and Methods

Kenland plants and plants grown from seed harvested from the 8th cycle of polycross recurrent selection for 2,4-D tolerance, performed in 2013 (UK2013), were utilized for the forage quality evaluations. Plants were grown from seed scarified with a medium emery cloth. The seed was then coated with thiram, and placed on 9.0 cm Whatman filter paper circles in a labeled Petri plate containing 8ml of distilled water. For each seed source, this was completed once, with approximately 50 seeds, each time the experiment was replicated. The Petri plates were then sealed with Parafilm®. Petri plates were placed in a growth room under a 24 h photoperiod using fluorescent bulbs ($0.56 \mu\text{mol m}^{-2} \text{sec}^{-1}$) and with temperatures maintained at 24C, until cotyledon emergence, approximately one week. Seedlings were then transplanted into Cone-tainers™ containing a 3:1 mixture of PRO-MIX and Maury (silt loam) soil, and placed in the greenhouse, where they were subirrigated as needed. The temperature in the greenhouse ranged from 21-30C and a 16h photoperiod was maintained with supplemental lighting when necessary.

Once the five to eight leaf stage was reached, a set of homogeneous healthy plants from each clover type was selected and treated with 0 or 1.12 kg ha⁻¹ of 2,4-D amine. The treatments were applied with a CO₂ pressurized spray chamber with a carrier rate volume of 280 L/ha-1 at 207 kPa spray pressure. Two weeks after the 2,4-D was applied,

the plants were harvested, air-dried to a constant weight, and dry weights were recorded. Samples were ground using a cutter mill (Thomas Wiley® mini mill. Thomas Scientific 1654 High Hill Road Swedesboro, NJ 08085) and five replicates for each treatment were homogenized. ADF and NDF contents were determined using a filter-bag method (Vogel et al., 1999). Each sample (0.45-0.55g) was sealed in a filter bag and the bags were placed in a fiber analyzer vessel with neutral detergent solution (ANKOM Technology 2052 O'Neil Road Macedon, NY 14502). Samples were processed with heat and agitation for 75 minutes. After processing, the samples were rinsed with hot distilled water and agitation for five minutes, repeated three times. The samples were rinsed with acetone and allowed to air-dry, followed by four hours in a drying oven at $102 \pm 2^{\circ}\text{C}$ before NDF weights were recorded. For ADF analysis, the samples were placed in the fiber analyzer vessel with acid detergent solution (ANKOM Technology 2052 O'Neil Road Macedon, NY 14502) for 60 minutes with heat and agitation before rinsing for five minutes, three times, with hot distilled water. Rinsed samples were then dried with acetone before being placed in a drying oven at $102 \pm 2^{\circ}\text{C}$ for four hours before ADF weights were recorded. CP content was determined with 100mg samples using a micro-Kjeldahl procedure utilizing a salicylic acid modification (Bradstreet, 1965; Chaney and Marbach, 1962). This experiment was replicated five times and experiments were pooled for statistical analysis. Data was subjected to an analysis of variance using ProcGLM in SAS® statistical software. Separation was performed using LSmeans at $\alpha=0.05$.

Results and Discussion

Neutral Detergent Fiber

The NDF content analysis was the only forage quality metric which was different between the Kenland cultivar and UK2013. There was no significant interaction between clover type and 2,4-D rate (Table 3.1), but Kenland had a higher NDF content than UK2013 over 2,4-D rates (Figure 3.1). As NDF is negatively related to intake potential, the potential intake of UK2013 red clover may be higher than that of Kenland. If this level of NDF is maintained until the release, the 2,4-D tolerant red clover may have higher forage quality than Kenland.

Acid Detergent Fiber and Crude Protein

There were no differences in ADF or CP content between Kenland and UK2013 (Figures 3.2 and 3.3). This indicates that UK2013 has equivalent forage quality to Kenland by these measures.

These results, from greenhouse grown plants, should be verified in field trials. However, these results are encouraging for a potential cultivar and supports proceeding with further development of the 2,4-D tolerant cultivar.

Yield

There was no interaction between 2,4-D rate and clover type for yield (Table 3.1), but over clover types yield was lower with 2,4-D treatment and UK2013 had a higher yield compared to Kenland, over 2,4-D rates (Figure 3.4). UK2013 out-performs Kenland with and without 2,4-D. However, these results are based on individual plant

measurements and on plants grown in a greenhouse environment. Although this is a promising result, further studies must be done in the field on the population scale in order to obtain a complete picture of UK2013 yield potential. However, just like the forage quality metrics, these are promising results.

Table 3.1. P-values for the forage quality and yield experiments^A

Data type	Source	P-Value
Neutral Detergent Fiber ^B	Herbicide	0.2021
	Clover	0.0375
	Clover* Herbicide	0.3570
Acid Detergent Fiber ^B	Herbicide	0.2740
	Clover	0.2448
	Clover* Herbicide	0.0886
Crude Protein ^B	Herbicide	0.0903
	Clover	0.3729
	Clover* Herbicide	0.6286
Yield	Herbicide	0.0235
	Clover	0.0019
	Clover* Herbicide	0.1491

^A All data analyzed via Analysis of Variance. ^BData analysis was performed using the arcsine of the square root transformation in SAS.

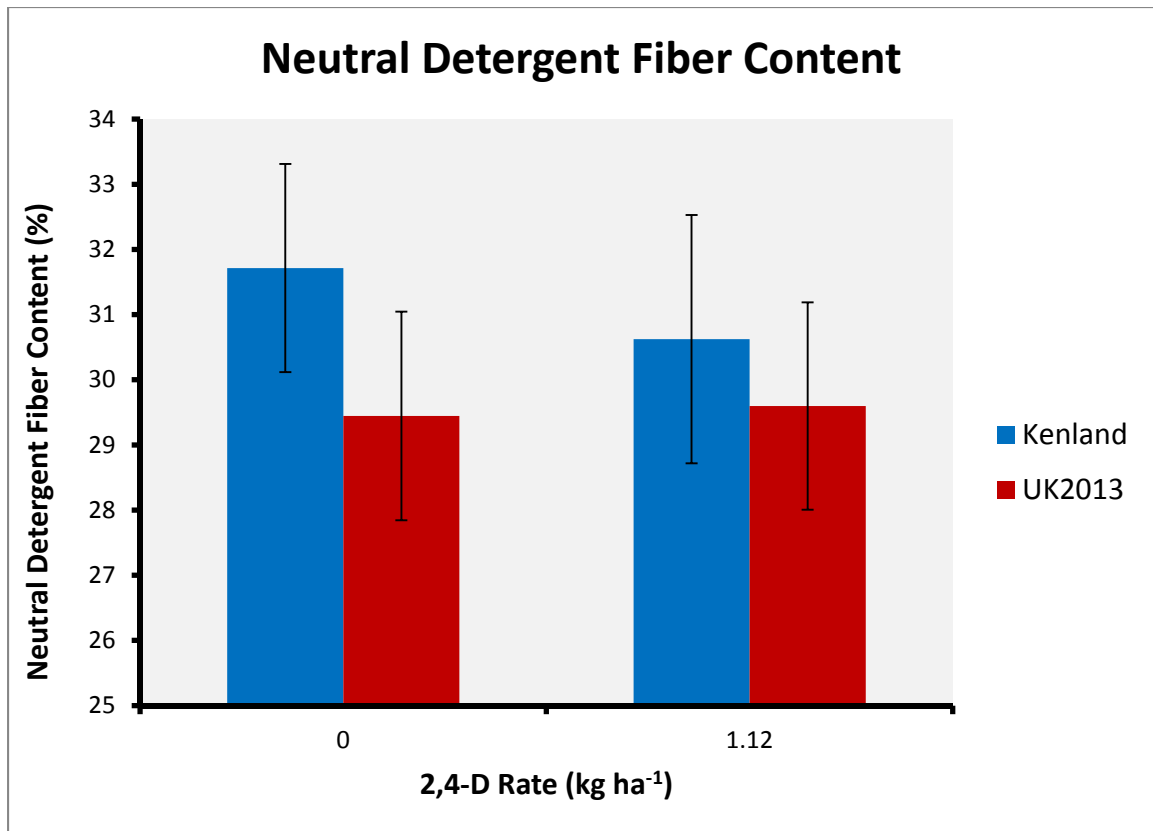


Figure 3.1. Neutral detergent fiber contents for Kenland and UK2013. There was no significant interaction between clover types and 2,4-D rates, but Kenland has a significantly higher NDF content compared to UK2013 over 2,4-D rates ($p < 0.05$).

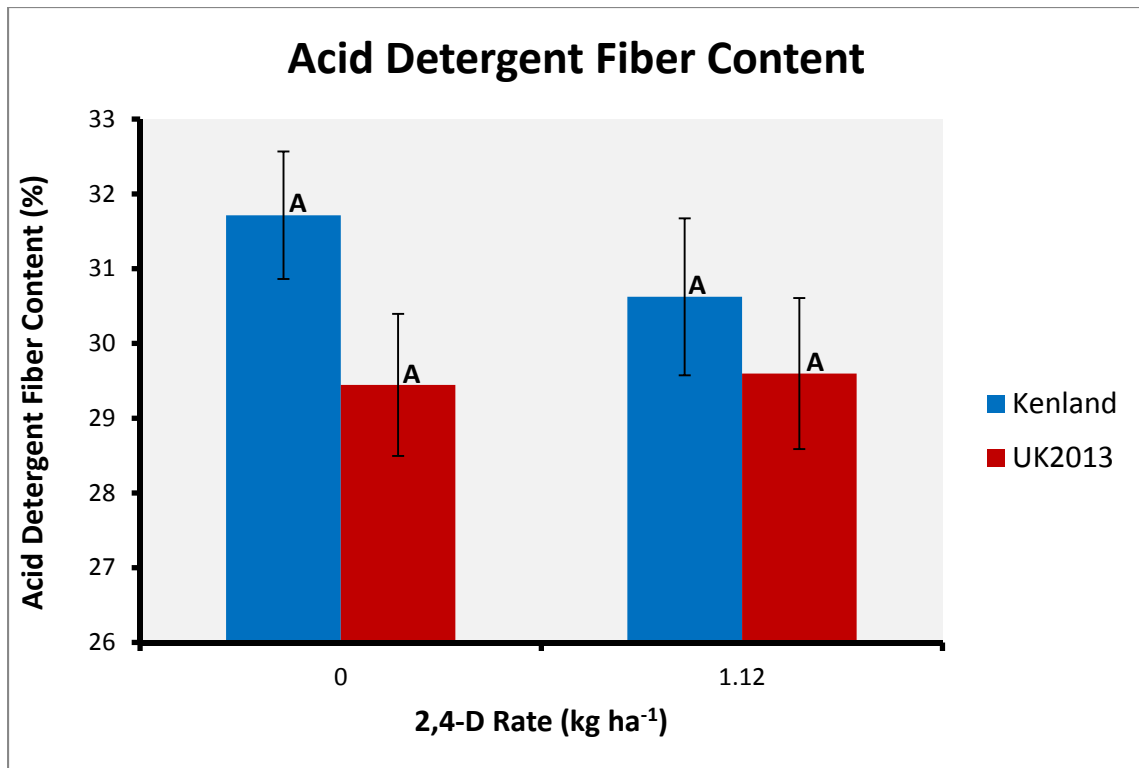


Figure 3.2. Acid detergent fiber contents for Kenland and UK2013. Bars with the same letter are not statistically different ($p < 0.05$).

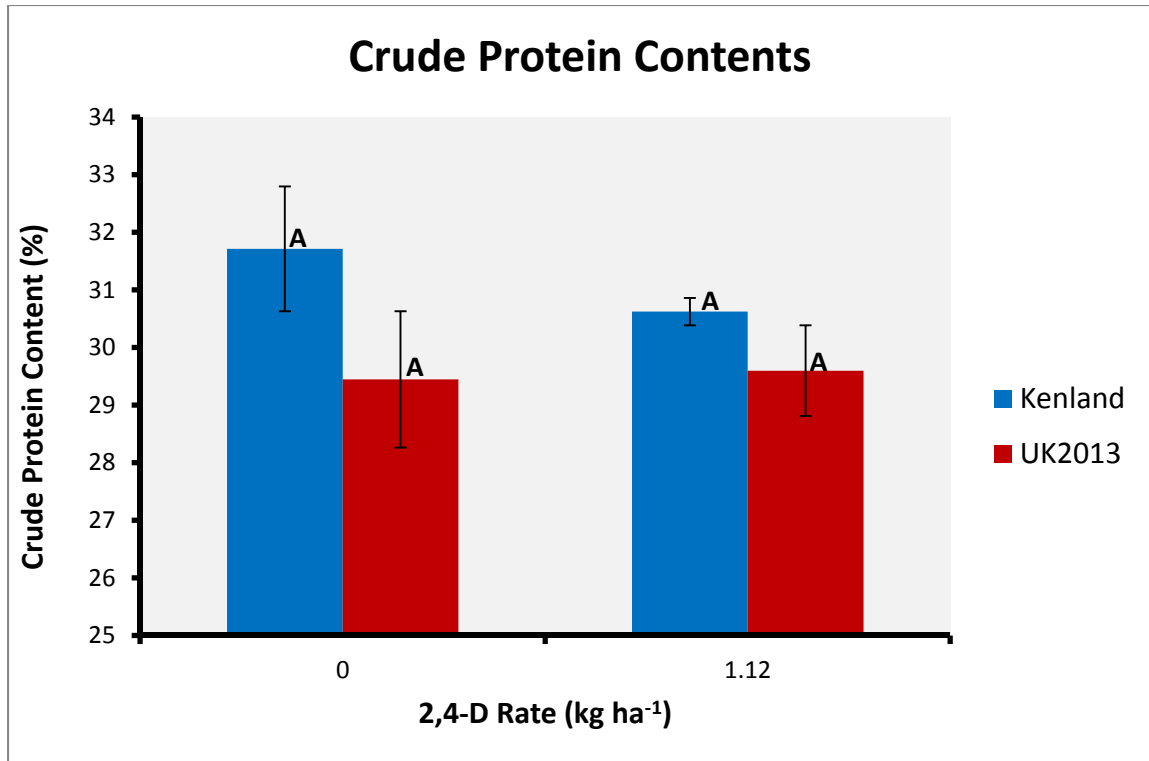


Figure 3.3. Crude protein contents for Kenland and UK2013. Bars with the same letter are not statistically different ($p < 0.05$).

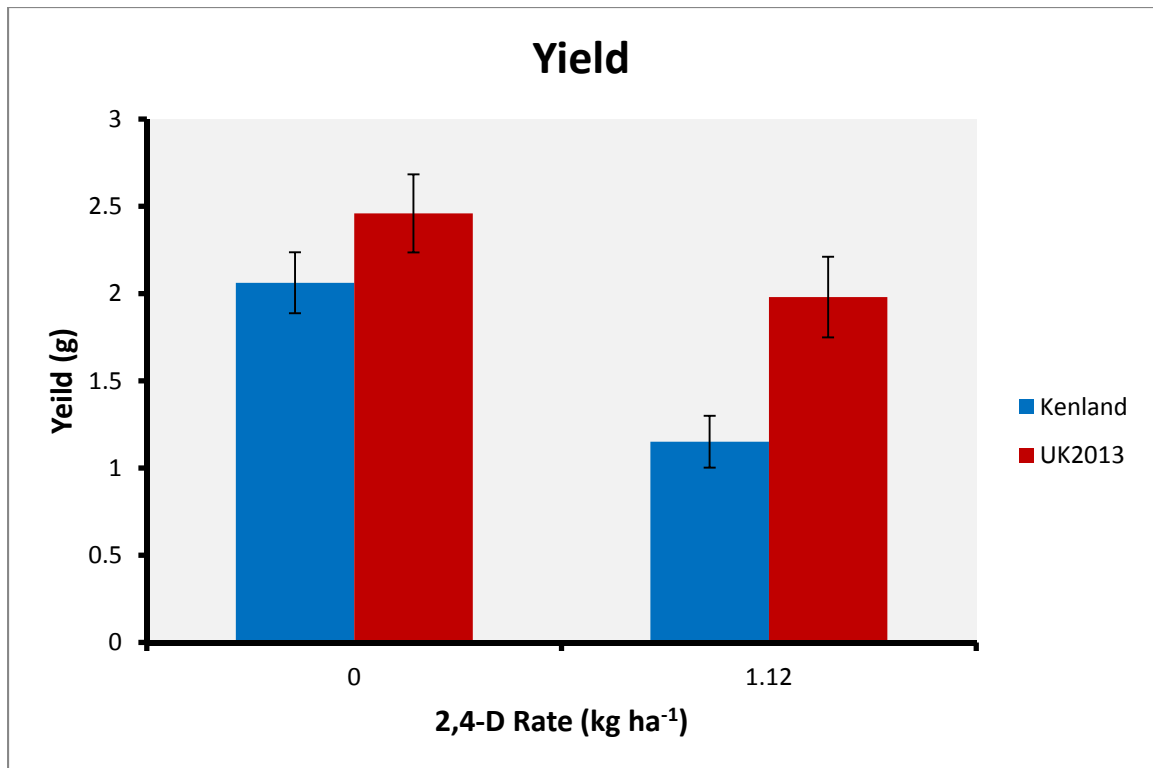


Figure 3.4. Individual plant yield of Kenland and UK2013 at 0 and 1.12 kg ha⁻¹ 2,4-D. There is no significant interaction between clover type and 2,4-D rate, but over 2,4-D rates, Kenland has a lower yield than UK2013 ($p < 0.05$).

Chapter Four

2,4-D Uptake, Translocation, and Metabolism in Sensitive and Tolerant Red Clover

Of all the herbicides on the market today, 2,4-D is one of the most commonly used in pastures and forage fields (Robinson et al. 2014; Anderson 1996). It is also frequently used in row crops and turf (Robinson et al. 2014; Anderson 1996). Discovered independently between 1940 to 1943 by four different research teams, 2,4-D and the related MCPA were the first selective herbicides (Mithila et al. 2011; Troyer 2001). Widely used since its initial release in 1945, 2,4-D is currently the third most commonly used herbicide in the United States, and the most commonly used herbicide worldwide (Anderson 1996; Colborn 1999; Joseph 2014; Peterson 1967).

Despite being both one of most commonly used and one of the oldest selective herbicides available, the exact mechanism and site of action of 2,4-D have not yet been completely elucidated. 2,4-D is a synthetic auxin herbicide in the phenoxy family and has been studied extensively since it was discovered (Audus, 1949; Fites et al. 1964; Grossmann 2000, 2003, 2010; Grossmann et al. 1996; Grossmann et al. 2004; Hagin et al. 1970; Richardson 1977; Weintraub 1953). As the regulation and action of auxin itself are not completely understood, it follows that auxin mimics like 2,4-D are not completely understood either. However, there is a good knowledge base on the general mechanism and site of action for synthetic auxins in general. Synthetic auxins like 2,4-D are known to bind to auxin receptors TIR1/AFB, which leads to ubiquitin-proteasome pathway targeted break down of transcriptional regulator proteins (Grossmann 2010). De-repression of transcriptional activator proteins, ARFs, then occurs due to the lack of transcriptional regulator proteins and this leads to activation and subsequent

overexpression of the auxin-responsive genes. These genes cause the overproduction of ethylene, ACC, and ABA, causing the characteristic symptomology associated with the synthetic auxin herbicides (Grossmann 2010). Because of the projected increase in 2,4-D use after the release of Enlist™ crops, which are genetically engineered to withstand both glyphosate and 2,4-D, understanding the uptake, translocation, and metabolism of 2,4-D in plants is more important than ever (Joseph 2014).

