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Title: GLYPHOSATE RESISTANCE AND TOLERANCE IN ITALIAN RYEGRASS (*LOLIUM PERENNE* SPP *MULTIFLORUM*): TERMINATION TREATMENTS, CULTIVAR, AND RESISTANCE EVALUATION

Abstract Approved:

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Annual ryegrass, or Italian ryegrass (*Lolium perenne* spp. *multiflorum*), has been the most stable, in terms of acres, grass produced for the past 25 years in Oregon. Increased interest in Italian ryegrass as a cover crop has increased demand for quality seed. Ideal Italian ryegrass cover crop cultivars are named cultivars, have winter hardiness and uniform germination, emergence, and growth to guarantee effective termination and reduce the potential for volunteer carryover. The typical method of cover crop termination is through chemical means with a non-selective herbicide or with tillage. In this study, nine termination treatments were used to determine effective treatments and explore potential differences in control between a diploid and a tetraploid cultivar. There was no difference in response based on ploidy level between the treatments. Glyphosate and glyphosate tank mixtures effectively controlled Italian ryegrass. A cultivar evaluation was also conducted to determine if there was a difference in response to glyphosate between ploidy levels. There was no difference in response to glyphosate due to ploidy level. Shikimate acid assays did suggest possible differences in the amount of glyphosate binding to EPSPS depending on the cultivar. An Italian ryegrass population (OR10) was found

to be glyphosate resistant. Along with screening the OR10 suspected resistant population, samples collected from seed production fields were collected and screened. No glyphosate resistant populations were found.

GLYPHOSATE RESISTANCE AND TOLERANCE IN ITALIAN RYEGRASS (*LOLIUM PERENNE* SPP *MULTIFLORUM*): TERMINATION TREATMENTS, CULTIVAR, AND RESISTANCE EVALUATION

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I understand that my thesis will become a part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Robert Chase MacPherran, Author

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Dr. Carol A. Mallory-Smith and Dr. Andrew G. Hulting proposed, advised, and revised writing for all aspects of the research conducted. Daniel Curtis was involved in conducting, preparation, and development of the termination treatment evaluation field project. Dr. Paul Marquardt was involved in the collection of the glyphosate resistant screening populations attached as Appendix A.

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CHAPTER 1:

GENERAL INTRODUCTION

Importance of Italian Ryegrass in Oregon

Annual ryegrass, or Italian ryegrass (*Lolium perenne* ssp. *multiflorum*), is one of the most important and versatile seed crops produced in Oregon. Italian ryegrass has been the most stable grass, in terms of the acres grown, for the past 25 years (Mellbye and Young, 2013). In 1991, legislation banned field burning and changed typical methods of seed establishment and pest management. This legislation resulted in Italian ryegrass seed production becoming more reliant on herbicides including glyphosate and flufenacet + metribuzin for stand establishment (Mellbye and Young, 2013). Reliance on herbicides rather than field burning for weed control has increased the selection pressure for herbicide resistant biotypes, particularly with Italian ryegrass. Conventional-tillage, no-tillage, and volunteer establishment methods implemented the use of flufenacet + metribuzin and glyphosate as primary weed management tools, thus increasing the selection pressure for resistance. Historically, *Lolium* spp. have had rapid evolution of resistance to herbicides with multiple mechanisms of resistance present worldwide (Powles & Preston, 2006).

Increased use of Italian ryegrass as a cover crop in Roundup Ready® cropping systems in the Midwest requires that Italian ryegrass can be killed with glyphosate. Seed production must implement practices that do not promote the evolution of glyphosate resistance. Fortunately, documented herbicide resistance has not been reported in commercial Italian ryegrass seed

production fields to date (Hulting, 2013). There is also concern that there may be differences in the response between diploid and tetraploid Italian ryegrass to herbicides. With this concern in potential variation in tolerance, the objectives of the following research were 1) to determine an optimal treatment for termination of commercially available Italian ryegrass cover crops, 2) to determine if there is a difference in glyphosate tolerance between the selected cultivars, 3) to determine if cultivars or ploidy level have an influence on the accumulation of shikimate acid following glyphosate application to the selected cultivars of Italian ryegrass, and 4) to screen and evaluate for potential resistant populations in seed production and other agricultural situations.

Cover Crops

Cover crops in the Midwestern United States are typically planted in the fall and winter months as an alternative to fallow (DeVinne et al. 1983). Cover crops may consist of various grasses, legumes, non-legume broadleaves, and some winter cereal crops. The crops are utilized for multiple reasons, but the use of cover crops often benefits the management and production of the primary cash crop. The benefits of planting cover crops include, but are not limited to, improved soil fertility, nutrient scavenging, water quality benefits, building soil quality and tilth, erosion prevention, and possible weed suppression (Plumer et al. 2013).

Italian ryegrass is an example of a cover crop that can reduce nitrate leaching and soil erosion. In a two year study by Bergström and Jokela (2001), nitrate leaching was reduced 50 to 66% in relation to a fallow field. Italian ryegrass also reduced erosion 37 to 64% depending on the stand development during critical times of erosion (Malik et al. 2000). Italian ryegrass is not reported to have a strong allelopathic ability to suppress weeds (Smith and Martin, 1994). However, Italian ryegrass can reduce weed densities as a cover crop through competition

(Reddy, 2001; Weston, 1990).

Although Italian ryegrass is capable of reducing weed densities, it can also reduce yields in the cash crop if it becomes a volunteer and is not adequately controlled when used as a cover crop. For example, soybean has been documented to have stand reduction up to 17% with the use of an Italian ryegrass cover crop in comparison to a no-tillage fallow field (Reddy, 2001). Corn yield can also be reduced with an Italian ryegrass cover crop in comparison to no cover crop (Hively and Cox 2001). Italian ryegrass densities of 0.7 to 93 plants m^{-2} reduced wheat yields by 4100 kg ha^{-1} and winter wheat yields can be reduced up to 92% through competition with Italian ryegrass (Appleby et al. 1976; Hashem et al. 1998). As a result, the removal or control of the cover crop is essential prior to rotating back into a cash crop. Control is typically accomplished with non-selective herbicides and tillage (Duke and Powles, 2008; Woodburn, 2000). In the Midwestern United States cover crop systems, glyphosate or herbicide mixtures utilizing glyphosate are the primary herbicides for cover crop control (Plumer et al. 2013).

Italian Ryegrass

The production of Italian ryegrass in Oregon represents a 2014 farm gate value of $73 million and supplies most of the seed used in the United States (Mellbye and Young, 2013). Italian ryegrass is used for a variety of applications, ranging from overseeding pastures to use as winter cover crops in the Midwest United States (Clark, 2007). An increased interest in the cover crop use results in pressure on the industry to provide high quality Italian ryegrass seed (Hulting, 2013).

The Oregon Ryegrass Commission (2013) recommends use of a named Italian ryegrass cultivar with winter hardiness, uniformity of growth and emergence for the most optimal use as a

cover crop. Italian ryegrass cover crops need uniform growth habit primarily to aid in spring termination and for minimization of volunteer carryover. However, Italian ryegrass is a cross-pollinated crop and genetic purity is difficult to maintain because of inbreeding depression (USDA 2015; Hughes et al. 1962). Therefore, synthetic cultivar breeding practices are used to gain uniformity.

A synthetic cultivar is the recombination of selected, non-inbred lines, with superior characteristics of interest. As pollination is not controlled in the production of synthetic cultivars, the clones or other parental lines are maintained to re-form synthetic cultivars with some uniformity from known selected crosses of interest (Hughes et al. 1962). Certified Italian ryegrass cultivars are possible following a ploidy test, florescence test, and field inspections prior to bloom for tolerances of other ryegrass types and off types (Oregon Seed Certification Service, 2015). Only 13,984 of the total 120,830 acres of Italian ryegrass seed harvested were certified in 2014 (Oregon Seed Certification Service, 2015; Anderson and Young III, 2014). Therefore, many named cultivars that could be used in the cover crop market are not certified.

Italian ryegrass cover crops can result in significant benefits or great losses in yield if management is not timely or correctly done. Corn and soybean rotations are recommended when using Italian ryegrass as a cover crop in the Midwest United States, while winter wheat rotations are not recommended due to the potential competition between wheat and Italian ryegrass (Plumer et al. 2014). A 1.1-1.4 kg ae ha^{-1} rate of glyphosate with the labeled rate of ammonium sulfate and non-ionic surfactant are effective for control of Italian ryegrass (Plumer et al. 2014). Yet, reliance on a single herbicide and mechanism of action (MOA) for both the seed producer and cover crop producer could lead to the selection of resistant populations (Hulting, 2013).

