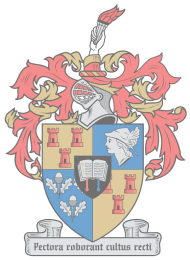
The influence of manganese on glyphosate efficacy in ryegrass (*Lolium spp*.)

by

Enrike Verster



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Supervisor: Dr. P.J. Pieterse

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Declaration

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Abstract

In the Western Cape of South Africa, ryegrass (*Lolium* spp.) developed resistance to the herbicide glyphosate within wheat fields, orchards and vineyards. The influence that plant available Mn might have on glyphosate resistance in ryegrass were investigated by measuring percentage survival and dry matter production in response to Mn treatments and glyphosate dosage rates.

Four different trials were conducted in this study. In the first trial, glyphosate dosage response was tested against the effect of added Mn treatment levels on glyphosate susceptible and resistant ryegrass populations. In the second trial, glyphosate dosage response was tested against the effect of soil types of differing Mn contents. In the third trial, glyphosate dosage response was tested against ryegrass species found in agricultural setting in soils of different Mn content from the same farm where it naturally grew. Glyphosate dosage response in ryegrass were lastly also tested under varying temperature ranges and Mn treatment levels.

Glyphosate efficacy was found to exhibit trends of decline at higher Mn treatment levels, especially at sublethal dosage rates or soils of naturally higher Mn content. Ryegrass originating from a soil of higher Mn content (biotype R3) as opposed to their counterparts seemed to be naturally more inclined to a magnified resistance, regardless of the soil into which it was transplanted.

When testing glyphosate dosage response against Mn treatment level and temperature conditions, trends showed that regardless of the temperature conditions ryegrass were subjected to, Mn levels played a distinct role in glyphosate efficacy. Added Mn exhibited trends of better survival rates than control treatments throughout the temperature conditions. At 25-30°C, glyphosate efficacy was reduced compared to the lower temperature ranges.

Generally, it was found that Mn treatments seemed to exert some effect of decreased glyphosate efficacy in ryegrass albeit only at low, sub-lethal dosage rates. Trends indicated that some levels of added plant available Mn seemed to curb glyphosate efficacy at low dosage rates. It was concluded that some effect must exist where plant available Mn influences the herbicidal activity of glyphosate in a negative manner. Reduced translocation is the most likely explanation for this trend. This reduced efficacy probably occurs as a result of either complexation of glyphosate with Mn and subsequent reduced uptake or vacuolar sequestration. However, Mn did not affect glyphosate efficacy at recommended dosage rates. Results of this study emphasize the importance of applying glyphosate at recommended dosage rates and never to apply below recommended dosage rates.

Uittreksel

Raaigras (*Lolium* spp.) het in die Wes-Kaap van Suid-Afrika oor die jare heen weerstandigheid ontwikkel in koringlande, wingerde en boorde teen die onkruiddoder glifosaat. Die invloed wat plant beskikbare Mn mag hê op glifosaat weerstandigheid in raaigras, is ondersoek deur oorlewingspersentasie en droëmassaproduksie te meet teenoor Mn behandelings en glifosaat dosisse.

Vier verskillende proewe is in hierdie studie afgehandel. In die eerste proef is glifosaat se reaksie getoets teen die effek van toegediende Mn vlakke op glifosaat weerstandige en -vatbare raaigras populasies. In die tweede proef is glifosaat se werking getoets teen die effek van verskillende grondtipes wat verskillende natuurlike Mn vlakke bevat. In die derde proef is die werking van glifosaat getoets teen raaigras populasies wat in landbougrond van verskillende Mn vlakke gevind is op die plaas waar dit natuurlik gegroei het. Laastens was die werking van glifosaat ook getoets in verskeie temperature in kombinasie met Mn behandelings.