There have been numerous studies involving the uptake, translocation, and metabolism of 2,4-D in various plant species since its release as a commercial herbicide in 1945. By 1960, when the first comprehensive review of 2,4-D movement in plants was published, research of this type included 54 different crop, weed, and ornamental plant species (Hull 1960). Most of the research during the period from 1945-1960 was conducted on 2,4-D translocation, the direction, destination, speed, and duration of which was determined to be species dependent (Ashton 1958; Day 1951; Fang 1958; Hull 1960; Vernon and Aronoff 1951). Some research linked species susceptibility to translocation, while others pointed to metabolism (Ashton 1958; Hull 1960; Williams 1956). The effects of many abiotic and biotic factors on translocation were also measured. Translocation was shown to increase with factors such as temperature, soil moisture, light, humidity, and plant carbohydrate content, while increases in herbicide contact injury, the age of the application site, pH, and plant stress level decreased translocation. Factors such as the use of different surfactants, carrier solutions, and additives such as sugar, boron, and gibberellic acid, were also explored, with differing effects depending on the plant species and particular surfactant, carrier, or additive used (Hull 1960). By 1977, Richardson had laid out the effects of most of these abiotic and biotic factors and

was able to determine through a review of the research how each affected 2,4-D absorption and translocation (Richardson 1977). Factors, such as translocation, excretion, immobilization, and metabolism, are still implicated as bases for 2,4-D tolerance in plants, with many authors suggesting that tolerance may be expressed in a species-dependent manner (Dexter et al. 1971; Morgan and Hall 1963; Neidermyer and Nalewaja 1969; Pallas 1963; Fites et al. 1964; Slife et al. 1962). The mechanism of 2,4-D tolerance in different species is still being studied and, in some cases, the inheritance of the tolerance mechanism is known (Di Meo 2012; Lym and Moxness 1989; Riar et al. 2011; Sunohara et al. 2010; Wyrill and Burnside 1976; Zheng and Hall 2001). However, no research concerning the movement and fate of 2,4-D in red clover and their contributions to 2,4-D sensitivity and tolerance in this species has been published to date.

A 2,4-D tolerant red clover type is in development at the University of Kentucky. Red clover is an excellent forage legume which is currently underutilized, partially due to its inability to withstand the majority of herbicides used for weed control in pastures (Ball et al. 2002; Cai et al. 2014; Green et al. 2006; Isobe et al. 2014). A 2,4-D tolerant red clover could be very beneficial to farmers, and progress to that end has been made.

The objective of this study is to determine the basis for the improved 2,4-D tolerance in the red clover under development. Uptake, translocation, and metabolism of 2,4-D will be compared between the cultivar Kenland (2,4-D sensitive), and the 2,4-D tolerant red clover (UK2013).

Materials and Methods

Kenland (2,4-D sensitive) and UK2013 (2,4-D tolerant) red clover plants were started from seed scarified with a medium emery cloth. Approximately 100 seeds were coated with thiram and placed on 9.0 cm Whatman filter paper circles in a Petri plate containing 8ml of distilled water. This was repeated 3 times for each seed source, such that approximately 300 seeds were sown across 3 Petri plates for both Kenland and UK2013. The Petri plates were then sealed with Parafilm® and placed in a growth room for one week under a 24 h photoperiod using fluorescent bulbs ($0.56 \mu\text{mol m}^{-2} \text{sec}^{-1}$) and with temperature maintained at 24C. Individual seedlings were transplanted into Cone-tainers™ containing a 3:1 mixture of PRO-MIX and Maury silt loam.

Seedlings were then moved to a greenhouse, where moisture was maintained through subirrigation. The temperature in the greenhouse ranged from 21-30C with a 16h photoperiod maintained with supplemental lighting as needed. Once the 5-8 leaf stage was reached, a set of 16 homogeneous plants from each type was selected and moved to a laboratory, where they were subirrigated with distilled water and acclimated for 3 days with temperature maintained at approximately 28C and a 16 h photoperiod provided by fluorescent bulbs (F20T12-D 20 watt fluorescent bulbs, Philips North America Corporation 3000 Minuteman Road M/S 109 Andover, MA 01810) ($0.25 \mu\text{mol m}^{-2} \text{sec}^{-1}$) (Figure 4.1).

After acclimation, 2,522 Bq in 10 μl of a 1.24 nmolar solution of uniformly ring labeled ^{14}C 2,4-D (American Radiolabeled Chemicals, Inc., 101 Arc drive, St. Louis MO 63146) suspended in a 0.1% v/v solution of crop oil concentrate (Maximizer, Loveland Products, 3005 Rocky Mountain Ave. Loveland, CO 80538) in water was applied to the

youngest completely unfurled leaf of each individual plant. The application consisted of twenty 0.5µl droplets evenly spaced across the adaxial surface of all three leaflets of each leaf treated (Figure 4.2). Plants were harvested at 8, 24, 48, and 72 hours after treatment (HAT). At harvest, four plants each of Kenland and UK2013 were separated into treated leaf (TL), untreated shoot (US), and root (R) sections. The treated leaf sections were rinsed twice with 5ml of methanol to remove any unabsorbed ^{14}C (Devine et al. 1984). These rinsates were combined and stored at 8C until analyzed. Radioactivity in the rinsates was measured by liquid scintillation spectroscopy (LSS) (TriCarb® 2200CA, Perkin Elmer™ Life Sciences, 2200 Warrenville Rd, Downers Grove, IL 60515). For this purpose, 5ml of the rinsate was mixed with 15ml of scintillation cocktail (Bio-Safe II™, Research Products International Corp. 410 N Business Center Drive, Mount Prospect, IL 60056). Fresh weights of the three harvested sections were determined and the tissues were stored at -20C until extraction.

Extractions were performed in a similar manner to Riar et al. (2011) by grinding each sample in liquid nitrogen in a mortar and pestle until completely pulverized. The sample was then suspended in methanol and centrifuged at 7650 g for 6 min. The supernatant was removed, the pellet resuspended in methanol, recentrifuged, and the two supernatants were combined and brought to volume. The pellet was air-dried and oxidized (Packard Sample Oxidizer model #307, Perkin Elmer™ Life Sciences, 940 Winter Street Waltham, MA 02451). Released $^{14}\text{CO}_2$ was quantified by LSS.

The extract was concentrated by evaporation in vacuo to a volume of 1.5ml. The 1.5ml samples were centrifuged for 2 minutes at 4550 g and then filtered through a sterile, nylon 0.45 µm filter (Fisher Scientific, 300 Industry Drive Pittsburgh, PA 15275).

Preliminary studies showed 90% recovery of the radioactivity originally present in the extracts following this procedure. To quantify total radioactivity in the extracts, the ^{14}C in an aliquot was measured by LSS.

Unmetabolized 2,4-D and metabolites in the extracts were separated and quantified using a high performance liquid chromatography (HPLC) (Prominence UFLC, Shimadzu, 1, Nishinokyo-Kuwabara-cho, Nakagyo-ku, Kyoto 604-8511, Japan) system coupled to a radioactivity detector (Radiomatic Flo-One ® Beta Series A-500, Canberra Industries, Inc. 800 Research Parkway, Meriden, CT 06450). The HPLC was equipped with a C18 5 μm 4.6 x 250 mm reverse phase column (GL Sciences Inc. Shinjuku Square Tower 30F, 6-22-1 Nishi Shinjuku, Shinjuku-ku, Tokyo, 163-1130 Japan). Elution was accomplished using a gradient beginning with an 8:2 ratio of 0.1% v/v phosphoric acid to acetonitrile (ACN) (Table 4.1) (Joshua J. Skelton, personal communication). The flow rate was 1 ml min $^{-1}$ and the sample injection volume was 500 μl . Parent 2,4-D eluted at 23 minutes (Figure 4.3). Radiochromatographs were integrated using the Flo-One software and the percentage of parent 2,4-D and metabolites as a fraction of the total radioactivity in each sample was calculated from these radiochromatographs. The plants were arranged in a split-split plot design, with harvest interval as the main plot, red clover type as the subplot, and harvested section as the sub-subplot. Data was subjected to an analysis of variance using ProcGLM in SAS® statistical software. The percent radioactivity

recovered was calculated as:
$$\frac{(\text{total radioactivity recovered from plant sections})+(\text{wash})}{(\text{total radioactivity applied})} \times 100.$$

The percent uptake of ^{14}C from ^{14}C 2,4-D was calculated as:

$$\frac{(\text{total radioactivity applied})-(\text{wash})}{(\text{total radioactivity applied})} \times 100.$$
 The percentage of radioactivity remaining

unextracted within each sample was calculated as

$\left(\frac{\text{total radioactivity unextracted}}{(\text{total radioactivity extracted})+(\text{total radioactivity unextracted})} \times 100 \right)$. The percentage of parent 2,4-D and metabolites are reported as a percentage of the total radioactivity in peaks on the radiochromatograph.. The percent unextracted radioactivity, percent of absorbed radioactivity translocated from the treated leaf to the untreated shoots and roots, parent and metabolite percentages, percent ^{14}C recovery, and the percentage of applied ^{14}C absorbed values were all transformed using the arcsine of the square root transformation. Separation was performed using LSmeans at $\alpha=0.05$. The entire experiment was repeated three times and experiments were pooled for statistical analysis.

Results and Discussion

Uptake and Translocation

There was a significant interaction between HAT and clover type for the amount of applied radioactivity recovered (Table 4.2). However, the main effects of clover and HAT were individually not significant. The percentage of radioactivity recovered at 8 HAT was greater from Kenland than UK2013 while at 24 HAT it was greater from UK2013 than Kenland (Figure 4.4). At 48 HAT, recovery was greater from Kenland than UK2013 and at 72 HAT there was no difference between Kenland and UK2013. Besides being inconsistent, the differences between the clover types were small, with overall recovery ranging from 50 to 70%. This is much lower than the recovery found in previous studies with birdsfoot trefoil and perennial glycine species, which were approximately 90% (Davis and Linscott 1986; White et al. 1990). Two possibilities for 2,4-D loss not accounted for in this study are volatilization from the treated leaf and excretion from the roots. Volatilization is not likely to occur after absorption by the plant

and the application was engineered to minimize the surface area susceptible to pre-absorption volatilization by applying the ^{14}C labeled 2,4-D in 0.5 μl droplets across the entire leaf surface. Had pre-uptake volatilization occurred, it would have been consistent for both clover types. If post-uptake volatilization occurred, it would have been a loss of ^{14}C as $^{14}\text{CO}_2$, which has been shown in other studies with absorbed 2,4-D (Morgan and Hall 1963; Schultz and Burnside 1980; Slife et al. 1962). However, such losses were minimal in other studies and would not account for the nearly 30% loss in recovery shown in this study (Morgan and Hall 1963; Schultz and Burnside 1980; Slife et al. 1962). Additionally, in species related to red clover, like soybean, there was no reported loss of ^{14}C though $^{14}\text{CO}_2$ (Sargent and Blackman 1962). Loss of $^{14}\text{CO}_2$ from ^{14}C 2,4-D has also been shown to increase substantially over time, well past 72 HAT, while we observed a leveling off of unrecovered ^{14}C by 48 HAT for UK2013 and a consistent amount of unrecovered ^{14}C at all time points for Kenland (Morgan and Hall 1963; Shultz and Burnside 1980; Slife et al. 1962) (Figure 4.4).

The second route by which radioactivity may have been lost is through translocation to the roots followed by excretion to the root medium. This has been identified as a mechanism of tolerance to 2,4-D in jimsonweed (*Datura stramonium*) and wild radish (*Raphanus raphanistrum*) and has also been reported in other species (Di Mio 2012; Fites et al. 1964; Hull 1960; Lym and Moxness 1989; Schultz and Burnside 1980). Root exudation of 2,4-D occurs in birdsfoot trefoil, a species related to red clover (Davis and Linscott 1986). Plants which form a symbiotic association with nitrogen fixing rhizobia, like birdsfoot trefoil and red clover, often release glycosides and other material as root exudates in order to attract nitrogen fixing symbionts (Sugiyama and Yazaki

2012). Glycosides of 2,4-D have been detected in several studies, including in birdsfoot trefoil (Davis and Linscott 1986; Lym and Moxness 1989; Slife et al. 1962).

There was a significant interaction between clover type and harvest interval for uptake of ^{14}C from the ^{14}C 2,4-D applied (Table 4.2). At every time point except 24 HAT, Kenland absorbed less ^{14}C than UK2013 (Figure 4.5). At 24 HAT, Kenland absorbed more ^{14}C than UK2013. There are also differences in uptake over time for both Kenland and UK2013. UK2013 at 8 HAT has already taken up as much ^{14}C 2,4-D as Kenland had by 72 HAT, which shows that 2,4-D uptake by the tolerant red clover is more rapid than uptake in the sensitive clover. Differential 2,4-D uptake would not explain the tolerance difference between and UK2013. Kenland also had a different pattern of 2,4-D uptake than UK2013. Where UK2013 ^{14}C uptake only increased between 24 and 48 HAT, with no increases previous to 24 or after 48 HAT, Kenland ^{14}C uptake increased between 8 and 24 HAT, decreased between 24 and 48 HAT, and then remained constant thereafter. The increase in uptake from 8 to 72 HAT is similar in amount for the two clover types. (Figure 4.5) It is difficult to assign a role for differential 2,4-D uptake to the difference in 2,4-D tolerance between Kenland and UK2013, although there are differences in both the speed and pattern of uptake between 2,4-D tolerant and 2,4-D sensitive red clover.

There was a significant interaction between clover type and harvest interval for the amount of radioactivity present in the treated leaf section (Table 4.2). However, this interaction was not detected for the untreated shoot and root sections. The amount of radioactivity in the treated leaf section of Kenland declined over time reaching its lowest level 72 HAT as compared to the other harvest intervals, but this pattern was not present

in UK2013 (Figure 4.6). Kenland also had less radioactivity in the treated leaf than UK2013 at both 24 and 72 HAT, which would suggest that ^{14}C is being fixed in the UK2013 treated leaf, but remains mobile in the treated leaf of Kenland. For the untreated shoot section there is no difference between clover types, but the main effect of harvest interval is significant, and over clover types there is more ^{14}C over time, indicating no difference in free or fixed ^{14}C in between the untreated shoot sections of the 2,4-D tolerant and sensitive red clovers (Figure 4.7). This also indicates a continued movement of ^{14}C from the treated leaf to the untreated shoot. For the root section, the main effects of clover type and harvest interval are significant. Over clover types, there is an increase in the amount of radioactivity in the roots over time and over harvest intervals there is more radioactivity in the roots of Kenland compared to the roots of UK2013 (Figure 4.8).

There is no significant interaction between HAT and clover type for the radioactivity, expressed as a percentage of the total ^{14}C absorbed, translocated to either the untreated shoot or roots (Table 4.2). More ^{14}C was translocated to the untreated shoots and roots of both Kenland and UK2013 over time (Figures 4.9 and 4.10). And, while there was no difference in translocation of ^{14}C to the untreated shoot between clover types more radioactivity was translocated to the Kenland roots compared to those of UK2013. This suggests that reduced translocation to the roots plays a role in the 2,4-D tolerance of UK2013.

Metabolism of ^{14}C 2,4-D

Over clovers and harvest times, roots had the highest amount of unextracted radioactivity followed by the untreated shoot and treated leaves (Figure 4.11). When

individual plant sections were analyzed separately, the treated leaf of UK2013 had higher levels of extracted ^{14}C compared to that of Kenland although there was no interaction with harvest time and clover (Figure 4.12). In contrast, there was no difference in unextracted radioactivity between the untreated shoot and roots of the two clovers (Table 4.2, Figures 4.13 and 4.14).

Although there is little research reported on the behavior of 2,4-D in red clover specifically with which to compare these results, studies were conducted by Davis and Linscott (1986) on the related species birdsfoot trefoil (*Lotus corniculatus*). They stated that “binding of 2,4-D to insoluble components, such as lignin, which resulted in decreased levels of free 2,4-D, has been reported in other species, and may be a factor in the differential tolerance of the trefoils to 2,4-D.” They go on to note that one variety of trefoil “appears to bind slightly more 2,4-D in the roots and stems and that another researcher, Blacklow, reported “about 50% more binding to lignaceous components in roots” (Blacklow 1968). This could explain the higher percentage of unextracted radioactivity in the root section for our experiment and, furthermore, the higher percentage of unextracted radioactivity in the untreated shoot section as the red clover stem was harvested with the untreated shoot section. 2,4-D tolerance was found to be partially due to immobilization in some species, which involves binding to insoluble components, and the hypothesis of Davis and Linscott indicates differential binding of 2,4-D to insoluble components in the plant may be a factor contributing to the differential tolerance of trefoils to 2,4-D (Davis and Linscott 1986; Dexter et al. 1971). This may be also be a contributing factor to 2,4-D tolerance in red clover, as indicated by the treated leaf of Kenland containing a lower percentage of unextracted radioactivity compared to

the treated leaf of UK2013 (Figure 4.12). However, this trend is not present for all sections, and the percent of unextracted radioactivity in Kenland as compared to UK2013 amounts to a maximum difference averaging at 10%, which is not large enough to completely account for the differential tolerance, but may indicate a function for differential immobilization within a multifactorial tolerance mechanism. Further analysis of differences between the amounts of radioactivity found in insoluble versus soluble components of these red clovers using toxic levels of 2,4-D is necessary in order to confirm or reject this hypothesis.

There was parent 2,4-D detected in all plant parts of both Kenland and UK2013 at all harvest intervals (Figures 4.15, 4.16, 4.17). Additionally, six unknown metabolites of 2,4-D were detected, designated M1 through M6. All are more polar than 2,4-D, eluting ahead of the 23 minute retention time for the parent 2,4-D peak, at 5, 6, 10, 15, 18, and 19 minutes for M1-M6, respectively (Figure 4.3). These metabolites were found in varying concentrations, with M1 found in the highest amounts. The primary consistent differences between Kenland and UK2013 are in the amount of parent 2,4-D and M1 (Table 4.2).

Unaltered 2,4-D remains in all sections of both clover types at all harvest intervals, and for all sections there is no significant interaction between harvest interval and clover type (Table 4.2). While there is no interaction between time and clover type for the amount of 2,4-D found in the plant sections, there is more 2,4-D found in all sections of Kenland compared to UK2013 (Figures 4.15, 4.16, and 4.17). For the treated leaf section, the main effect of harvest interval is also significant and over clover types the amount of 2,4-D decreased over time (Figure 4.15). For the untreated shoot section,

the model is only significant at the $p < 0.1$ level but at this level the untreated shoots of Kenland contain more 2,4-D than UK2013 (4.16). There is no difference over time for the amount of 2,4-D found in either the untreated shoot section or the root section (Figures 4.16 and 4.17). The root section, like the treated leaf section, has more 2,4-D in Kenland as compared to UK2013. Overall, there is more 2,4-D in Kenland than UK2013 and a trend of less 2,4-D over time in the treated leaf section of both clovers.

Metabolite M1 also occurs in all sections of both clover types at all harvest times. There was no significant interaction between clover type and harvest time for the amount of M1 in any section (Table 4.2), but more M1 is found in UK2013 sections relative to Kenland (Figures 4.18, 4.19, and 4.20). Over clover types, M1 increases over time in the treated leaf of both clovers (Figure 4.18). This is the reverse of what was observed for 2,4-D (Figure 4.15). Similar to the amount of 2,4-D in the untreated shoot (Figure 4.16), Kenland has less M1 than UK2013 in the untreated shoot is but this is only significant at the $p < 0.1$ level (Figure 4.19). There is no difference over time for the amount of M1 found in the untreated shoot section. For the root section, at either the $p < 0.05$ or $p < 0.1$ levels (Figure 4.20). Overall, M1 was found in higher proportions in UK2013 than Kenland in both the treated leaf and untreated shoot section and the amount of M1 increased over time in the treated leaf section of both clovers. The clear differences in the amounts of both M1 and unmetabolized 2,4-D between the UK2013 (tolerant) and Kenland (sensitive) indicates that 2,4-D metabolism is an important component in the mechanism of tolerance.