To prevent the evolution of resistance, multiple management practices need to be

implemented (Hulting, 2013). In corn production, glyphosate plus the addition of atrazine, mesotrione, simazine, isoxaflutole, rimsulfuron, rimsulfuron + thifensulfuron, pyroxasulfone, or flufenacet + metribuzin have provided good control of Italian ryegrass cover crops (Plumer et al. 2014). Good control was quantified in conventional soybean production with clethodim, sethoxydim, and fluazifop use with their respective recommended surfactants (Plumer et al. 2014). Although risk of selecting for resistant populations is possible with the use of one mechanism of action, control of weeds with glyphosate in Roundup Ready® crops is much easier than with other herbicides. If glyphosate is used both in Italian ryegrass seed production and cover crop termination, resistance is a concern for the Italian ryegrass cover crop industry.

Herbicide Resistance

Herbicide resistance is the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type (Vencil et al. 2012). Herbicide resistant weed populations are capable of evolving when there is sufficient selection pressure on a population with a resistance mechanism present within a susceptible population (Powles et al. 1998; Roux et al. 2008). Therefore, continual application of herbicides with the same MOA could result in the evolution of a resistant population (Christoffers 1999; Roux et al. 2008).

Mechanisms of resistance can be organized into target site mutation and non-target site mutations. Target site resistance is the modification of a nucleotide in the gene encoding the target site enzyme. The mutations in the target site enzyme can cause a reduction in the affinity between the altered target site enzyme and herbicide. Non-target site resistance may be the result of differential herbicide uptake and/or translocation, increased metabolic detoxification, or sequestration to maintain separation of the herbicide molecule and the target site (Devine and

Shukla 2000; Preston and Mallory-Smith 2001). Cross-resistances and multiple resistances can lead to interactions between these key resistant mechanisms.

Cross-resistance enables resistance to two or more herbicides with the same MOA (Beckie and Tardif, 2012). Multiple-resistance is two or more distinct resistance mechanisms enabling the plant to exhibit resistance to two or more herbicide classes (Vencil et al. 2012). Both target site and non-target site resistant mechanisms can be present within a resistant population (Christopher et al. 1992; Preston and Mallory-Smith, 2001).

Tolerance is the inherent ability of a species to survive and reproduce after herbicide treatment (Vencill et al. 2012). It is distinguished from resistance in that no selection or genetic manipulation was applied to make the plant tolerant; it is naturally tolerant. Although resistant populations have not been discovered in Italian ryegrass seed production fields, a difference in glyphosate tolerance due to polyploidy could affect the cultivar choice used for cover crop applications.

Importance of Glyphosate

Glyphosate is a non-selective, systemic, broad-spectrum herbicide that is useful in a number of cropping and non-cropping situations (Dill et al. 2008). Glyphosate was developed and tested by Monsanto in 1970. The herbicide was available for commercial use in 1974 under the trade name Roundup ® (Duke and Powles, 2008). In 1996, the production of glyphosate-resistant (GR) crops lead to a change in how glyphosate could be used (Duke and Powles, 2008). The use of GR crops allowed for post-emergent application of glyphosate without injury to the GR crops (Dill et al. 2008).

Glyphosate is an ambimobile herbicide with symplastic movement and sufficient

apoplastic movement through the plants (Franz et al. 1997). Once at the site of action, glyphosate inhibits the chloroplast protein EPSPS (5-enolpyruvylshikimate 3-phospate synthase) that catalyzes shikimate-3-phosphate and phosphoenol-pyruvate (PEP) to produce an inorganic phosphate and EPSP (5-enolpyruvyl-shikimate-3-phosphate) (Duke and Powles, 2008; Schönbrunn et al. 2001). EPSP is a required intermediate state in the production of the aromatic amino acids phenylalanine, tyrosine, tryptophan, and other aromatic secondary metabolites (Herrmann 1995; Kishore and Shah 1998; Geiger and Fuchs 2002).

With the inhibition of aromatic amino acids required for protein synthesis, increased levels of shikimic acid and shikimate-derived benzoic acids are also accumulated (Siehl 1997). These increased levels of shikimic acid leads to a reduction in carbon fixation intermediates, such as ribulose bisphosphate, and photosynthesis (Duke et al. 2003). Therefore, the loss of the essential plant compounds and biochemical pathways eventually kills the plant (Duke and Powles, 2008; Pline-Srnic, 2005; Shieh et. al 1991). Singh and Shaner (1998) determined that shikimate acid spectrophotometric measurements were correlated with glyphosate resistant populations. Therefore, differences in the amount of shikimate acid accumulation can be used to determine potential differences between Italian ryegrass cultivars in response to glyphosate.

Glyphosate-Resistant Weeds

Although glyphosate resistance was predicted to not evolve due to its unique mode of action, 32 weed species have evolved resistance to glyphosate at present (Bradshaw et al. 1997; Heap, 2015). Three resistance mechanisms have been discovered in many weed species that have evolved glyphosate resistance (Feng et al. 1999; Jasieniuk et al. 2008; Sammons and Gaines, 2014). Vascular sequestration, enzyme target-site mutations, and gene duplication are

the resistances currently known to exist for glyphosate resistant populations (Feng et al. 1999; Sammons and Gaines, 2014). Ultimately, identification of glyphosate resistance mechanisms for resistant populations will continue to be the primary goal of research to understand the evolution of these biotypes and how to manage them (Owen and Powles, 2010).

The first report of glyphosate resistance was in rigid ryegrass (*Lolium rigidum* L.) in Australia (Pratley et al. 1996). The population had no difference in expression of EPSPS or in sensitivity of glyphosate to EPSPS. Yet, the accumulation of glyphosate was notably different between the susceptible plants and resistant plants (Lorraine-Colwill et al. 2002). This reduced glyphosate translocation resistance mechanism allows for increased glyphosate accumulation in the treated leaves and stems, with decreased accumulation in the actively growing roots and meristematic tissue (Powles and Preston, 2006; Wakelin et al., 2004).

Since the original emergence of glyphosate resistance in 1996, similar rapid vacuole sequestration resistant populations have demonstrated the importance of the exclusion mechanism within *Lolium* spp. (Ge et al. 2012). Similar mechanisms have been observed between glyphosate-resistant populations on separate continents, suggesting that the resistant trait is present within *Lolium* spp. and can be selected for independently (Ge et al. 2012; Sammons and Gaines, 2013). Another unique trait of the vacuole sequestration glyphosate resistance in *Lolium* spp. is an increased sensitivity under colder temperature, making the resistant population more susceptible (Vila-Aiub et al. 2012). Although there is variation in response to glyphosate at suboptimal temperatures with vacuole sequestration, target site resistance was not affected by temperature (Collavo and Sattin, 2011)

The difference in response to glyphosate with vacuole sequestration and target site resistance supports the idea that multiple mechanisms of resistance in *Lolium* spp. may be

involved (Sammons and Gaines, 2014). The first evolved population with target site resistance was identified in goosegrass (*Eleusine indica* L.Gaertn.) from Malaysia (Baerson et al., 2002a; Lee and Ngim, 2000). This target site resistance was characterized by a proline to serine, threonine, or alanine substitution at the 106 amino acid in the target enzyme (Baerson et al. 2002a; Ng et al. 2003). The substitiution of proline to serine, threonine, or alanine at the 106 amino acid was similar to the glyphosate resistant populations of *Lolium* spp. found in California, Spain, Chile, and Australia (Jasieniuk et al. 2008; Gonzalez-Torralva et al. 2012; Michitte et al. 2007; Wakelin and Preston, 2006).

Variation in the strength of target-site mutations endowing herbicide resistance are present (Powles and Preston, 2006). Target site mutations providing high-level (>20-100-fold compared to susceptible) herbicide resistance have been examined in acetolactate synthase (ALS)-inhibiting, and acetyl coenzyme A carboxylase (ACCase)-inhibiting resistant populations (Powles and Preston, 2010; Preston and Mallory-Smith 2001). Weak resistances provide marginal levels (2-5 fold compared to susceptible) of herbicide resistance (Powles and Preston, 2010). The *Lolium* spp. Pro-106 mutations in the EPSPS gene provided a weak 2-fold to moderate 15-fold resistance (Preston et al. 2009). However, target site mutation and reduced translocation have been observed in similar populations of *L. rigidum* to provide greater resistance (Bostamam et al. 2012; Kaundun et al. 2011). As it is common for cross-pollinated species to develop multiple mechanisms of resistance under selection (Sammons and Gaines 2014), the moderately resistant target site mutation in *Lolium* spp. may not be observed in the field until the evolution of multiple resistant mechanisms.