Daar is bevind dat die effektiwiteit van glifosaat afneem by hoër vlakke van Mn behandelings of grondtipes van natuurlike hoër Mn inhoud – veral by sub-letale dosisse. Raaigras afkomstig van grondtipes met ‘n hoër Mn inhoud (biotipe R3), blyk meer geneigd te wees tot ‘n verhoogde weerstandigheid in vergelyking met ander raaigras populasies; ongeag van die grond tipe waarin dit oorgeplant is. Tendense het getoon dat Mn by alle temperatuurvlakke ‘n wesenlike rol speel in die effektiwiteit van glifosaat. Behandelings van toegevoegde Mn het tendense getoon van beter oorlewing by al die temperatuurvlakke teenoor die kontrolebehandelings. By 25-30°C was die werking van glifosaat verminder vergeleke met die laer temperatuurvlakke.

Oor die algemeen is daar bevind dat Mn behandelings tog ‘n effek van verlaagde effektiwiteit uitoefen op die werking van glifosaat op raaigras alhoewel slegs by lae, subletale dosisse. Tendense het getoon dat veral sekere vlakke van toegevoegde plant beskikbare Mn die werking van glifosaat stu. Die gevolgtrekking is gemaak dat plant beskikbare Mn heel moontlik die onkruiddodende aktiwiteit van glifosaat negatief beïnvloed. Verminderde translokasie deur die plant is heel moontlik die mees voor die hand liggende verduideliking van hierdie tendens. Die verlaagde effektiwiteit is moontlik as gevolg van kompleksasie van die glifosaat molekule met Mn en die daaropvolgende verlaagde opname of vakuolêre sekwestrasie. Mangaan het egter nie die effektiwiteit van glifosaat benadeel wanneer dit teen geregistreerde dosisse gespuit is nie. Die resultate van hierdie studie beklemtoon weereens die belangrikheid om glifosaat teen aanbevole dosisse toe te dien en nie teen laer as geregistreerde dosisse nie.

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- Soli Deo Gloria.

Abbreviations

°C	Degrees Celsius
ANOVA	Analysis of variance
DM	Dry matter
g ai L^{-1}	Gram active ingredient per litre
g	Gram
L ha^{-1}	Litre per hectare
LSD	Least significant difference values
mM	Milli Molar
Mn	Manganese
MN1	Soil collected from Altona farm (5.26 mg/kg manganese content)
MN2	Soil collected from Welgevallen experimental farm (65.98 mg/kg Mn content)
MN3	Soil collected from Langgewens farm (147.3 mg/kg Mn content)
MN4	Soil collected from Murludi fruit farm, Tulbagh area (215.20 mg/kg Mn content)
MN5	Soil collected from Murludi fruit farm, Tulbagh area (13.7 mg/kg Mn content)
R1	Moderately resistant ryegrass biotype (wheat field, Malmesbury)
R2	Strongly resistant ryegrass biotype (Welgevallen experimental farm)
R3	Putative resistant ryegrass population obtained from soil MN4
S1	Susceptible ryegrass biotype (Mach1, Agricol)
S2	Susceptible ryegrass biotype (Rondebosch Common)
S5	Putative susceptible ryegrass population obtained from soil MN5

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Chapter 1

Introduction

Ryegrass (*Lolium spp.*) is a well-known and widely cultivated plant species. Perennial ryegrass (*Lolium perenne*) in particular is globally cultivated for livestock grazing as well as fodder. It is also used in the stabilisation of soil and pasture improvement as an efficient cover crop and is widely known for its suitability as a lawn and turf grass (Anon 2015b).

Ryegrass had in the past years become infamous in the Western Cape of South Africa as well as in various places across the globe as a notorious weed invading orchards, vineyards and cereal crops. The innate weedy characteristics of ryegrass include rapid adaptation to its environment, copious seed production and easy dispersion (Anon 2015c, Anon 2015a). The number of plants treated over a period of time and the fitness, frequency and number of genes involved in conferring resistance to each site of action by the herbicide also plays an important role in selection for resistance (Heap 2014). These characteristics put ryegrass in the ideal position to rapidly spread and develop herbicide resistance, but in particular; glyphosate resistance.