There are fewer consistent difference between Kenland and UK2013 for the other metabolites (Figures 4.21-4.32). There are no differences between them for M2. M3

occurs in higher proportions in the untreated shoot section of UK2013 relative to Kenland, over harvest intervals (Figure 4.22). There was a significant interaction between clover type and harvest interval for M4 in the root section, M4 was present in higher proportions in the root section of UK2013 relative to Kenland, but only at 8 HAT (Figure 4.26). Kenland contains less M5 than UK2013 over harvest intervals, and the amount of M5 in the treated leaf increases with time over clover types (Figure 4.28). Thus, M5 follows a similar trend to M1 in the treated leaf of the two clovers. There are also several trends observed in the proportion of metabolites 3-6 which fall short of being statistically significant between the clover types, but support the overall trend of UK2013 containing a higher proportion of metabolites relative to Kenland, while Kenland contains a higher proportion of 2,4-D relative to UK2013 (Figures 4.21-4.32). Differences in metabolites 3-6, as well as especially M1 and 2,4-D, support the involvement of metabolism in the mechanism of tolerance to 2,4-D in UK2013 red clover. This is apparent in the lower proportion of parent 2,4-D present in all sections of the tolerant UK2013 red clover compared to the sensitive Kenland red clover. However, the presence of the same 6 metabolites in both clover types indicates that the difference in the rate of 2,4-D metabolism between the two clovers is not due to a difference in the 2,4-D metabolism pathways therein. Rather, the same 2,4-D metabolites are being formed in UK2013 as Kenland but at a faster rate. We would hypothesize that M1 may be a glucose conjugate of hydroxylated 2,4-D and that M5 may be hydroxylated 2,4-D. This would further suggest that increased cytochrome P450 activity is responsible for the enhanced 2,4-D metabolism and tolerance. Further, the enhanced 2,4-D metabolism leads to decreased

movement of the herbicide from the treated leaves to other untreated plant parts such as the roots.

Table 4.1. HPLC solvent gradient used for 2,4-D analysis.

Time	Flow	0.1% Phosphoric acid Solution in Water (%)	Acetonitrile (%)
0	1.00	80	20
12	1.00	60	40
17	1.00	30	70
19	1.00	10	90
22	1.00	80	20

Table 4.2. P-values for each data type – the model was significant at the $p < 0.05$ level unless otherwise noted. ^A

Data Type	Source	P-Value
Percent Recovery	HAT	0.1445
	Clover	0.9627
	HAT*Clover	<0.0001
Uptake ^B	HAT	<0.0001
	Clover	0.0134
	HAT*Clover	<0.0001
Total Radioactivity:		
Treated Leaf Section	HAT	0.0220
	Clover	0.0010
	Clover *HAT	0.0443
Total Radioactivity:		
Untreated Shoot Section	HAT	<0.0001
	Clover	0.2050
	Clover *HAT	0.4605
Total Radioactivity:		
Root Section	HAT	<0.0001
	Clover	0.0139
	Clover *HAT	0.1347
Unextracted Radioactivity:		
All Sections	HAT	0.1723
	Clover	0.0057
	Section	<0.0001
	Clover and HAT*Section	0.3940
	Clover *HAT	0.0201
	Clover*Section	0.7384

	HAT*Section	0.1415
Unextracted Radioactivity:		
Treated Leaf Section ^B	HAT	<0.0001
	Clover	<0.0001
	Clover *HAT	0.4999
Unextracted Radioactivity:		
Untreated Shoot Section ^B	HAT	0.1676
	Clover	0.1221
	Clover *HAT	0.6183
Unextracted Radioactivity:		
Root Section ^B	HAT	0.8787
	Clover	0.3833
	Clover *HAT	0.1528
Shoot Translocation ^B	HAT	<0.0001
	Clover	0.0645
	HAT*Clover	0.2566
Root Translocation ^B	HAT	<0.0001
	Clover	0.0113
	HAT*Clover	0.0687
Metabolism in the Treated Leaf:		
2,4-D ^B	HAT	<0.0001
	Clover	<0.0001
	Clover *HAT	0.4019
Metabolism in the Treated Leaf:		
Metabolite M1 ^B	HAT	<0.0001
	Clover	<0.0001
	Clover *HAT	0.1477
Metabolism in the Treated Leaf:		
Metabolite M2 ^B	HAT	0.1451

	Clover	0.1090
	Clover *HAT	0.2341
Metabolism in the Treated Leaf:		
Metabolite M3 ^B	HAT	0.0487
	Clover	0.1600
	Clover *HAT	0.1308
Metabolism in the Treated Leaf:		
Metabolite M4 ^B	HAT	0.0029
	Clover	0.3379
	Clover *HAT	0.8520
Metabolism in the Treated Leaf:		
Metabolite M5 ^B	HAT	0.0077
	Clover	0.0058
	Clover *HAT	0.1368
Metabolism in the Treated Leaf:		
Metabolite M6 ^B	HAT	0.3124
	Clover	0.9993
	Clover *HAT	0.0603
Metabolism in the Untreated Shoot:		
2,4-D ^{BD}	HAT	0.8127
	Clover	<0.0001
	Clover *HAT	0.4554
Metabolism in the Untreated Shoot:		
Metabolite M1 ^{BD}	HAT	0.7724
	Clover	<0.0001
	Clover *HAT	0.4219
Metabolism in the Untreated Shoot:		
Metabolite M2 ^{BC}	HAT	0.4244
	Clover	0.9946
	Clover *HAT	0.5657

Metabolism in the Untreated Shoot:

Metabolite M3 ^B	HAT	0.0004
	Clover	0.0041
	Clover *HAT	0.6215

Metabolism in the Untreated Shoot:

Metabolite M4 ^B	HAT	0.0007
	Clover	0.7180
	Clover *HAT	0.9823

Metabolism in the Untreated Shoot:

Metabolite M5 ^{BC}	HAT	0.0442
	Clover	0.0083
	Clover *HAT	0.2528

Metabolism in the Untreated Shoot:

Metabolite M6 ^B	HAT	<0.0001
	Clover	0.3318
	Clover *HAT	0.8032

Metabolism in the Root:

2,4-D ^B	HAT	0.4427
	Clover	0.0002
	Clover *HAT	0.0722

Metabolism in the Root:

Metabolite M1 ^{BC}	HAT	0.3989
	Clover	0.0446
	Clover *HAT	0.4491

Metabolism in the Root:

Metabolite M2 ^{BD}	HAT	0.0206
	Clover	0.1746
	Clover *HAT	0.1786

Metabolism in the Root:

Metabolite M3 ^{BC}	HAT	0.2239
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	Clover	0.9394
	Clover *HAT	0.1258
Metabolism in the Root:		
Metabolite M4 ^B	HAT	<0.0001
	Clover	0.0025
	Clover *HAT	0.0014
Metabolism in the Root:		
Metabolite M5 ^{BC}	HAT	0.0369
	Clover	0.0270
	Clover *HAT	0.8809
Metabolism in the Root:		
Metabolite M6 ^{BC}	HAT	0.0119
	Clover	0.3540
	Clover *HAT	0.8133

^A Results of Analysis of Variance. ^B Data analysis was performed using the arcsine of the square root transformation in SAS. ^C Model was not significant at the $p < 0.05$ or $p < 0.1$ level. ^D Model was not significant at the $p < 0.05$ level, but was significant at the $p < 0.1$ level.



Figure 4.1. Treatment area for studies.



Figure 4.2. ^{14}C 2,4-D droplets of treatment solution on red clover leaf.

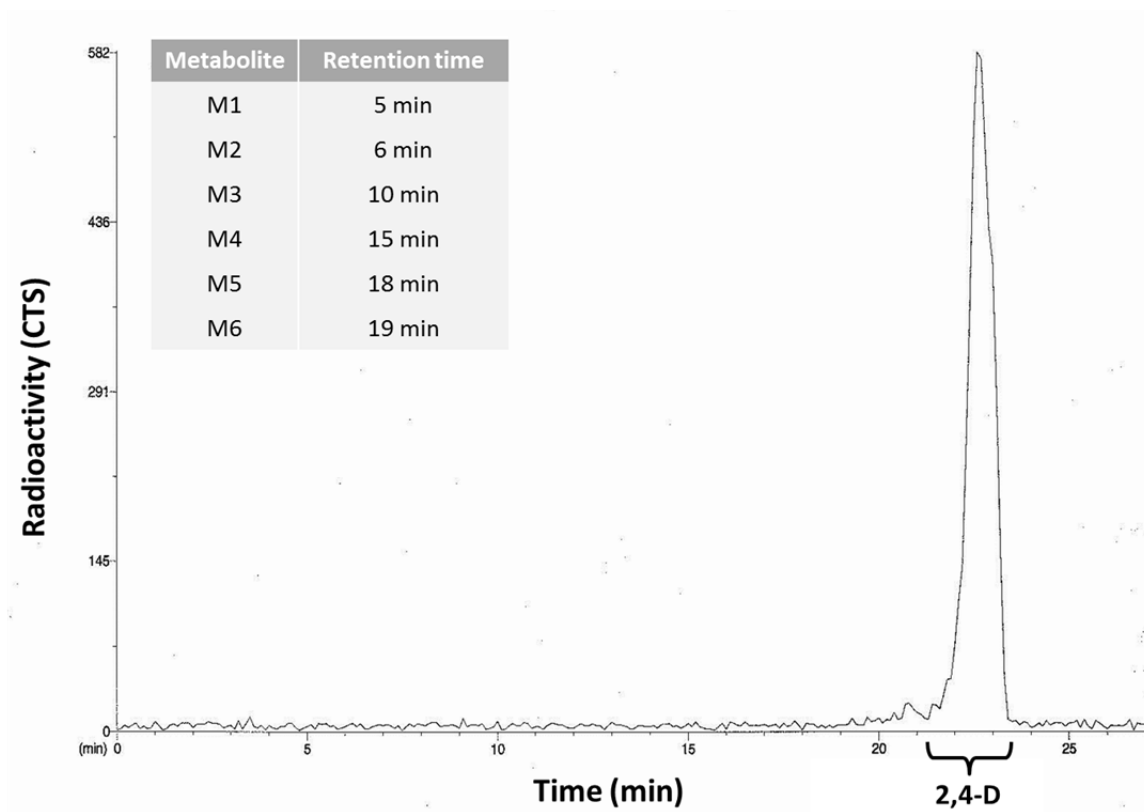


Figure 4.3. Sample radiochromatograph showing ^{14}C 2,4-D peak at 23 minutes and metabolite retention times.

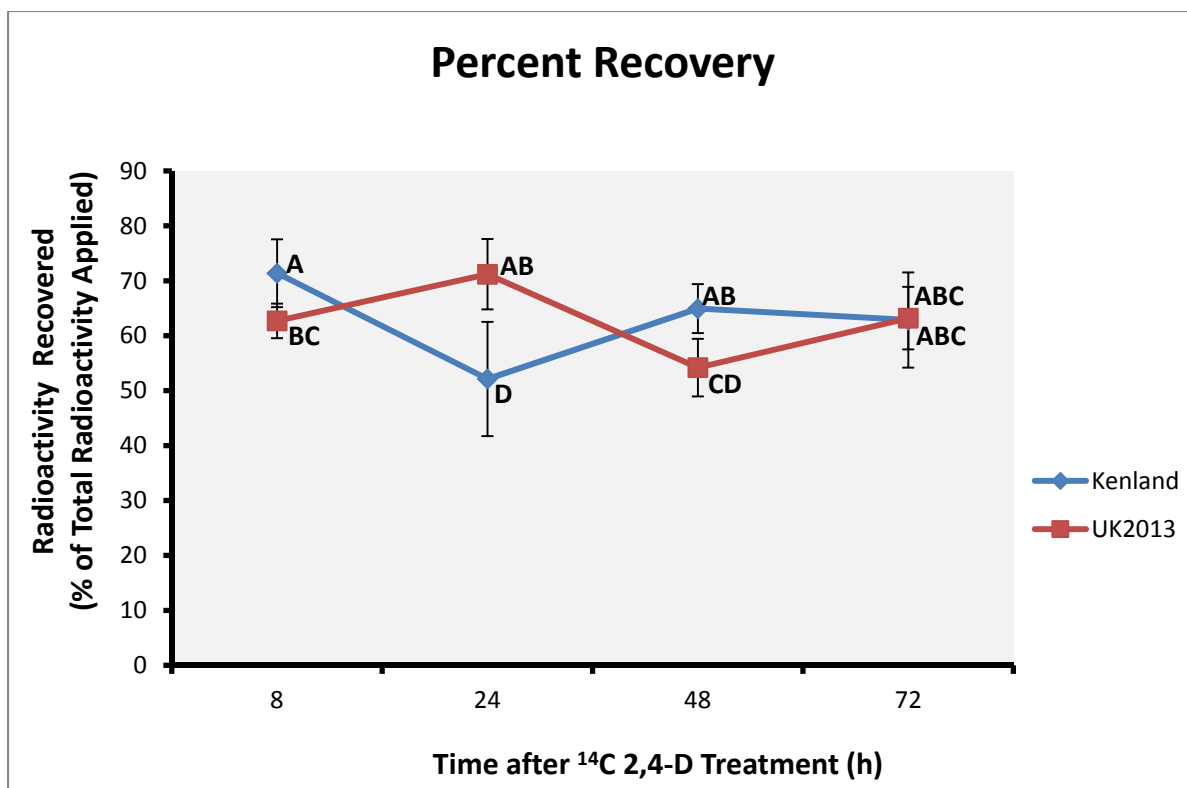


Figure 4.4. Radioactivity recovered from Kenland and UK2013 plants over time. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. Points with the same letter are not statistically different ($p < 0.05$).

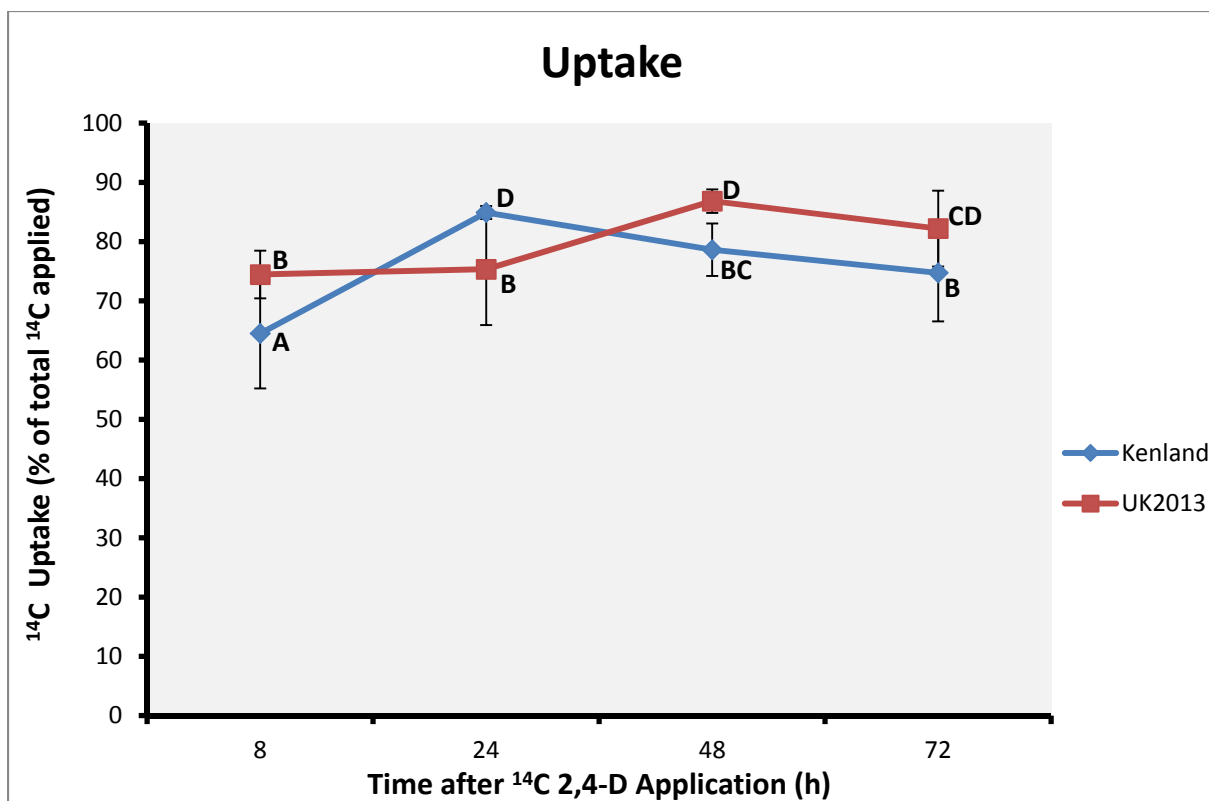


Figure 4.5. Uptake of ¹⁴C from ¹⁴C 2,4-D by Kenland and UK2013 plants over time. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. Points with the same letter are not statistically different ($p < 0.05$).

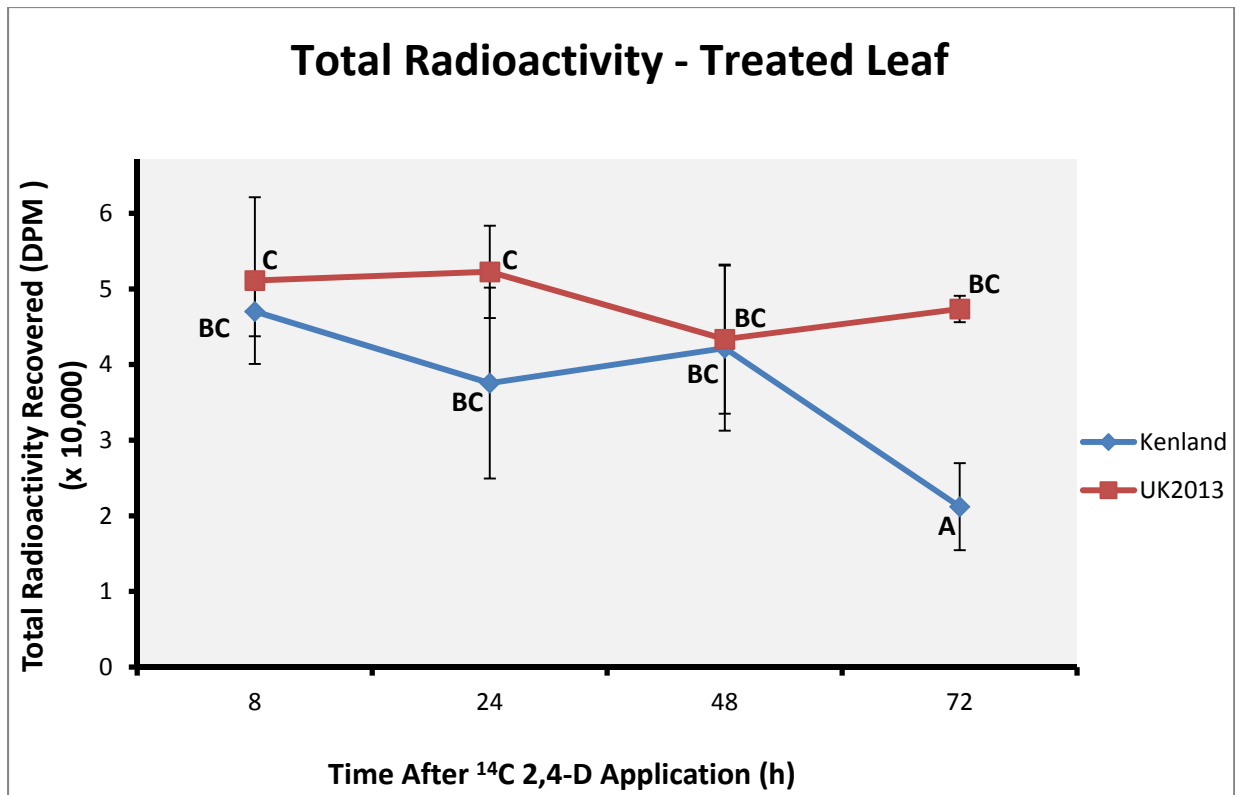


Figure 4.6. Total radioactivity in the treated leaf of Kenland and UK2013. Points with the same letter are not statistically different ($p < 0.05$).