Along with target site mutations, gene duplication is another mechanism of resistance that has been discovered. Gene duplication was first reported in an *Amaranthus palmeri*

population in Georgia, USA (Gaines et al. 2010). This resistant population of *A. palmeri* had increased EPSPS expression with increased EPSPS gene copy number (Gaines et al. 2010). Resistance in an Italian ryegrass population was reported to have a 12 to 13-fold resistance with a linear correlation between the genomic copy number, EPSPS mRNA expression, and EPSPS protein levels (Salas et al. 2011). Therefore, additional EPSPS enzyme activity in concert with increased genomic copies may provide a mechanism for survival.

Glyphosate sequestration and target site mutations in *Lolium* spp. have indicated that these mechanisms are present within separate populations and can be selected independently (Ge et al. 2012; Sammons and Gaines, 2013; Baerson et al. 2002b; Perez-Jones et al. 2007; Yu et al. 2007; Jasieniuk et al. 2008; Wakelin and Preston 2006). The correlation of genomic copy number of EPSPS with gene amplification and EPSPS enzyme activity in Italian ryegrass shows the ability of *Lolium* spp. to evolve and survive (Gaines et al 2010; Salas et al. 2011). In relating the gene duplication mechanism of resistance in the *Lolium* spp. to tolerance, the presence of polyploidy in Italian ryegrass could have an effect on survival following glyphosate applications due to increased genomic copies.

Polyploidy

Polyploidy species have more than two copies of the basic haploid number of chromosomes (DeVinne et al. 1983). Polyploidy is recognized as a major force affecting diversification and speciation in plant evolution (Otto and Whitton, 2000; Adams and Wendel, 2005; Soltis et al. 2009; Madlung, 2013). Polyploidy in *Spartina*, a model for Poaceae, exhibits a range of possible responses that vary among species (Ainouche et al. 2012; Salmon et al. 2005; Salmon and Ainouche 2010). Through AFLP (amplified fragment length polymorphism) and

MSAP (methylation sensitive amplification polymorphism) studies, genome doubling does exhibit changes in methylation (Salmon et al. 2005).

Changes in DNA methylation/histone methylations are directly correlated to gene dosage control and nucleolar dominance (Lawrence et al. 2004). In *Brassica*, Chen and Pickaard (1997a, 1997b) determined that there is an enforcement mechanism that regulates rRNA gene by DNA methylation and histone modifications. In *Arabidopsis* nucleolar dominance is a chromosomal phenomenon that can express or depress the silencing of rRNA genes (Chen et al. 1998). Current information suggests that polyploidization can produce shifts in the genetic systems and phenotypes, but the evidence is not conclusive enough to indicate that cause and effect based on polyploidy and evolutionary success are correlated (Madlung, 2013; Otto and Whitton, 2000). Polyploidy allows for the masking of deleterious recessive mutations, transgressive performance with stable heterosis, and duplicated genes acquiring a new or slightly varied function (Weiss-Schneeweiss et al. 2013). Thus, tolerance might be promoted if there are a greater number of copies of the target enzyme, unless regulation within the population of Italian ryegrass prevents enzymatic amplification.

Summary

The large plasticity in the response of populations of Italian ryegrass to glyphosate indicates the diversity present in the species and the necessity to understand how resistance evolved, and to understand and manage glyphosate resistance populations (Jasieniuk et al., 2008). It has also been discovered that glyphosate resistance is present in Oregon biotypes with multiple resistance mechanisms (Perez-Jones et al. 2005; Liu et al. 2013). As Oregon is the major producer of seed for Italian ryegrass, presence of resistant biotypes or differences in

tolerance in seed production would have a negative impact on the quality of seed being produced. Therefore, the objectives of this research were to conduct evaluations: 1) to determine the optimal treatment for control of an Italian ryegrass cover crop, 2) to determine if there is a difference in tolerance to glyphosate between selected cultivars or ploidy level, 3) to determine if cultivars or ploidy level have an influence on the accumulation of shikimate acid following glyphosate application, and 4) to screen for and evaluate resistant populations in seed production and other agricultural settings.

CHAPTER 2: Termination Treatments for Diploid and Tetraploid Italian Ryegrass Cover Crops

ABSTRACT

Italian ryegrass is a beneficial cover crop in the corn-soybean cropping system of the Midwest because of its low seed cost, rapid establishment and because it improves soil structure. However, the use of Italian ryegrass as a cover crop is under question because of the perception that it may become a weed which could reduce crop yields through competition. Studies conducted over two years at the Oregon State Hyslop Research Farm investigated the efficacy of different herbicides to control Italian ryegrass when used as a cover crop. Two cultivars of Italian ryegrass, Bounty® (diploid) and TAMTBO® (tetraploid), were planted in two randomized complete block design experiments. Herbicides were applied in the spring when the first node of the Italian ryegrass was 2.5 cm above the soil surface. Clethodim (0.17 kg ai ha^{-1}) reduced biomass for the diploid and tetraploid varieties, 90% and 86%, respectively. Paraquat (0.841 kg ai ha^{-1}) followed by clethodim (0.17 kg ai ha^{-1}) resulted in the greatest reduction (96%) in biomass among all treatments in both cultivars, but did not prevent regrowth. Glyphosate applied at 0.184, 0.229, and 0.367 kg ae ha^{-1} and tank mixed treatments of glyphosate (1.401 kg ae ha^{-1}) plus saflufenacil (0.025 kg ai ha^{-1}), rimsulfuron (0.017 kg ai ha^{-1}), or pyroxasulfone (0.179 kg ai ha^{-1}) reduced biomass by at least 90% for both cultivars.

Nomenclature: Annual ryegrass, Italian ryegrass, *Lolium perenne* ssp. *multiflorum*.; AMS, ammonium sulfate; bromoxynil; clethodim; ethofumesate; glyphosate, isopropylamine salt of glyphosate; MCPA Ester, 2-ethylhexyl ester of 2-methyl-4-chlorophenoxyacetic acid; paraquat; pyroxasulfone; R11, dimethylpolysiloxane; rimsulfuron; saflufenacil; pyrasulfotole

Keywords: Annual ryegrass; control; cover crop; diploid; tetraploid

INTRODUCTION

Benefits of cover crops have been well documented. Alfalfa (*Medicago sativa* L.), barrel medic (*Medicago lupulina* L.), hairy vetch (*Vicia villosa*), Italian ryegrass (*Lolium perenne* ssp. *multiflorum*), oat (*Avena sativa* L.), red clover (*Trifolium pratense* L.), rye (*Secale cereale* L.), triticale (*Triticosecale*), wheat (*Triticum aestivum* L.), and white clover (*Trifolium repens* L.) have utility as cover crops (Hively and Cox, 2001; Moore et al. 1994; Reddy, 2001, 2003; Teasdale et al. 1991). Currently, grass and legume winter cover crops can be used to maintain or increase corn and soybean yields (Miguez and Bollero, 2005; Moore et al. 1994).

Among the cover crop species, Italian ryegrass has been documented to provide several benefits. The positive aspects of Italian ryegrass include reducing nitrate leaching and improving soil properties. The estimated reduction in nitrate leaching in a two year study ranged from 50 to 66 % (Bergström and Jokela, 2001; Miguez and Bollero, 2005). Additionally, Italian ryegrass used as a cover crop can control erosion (Abdin et al. 1997; Malik et al. 2000). Erosion can be reduced 37 to 64 % depending on the stand development at critical times for erosion (Malik et al. 2000). In addition, Italian ryegrass has the ability to compete with weeds and the following crop. Therefore, in order to utilize these benefits, controlling the cover crop is essential (Hartwig and Ammon, 2010).

Insufficient control of an Italian ryegrass cover crop is an important concern because Italian ryegrass can reduce yields of both corn and soybean through competition (Hively and Cox 2001; Reddy, 2001). Typically, control of cover crops is achieved through applications of a non-selective herbicide and tillage (Duke and Powles, 2008; Woodburn, 2000). Glyphosate and glyphosate tank mixtures are the primary means used to control cover crops (Plumer et al. 2013).

Thus, development of effective treatments for termination of Italian ryegrass is a necessity when using it as a cover crop.

Reported differences in control between the differing ploidy levels necessitated the inclusion of both diploid and tetraploid Italian ryegrass used in this study. The diploid cultivar (Bounty®) has two sets of chromosomes and the tetraploid cultivar (TAMTBO®) has four sets of chromosomes. Differing gene expression is dependent on functional interconnections, fate of the duplicated copy, and abiotic and biotic changes (Salmon et al., 2005; Salmon and Ainouche, 2010). Glyphosate-resistance has been recognized to be correlated with increased EPSPS (5-enol-pyruvylshikimate-3-phophate synthase) enzyme activity and EPSPS copy number in Italian ryegrass (Salas et al., 2012). Thus, utilization of different ploidy levels could provide some information on differences in control between ploidy level.