Glyphosate had been deemed as the single most effective herbicide because of its broad-spectrum systemic properties. Glyphosate inhibits the biosynthesis of aromatic amino acids in plants and consequently the synthesis of proteins. Glyphosate blocks an important step in the shikimate pathway of plants. This shikimate pathway ultimately synthesizes aromatic amino acids and important plant metabolites (Székács and Darvas 2012). Therefore, inhibition of this pathway results in significant consequences for the plant body.

Glyphosate has been commercially available since 1974. As time went by and the selling price of glyphosate eventually decreased, farmers began to widely use it in systems of zero-tillage and for pre-emergence weed control. Roundup Ready® crops in the form of soybean entered the agricultural scene in 1996, and subsequently the use of glyphosate increased drastically as it became highly convenient in agronomic crops.

The first instance of glyphosate resistant rigid ryegrass (*Lolium rigidum*) was found in orchards in Australia in 1996. The herbicide was applied several times per year consecutively for more than a decade (Heap 2014). Glyphosate resistance in rigid ryegrass was followed by resistance in Italian ryegrass (*Lolium multiflorum*) in Chile in 2001 and perennial ryegrass in Argentina in 2008. Hence after, resistance was recorded in the USA, New Zealand and several countries in Europe amongst many other

places globally (Heap 2009, Heap 2014). As pertaining to South Africa, glyphosate resistance in perennial ryegrass was first recorded in 2001 in a valley of the Boland in the Western Cape of South Africa (Heap 2009).

The process of natural selection quite understandably and predictably often results in herbicide resistance. According to (Heap 2014), rare mutations which confer herbicide resistance exist in plant species even before herbicide application. These mutations are amplified through repetitive use of a single herbicide up to a point where no control of the specific weeds are obtained at recommended dosages in agricultural systems (Heap 2014).

Five main mechanisms of herbicide resistance are prevalent: target site mutation, enhanced metabolism, altered translocation (decreased absorption), vacuolar sequestration and gene amplification (Heap 2014).

Environmental circumstances play a role in the development of glyphosate resistance – some to a lesser and some to a greater extent. The role of inherent soil characteristics and interactions within the rhizosphere cannot be downplayed. Soil can be deemed as a complex organism in its own right. Therefore, the attempt to isolate one specific trait or interaction as the sole reason for development of resistance would be near impossible. Any single factors examined should be interpreted in the perspective of a larger integrative whole.

When glyphosate resistance was first recorded in rigid ryegrass in the Western Cape of the South Africa, the contributing weed scientists involved made the observation that the particular valley where it was recorded had been cultivated for more or less 300 years. The soil was found to be very acidic and subject to manganese toxicity (Heap 2009).

Manganese had been known as one element amongst others that may play a role in the translocation of glyphosate throughout the plant. Manganese is known to form complexes with the glyphosate molecule. As a result, Mn indirectly inhibits systemic action of the herbicide by hindering the free transport of the glyphosate molecule throughout the plant (Hartzler 2010, Soltani et al. 2011). Limited translocation of glyphosate inevitably therefore leads to less necrosis of the plant and ultimately less mortality and unsatisfactory control of the population of weeds (Bailey et al. 2002).

It is a known soil chemistry fact that in the case of manganese, the amount of plant available manganese drastically decreases as soil pH increase. Therefore, the effect of the reduced efficiency of the glyphosate molecule may well easily be countered by the application of lime to the soils in question.

That said, soil pH should always remain within acceptable ranges in order to sustain satisfactory plant growth of crops.

During hot and dry summer conditions, available Mn is more likely to be toxic to plants and under these conditions unlimed soil is also more likely to be Mn toxic. These conditions catalyze chemical changes where oxidized Mn is converted to reduced Mn which is the plant available form (Upjohn et al. 2005).

As mentioned in the literature cited above, temperature certainly exerts an effect on the amount of plant available Mn in soil. The question arises if temperature will severely effect glyphosate resistance. An effect should also be found on plant growth and metabolism through increased transpiration. Would an increase in temperature cause more Mn to become available to plants in the soil and hence form complexes with the glyphosate molecule; or would an increase in temperature significantly boost plant metabolism and affect glyphosate efficiency; or would a combination of these factors play a visibly significant role?