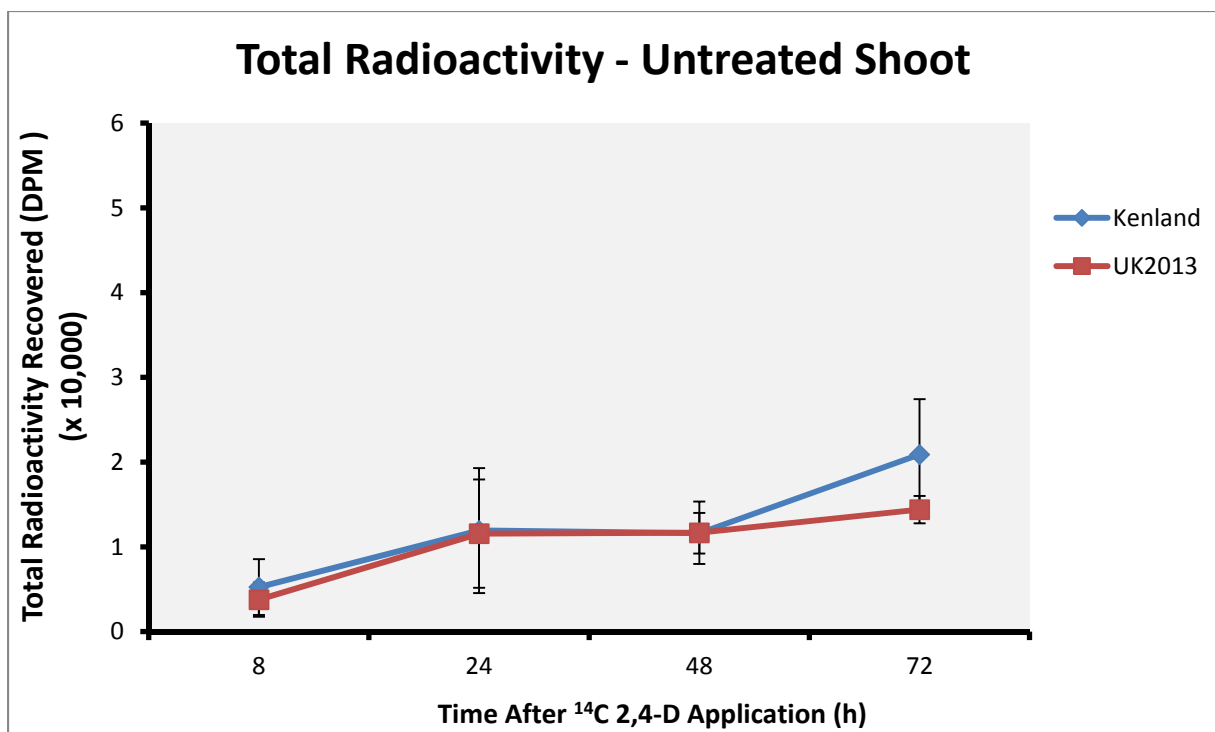


Figure 4.7. Total radioactivity in the untreated shoot of Kenland and UK2013. There is no significant interaction between clover type and harvest interval, but over clover types recovered radioactivity increased with time ($p < 0.05$).

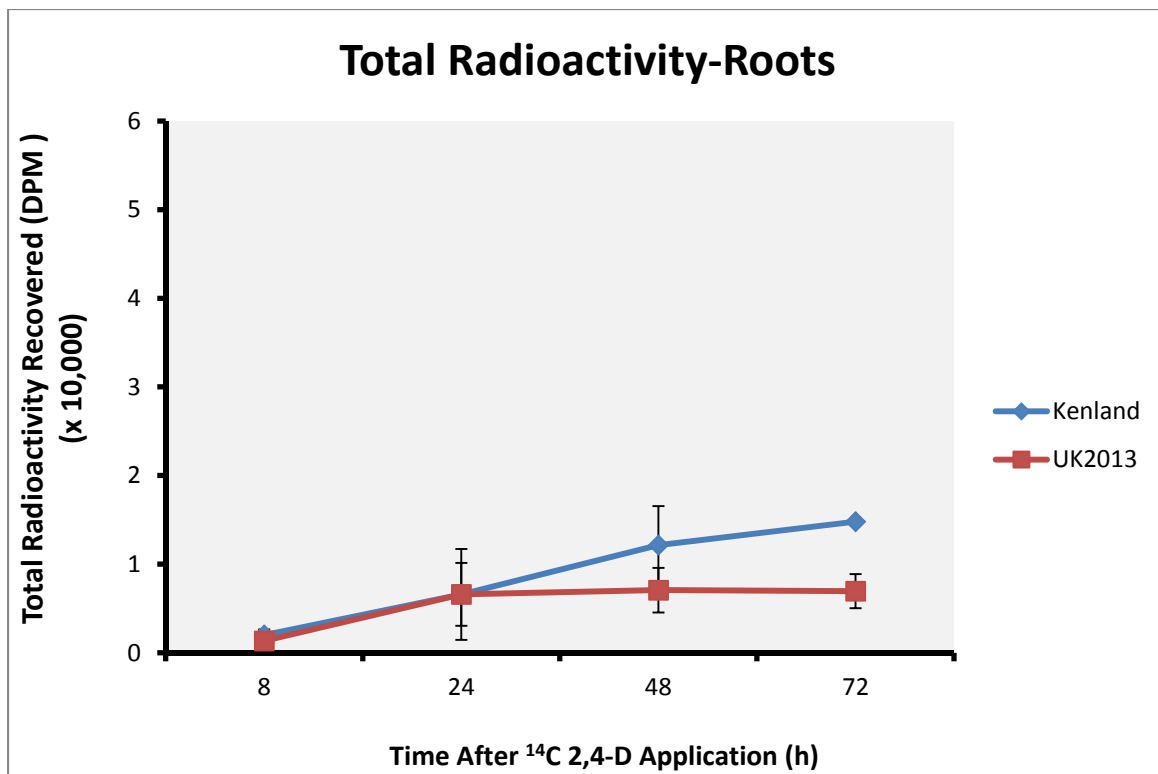


Figure 4.8. Total radioactivity in the root section of Kenland and UK2013. There is no interaction between clover types and harvest intervals, but over clover types, recovered radioactivity increased with time, and over harvest intervals Kenland has significantly more radioactivity compared to UK2013 ($p < 0.05$).

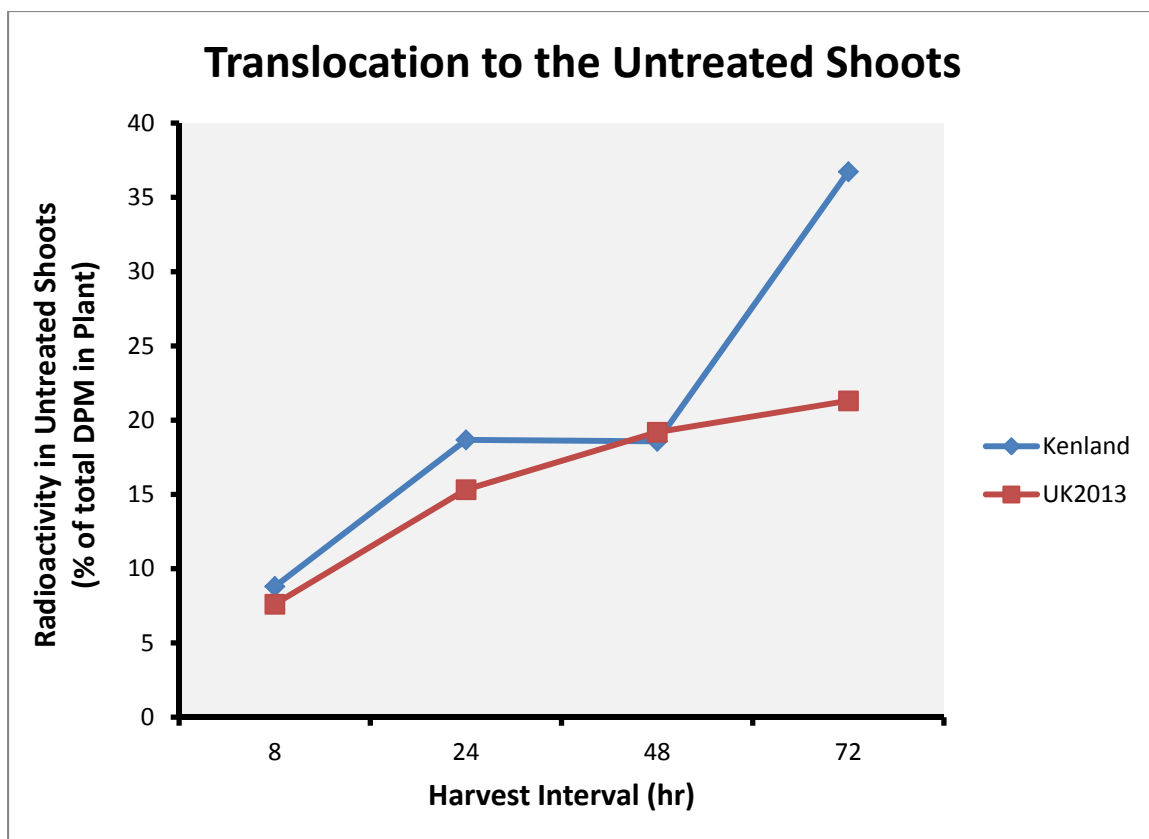


Figure 4.9. Translocation of ^{14}C from ^{14}C 2,4-D to the untreated shoots across 8 to 72 HAT. Data analysis was performed using the arcsine of the square root transformation in SAS. The values in here are back-transformed. There was no significant interaction between clover type and harvest interval but over clover types, translocation to the untreated shoots increased with time ($p < 0.05$).

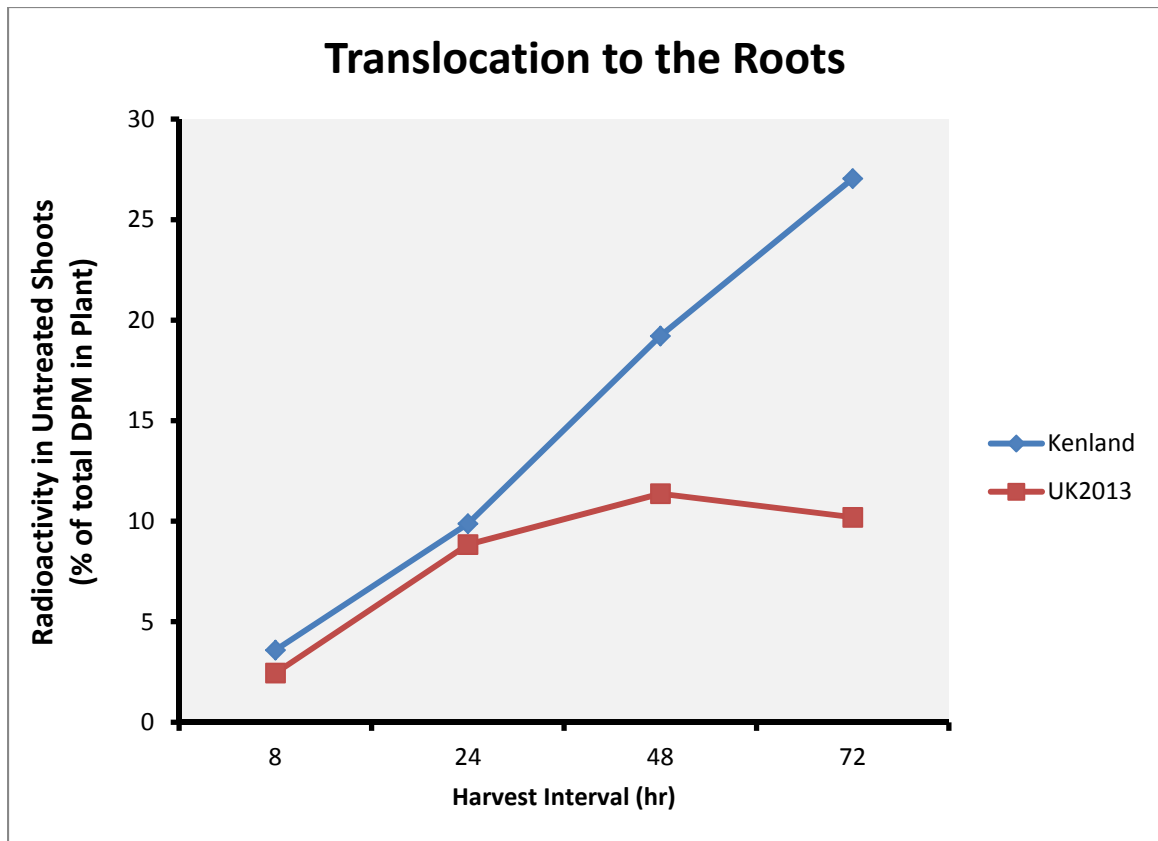


Figure 4.10. Translocation of ^{14}C from ^{14}C 2,4-D to the roots from 8 to 72 HAT. Data analysis was performed using the arcsine of the square root transformation in SAS. The values in here are back-transformed. There was no significant interaction between clover type and harvest interval, but over clover types there is a trend of increasing translocation over increasing time, and over harvest intervals Kenland has significantly more translocation to the roots than UK2013 ($p < 0.05$).

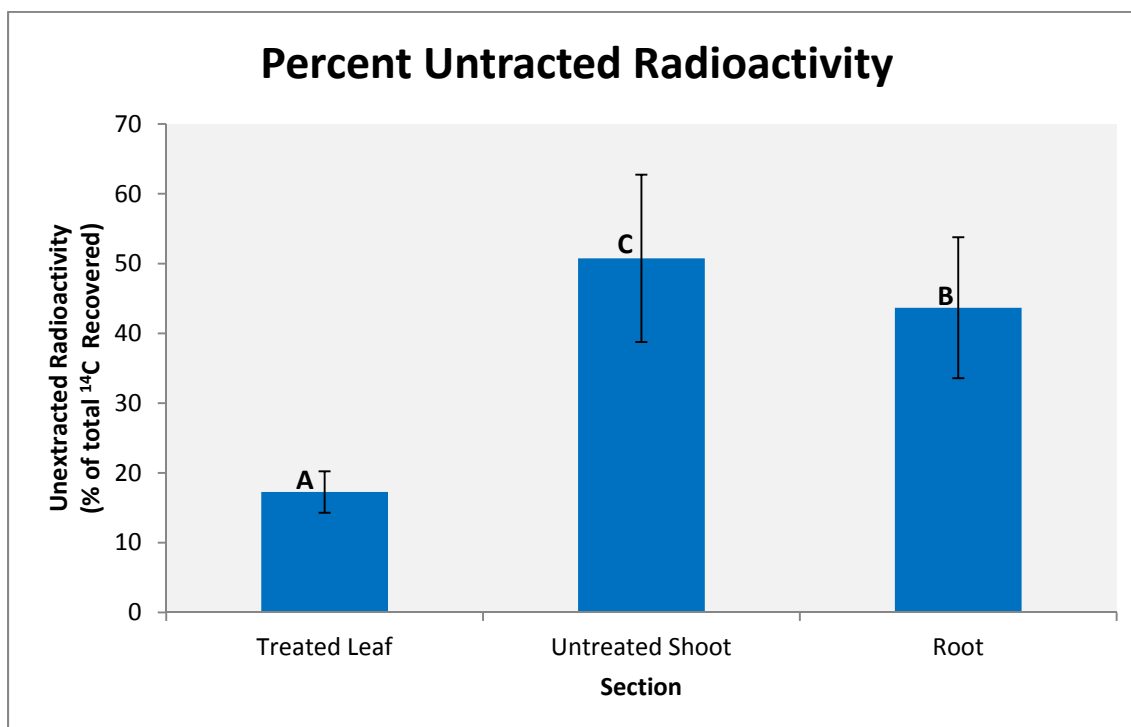


Figure 4.11. Radioactivity extracted from the treated leaf (TL), untreated shoots (US), and roots (R), over clover types and harvest intervals. Bars with the same letter are not significantly different ($p < 0.05$).

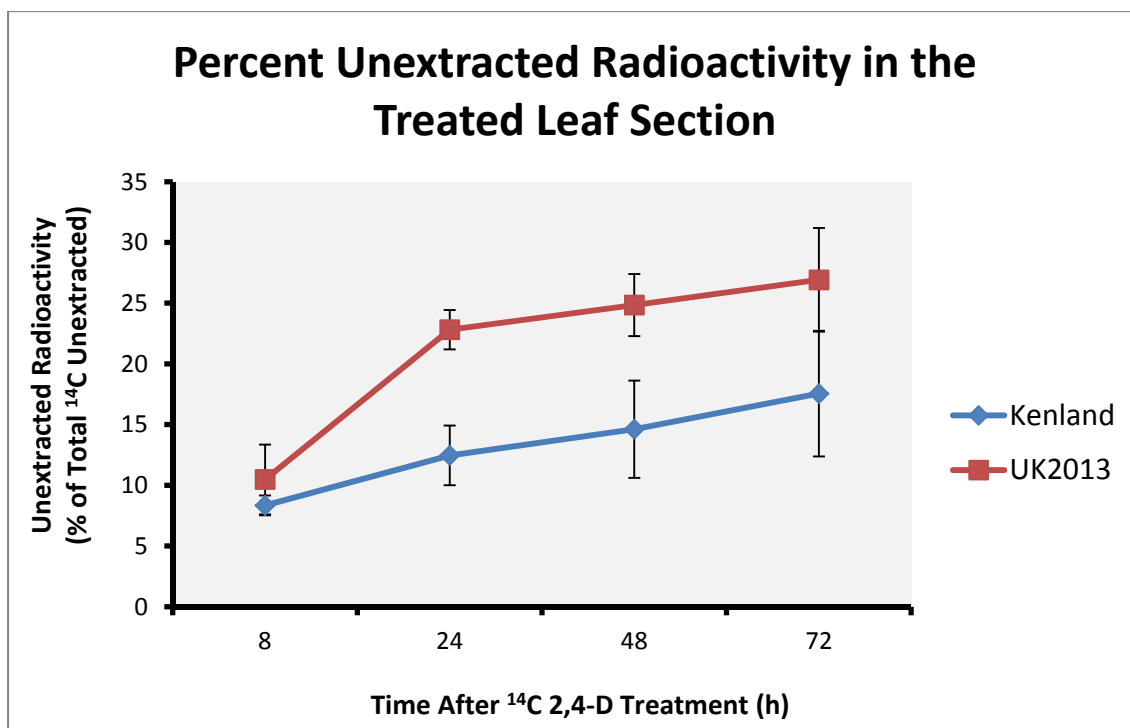


Figure 4.12. Radioactivity unextracted from Kenland and UK2013 at 8, 24, 48, and 72 HAT, in the treated leaf section. There was no significant interaction between clover type and harvest interval, but over harvest intervals Kenland has a lower percentage of unextracted radioactivity compared to UK2013, and over clover types 8 HAT has a lower percentage of radioactivity unextracted compared to all other harvest intervals ($p < 0.05$).