There have been studies that determined the timing and rates of herbicides for termination of cover crops (Lins et al., 2007; Reddy, 2001, 2001). However, there has not been extensive analysis of termination of Italian ryegrass cultivars with different ploidy levels. Therefore, the objective of this research was to evaluate the effect of glyphosate, one of the most commonly used herbicides for cover crop termination, glyphosate tank mix treatments, and herbicides with other mechanisms of action for the control of a diploid and tetraploid Italian ryegrass cultivar.

MATERIALS AND METHODS

Field

Field experiments were planted in the fall of 2012 and 2013 at the Oregon State University Hyslop Field Research Farm near Corvallis, Oregon. The soil type was a Woodburn

silt clay loam containing 14.6% sand, 58% silt, 27.5% clay, and 2.52% organic matter, with pH of 6.1, and a cation exchange capacity of 15.4. The field was plowed, disked, harrowed, and fertilized with 16-16-16 at a rate of 224.2 kg ha^{-1} prior to planting the Italian ryegrass.

Planting and Maintenance

A gravity fed grain drill was calibrated for a seeding rate of 16.8 kg ha^{-1}. Weights of the seed were measured prior to planting and after planting. The seeding rate was calculated with the measurements taken. The first year, Bounty® (16.8 kg ha^{-1}) and TAMTBO® (17.4 kg ha^{-1}) were planted on September 27, 2012. The second year, Bounty® (18.8 kg ha^{-1}) and TAMTBO® (16.4 kg ha^{-1}) were planted on October 23, 2013. On December 18 of both years, 1.1 kg ai ha^{-1} ethofumesate, 583.2 g ai ha^{-1} MCPA Ester, 40.7 g ai ha^{-1} pyrasulfotole and 229.9 g ai ha^{-1} bromoxynil were applied for general grass and broadleaf weed control.

Treatments

The termination treatments were applied the following spring when the first node elongated 2.5 cm above the soil surface. Treatments were applied on March 7, 2013, and March 23, 2014. Nine herbicide treatments were used each year (Tables 2.1 and 2.2). Herbicides were applied with a unicycle sprayer, operated at 241 kPa, using flat fan 11001 nozzles, spaced 20.3 cm apart, with a boom length of 2.3 m, average boom height of 71.1 cm, and a spray volume of 1050 L ha^{-1}.

Analysis

Experiments were a randomized block design with four replications. Cultivars were grown in separate experiments. The plots were 2.4 by 7 m and were surrounded by a 0.6 m border. A visual rating was taken to estimate the control on a scale of 0 (no control) to 100 (complete control) at 28 and 56 days after treatment (DAT). Quarter meter square quadrats were used to harvest aboveground biomass at 28 and 56 DAT. Samples were cut, dried at 70 C for 72 h, and weighed.

Data for above ground biomass and visual ratings for both years were combined and analyzed. The Italian ryegrass biomass and visual control were analyzed for normality using the Shapiro-Wilk normality test. Biomass was logistically transformed due to absence of normality. The nine treatments were analyzed using a Tukey multiple comparison of means test and separated at a significant difference of $P < 0.05$.

RESULTS AND DISCUSSION

Visual Rating

Visual ratings for control were similar between cultivars (Table 2.1). All treatments provided control compared to the untreated check. At 28 DAT, the three rates of glyphosate and the glyphosate tank mixes resulted in 98-100% control. The clethodim applied alone and the paraquat followed by clethodim 10 days later resulted in less control compared to the other treatments. At 56 DAT, there was less control in the 1.1 kg ha^{-1} glyphosate rate treatment compared to all other treatments.

Biomass

There was a difference in biomass between all the treatments and the untreated check (Table 2.2). At the 28 day collection, Bounty had a greater reduction in biomass in the paraquat followed by clethodim treatment, the 1.1 kg ha^{-1} rate of glyphosate, and glyphosate with saflufenacil compared to the clethodim and the 2.2 kg ha^{-1} rate of glyphosate. For TAMTBO, paraquat followed by clethodim and glyphosate plus saflufenacil resulted in a greater reduction in biomass compared to clethodim, and the 1.1 kg ha^{-1} and 1.4 kg ha^{-1} rates of glyphosate.

At 56 DAT, paraquat followed by clethodim resulted in a greater reduction in biomass compared to the 1.1 kg ha^{-1}, 1.4 kg ha^{-1}, and 2.2 kg ha^{-1} rates of glyphosate, glyphosate with rimsulfuron, glyphosate with pyroxasulfone, and the clethodim treatments for Bounty®. At 56 DAT for TAMTBO®, the only difference in biomass reduction was between the paraquat followed by clethodim treatment compared to the clethodim alone treatment.

Visual control was generally lower with the lowest rate of glyphosate (Table 2.1). Although there was no evidence that varying rates of glyphosate had an effect on biomass reduction at 56 DAT, the visual rating results indicated a reduced ability to terminate the cover crop at the lower rates of glyphosate.

Within each cultivar, the paraquat followed by clethodim 10 days later resulted in the greatest reduction in biomass. However, there was re-growth in the treatment of paraquat followed by clethodim 10 days later (personal observation). A difference in biomass reduction was observed between the two cultivars for the glyphosate treatments at 28 DAT. There was a greater reduction in Bounty® biomass at the lower rate than the higher rate of glyphosate. TAMTBO® had greater reduction in biomass at the higher rate than the lower rate of glyphosate. This difference in response to glyphosate rate could be a result of differences in the interaction of

glyphosate between the ploidy levels. However, no difference between the biomass, at the later evaluation date, was observed to substantiate an influence of ploidy level on the control.

The inability to control Italian ryegrass with low rates of glyphosate, the regrowth in the paraquat followed by clethodim treatment, and the slow biomass reduction with clethodim alone make these treatments unacceptable in relation to the other treatments. In conclusion, the higher rates of glyphosate or glyphosate in combination with either saflufenacil, rimsulfuron, or pyroxasulfone are recommended for the most effective Italian ryegrass control. There was no difference in treatment responses between the diploid and tetraploid cultivars at the final measurements dates. To determine if differences between tetraploid and diploid control with glyphosate are possible, more cultivars of each ploidy level should be tested in the future.

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Table 2.1: Annual ryegrass cover crop average visual rating for % control of Bounty and TAMTBO at 28 and 56 days after initial treatment. Data combined for 2012 and 2013.

	Rate	28 DAT Visual Ratings (% control)		56 DAT Visual Ratings (% control)	
Treatments	kg ae/ha	Bounty	TAMTBO	Bounty	TAMTBO
1) untreated check	-	0 a[2]	0 a	0 a	0 a
2) glyphosate	1.121	96 b	95 b	88 b	88 b
3) glyphosate	1.401	98 b	98 b	94 c	95 c
4) glyphosate	2.242	99 b	99 b	98 c	98 c
5) glyphosate + saflufenacil	1.401 0.025	99 b	99 b	97 c	98 c
6) glyphosate + rimsulfuron	1.401 0.017	100 b	99 b	99 c	99 c
7) glyphosate + pyroxasulfone	1.401 0.179	98 b	99 b	99 c	99 c
8) paraquat + clethodim*	0.841 0.170	83 c	86 c	97 c	96 c
9) clethodim	0.170	67 d	71 d	98 c	94 c

[1]10 Days after first treatment

[2]Numbers in same column with the same letter are not significantly different (p=0.05), Tukey multiple comparison of means.

Table 2.2: Annual ryegrass cover crop dry above ground biomass of Bounty and TAMTBO at 28 and 56 days after initial treatment for Bounty and TAMTBO.

		28 DAT				56 DAT			
	Rate	Bounty		TAMTBO		Bounty		TAMTBO	
Treatments	kg ae/ha	Average biomass (g 0.25m^{-2})	Percent reduction (% of control)	Average biomass (g 0.25m^{-2})	Percent reduction (% of control)	Average biomass (g 0.25m^{-2})	Percent reduction (% of control)	Average biomass (g 0.25m^{-2})	Percent reduction (% of control)
1) untreated check	-	73.4 a[2]	-	105.1 a	-	209.7 a	-	249.5 a	-
2) glyphosate	1.121	24.6 b	67	38.4 c	64	17.2 c	92	24.7 bc	90
3) glyphosate	1.401	26.5 bc	64	38.2 c	64	17.7 c	92	25.7 bc	90
4) glyphosate	2.242	27.8 c	62	33.2 bc	68	15.5 c	92	24.3 bc	90
5) glyphosate + saflufenacil	1.401 0.025	25.0 b	66	29.3 b	72	12.6 bc	66	22.2 bc	91
6) glyphosate + rimsulfuron	1.401 0.017	26.3 bc	64	35.7 bc	66	14.9 c	93	21.5 bc	91
7) glyphosate + pyroxasulfone	1.401 0.179	26.3 bc	64	36.5 bc	65	17.2 c	92	22.1 bc	91
8) paraquat + clethodim*	0.841 0.17	18.5 b	75	23.1 b	78	7.8 b	96	9.0 b	96
9) clethodim	0.17	39.6 c	46	54.9 c	48	20.3 c	90	34.2 c	86

[1]10 Days after first treatment

[2]Numbers in same column with the same letter are not significantly different (p=0.05), Tukey multiple comparison of means.