Furthermore, it is a well-known and researched fact that the performance of glyphosate resistance is temperature dependent (Tanpipat et al. 1997, Adkins et al. 1998, Sammons and Gaines 2014). In the study of Ghanizadeh et al. (2015), it was found that a glyphosate resistant population of perennial ryegrass with the mechanism of restricted herbicide translocation exhibited suppressed glyphosate resistance under sub-optimal (below 10°C) growing temperatures. Similar results have been found in rigid ryegrass, (Vila-Aiub et al. 2013), horseweed (Ge et al. 2011) and barnyard grass (Nguyen et al. 2016). Therefore, lower growing temperatures appear to counteract some of the resistance mechanisms, in particular the mechanism of vacuolar sequestration.

Assuming that the effects of plant available Mn and temperature are as severe on glyphosate efficiency in the context of this research as mentioned above, the question arises on how to manage the prevalence of said effects.

Lime application in order to lift the pH of soil may prove to be a very helpful method to decrease plant available manganese in the soil (Upjohn et al. 2005), but further research is to be done on the topic.

A very prevalent solution researched and posed by several authors is the recommendation of spraying glyphosate on emerged weeds at temperatures as low as possible (Tanpipat et al. 1997, Adkins et al. 1998, Ge et al. 2011, Vila-Aiub et al. 2013, Sammons and Gaines 2014, Ghanizadeh et al. 2015, Nguyen et al. 2016).

The main objectives of this study

The main objectives of this study were:

1. To determine whether plant available manganese in soils of the Western Cape decreases the efficacy of glyphosate in different ryegrass biotypes under glasshouse conditions.

2. To determine the effect of a range of controlled temperature conditions in glasshouses on the efficacy of glyphosate on manganese treated ryegrass.

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Chapter 2

Literature Review

2.1. Introduction

Glyphosate [N-(phosphonomethyl)glycine] is arguably the most widely used and important herbicide worldwide. Glyphosate has been in commercial use for 40 years now, after being introduced to the market in 1974. In 1995, transgenic glyphosate resistant crops (soybean, cotton, maize etc.) were introduced to agriculture. These genetically modified crops allowed for glyphosate to be used in weed management as a selective herbicide (Yu et al. 2007).

Glyphosate is very widely known and applied as a highly effective, systemic, broad spectrum herbicide controlling annual and perennial weeds (Yu et al. 2007; Duke and Powles 2008). It is commonly used as a foliar-applied, post-emergence herbicide.

Glyphosate is environmentally safe as it can be rapidly inactivated in soils and therefore exhibits little residual soil activity (Yu et al. 2007; Duke and Powles 2008; Borggaard 2011). The glyphosate molecule exhibits outstanding water solubility characteristics which makes its systemic action possible (Székács et al. 2012). Translocation takes place via the phloem from leaves towards delicate sink tissues (Ge et al. 2012). Gradual necrosis and eventual death of the whole plant occurs.

Ryegrass (*Lolium spp.*) is a notorious plant when mentioned alongside glyphosate. It is a widely established glyphosate resistant weed. Some species of ryegrass are grown as pasture and feed for livestock. However, in orchards, vineyards and some field crops it is a weed which can lead to vast yield reductions if left to proliferate freely (Baerson et al. 2002). The first reported case of glyphosate resistance in ryegrass was reported in Australia in *Lolium rigidum* (rigid ryegrass) around 1996 (Powles et al. 1998; Yu et al. 2007).

Ryegrass exhibits several characteristics which contribute toward the evolutionary development of its herbicide resistance. According to Feng et al. (1999) and Baerson et al. (2002) wide distribution, allogamous reproduction, abundant seed set as well as substantial genetic variability are traits contributing to herbicide resistance of a rigid ryegrass population from Australia in particular. On top of that, continuous and persistent use of one herbicide in any area naturally creates a high selection pressure. All these factors combined apply to and work together in facilitating the development of herbicide resistance in herbicide resistant ryegrass species around the world.