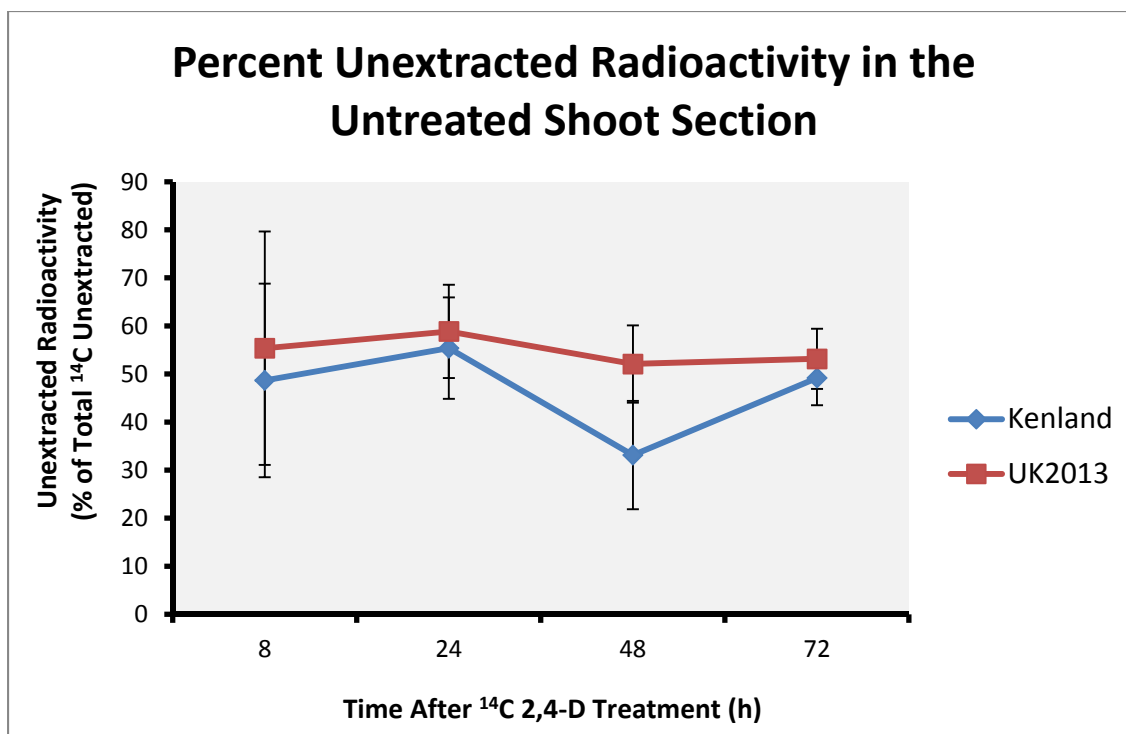


Figure 4.13. Radioactivity extracted from Kenland and UK2013 at 8, 24, 48, and 72 HAT, in the treated leaf section. There was no significant interaction between clover type and harvest interval, and the main effects of clover type and harvest interval are also not significant ($p < 0.05$).

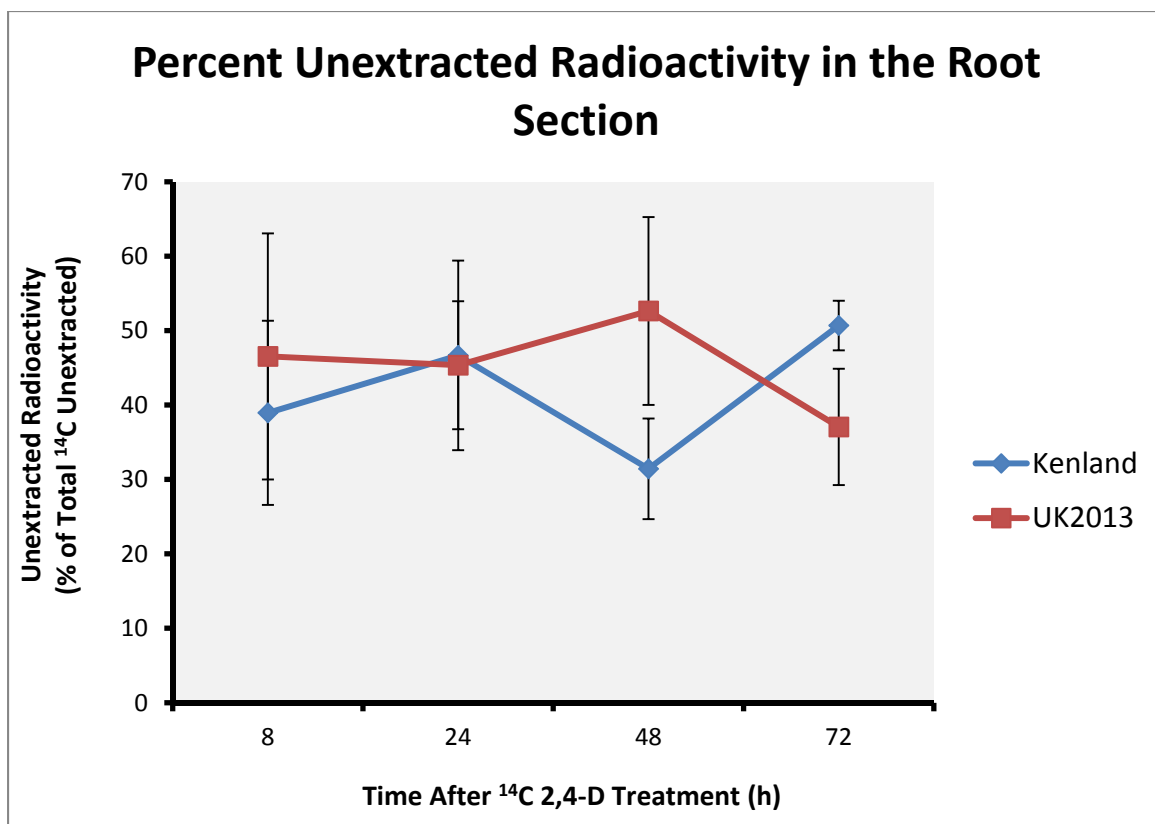


Figure 4.14. Radioactivity extracted from Kenland and UK2013 at 8, 24, 48, and 72 HAT, in the treated leaf section. There was no significant interaction between clover type and harvest interval, and the main effects of clover type and harvest interval are also not significant ($p < 0.05$).

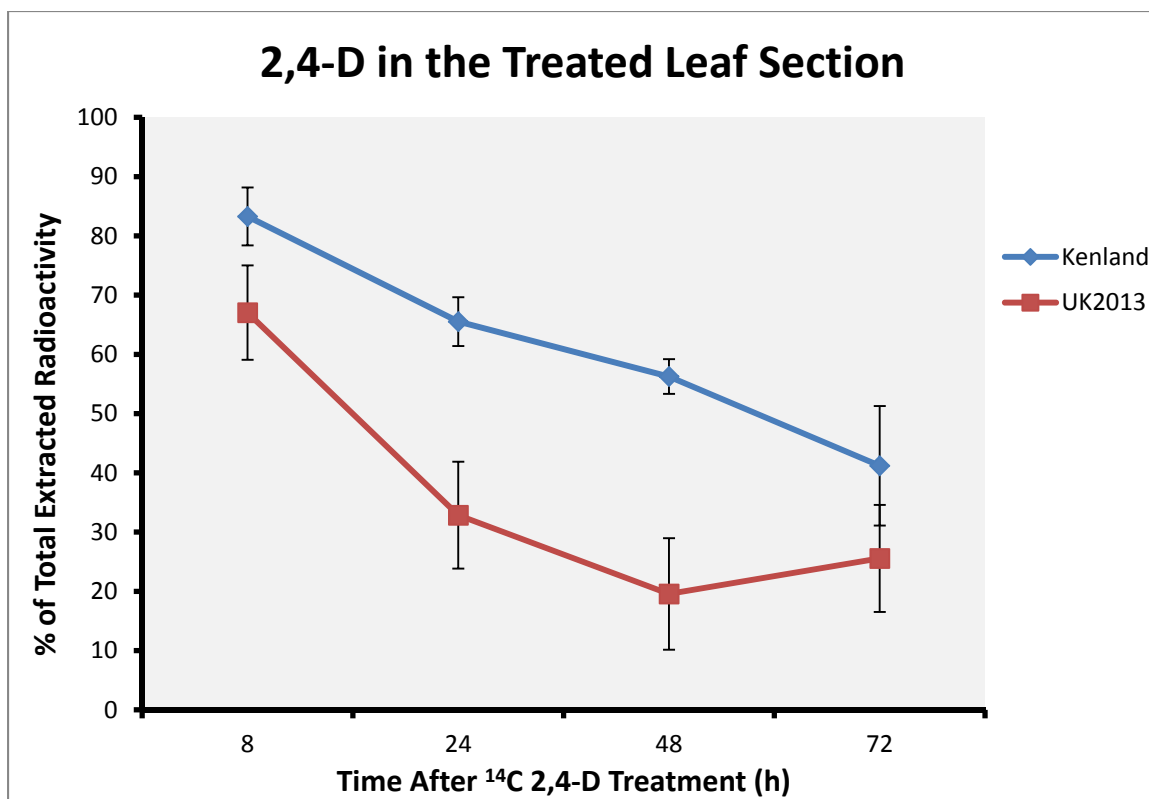


Figure 4.15. Metabolism of ¹⁴C from ¹⁴C 2,4-D: 2,4-D remaining in Kenland and UK2013 treated leaf sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. There is no significant interaction between clover type and harvest interval, but over harvest intervals the treated leaf of UK2013 contains less 2,4-D compared to Kenland, and over clover types the treated leaf at 8 HAT has more 2,4-D compared to all other harvest intervals, and the treated leaf at 24 HAT contains more 2,4-D than at 72 HAT ($p < 0.05$).

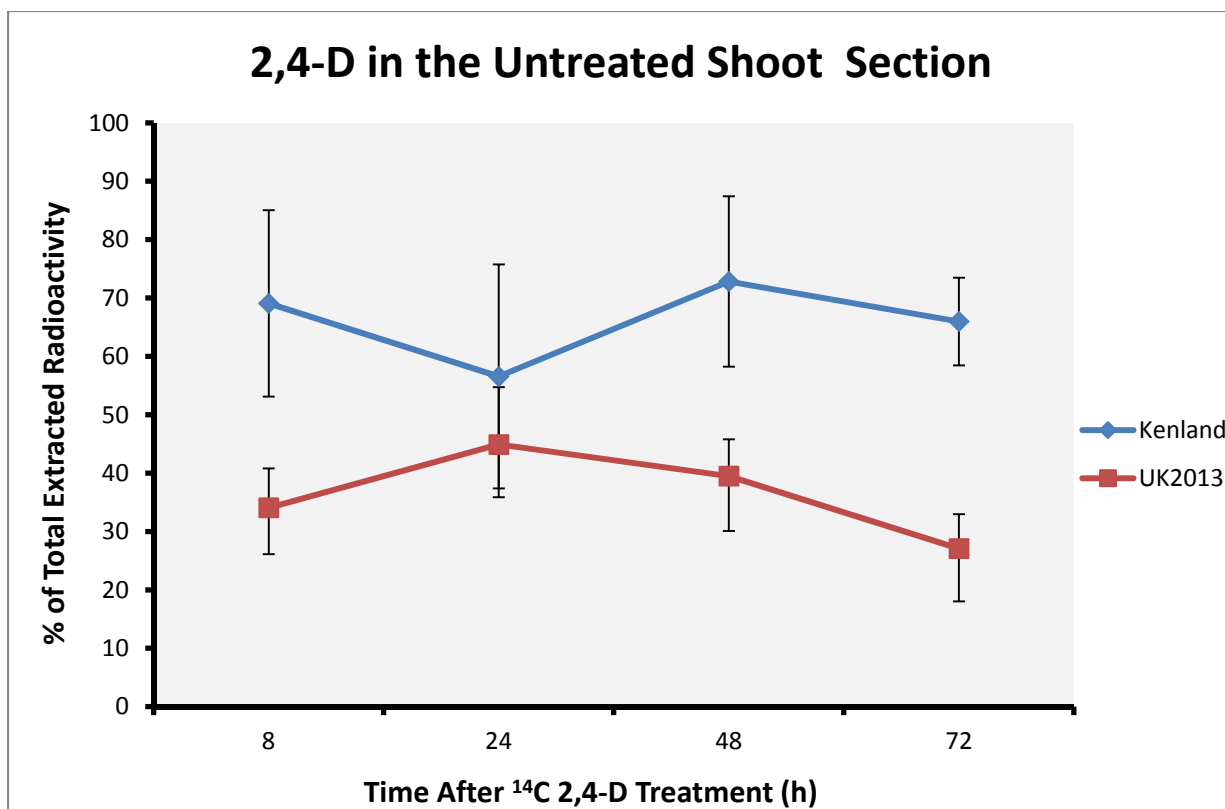


Figure 4.16. Metabolism of ¹⁴C from ¹⁴C 2,4-D: 2,4-D remaining in Kenland and UK2013 untreated shoot sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. The model for this comparison is not significant ($p < 0.05$).

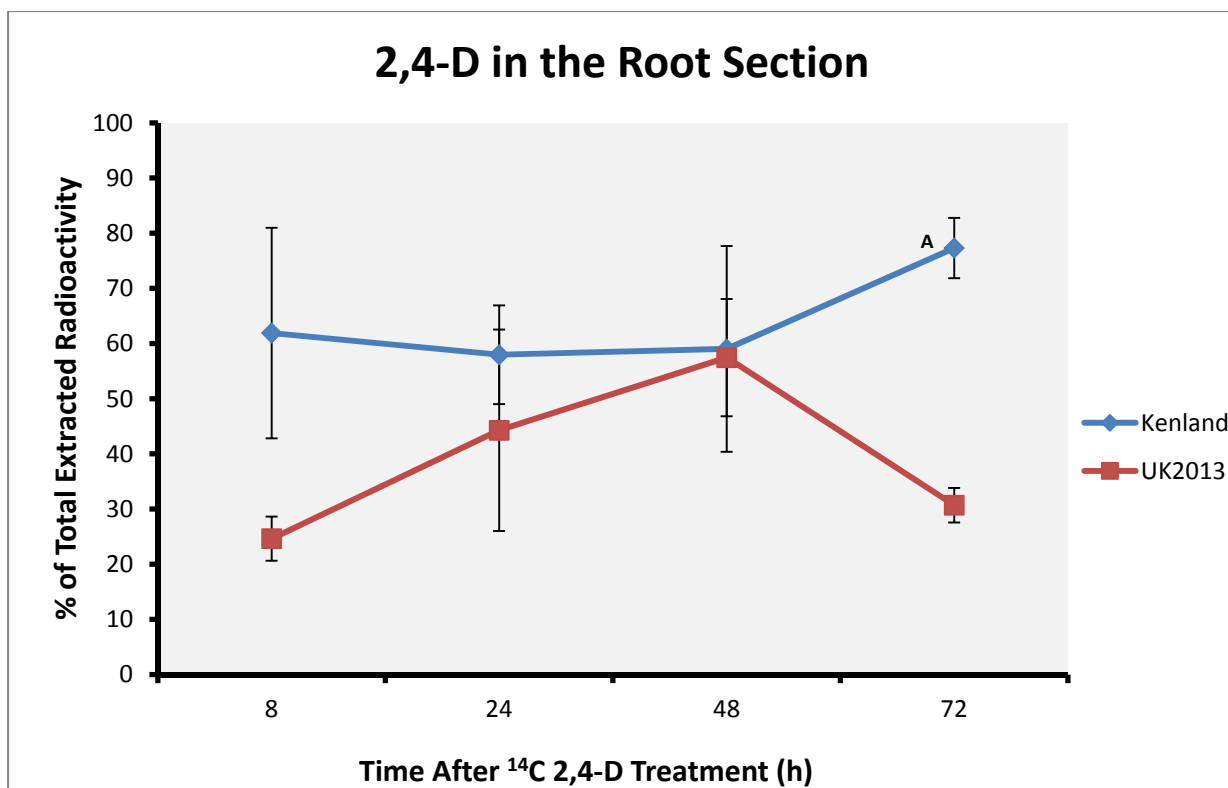


Figure 4.17. Metabolism of ¹⁴C from ¹⁴C 2,4-D: 2,4-D remaining in Kenland and UK2013 root sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. There is no significant interaction between clover type and harvest interval, but over harvest intervals the roots of UK2013 contain less 2,4-D than the roots of Kenland ($p < 0.05$).

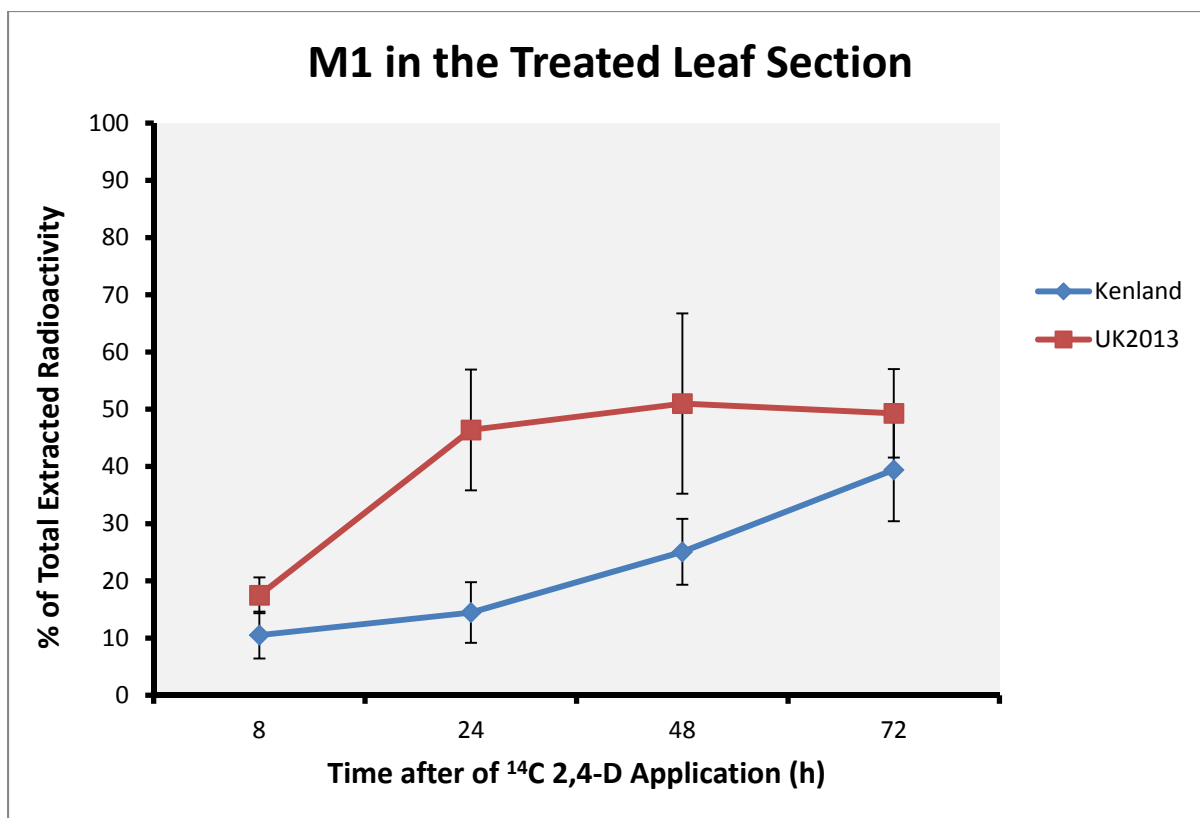


Figure 4.18. Metabolism of ¹⁴C from ¹⁴C 2,4-D: Metabolite 1 in Kenland and UK2013 treated leaf sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. There is no significant interaction between clover type and harvest interval, but over harvest intervals the treated leaf of Kenland contains less M1 than the treated leaf of UK2013, and over clover types the treated leaf at 8 HAT has more 2,4-D compared to all other harvest intervals, and the treated leaf at 24 HAT contains more 2,4-D than at 72 HAT ($p < 0.05$).