CHAPTER 3: Tolerance of Diploid and Tetraploid Italian Ryegrass to Glyphosate

ABSTRACT

Italian ryegrass is used for forage production, overseeding, and as a cover crop. Possible differences in tolerance to glyphosate due to ploidy levels are of interest to growers using Italian ryegrass as a cover crop. Studies conducted for one year in a field setting accompanied with laboratory measurements were used to investigate the response of diploid and tetraploid cultivars to glyphosate. The field experiment indicated the biomass for the tetraploid cultivars was greater than the diploid for the untreated control at the first date of evaluation. There was no difference in biomass between the ploidy levels for any of the treatments at any of the later evaluation dates. The shikimate acid measurements conducted in the laboratory were not different among ploidy levels at any of the incubation times. Accumulation of shikimate acid did vary between cultivars at 20 and 24 hours of incubation. Although the average biomass of the tetraploid cultivar was greater than the diploid cultivars and there were some differences between the cultivars for shikimate acid accumulation, control of the Italian ryegrass cover crop did not differ between ploidy levels or cultivars in the field.

INTRODUCTION

Italian ryegrass (*Lolium perenne* ssp. *multiflorum)* is both a useful cover crop and a problematic weed. When used as a cover crop, soil erosion and nitrate leaching can be reduced (Bergström and Jokela, 2001; Malik et al. 2000). Italian ryegrass does not have allelopathic ability to suppress weeds, but weed densities can be reduced through competition (Reddy, 2001; Smith and Martin, 1994; Weston, 1990). Unfortunately, competition from Italian ryegrass can reduce yields in following crops if volunteers from the cover crop are not controlled (Hively and Cox 2001; Reddy, 2001). To reduce volunteers, cultivars that grow uniformly are suggested to ensure equal germination and emergence when used as a cover crop (Oregon Ryegrass Commission, 2013).

Although Italian ryegrass is a cross-pollinated crop that is susceptible to inbreeding depression which results in loss of vigor for progeny, many cultivars have been developed (Hughes et al. 1962; USDA, 2015). The majority of seed production for Italian ryegrass is located in the Willamette Valley of Oregon (Mellbye and Young, 2013). Certified Italian ryegrass seed is available, but is produced in low quantities compared to uncertified production (Oregon Seed Certification Service, 2015; Anderson and Young III, 2014). However, through maintenance of clones or other parental lines, synthetic lines can be reliably produced through known recombination of non-inbred lines with characteristics of interest (Hughes et al. 1962).

The increased interest in using Italian ryegrass as a cover crop has resulted in scrutiny on the production practices of Italian ryegrass used in the seed industry (Hulting, 2013). The characteristics of interest for a cover crop are uniformity and winter hardiness. These traits allow uniform growth for efficient termination and minimization of volunteer carryover (Oregon Ryegrass Commission, 2013).

Prior to 1991, field burning was the standard practice for pest management in Italian ryegrass and other grass seed production (Mellbye and Young, 2013). The field burning ban resulted in a greater reliance on herbicides such as glyphosate and flufenacet + metribuzin for weed management (Mellbye and Young, 2013). Although it is recommended to utilize herbicides with different sites of actions to prevent the evolution of resistance, glyphosate is still the recommended and best herbicide for control of Italian ryegrass cover crops (Plumer et al. 2014). Utilizing a single herbicide or herbicide group in both seed production and within the cover crop market could lead to the evolution of resistant populations (Hulting, 2013).

Glyphosate is a non-selective, broad-spectrum herbicide that is useful in many commercial and non-commercial situations (Dill et al. 2008). Glyphosate is a systemic herbicide that inhibits the chloroplast protein EPSPS (5-enolpyruvylshikimate 3-phosphate synthase), preventing the formation of EPSP (5-enolpyruvyl-shikimate-3-phosphate) (Duke and Powles, 2008). The inhibition of EPSP causes an increase in the levels of shikimic acid and shikimate-derived benzoic acid accumulation (Siehl, 1997). The inhibition of EPSPS, which stops the production of aromatic amino acids, and the accumulation of shikimic acid, reduces carbon fixation, and ultimately kills the plant (Duke and Powles, 2008; Duke et al. 2003; Shieh et al. 1991). It was determined by Singh and Shaner (1998) that shikimate acid spectrophotometric measurements could be used to determine differences in resistance to glyphosate, which may also be useful for determining differences in tolerance to glyphosate in Italian ryegrass based on ploidy levels.

In *Spartina*, a model for Poaceae, polyploids are capable of expressing a range of responses that can be different between species (Ainouche et al. 2012; Salmon et al. 2005; Salmon and Ainouche 2010). Although there is variation among species, genome doubling can

cause changes in methylation (Salmon et al. 2005). Polyploidization can produce shifts in the genetic systems and phenotypes due to the changes in DNA methylation correlated to gene dosage control (Madlung, 2013; Otto and Whitton, 2000; Lawrence et al. 2004). These shifts in genetic systems and phenotypes warrant an evaluation of the differences in polyploidy of Italian ryegrass cultivars. Thus, the objectives of this experiment were to evaluate the effects of glyphosate and a glyphosate tank mix treatment in the field as well as shikimate acid accumulation measurements in the laboratory between diploid and tetraploid Italian ryegrass cultivars.

MATERIALS AND METHODS

Plant Material Selection

Commercial Italian ryegrass cultivars were selected based on ploidy levels, diploid and tetraploid. The diploid cultivars were Bounty, Royal, Bruiser, and Kodiak. The tetraploid cultivars were Maximus, FLX 1995, TAMTBO, and Andes. The plants for leaf-segment shikimate acid bioassays were grown in 25.4 by 25.4 cm pots with Metro-mix® soil. These plants were grown in the greenhouse under a 16 hour photoperiod within a 25/20 C day/night temperature range.

Field Experiment

The field experiment was planted in the fall of 2014 at Oregon State University Hyslop Field Research Farm in Corvallis, Oregon. The soil type was a Woodburn silt clay loam containing 13.8% sand, 57.5% silt, and 28.8% clay with 2.45% organic matter, a pH of 5.2, and a

cation exchange capacity of 14.3. The field was plowed, disked, harrowed, and fertilized with 16-16-16 at a rate of 224.2 kg ha^{-1} prior to planting.

The experiment was designed as a strip plot. A gravity fed grain drill was calibrated to deliver a seeding rate of 16.8 kg ha^{-1}. The cultivars were planted in 4 replications with the four diploid and four tetraploid cultivars randomly assigned. The individual cultivars were planted in 12.2 m by 4.6 m strips with a 0.9 m separation between the cultivars to allow for equipment access. Within the cultivar strips, three subplots 3 m by 4.6 m with a 1.5 m border at each end were maintained. Following planting, on December 15, 2014, 1.1 kg ai ha^{-1} ethofumesate, 583.2 g ai ha^{-1} MCPA ester, 40.7 g ai ha^{-1} pyrasulfotole and 229.9 g ai ha^{-1} bromoxynil were used for general grass and broadleaf weed control.

Treatments were applied on February 10, 2015, when the first node of the Italian ryegrass had elongated 2.5 cm above the soil surface. Herbicides were applied with a unicycle sprayer, operated at 241 kPa, using flat fan 11001 nozzles, spaced 20.3 cm apart, with a boom length of 2.3 meters, average boom height of 71.1 cm, and a spray volume of 1050 L ha^{-1}. Treatments included a control, 1.4 kg ae ha^{-1} glyphosate, and 1.4 kg ae ha^{-1} of glyphosate plus 0.025 kg ai ha^{-1} saflufenacil, which were randomly assigned to the three subplots. The treatments included the use of the label recommended surfactants, NIS at 0.25% or MSO at 1% v/v.

A visual rating was taken to estimate the control on a scale of 0 (no control) to 100 (complete control) at 28, 56, and 84 days after treatment (DAT). Quarter meter square sections of aboveground biomass were cut at 28, 56, and 84 DAT, dried at 70 C for 72 h, and weighed. The biomass and visual control were analyzed for normality using the Shapiro-Wilk normality test. Data were analyzed separately by ploidy level and cultivar. The three treatments were

analyzed using a Tukey multiple comparison of means test and separated at a significant difference of $P < 0.05$.