As mentioned, ryegrass has an allogamous reproductive habit, i.e., it is an obligate cross-pollinated species which means that a diversity of genetic material from two separate plants from the same species are involved in successful pollination. Therefore it should certainly have been expected that a variety of mechanisms of glyphosate resistance and consequent multiple resistance were realistically bound to arise in ryegrass (Preston et al. 2009). Such cases involving a variety of herbicides are already widely documented (Yu et al. 2007).

Two broad types of resistance mechanisms have been identified; target site resistance and non-target site resistance. According to Powles and Preston (2006) both the above mentioned mechanisms (target as well as non-target site) are inherited as nuclear traits on one single gene. Target site resistance comprises of the physical mutational changes that the EPSPS target site undergoes, aiding in the development of resistance. Non-target site resistance on the other hand includes phenomena such as enhanced metabolism, altered translocation, herbicide sequestration and gene amplification or overexpression (Heap 2014).

The complex role of soil in any agricultural and agronomic research is an always included factor of paramount importance (Gerritse et al. 1996; Beltrão et al. 2013; Ololade et al. 2014). Soil composition, fertility, pH, nutrients, CEC, moisture and organic matter content are some of the many important factors which should be taken into careful consideration when researching glyphosate efficiency in any weed species.

2.2. Glyphosate

The glyphosate molecule and EPSPS

The glyphosate molecule [N-(phosphonomethyl)glycine] in Figure 1.1 is a derivative of the amino acid glycine (Nandula 2010). This soluble molecule is usually formulated in the form of ammonium, isopropylammonium, potassium, sodium or trimesium salts in order to further increase its solubility in water (Székács et al. 2012) to aid optimal translocation in the plant.

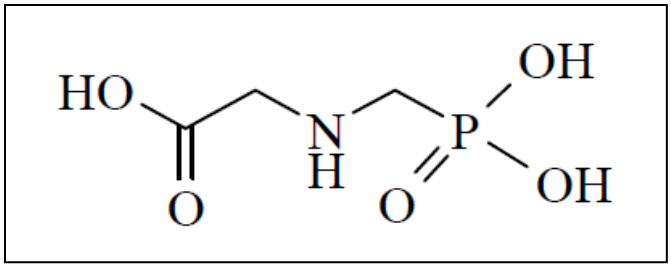

Figure 1.1: The glyphosate molecule; from Székács and Darvas (2012)

EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) is an enzyme at work within the chloroplasts of plants. It forms a crucial part of the shikimate biosynthesis pathway (Figure 1.2) which produces shikimic acid. Shikimic acid precedes the formation of the aromatic amino acids tryptophan, tyrosine and phenylalanine (Yu et al. 2007; Preston et al. 2009; Maeda and Dudareva, 2012).

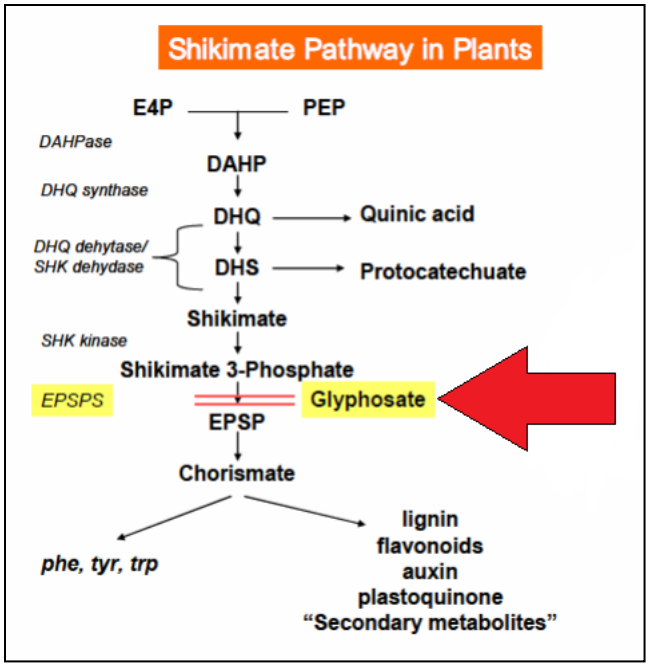

Figure 1.2: The shikimate pathway; from Kretzmer et al. (2007)