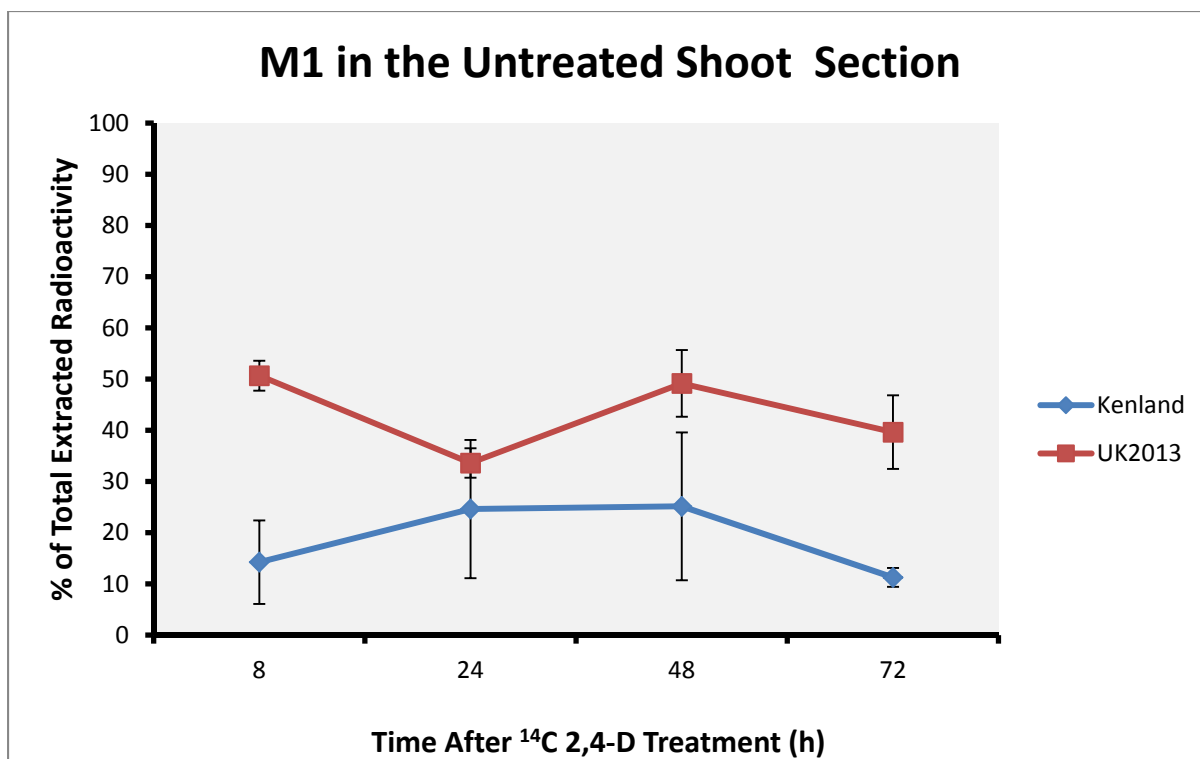


Figure 4.19. Metabolism of ¹⁴C from ¹⁴C 2,4-D: Metabolite 1 in Kenland and UK2013 untreated shoot sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. The model for this comparison is not significant ($p < 0.05$).

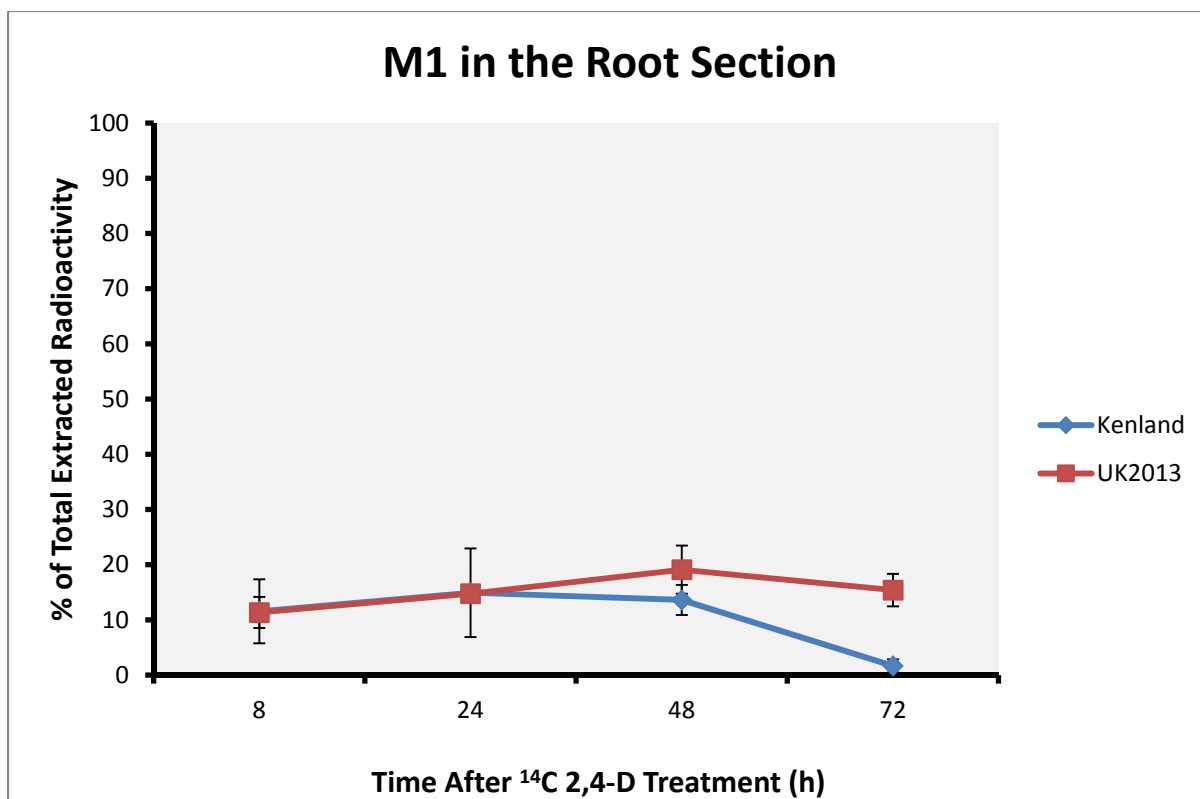


Figure 4.20. Metabolism of ¹⁴C from ¹⁴C 2,4-D: Metabolite 1 in Kenland and UK2013 root sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. The model for this comparison is not significant ($p < 0.05$).

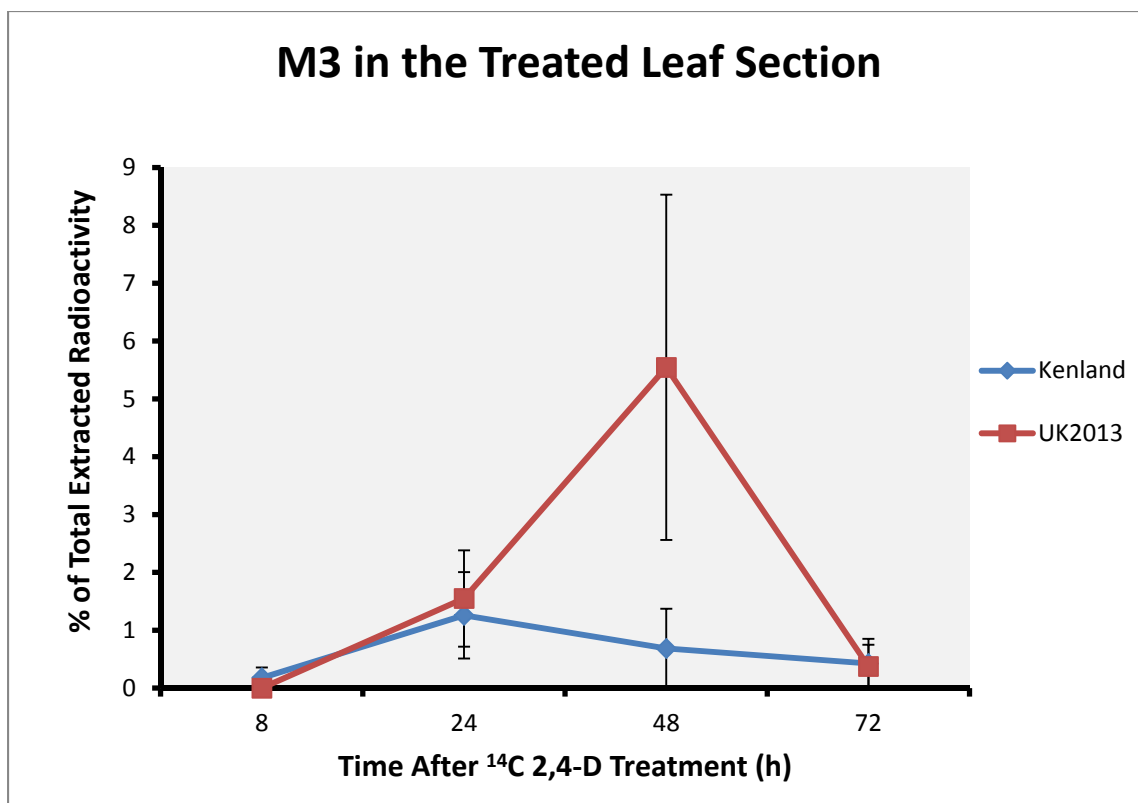


Figure 4.21. Metabolism of ¹⁴C from ¹⁴C 2,4-D: Metabolite 3 in Kenland and UK2013 treated leaf sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. There is no significant interaction between clover type and harvest interval, but over clover types, the treated leaf at 48 HAT contains more M3 than at both 8 and 72 HAT ($p < 0.05$).

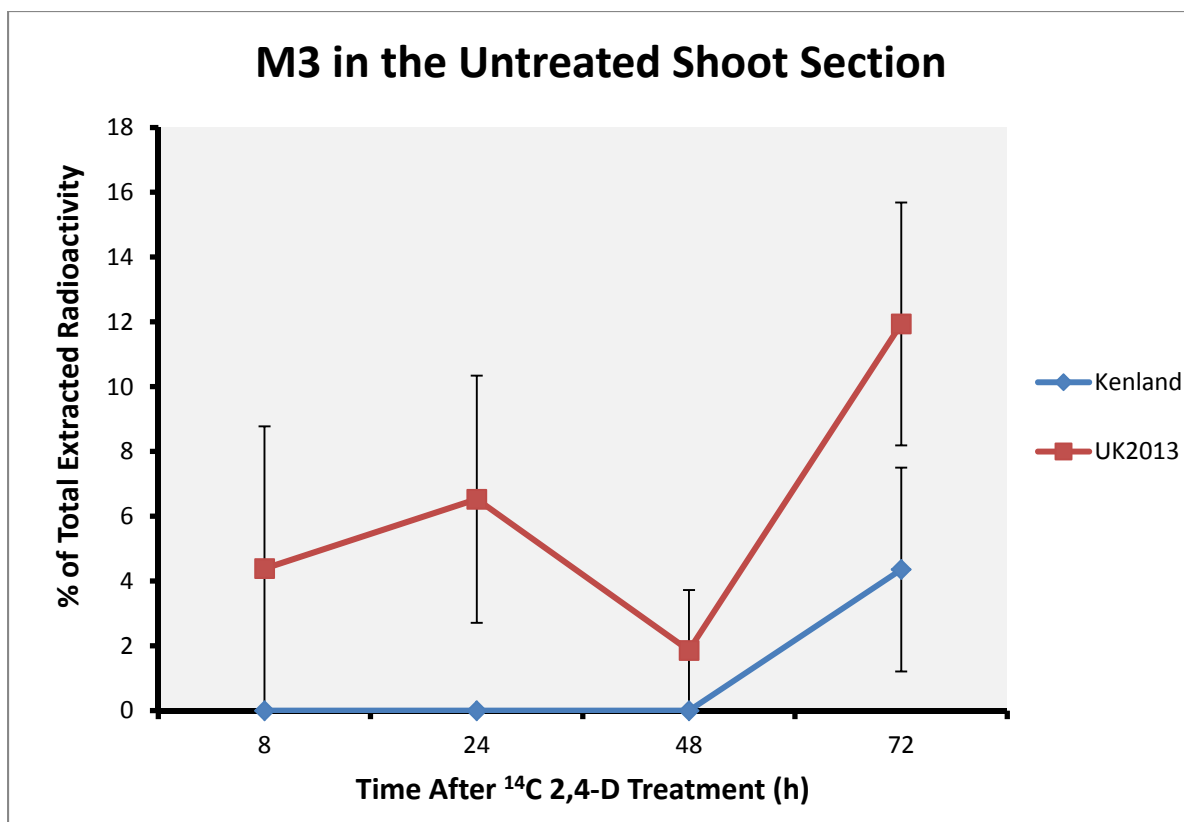


Figure 4.22. Metabolism of ¹⁴C from ¹⁴C 2,4-D: Metabolite 3 in Kenland and UK2013 untreated shoot sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. There is no significant interaction between clover type and harvest interval, but over harvest intervals the untreated shoot of Kenland contains less M3 compared to the untreated shoot of UK2013, and over clover types the untreated shoot at 72 HAT contains more M3 compared to all other harvest intervals ($p < 0.05$).

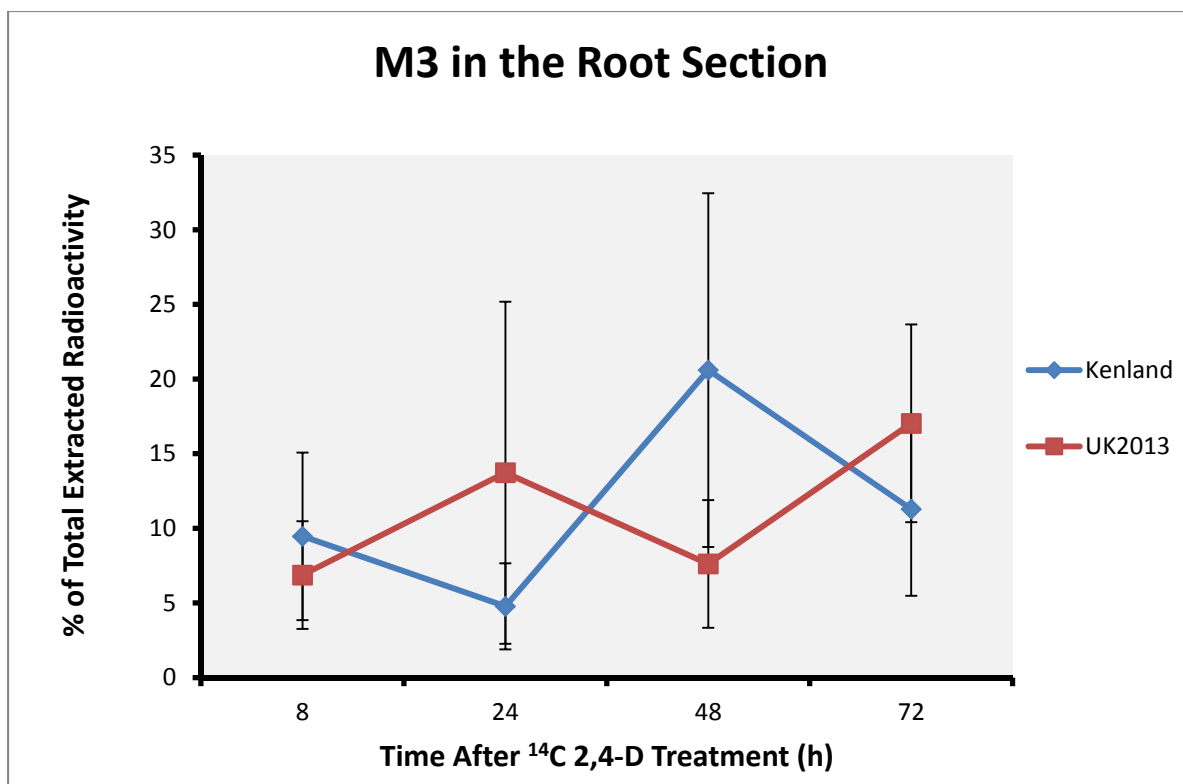


Figure 4.23. Metabolism of ¹⁴C from ¹⁴C 2,4-D: Metabolite 3 in Kenland and UK2013 root sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. The model for this comparison is not significant ($p < 0.05$).

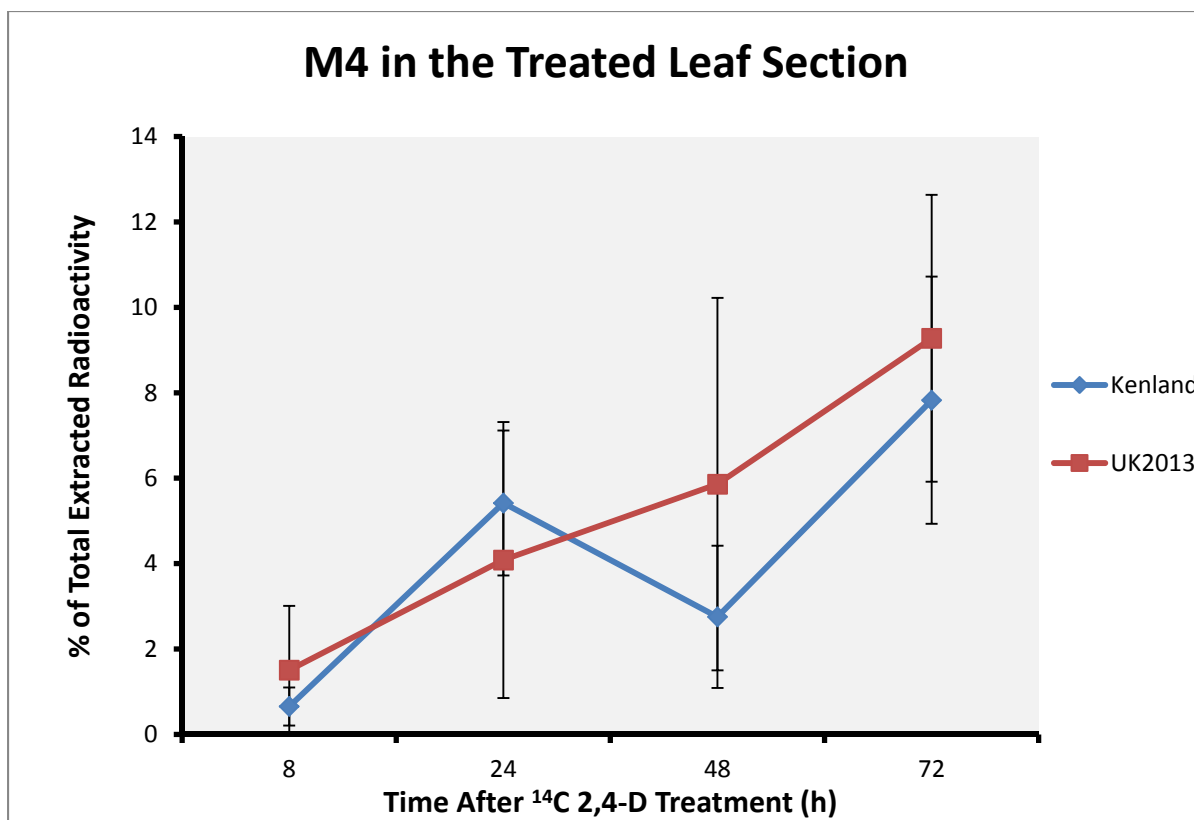


Figure 4.24. Metabolism of ¹⁴C from ¹⁴C 2,4-D: Metabolite 4 in Kenland and UK2013 treated leaf sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. There is no significant interaction between clover type and harvest interval, but over clover types, the treated leaf at 8 HAT contains less M4 compared with at 24 and 72 HAT, and the treated leaf at 72 HAT also contains more M4 compared with at 48 HAT ($p < 0.05$).