Leaf-Segment Shikimic Acid Bioassay

The effects of glyphosate on shikimic acid accumulation in the differing ploidy levels of Italian ryegrass cultivars were determined using the methods developed by Shaner et al. (2005). The plant material grown in the greenhouse was cut into 0.5 cm leaf segments at the 3 leaf stage. The leaf segments were placed in 96-well plates containing 100 µl 10 mM $NH_4H_2PO_4$ (pH 4.4) and differing rates of glyphosate (0, 0.5, 1, 5, 10, 50, 100, 250, 500, 1000, 2000, and 5000 µM). The leaf segments were incubated for 12, 16, 20, and 24 hours under continuous light at 25 C. Samples were then placed at -20 C for at least one hour. Samples were then thawed at room temperature for leaf tissue disruption. Then, 50 µl 1.25 M HCl was added to the thawed samples and was incubated for 20 minutes at 60 C for tissue digestion. After digestion, 25 µl of the solution was transferred to EIA/RIA 96 well plates with a 100 µl solution of 0.25% periodic acid and 0.25% sodium(meta)periodate and incubated for 40 minutes at 37 C. After incubation, a 100 µl quench buffer of 0.6 M NaOH and 0.22 M Na_2SO_3 was added to the solution. Optical densities were measured spectrophotometrically at 380 nm. Data for absorbance of shikimate acid were calculated as a percent of the control. The LD_{50}s were calculated for the shikimate acid using R and drc package (Ritz and Strebig, 2015). An ANOVA and analysis of means were used to determine influence of variables.

RESULTS AND DISCUSSION

Visual Ratings

Twenty-seven DAT, no difference in visual ratings was observed between the ploidy levels, but a difference was observed between the cultivars. There was greater control of Kodiak (4n) and Andes (2n) than Royal (2n) and TAMTBO (4n) (Table 3.1). The treatments were different from one another for both cultivar and ploidy level. At 56 DAT there was no difference in control between ploidy levels. There was an interaction between treatments and the cultivars. Eighty-four DAT, there was no difference in control between ploidy levels, while a difference between cultivars was observed. Greater control was observed for Kodiak (4n) and Andes (2n) than Maximus for both treatments. There were no differences in control between glyphosate and the glyphosate tank mix treatment for either cultivar or ploidy level (Table 3.2).

Biomass

Twenty-seven DAT there was an interaction between the ploidy level and treatment. A difference was observed between the ploidy levels for the untreated control with no differences between ploidy levels for the other treatments. Tetraploid cultivars had a greater biomass than the diploid cultivars. An interaction between the cultivars and treatments was observed similar to analysis between ploidy levels. The untreated control produced greater biomass than the other treatments. There were no differences exhibited between the other treatments for any of the cultivars or either ploidy level.

Fifty-six DAT, there was no difference between ploidy levels or cultivars for biomass. The control treatment had greater biomass than the glyphosate and tank mix treatments. No

differences between the glyphosate and tank mix treatment were observed. No difference between ploidy levels or cultivar was observed 84 DAT. The only difference observed was the control had greater biomass than the other treatments. Again, there was no difference between the treatments.

Leaf-Segment Shikimic Acid Bioassays

The ploidy level did not result in an effect on the response to glyphosate at any of the incubation times. The 12 and 16 hr incubation resulted in no difference between ploidy level or cultivar. The 20 hr incubation resulted in Andes and Kodiak having a greater concentration of shikimate acid than Bruiser, FLX, and TAMTBO. At 20 hr incubation Bounty had a greater concentration of shikimate acid than TAMTBO (Table 3.3). The 24 hr incubation resulted in Maximus having a greater concentration of shikimate acid than Bounty (Table 3.4).

In summary, there was no evidence ploidy level caused a difference in response to glyphosate. The initial difference in biomass observed was that the untreated controls for tetraploid cultivars had a greater biomass than the untreated controls for diploid cultivars. There were no differences between treatments for ploidy levels or cultivar for biomass on the other collection dates. The shikimate acid measurements did indicate difference in response between the cultivars at 20 and 24 hr of incubation, but not at the other incubation times. The resulting differences in the shikimate acid measurements between cultivar were not consistent and are inconclusive. Although there were differences in shikimate acid accumulation, the field experiment did not show any difference in response to ploidy level or cultivar. Variation of shikimate acid accumulation could represent a difference in the amount of glyphosate bound to

the target enzyme between cultivar. However, this difference did not have an effect on tolerance to glyphosate in the field.

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Table 3.1: Comparison of visual injury by cultivar at 27 DAT.

		Treatment			
		1.4 kg ae ha^{-1} glyphosate		1.4 kg ae ha^{-1} glyphosate + 0.025 kg ai ha^{-1} saflufenacil	
Cultivar	Ploidy	Visual rating of injury (0-100)	SE	Visual rating of injury (0-100)	SE
Andes	4	87.5	2.5	90.0	0.0
Maximus	4	82.5	2.5	85.0	2.9
TAMTBO	4	80.0	0.0	82.5	2.5
FLX	4	82.5	2.5	87.5	2.5
Kodiak	2	87.5	2.5	90.0	0.0
Royal	2	80.0	0.0	82.5	2.5
Bounty	2	80.0	0.0	85.0	2.9
Bruiser	2	85.0	2.9	85.0	2.9

Table 3.2: Comparison of visual injury by cultivar at 84 DAT.

		Treatment			
		1.4 kg ae ha^{-1} glyphosate		1.4 kg ae ha^{-1} glyphosate + 0.025 kg ai ha^{-1} saflufenacil	
Cultivar	Ploidy	Visual rating of injury (0-100)	SE	Visual rating of injury (0-100)	SE
Andes	4	100.0	0.0	99.3	0.3
Maximus	4	97.0	2.3	94.8	1.8
TAMTBO	4	97.0	1.2	98.8	0.3
FLX	4	99.3	0.3	99.0	0.4
Kodiak	2	99.5	0.3	100.0	0.0
Royal	2	97.5	0.9	95.8	0.8
Bounty	2	97.8	0.9	95.8	2.1
Bruiser	2	99.0	0.4	98.0	1.1

Table 3.3: Comparison of shikimate acid measurements as percent of control for the 20 hr incubation.

Glyphosate Treatments	Cultivar															
	Andes		Maximus		TAMTBO		FLX		Kodiak		Royal		Bounty		Bruiser	
(μM)	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
0.5	70.6	56.6	16.4	6.6	8.6	2.5	19.4	5.5	28.6	15.6	22.5	10.6	16.7	3.9	26.6	7.4
1	36.5	11.9	12.3	4.9	11.3	4.0	22.4	8.2	51.9	30.7	26.7	8.0	21.1	9.5	18.5	8.8
5	34.2	27.6	11.8	7.1	14.5	7.1	15.1	5.3	68.5	31.2	14.1	3.5	25.9	9.9	17.4	3.1
10	46.8	20.1	17.2	6.1	15.0	7.8	15.1	5.9	67.4	32.8	37.0	11.0	16.5	3.6	36.2	8.9
50	35.9	28.0	44.4	17.9	16.4	6.5	9.8	3.8	37.8	24.5	18.0	6.2	41.1	16.8	13.2	5.5
100	46.4	31.3	9.0	4.5	24.7	7.2	18.3	6.5	35.5	15.7	31.1	12.6	26.0	13.5	19.5	7.2
250	72.3	38.9	27.4	7.2	24.4	8.9	20.8	4.9	51.2	32.0	34.5	13.5	22.8	3.3	19.2	4.1
500	82.7	38.0	65.5	25.9	34.7	16.4	35.9	10.7	69.6	31.1	34.6	7.8	19.1	5.0	34.8	9.9
1000	81.9	49.9	41.7	7.3	37.4	19.4	42.1	16.3	60.6	41.7	42.2	18.1	74.6	31.5	40.4	8.8
2000	181.1	69.6	88.7	26.6	37.7	13.2	55.1	19.4	162.7	81.8	102.6	20.2	120.1	23.2	74.0	11.1
5000	**284.5**	**122.4**	206.1	42.2	126.5	42.4	187.4	59.0	170.3	40.0	139.4	14.4	216.8	46.6	**101.0**	**25.3**

Bold values represent a difference in the means within treatments at a significance of $P<0.05$

Table 3.4: Comparison of shikimate acid measurements as percent of control for the 24 hr incubation.