These amino acids eventually play pivotal roles in plant essential functions such as growth and development, propagation, defence against pathogens and responses to the environment (Maeda and Dudareva 2012). The shikimate pathway also produces a range of aromatic products besides the above-mentioned amino acids which are used for protein synthesis. Such products are plant hormones, alkaloids, lignins and benzoic acids (https://passel.unl.edu/pages/; Nandula, 2010), each of which plays an important part in healthy plant metabolism.

Mode of action of glyphosate

Glyphosate goes to action by inhibiting EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) which is an important enzyme in the shikimate pathway of plants as illustrated in Figure 1.2 (Ge et al. 2012). This enzyme catalyses the second last step in the shikimate pathway which forms EPSP. EPSP in turn eventually synthesises a side chain in the structures of each of the essential aromatic amino acids phenylalanine, tyrosine and tryptophan (Maeda and Dudareva 2012).

Glyphosate achieves this inhibition of EPSPS by interfering with the shikimate pathway by competing with PEP (phosphoenolpyruvate) for a binding site on the EPSPS enzyme (Pavlović et al. 2011; Maeda and Dudareva 2012). Phosphoenolpyruvate is the molecule that binds to EPSPS to successfully complete the shikimate pathway under normal circumstances. The EPSPS active site is a conservative area in higher plants, making glyphosate's herbicidal action global among plant species (Székács et al. 2012). Instead of PEP, the opportunistic glyphosate binds to the EPSPS active site and leads to an abortive complex that can't complete the shikimate pathway which produces the usual products (Shaner et al. 2012).

This inhibition of EPSPS leads to shikimic acid being accumulated in the chloroplast. As a result, too little EPSP and its resulting metabolic products are produced. This depletion leads to a detrimental decline in the essential aromatic amino acids normally produced by plants (Powles and Preston 2006; Yu et al. 2007).

These aromatic amino acids (tryptophan, phenylalanine and tyrosine) are crucial building blocks for protein synthesis in living animal and plant cells. Without these amino acids the production of natural plant products like phytoalexins, alkaloids and auxin plant hormones are cut short. These products are vitally important for plant growth, plant reproduction, development, defence and many environmental responses (Maeda and Dudareva 2012). Gradual necrosis and plant death follow.

On most annual weed species, discernible effects of glyphosate may be observed within two to four days, and up to seven days for most perennial weeds (Schuette 1998). These visible effects of glyphosate manifest as progressive wilting, stunted growth, leaf wrinkling and malformation and discolouration of the plant after which it turns into browning of all above-ground growth and degeneration of underground material (Schuette 1998).

Uptake and translocation

Glyphosate is a post-emergence, foliar applied, systemic herbicide. After glyphosate is sprayed onto leaves, glyphosate molecules are absorbed into plant cells from the leaf surface. From there the molecule is translocated via the phloem to the meristematic tissues where it gets to work (Schuette 1998).

The efficacy of glyphosate as a herbicide depends on the dose reaching living plant material. Good environmental conditions and plant health strongly influence phloem transport and delivery of glyphosate to meristematic tissues (Nandula 2010).

In field conditions, optimal uptake of glyphosate into the plants it is applied to is dependent on many integrative factors. The size and spread of glyphosate droplets, glyphosate concentration, humidity, type of cuticle of the target plant and the type and concentration of surfactant are only a few such examples (Nandula 2010).

Translocation efficiency on the other hand faces potential limiting factors of its own. According to Nandula (2010), glyphosate toxicity in a plant tend to have a self-limitation peculiarity. Self-limiting toxicity is said to occur when plants are treated at lethal dosages. Cytotoxic damages take place and photosynthesis and sugar metabolism are impaired, which in turn directly limits glyphosate translocation (Feng et al. 2004; Nandula 2010). Any steps taken to accelerate uptake before self-limitation sets in is said to be beneficial for glyphosate translocation and consequent efficacy.