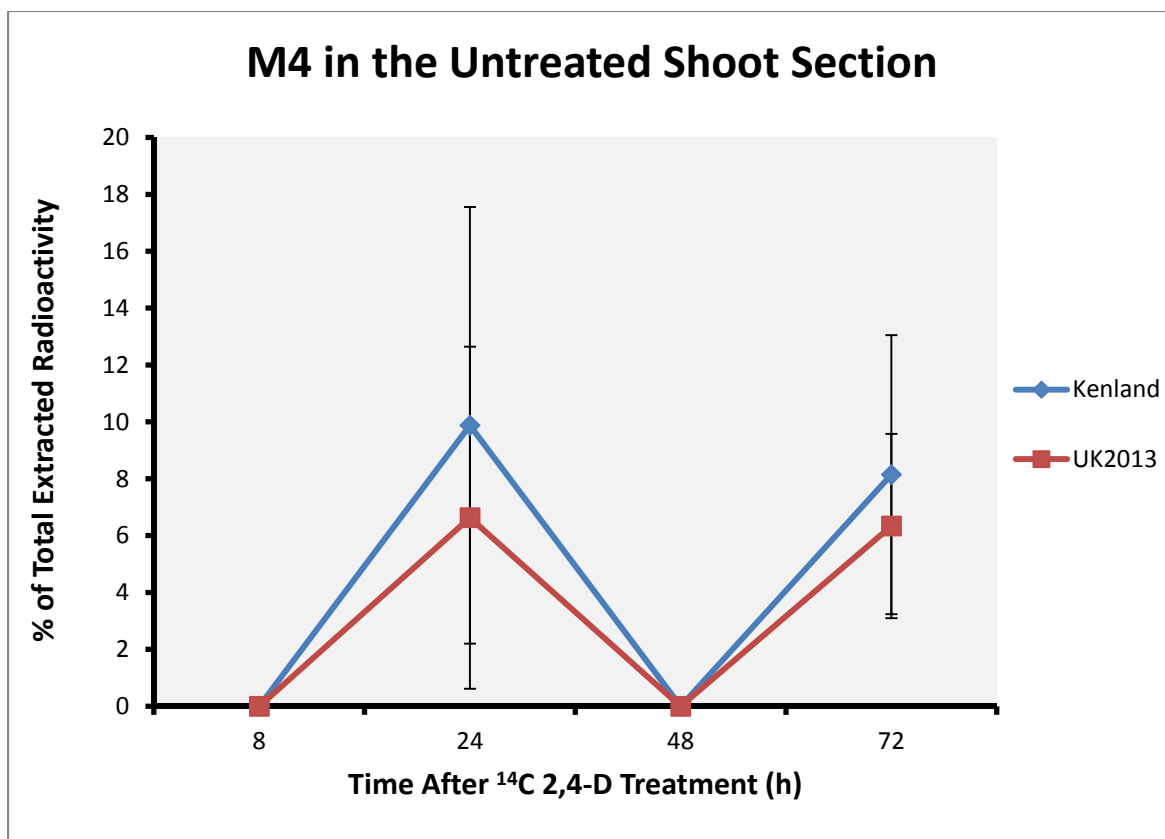


Figure 4.25. Metabolism of ¹⁴C from ¹⁴C 2,4-D: Metabolite 4 in Kenland and UK2013 untreated shoot sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. There is no significant interaction between clover type and harvest interval, but over clover types, the untreated shoot at 8 HAT contains less M4 compared with at 24 and 72 HAT, the untreated shoot at 24 and 72 HAT also contain more M4 compared with at 48 HAT ($p < 0.05$).

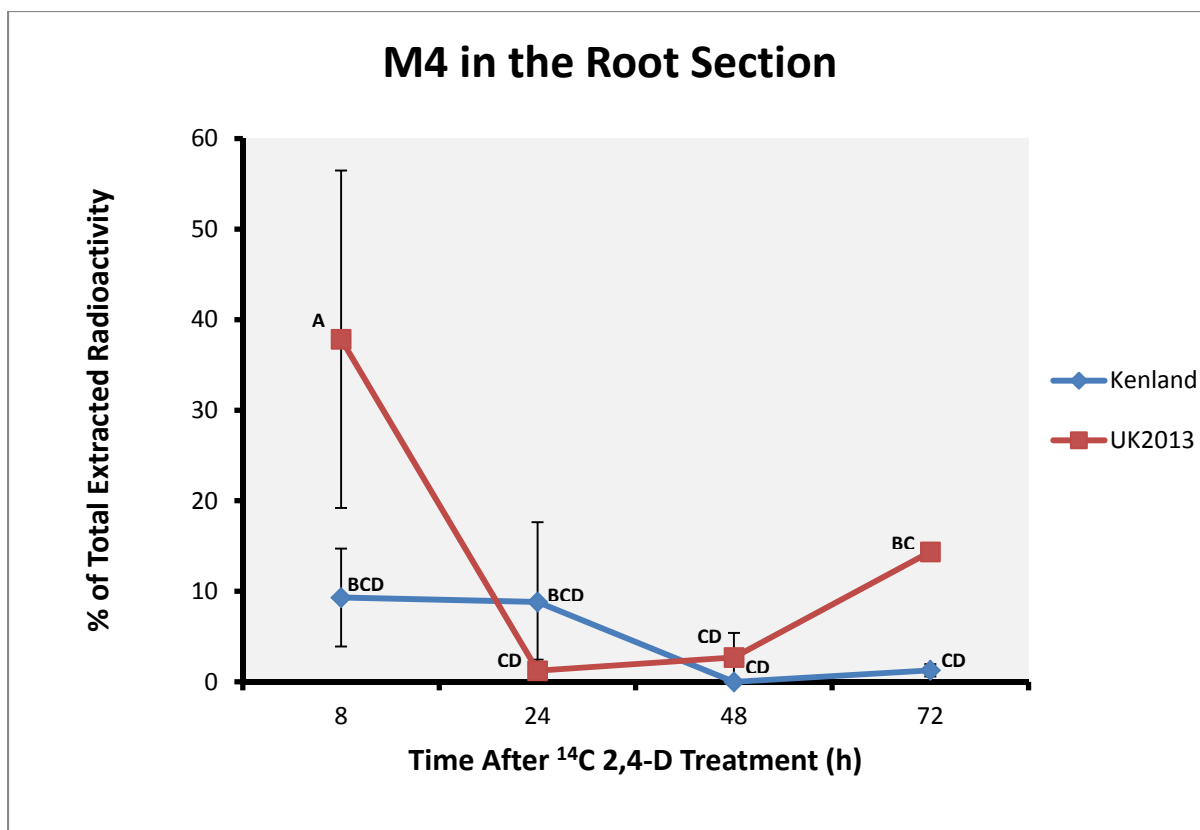


Figure 4.26. Metabolism of ¹⁴C from ¹⁴C 2,4-D: Metabolite 4 in Kenland and UK2013 root sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. Points with the same letter are not statistically different ($p < 0.05$).

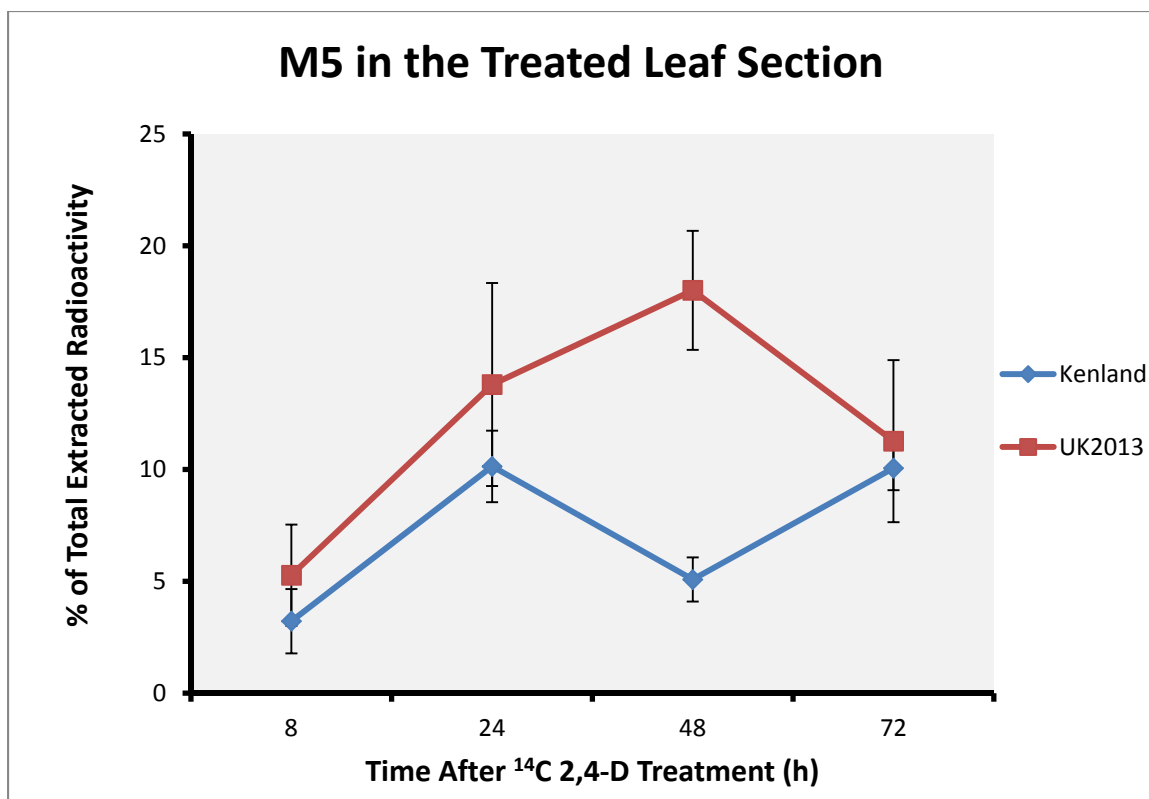


Figure 4.27. Metabolism of ¹⁴C from ¹⁴C 2,4-D: Metabolite 5 in Kenland and UK2013 treated leaf sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. There is no significant interaction between clover type and harvest interval, but over harvest intervals the treated leaf section of Kenland contains less M5 compared to the treated leaf section of UK2013, and over clover types the treated leaf at 8 HAT contains less M5 than at all other harvest intervals ($p < 0.05$).

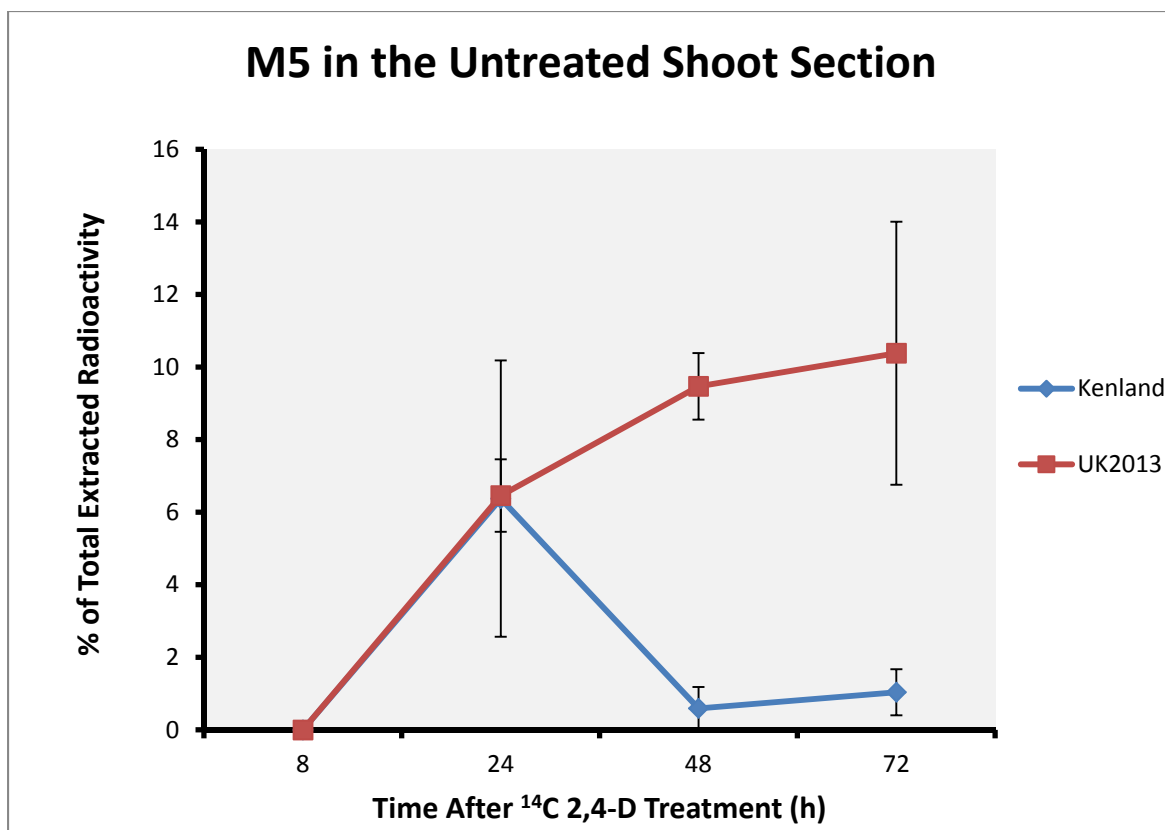


Figure 4.28. Metabolism of ¹⁴C from ¹⁴C 2,4-D: Metabolite 5 in Kenland and UK2013 untreated shoot sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. The model for this comparison is not significant ($p < 0.05$).

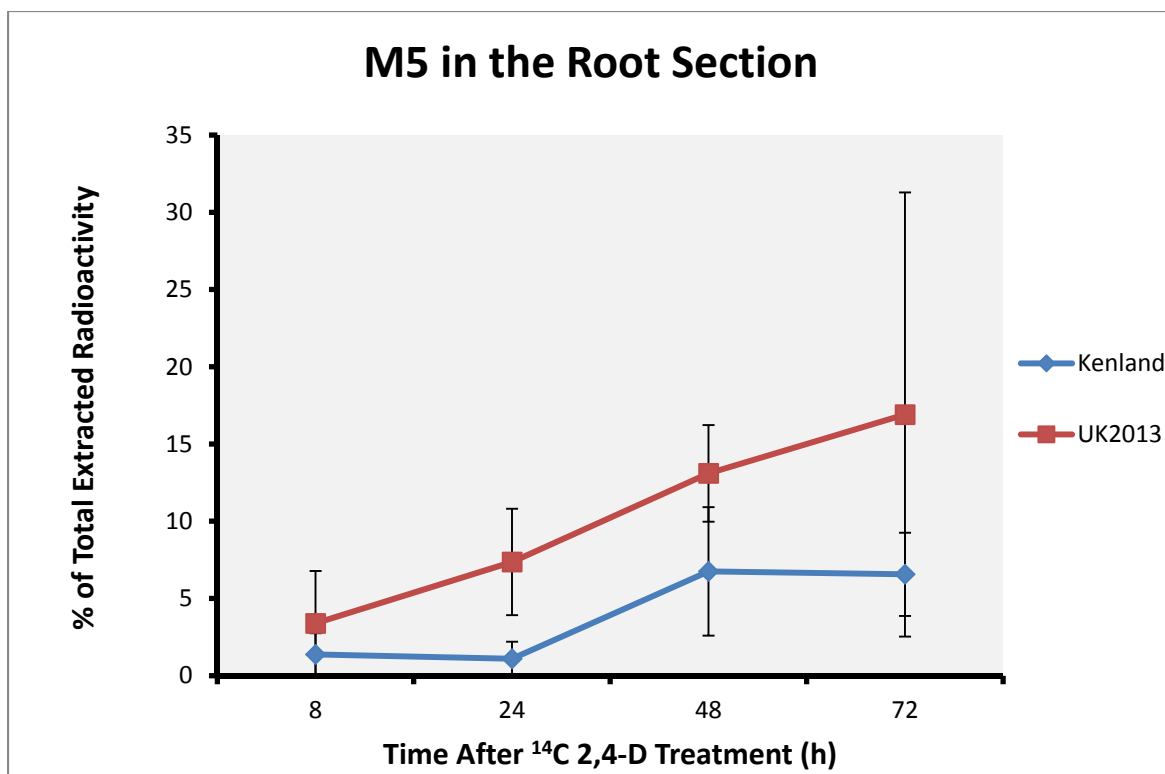


Figure 4.29. Metabolism of ^{14}C from ^{14}C 2,4-D: Metabolite 5 in Kenland and UK2013 root sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. The model for this comparison is not significant ($p < 0.05$).

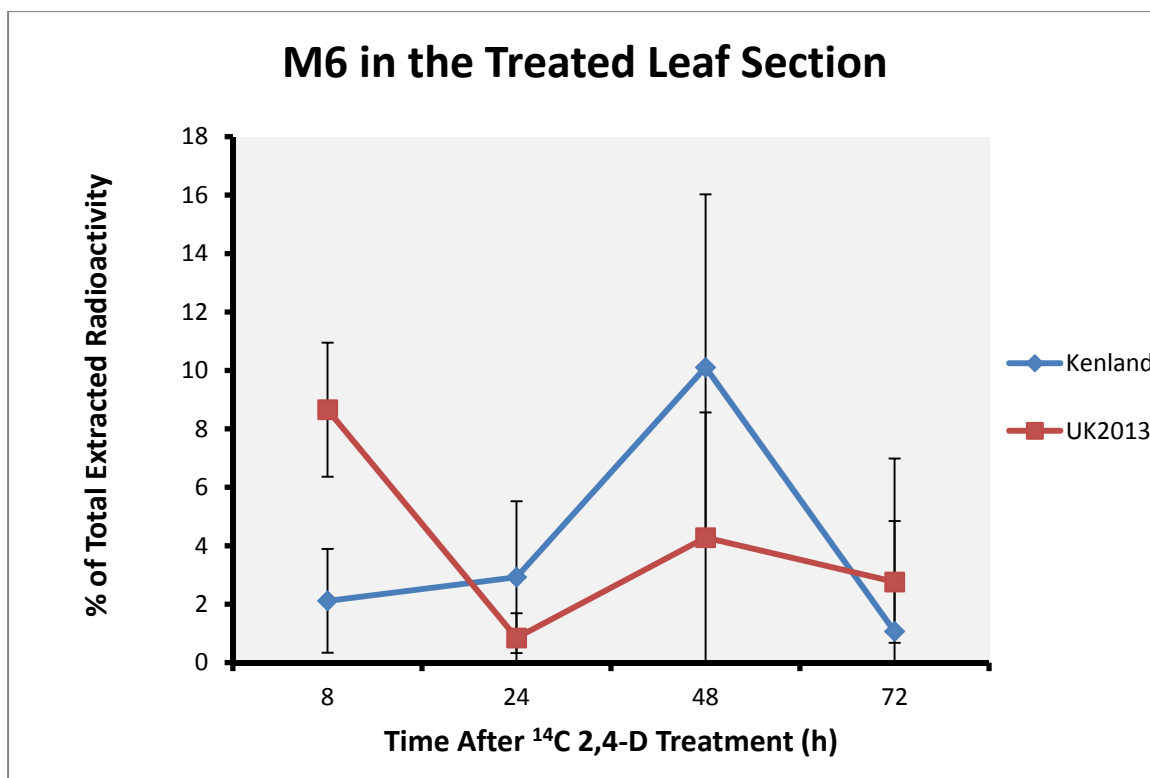


Figure 4.30. Metabolism of ¹⁴C from ¹⁴C 2,4-D: Metabolite 6 in Kenland and UK2013 treated leaf sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. The model for this comparison is not significant ($p < 0.05$).