Glyphosate	Cultivar															
Treatments	Andes		Maximus		TAMTBO		FLX		Kodiak		Royal		Bounty		Bruiser	
(μM)	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
0.5	18.0	7.2	12.2	4.2	7.9	4.8	18.9	11.3	18.7	7.4	24.5	6.6	23.3	9.9	14.4	6.0
1	12.9	3.4	30.2	6.4	17.0	7.8	35.8	9.7	11.2	5.4	12.1	4.1	25.2	10.5	19.3	8.0
5	26.4	12.2	51.3	16.9	30.9	10.4	18.3	7.6	16.1	7.1	14.6	8.5	24.6	9.3	18.4	3.9
10	43.8	13.2	53.2	30.4	16.2	7.5	16.3	5.6	17.8	7.9	14.8	3.8	22.3	14.1	21.8	5.1
50	63.8	25.2	17.1	4.8	12.3	7.4	19.1	4.3	23.3	8.7	11.7	5.6	19.2	4.9	19.3	6.9
100	19.2	16.4	35.8	5.8	17.4	6.6	23.0	5.0	17.7	11.8	16.4	7.6	17.7	6.5	39.2	12.4
250	19.2	3.6	52.7	18.6	10.1	3.4	27.4	8.2	24.7	10.1	18.6	8.7	23.3	10.9	23.3	11.3
500	22.8	6.3	38.3	26.1	22.6	9.3	31.4	8.6	11.0	3.3	26.4	12.1	32.6	8.4	21.5	4.4
1000	30.0	11.2	41.1	17.5	42.8	23.5	22.4	6.5	23.7	12.7	44.4	15.0	16.8	6.7	39.1	13.3
2000	48.8	14.8	67.5	23.6	32.9	7.2	67.2	16.3	54.5	17.6	41.7	13.3	46.0	14.9	86.2	20.7
5000	88.7	24.6	102.4	42.0	132.3	28.4	**55.5**	**13.6**	103.7	30.1	141.6	37.3	**60.1**	**13.6**	**167.3**	**72.9**

Bold values represent a difference in the means within treatments at a significance of $P<0.05$

CHAPTER 4: GLYPHOSATE-RESISTANT ITALIAN RYEGRASS (*LOLIUM* SPP *PERENNE MULTIFLORUM*) POPULATION FROM A CHERRY (*PRUNUS AVIUM)* ORCHARD

ABSTRACT

Glyphosate is a useful herbicide for vegetation management in many crop and non-crop settings. Repeated applications of a herbicide with a single mechanism of action are capable of providing the required selection pressure to select a resistant population. At this time, 32 weed species have been identified that have glyphosate resistance, including Italian ryegrass. A population of Italian ryegrass (OR10) could not be controlled with labeled rates of glyphosate in a cherry orchard in the Willamette Valley of Oregon. This orchard received several applications of glyphosate per year. The purpose of this experiment was to determine if this population was resistant to glyphosate. The biomass and the number of surviving plants were greater for the OR 10 population than the susceptible Gulf population following application of glyphosate in the greenhouse. The OR10 population was found to be resistant to glyphosate.

INTRODUCTION

Glyphosate is a non-selcective, systemic, broad-spectrum herbicide used for vegetation control in crop and non-crop settings (Dill et al. 2008). This herbicide was produced and commercialized in the 1970s under the trade name Roundup® (Duke and Powles, 2008). Glyphosate inhibits the sixth enzyme in the shikimic acid pathway, 5-enolpyruvylshikimate 3-phospate synthase (EPSPS) preventing the formation of 5-enolpyruvyl-shikimate-3-phosphate (EPSP) (Duke and Powles, 2008). EPSP is an intermediate compound in the production of the aromatic amino acids phenylalanine, tyrosine, tryptophan, and other aromatic secondary metabolites essential to plant survival (Herrmann, 1995; Kishore and Fuchs, 2002; Geiger and Fuchs, 2002). The inhibition of EPSPS results in increased levels of shikimic acid and shikimate-derived benzoic acid, which reduce carbon fixation and photosynthesis (Duke et al. 2003; Siehl, 1997). Plant death is the result of the loss of essential plant compounds and biochemical pathways (Duke and Powles, 2008; Pline-Srnic, 2005; Shieh et. al 1991).

The first report of glyphosate resistance was in Australia within a population of rigid ryegrass (*Lolium rigidum*) (Pratley et al. 1996). This population received multiple applications of glyphosate per year as a vegetation management practice in an orchard. The population had reduced glyphosate translocation (non-target site resistance) and accumulation in the actively growing roots and meristematic tissue (Powles and Preston, 2006; Wakelin et al. 2004). Target-site mutations are typically observed as a substitution of the proline 106 amino acid to serine, threonine, or alanine (Baerson et al. 2002; Ng et al 2003). Along with target site mutations, gene duplication is another mechanism of resistance that correlates to the genomic copy number, EPSPS mRNA expression, and EPSPS protein levels (Salas et al. 2011). Therefore, as cross-

pollinated species commonly develop multiple mechanisms of resistance under selection (Sammons and Gaines 2014), resistant biotypes need to be prevented in *Lolium* spp.

Continual application of herbicides with similar mechanisms of action could result in the evolution of a resistant population (Christoffers 1999; Roux et al. 2008). The first glyphosate resistant population of Italian ryegrass (*Lolium perenne* ssp. *multiflorum*) was found in a Chilean fruit orchard that received multiple glyphosate applications a year (Perez and Kogan, 2003). Similarly, the glyphosate resistant Italian ryegrass populations that have been found in Oregon have also received multiple applications of glyphosate as part of general vegetation control (Perez-Jones et al. 2005; Liu et al. 2013). A population of Italian ryegrass found in a cherry orchard (OR10) that received multiple applications of glyphosate was suspected to be resistant. Therefore, to determine if the OR10 population was resistant, a dose-response experiment was conducted.

MATERIALS AND METHODS

Plant material

Plants from the OR10 Italian ryegrass population were collected in 2014 from a cherry tree orchard in Oregon. Seed was produced in the greenhouse from the parental lines collected in the field. The herbicide use history of the orchard consisted of multiple applications of glyphosate per year. Gulf, a known herbicide-susceptible Italian ryegrass cultivar (S), was used as the control.

Greenhouse dose-response procedures

Environmental conditions during the growth of the susceptible and OR10 populations were 25/20 C day/night temperatures and ambient sunlight supplemented with grow lights at 25 mW cm^{-2} for 14 hours of light a day in the greenhouse. Plants were grown in 25.4 by 25.4 cm pots filled with Metro-mix® soil. A Generation III Research Sprayer® with 8004 nozzles and calibrated to deliver 187 L ha^{-1} at 276 Kpa was used to apply treatments.

Thirty seeds from each population were placed in each Metro-mix® soil filled tray. The plants were grown to the 2 to 3 leaf stage and thinned to an even number plants (15 and 20 plants) per tray 4 days prior to treatment. The late emerging plants, after treatment applications, were removed. The populations received nine treatments including a control and 8 glyphosate treatments (Touchdown Hitech®) with the surfactants of 1.80 kg L^{-1} ammonium sulfate (AMS) and 0.25% v/v non-ionic surfactant (R11) (Table 4.1). Based on preliminary experiments, the two populations received different rates of glyphosate with four overlapping concentrations to obtain a full dose-response.

Twenty-four days after treatment (DAT), the number of surviving plants was counted and the above ground biomass was collected and dried at 70 C for 72 hours. Percent dry weight of the treated plants relative to the untreated controls for both the susceptible and suspected resistant populations was calculated. The experiment had 4 replications and was repeated in two studies and the data were pooled. Homogeneity of variance between populations survival was compared with similar treatments. An ANOVA and analysis of means were used to determine influence of variables at a significance of $P < 0.05$.

RESULTS AND DISCUSSION

The number of survivors for the common treatments, 1.680 kg ae ha^{-1}, 3.360 kg ae ha^{-1}, 5.040 kg ae ha^{-1}, and 6.720 kg ae ha^{-1}, indicated a greater number of survivors for the OR10 population than the susceptible Gulf (Table 4.3). Similarly, the biomass for the OR10 population was greater for all of the glyphosate treatments than the susceptible Gulf population (Table 4.2). The dose response data did not fit the nonlinear dose response curve. However, the resistance was estimated to be greater than a 100-fold.

The level of glyphosate resistance in most rigid ryegrass and Italian ryegrass populations is low. These populations mostly have the reduced translocation mechanism of resistance and target-site mutations conferring resistance ranging from 2- to 15-fold (Perez-Jones et al. 2005; Powles and Preston, 2006; Powles et al. 1998; Jasieniuk et al. 2008; Wakelin and Preston, 2006; Wakelin et al. 2003). Glyphosate resistance has been reported to be upward of 100-fold with EPSPS gene amplification resistance (Gaines et al. 2010; Preston et al. 2009). Because the resistance levels found in this OR10 population resulted in survivors at the highest 26.880 kg ha^{-1} rate and no survivors were found at the highest 6.720 kg ha^{-1} in Gulf, gene amplification should be investigated as a source of resistance.