2.3. Glyphosate resistance in *Lolium spp.*

Background

Herbicide resistance is a predictable consequence of natural selection. According to Heap (2014), 220 weed species globally has now evolved to become resistant to at least one herbicide. In 1996, rigid ryegrass from an orchard was found and said to be the first reported glyphosate resistant weed (Heap 2014).

Preston et al. (2009) states that glyphosate resistance in particularly Italian ryegrass (*L. multiflorum*) and rigid ryegrass has spread to several countries over as much as five continents across the world. Agriculture is an intensive in terms of survival pressure exerted on any given weed species. Ryegrass species are widely spread throughout the world in many agricultural settings. Intensive agricultural weed management practices frequently bring about facilitation for the evolution of glyphosate resistance – especially when these practices are mismanaged. In Italian and rigid ryegrass, Preston et al. (2009) points out that at least two mechanisms of resistance have been identified.

A population of glyphosate resistant rigid ryegrass biotype in Australia in 1996 was the first reported case of glyphosate resistance in ryegrass. Since then, the problem has been reported worldwide including USA, Spain, France, South Africa, Israel, Italy and even new occurrences in Australia. Glyphosate resistant Italian ryegrass biotypes have been reported in Chile, Brazil, Argentina, USA, Spain and France (Yanniccari et al. 2012). Cases of resistant perennial ryegrass (*L. perenne*) have been recorded in Argentina.

Selection pressure

The characteristics of a weed species under herbicide treatment largely determine the selection of resistance in that species. As previously mentioned, ryegrass species comply to many of these required selection characteristics (e.g. fitness, breeding system, fecundity) (Heap 2014).

Regular applications of glyphosate and the widespread use of glyphosate tolerant crops (which further expands the use of glyphosate) intensify the incidence of glyphosate resistance in the environment. These circumstances exercise heavy selection pressure on any weed species, let alone one like ryegrass with exceptional proliferation characteristics (Székács et al. 2012).

Herbicide tolerance can be described as an innate ability of a species to survive and proliferate after treatment with herbicides. Resistance is the failure of herbicides to proficiently control a weed species that has previously been successfully controlled by the same herbicide. When a weed biotype with a resistant trait reproduces profusely whilst susceptible biotypes are controlled, resistance are manifested. Resistant traits are inheritable and are therefore genetically passed to the progeny of following generations (Bhatti et al. 2013).