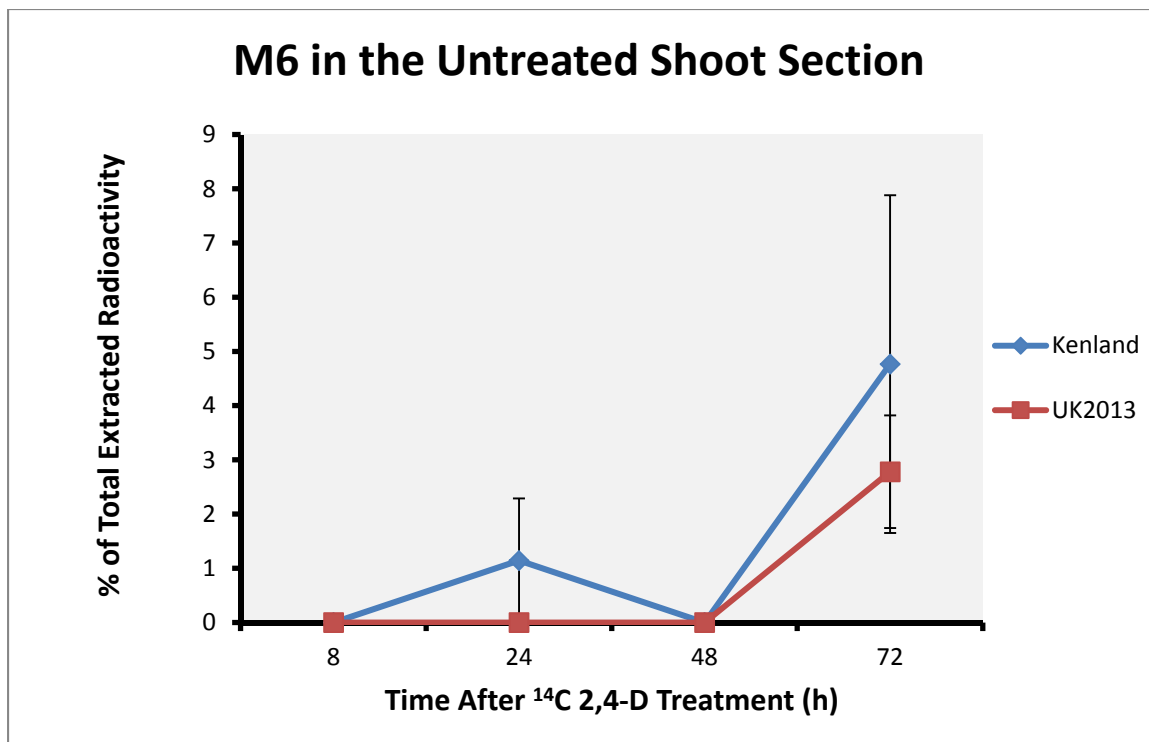


Figure 4.31. Metabolism of ¹⁴C from ¹⁴C 2,4-D: Metabolite 6 in Kenland and UK2013 untreated shoot sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. There is no significant interaction between clover type and harvest interval, but over clover types the untreated shoot section at 72 HAT contains more M6 than at all other harvest intervals ($p < 0.05$).

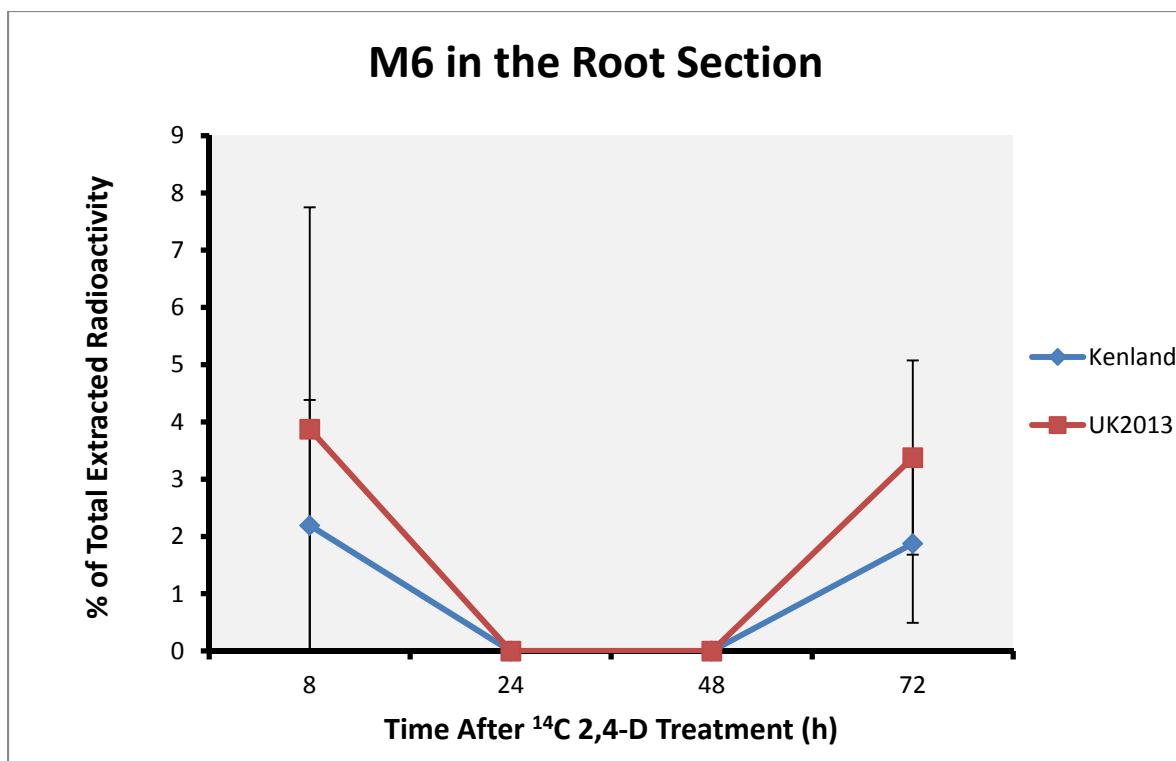


Figure 4.32. Metabolism of ¹⁴C from ¹⁴C 2,4-D: Metabolite 6 in Kenland and UK2013 root sections. Data analysis was performed using the arcsine of the square root transformation in SAS. The values here are back-transformed. The model for this comparison is not significant ($p < 0.05$).

Chapter Five

Conclusions

Red clover is a highly valuable forage crop that could benefit greatly by adding herbicide tolerance to its list of useful qualities. 2,4-D is one of the most common herbicides used worldwide to manage broadleaf weeds in various situations, including pastures, and increased tolerance to this herbicide in red clover would be useful.

Breeding work done by Dr. Norman Taylor to this objective laid the groundwork for this thesis.

The recurrent, field based, polycross selection for 2,4-D tolerance initiated by Dr. Taylor, was continued but included, starting in 2012, removal of plants which had visible damage from 2,4-D. Plants grown from seed from the 2012 and 2013 polycross recurrent selections had improved 2,4-D tolerance. In 2013, the rate of 2,4-D applied was increased to double the recommended use rate for pastures. Also initiated in 2013 was the use of a second year polycross plot, using 70 of the most tolerant, overwintered clover plants from the 2012 polycross recurrent selection field. These plants were tested for tolerance levels in the spring of 2013 by applying 2.24 kg ha^{-1} 2,4-D at double the recommended use rate for pastures and the more tolerant plants were transplanted and allowed to intercross, separate from the polycross recurrent selection field for 2013. After one year, the plants grown from seed harvested from the second year polycross plot did not have higher 2,4-D tolerance than those from the 2013 polycross recurrent selection, although both populations had improvements from the selection of the previous year. It is possible that, had a larger population been used as the basis for this second year polycross plot, more significant gains could have been made. Also, after more than

one year of selection using this method, increased gains could have occurred above that of the polycross recurrent selection field but, after only one year, the second year polycross method was not superior to the polycross recurrent selection method for this breeding population. Overall, the gains in 2,4-D tolerance made in this red clover population are sufficient to proceed with cultivar development trials. In greenhouse studies, visual injury was found to be far superior compared to both fresh and dry weight for evaluating red clover 2,4-D tolerance. In future work, injury ratings are the preferred evaluation method to combine with other methods, such as percent survival and top growth harvest on the population scale. However, any new metrics to be utilized on the population scale will necessitate new methodology.

Forage quality must be maintained throughout the process of selecting for 2,4-D tolerance in red clover. To ensure this, forage quality and yield were compared between the popular and high-performing Kenland red clover cultivar and the 2,4-D tolerant red clover from the 2013 polycross recurrent selection. UK2013 was found to have comparable acid digestible fiber and crude protein levels to Kenland. UK2013 had higher neutral digestible fiber content and yield than Kenland. Selecting for 2,4-D tolerance in red clover has evidently not come at the price of reduced quality or yield. These are promising results, but should be confirmed through forage quality and yield testing performed on plants grown in the field.

Understanding the basis for 2,4-D tolerance in the red clover being developed at UK could give insight into the genetic control of this trait and help in its further improvement. This understanding would also contribute to our current understanding of 2,4-D which, despite being one of the oldest selective herbicides, still is relatively

mysterious in terms of its mechanism and site of action. Numerous studies have been performed to determine how tolerance to 2,4-D in various plant species occurs. Each new method of tolerance discovered, as well as each new plant species whose tolerance mechanism is determined, allows for a more complete understanding of this fascinating herbicide. 2,4-D is one of the most commonly used herbicides in the world and, thus, a complete understanding of it can better inform its safe and effective use. Examining the behavior of 2,4-D in the sensitive Kenland compared to a more tolerant red clover (UK2013) developed from a two parent cross of Kenland to a 2,4-D tolerant red clover gave a more complete view of what has changed from the Kenland background to produce the 2,4-D tolerance.

Experiments were performed to evaluate the potential contributions of differential 2,4-D uptake, translocation, and metabolism to the tolerance difference between Kenland and UK2013. Immobilization of ^{14}C from radioactive 2,4-D was found to occur in different amounts depending on the section harvested, and in the treated leaf section, where immobilization was lowest, it was higher in UK2013 compared to Kenland. Also, the amount of radioactivity recovered varied over time and between clover types, with more recovered from Kenland at two time points and more from UK2013 for one time point. Low levels of recovery indicate radioactive compounds were lost from the plant, possibly either through volatilization or excretion from the roots, and this occurred more in UK2013. Future studies which measure volatilization of ^{14}C and loss of ^{14}C as root exudates would be required to determine by which mechanism ^{14}C is lost.

Neither immobilization nor loss through volatilization/exudation is broad enough in scope to be responsible for the entire tolerance difference between Kenland and

UK2013. Rather, this tolerance seems to be largely the result of differential 2,4-D translocation and metabolism. Translocation to the roots of UK2013 clover was reduced relative to Kenland. Metabolism of 2,4-D is faster in UK2013 compared to Kenland. In addition to differences in speed, differences in the pattern of metabolism are also observed. All six metabolites occur in both clover types, and of those six, M1, M3, M4, M5, and M6 seem to play a role in the increased tolerance found in UK2013, with clear differences in the amounts of 2,4-D and M1 between the two clover types. Based on this study, the basis for the 2,4-D tolerance in the UK developed red clover population (UK2013) includes metabolic changes resulting in quickened 2,4-D metabolism, as well as increased immobilization, to the same compounds found in sensitive clover. This, in turn, leads to reduced translocation of 2,4-D in the tolerant compared to sensitive clover. There may also be increased 2,4-D exudation and other volatilization from the tolerant clover. Further studies with the 2,4-D tolerant and 2,4-D sensitive red clovers could clarify the mechanism of tolerance further. To determine the genetic basis for the tolerance to 2,4-D, RT-PCR or sequencing experiments could be performed on these populations after applying 1.12 kg ha⁻¹ or more of 2,4-D. Ideally, such studies would utilize an additional early harvest interval, prior to 8 HAT, to elucidate genetic changes that occur prior to the uptake of over 50% of the 2,4-D applied, which has occurred by 8 HAT. Furthermore, mass spectrometry studies of the metabolites discovered here could illuminate the identities of the metabolites and clarify their purpose in the mechanism of 2,4-D tolerance.

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Vita

Education

- **University of Kentucky - Lexington, KY 2004-2008**

Bachelors Degree in Agricultural Biotechnology (Minor in Biology)

Thesis: Expression of the *Pleurotus ostreatus* Δ -9 Desaturase in *Nicotiana tabacum* and *Arabidopsis thaliana*.

Research Mentor: Dr. David Hildebrand

- **Thomas Edison High School of Technology - Silver Spring, MD 2002-2003**

Biotechnology Vocational Program Graduate

Professional Positions Held

- **Graduate Research Assistant-** Weed Science Laboratory, University of Kentucky, Department of Integrated Plant and Soil Science, 2012-2015
- **Graduate Teaching Assistant-** University of Kentucky, Department of Integrated Plant and Soil Science, 2013-2014
- **Lab Technician-** Tobacco and Tall Fescue Analytical Chemistry laboratory, University of Kentucky, Department of Plant and Soil Science, 2011-2012
- **Lab manager/Senior Lab Technician-** Plant Protein Laboratory, University of Kentucky, Department of Plant and Soil Science, 2009-2011
- **Student Lab Technician-** Plant Genetics Laboratory, University of Kentucky, Department of Plant and Soil Science, 2005-2009
- **Biological Safety level 3 Student Lab Technician-** Biocontrol Laboratory, United States Department of Agriculture, Foreign Disease Weed Science Research Unit, 2004-2005

- **Biological Safety Level 3 Student Lab Technician**- Plant Virology Laboratory, United States Department of Agriculture, Foreign Disease Weed Science Research Unit, 2006-2007
- **Sigma-Tau Lab Intern**- Microbiology Laboratory, Food and Drug Administration, Center for Food Safety and Applied Nutrition, 2003

Scholastic and Professional Honors

- Achievement Rewards for College Scientists (ARCS) **Fellowship** (Offered) (2015-2017) (Department of Crop Science and Agronomy, Washington State University)
- Weed Photography Contest- Reproductive Structure Category **Second Place** (2015) (Weed Science Society of America Annual Meeting)
- Weed Photography Contest- Vegetative Structure Category **First Place** (2015) (Weed Science Society of America Annual Meeting)
- Donald Sparks Graduate Student Symposium Presentation Competition **Third Place** (2014) (Integrated Plant and Soil Science Department, University of Kentucky)
- Departmental **Travel Award** (2014) (Integrated Plant and Soil Science Department, University of Kentucky)
- Forage Section Student Poster Competition **Second Place** (2013) (North Central Weed Science Society Annual Meeting)
- “Moving Up!” Student Elevator Speech Competition **First Place** (2013) (Agronomy Society of America/Crop Science Society of America/Soil Science Society of America Annual Meeting)

- Weed Science Society of America Graduate **Travel Award** (2013) (Weed Science Society of America Annual Meeting)
- Donald Sparks Graduate Student Symposium Presentation Competition **Second Place** (2013) (Integrated Plant and Soil Science Department, University of Kentucky)
- L.H. May **Scholarship** (2004)(College of Agriculture, University of Kentucky)
- 3 Spot **Awards** for Excellence in Performance and Service (2004-2006) (USDA - Foreign Disease Weed Science Research Unit)
- Sigma Tau **Scholarship/Internship** (2004) (FDA)
- Local and Regional Job Skills Demonstration Competition **First Place** (2003) (SkillsUSA)
- State Job Skills Demonstration Competition **Second Place** (2003) (SkillsUSA)
- **Student of the Year Award** for exemplary research and academic performance (2003) (Thomas Edison High School of Technology)

Professional Publications

- John V. Thoguru, **Tara L. Burke**, Suryadevara S. Rao, David F. Hildebrand. Expression of the *Pleurotus ostreatus* $\Delta 9$ desaturase in *Glycine max*, *Arabidopsis thaliana*, *Nicotiana tabacum* and *Petunia floribunda* .Manuscript in preparation
- Sufian F. Al-Khaldi, Magdi M. Mossoba, **Tara L. Burke**, Frederick S. Fry. 2009. Differentiation of Whole Bacterial Cells Based on High-Throughput Microarray Chip Printing and Infrared Microspectroscopic Readout. Foodborne Pathogens and Disease. October 2009, 6(8): 1001-1007

- Jasmina Kurepa et. al. 2010. Uptake and Distribution of Ultrasmall Anatase TiO₂ Alizarin Red S Nanoconjugates in *Arabidopsis thaliana*. Nano Letters. 2010, 10 (7), 2296–2302 (**in acknowledgements**)
- D.H. Boucher et. al. 2005. Hurricane Isabel and the Forests of the Mid-Atlantic Piedmont and Blue Ridge: Short-Term Impacts and Long-term Implications. In: Hurricane Isabel in Perspective. K.G. Sellner (ed.). Chesapeake Research Consortium, CRC Publication 05-160, Edgewater, MD. 201-208 (**in acknowledgements**)
- J.L. Snitzer, D.H. Boucher, and K.L. Kyde. 2005. Response of Exotic Invasive Plant Species to Forest Damage Caused by Hurricane Isabel. In: Hurricane Isabel in Perspective. K.G. Sellner (ed.). Chesapeake Research Consortium, CRC Publication 05-160, Edgewater, MD. 209–214. (**in acknowledgements**)

Professional Society Memberships

- North Central Weed Science Society **Member** (2012-present)
- Soil Science Society of America **Member** (2012-2014)
- Crop Science Society of America **Member** (2012-2014)
- Agronomy Society of America **Member** (2012-2014)
- Weed Science Society of America **Member** (2013-present)
- Crop Science Society of America Clover and Special Purpose Legume Crop Germplasm **Committee Member** (2013- 2014)
- North Central Weed Science Society **Graduate Student Representative** for the University of Kentucky (2013-2014)

- Weed Science Society of America Public Awareness Committee **Graduate Student Representative** (2014- 2015)
- Weed Science Society of America Local Arrangements **Committee Member** (2015)

Presentations/Abstracts at Professional Meetings

- Translocation and Metabolism of 2,4-D in Sensitive and Tolerant Red Clover (*Trifolium pratense*) Lines. Weed Science Society of America 2015 Annual Meeting (**Lecture**)
- 2,4-D Translocation and Metabolism in Sensitive and Tolerant Red Clover (*Trifolium pratense*) Lines. 2014. North Central Weed Science Society 2014 Annual Meeting (**Lecture**)
- Behavior of 2,4-D in Sensitive and Tolerant Red Clover (*Trifolium pratense*) Lines. Weed Science Society of America/Canadian Weed Science Society Joint 2014 Annual Meeting (**Lecture**)
- Selection Based Breeding Improvement for 2,4-D Tolerance in Red Clover (*Trifolium pratense*). North Central Weed Science Society 2013 Annual Meeting (**Poster**)
- Progress Towards a 2,4-D Tolerant Red Clover (*Trifolium pratense*). Agronomy Society of America/ Crop Science Society of America/ Soil Science Society of America 2013 International Annual Meeting (**Poster**)
- Selection for Improved 2,4-D Tolerance in Red Clover (*Trifolium pratense*). Weed Science Society of America 2013 Annual Meeting (**Poster**)

Extension and Other Presentations

- Selection Based Improvement for 2,4-D Tolerance in Red Clover (*Trifolium pratense*). 2015 University of Kentucky Graduate Student Day at the Capitol-Posters at the Capitol Event (**Poster**)
- Weed Identification and Control in Turf and Landscape Situations. 2015 Annual Kentucky Turf and Landscape Management Short Course (**Lecture**)
- Weed Identification and New Herbicides on the Market. 2014 Annual Kentucky Turf Short Course (**Lecture**)
- 2,4-D Behavior in Tolerant and Sensitive Red Clover (*Trifolium pratense*) Lines. University of Kentucky Department of Integrated Plant and Soil Science 2014 Donald Sparks Semi-Annual Graduate Student Symposium (**Lecture**)
- Broadleaf Weed Identification. 2014 Level 2 Kentucky Certified Professional Turf Managers Workshop (**Lecture**)
- Improvement of Tolerance to 2,4-D in Red Clover (*Trifolium pratense*) through Selection Based Breeding. Kentucky Turfgrass Council 2013 Annual Meeting (**Lecture**)
- Improving 2,4-D Tolerance in Red Clover (*Trifolium pratense*) with Selection Based Breeding. University of Kentucky Turfgrass 2013 Annual Field Day (**Lecture**)

- Selection Based Improvement of Tolerance to 2,4-D in Red Clover (*Trifolium pratense*). University of Kentucky Department of Integrated Plant and Soil Science 2013 Donald Sparks Graduate Student Symposium (**Lecture**)
- Expression of the *Pleurotus ostreatus* Δ -9 Desaturase in *Nicotiana tabacum* and *Arabidopsis thaliana*. University of Kentucky Agricultural Biotechnology 2008 Undergraduate Research Symposium.(**Lecture**)