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Table 4.1: Dose-response glyphosate rates and the respective populations (kg ha^{-1})

Gulf	0.105	0.210	0.420	0.840	1.680	3.360	5.040	6.720
OR10	1.680	2.100	2.520	3.360	5.040	6.720	13.440	26.880

Table 4.2: Comparison of per plant weights for the untreated control and the four glyphosate treatments.

	Treatments (kg ha^{-1})									
	Untreated Control		1.68		3.36		5.04		6.72	
Population	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Gulf (S)	0.159	0.019	0.020	0.002	0.018	0.003	0.017	0.003	0.017	0.004
OR10 (R)	0.153	0.009	0.077	0.002	0.054	0.006	0.042	0.003	0.037	0.004

Table 4.3: Comparison of percent of surviving plants for the untreated control and the four glyphosate treatments.

	Treatments (kg ha^{-1})				
Population	Untreated Control	1.68	3.36	5.04	6.72
Gulf (S)	99	1	0	0	0
OR10 (R)	100	85	72	39	46

CHAPTER 5

GENERAL CONCLUSIONS

The studies described in this thesis were conducted to determine if there were differences in response to termination treatments between diploid and tetraploid Italian ryegrass cultivars intended for the cover crop market. The treatment evaluation investigated possible herbicide applications that could be effective for controlling Italian ryegrass and the cultivar evaluation examined possible variation in response to glyphosate depending on ploidy level or cultivar. An evaluation of the suspected resistant population OR10 was conducted to determine if the population collected was resistant to glyphosate.

The treatment evaluation indicated that glyphosate and glyphosate in combination with either saflufenacil, rimsulfuron, or pyroxasulfone are effective treatments for the control of Italian ryegrass. Although paraquat followed by clethodim provided the greatest reduction in biomass, the regrowth that was observed could result in management problems in the following cash crop. The slower rate of control with the clethodim alone treatment would be undesirable for a cover crop control treatment. Visual ratings of glyphosate treatments suggested that lower rates of glyphosate did not control the cover crop as effectively as the higher rates. Therefore, higher rates of glyphosate would be optimal to ensure cover crop termination even though there were no observable differences in biomass reduction between cultivar or ploidy level.

Overall, there was no significant difference between the response in treatment with glyphosate for the diploid Bounty® cultivar and the tetraploid TAMTBO® cultivar. The only difference observed was between the highest and lowest rates of glyphosate. The unique

difference was in the response of glyphosate 27 DAT, were TAMTBO had a greater reduction in biomass at the higher rate than the lower rate of glyphosate and Bounty had greater reduction in biomass at the lower rate than the higher rate of glyphosate.

The cultivar evaluation further supported an absence of the effect of ploidy level on the ability to control an Italian ryegrass cover crop with glyphosate. The only observable difference in response due to ploidy level for biomass was exhibited between the untreated controls at the earliest date of evaluation. The tetraploid cultivars had a greater average biomass than the diploid cultivars. There were no other differences in response for biomass at any of the later evaluation dates or between treatments. The visual ratings did not indicate that ploidy level had any effect in response to glyphosate; however, there was an indication that there was a difference in response dependent on the cultivar. Visual results indicated that both Kodiak and Andes were controlled greater than Royal and TAMTBO at 27 DAT and Maximus 84 DAT. By utilizing the shikimate acid bioassay measurements we determined that FLX 1995 had greater shikimate acid than Royal and TAMTBO 27 DAT and Maximus 84 DAT. Therefore, the absence of differences in the biomass, visual rating, and the inconsistency in bioassay measurements seem to indicate there is not a significant difference in control of diploid and tetraploid Italian ryegrass cover crops with glyphosate.

The specific differences in shikimate acid accumulation between the cultivars could represent a difference in the number of target enzymes binding with glyphosate. The differences between cultivars of Italian ryegrass could provide an evolutionary force for survival under selection pressure such as herbicide applications. As polyploidization allows for the masking of deleterious recessive mutations, transformative properties of duplicated genes, and transgressive

performance with stable heterosis (Weiss-Schneeweiss et al. 2013), the differences in the evolutionary mechanisms for survival could have no observable differences without selection.

Results from the herbicide evaluation and the cultivar evaluation indicate there could be possible differences in the visual ratings of control for an Italian ryegrass cover crop. Less control was observed with the lowest rate of glyphosate for the herbicide evaluations. Because the tetraploid cultivars have a greater initial biomass than the diploid cultivars, this might provide some difference in control between the ploidy levels due to an insufficient rate and coverage on cultivars with greater amounts of biomass. The bioassay indicated no difference in response between ploidy levels but there were possible differences between cultivar. The recommended field rate of glyphosate was sufficient to control the cover crop.

Although there was no difference in response for control with glyphosate in Italian ryegrass, glyphosate resistance is still readily selected, as demonstrated by our results screening the OR10 population. Because the *Lolium* spp. have shown the ability to evolve resistance rapidly in the presence of herbicide application (Powles and Preston, 2006), it is important to reduce the selection pressure of herbicides. Therefore, management practices should be taken as increased reliance on glyphosate worldwide will inevitably result in a greater selection for more glyphosate resistant weeds (Hulting, 2013; Powles and Preston, 2006).

One practice that could be utilized is reducing the use of glyphosate in either seed production or in the cover crop market. Unfortunately, glyphosate is a useful tool for total vegetation control in both agricultural situations. Within this study and based on previous observations, glyphosate resistance has not been discovered in seed production fields (Appendix A), but it has been found in multiple orchards receiving several applications of glyphosate for total vegetation control. Therefore, to delay the spread of glyphosate resistance in an obligate

cross-pollinating species, reduced glyphosate applications in these locations could help delay the emergence of resistance in seed production. As vegetation control is still a necessity prior to harvest of many orchards, alternating herbicide MOA in the orchards would reduce the selection pressure for glyphosate resistant weed species.

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APPENDIX A

This experiment was designed and planted on March 3, 2015 as a strip plot design with the 18 seed samples (Table 1) randomized in single 30.5 meter strips per each individual planting unit with 4, 7.6 meter replications. Within each replication 2 strips of a 1.68 kg ae ha-1 glyphosate rate, with 0.25% v v^{-1} NIS R11, and 1.02 kg L^{-1} AMS were applied. The treatment was initiated on April 2, 2015 at the third leaf growth stage. Application of the treatments was completed with a unicycle sprayer, operated at 241 kPa, using a flat fan 11002 nozzle, spaced 20.3 cm apart, with a boom length of 2.3 meters, average boom height of 71.12 cm, and a spray volume of 1050 L ha^{-1}. The number of Italian ryegrass seedlings germinated and survivors were quantified 28 days after treatment (DAT) and analyzed for normality using the Shapiro-Wilk normality test. The differences between the cropping histories of the seed lots were determined through ANOVA. The separate samples were analyzed using a Tukey's comparison of means test and at a significant difference of $P < 0.05$.

None of the samples collected were completely controlled at the initial application. To ensure this was not a function of late emergence, a second application of glyphosate at a rate of 1.68 kg ae ha^{-1} was applied 35 days after the initial application. There was no difference in the number of surviving plants at both dates after treatment. The only difference observed was in the initial germination. TAMTBO and Flying A cultivars both resulted in a lower amount of initial seedlings prior to glyphosate applications in comparison to Gulf. No discernable difference between surviving plants and cropping history was observed. The absence of survivors following the second treatment suggests glyphosate resistance is not present in the seed production samples collected.

Table 6.1: Italian ryegrass seed samples from production fields screened for resistance screening samples.

Sample	Cultivar	Field History	Ploidy
1	Assist	Volunteer	Diploid
2	Bounty	Volunteer	Diploid
3	Royal	Volunteer Row Spray	Diploid
4	Royal	Conventional	Diploid
5	Royal	No-Till	Diploid
6	Assist	No-Till	Diploid
7	Gulf	Volunteer	Diploid
8	Bounty	No-Till	Diploid
9	Royal	No-Till	Diploid
10	Royal	No-Till	Diploid
11	Royal	Conventional	Diploid
12	Carreys Marshall	Mixed	Diploid
13	Flying A	Mixed	Diploid
14	TAMTBO	Mixed	Tetraploid
15	Ribeye	Mixed	Diploid
16	Floyds Gulf	Mixed	Diploid
17	Bruiser	No-Till	Diploid