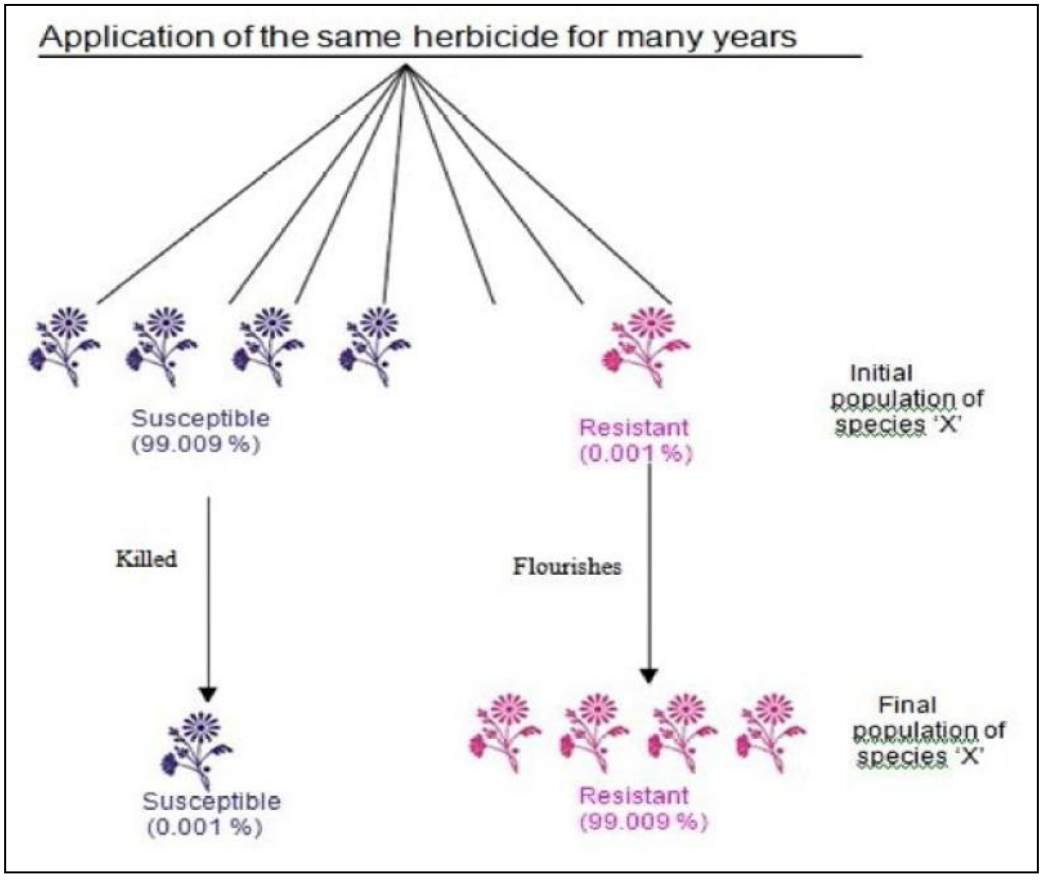

Figure 2.1: Evolution of herbicide resistance; from Bhatti et al. (2013)

Rigid ryegrass (*Lolium rigidum*)

Rigid ryegrass resistance was first reported in 1996 in Australia and in 2001 also in South Africa. Occurrence of resistance has ever since been widely reported in several other countries including USA, France, Israel and Italy (Heap 2009).

Glyphosate resistance was discovered in 1998 in a rigid ryegrass population in northern California, USA. Simarmata et al. (2005) conducted greenhouse trials in order to examine dose-responses and inheritance characteristics of the progeny of a cross between resistant and susceptible lines.

Rigid ryegrass reproduces by cross fertilization. Repeated selections through four and eight generations respectively had to be carried out by Simarmata et al. (2005) before finally a homozygous susceptible and a homozygous resistant parent was bred.

Ultimately, Simarmata et al. (2005) found that this rigid ryegrass from California had nuclear, semi-dominant inheritance characteristics. They found that resistance inheritance involves more than one mutant allele and that it is transmitted through pollen.

Wakelin and Preston (2006) studied several populations of rigid ryegrass in Australia. Their study also led to the conclusion that the nuclear genome was responsible for encoding glyphosate resistance in each of the eight populations of rigid ryegrass in their study. However, within the dosages tested, the level of dominance observed ranged from partial to total. Finally, in contrast with what Simarmata et al. (2005) reported, Wakelin and Preston (2006) concluded that a single dominant allele is responsible for the control of glyphosate resistance.

This study of Simarmata et al. (2005) preceded one by Simarmata and Penner (2008) that examined the role of glyphosate metabolism in the plants bred for resistance. They also evaluated the effect of glyphosate on the activity of EPSPS and the EPSPS gene in resistant and susceptible rigid ryegrass biotypes. No significant difference was found in the metabolism of a glyphosate molecule between the two biotypes. However, activity of EPSPS showed vastly more inhibition in the susceptible than in the resistant biotype – more than 90-fold (Simarmata and Penner 2008).

In the study of Pavlović et al. (2011), it was found that rigid ryegrass accumulated more than the normal amount of shikimic acid in susceptible populations than in resistant ones. When shikimate accumulates, it is a sign that glyphosate is reaching and inhibiting the target enzyme (Powles and Preston 2006).

The conclusion was therefore drawn that the EPSPS insensitivity of the resistant biotype was to be considered an important contributor to the resistance mechanism of this population of ryegrass in California (Simarmata and Penner 2008).

Isolation of fragments of the EPSPS gene was isolated from the biotypes. One nucleotide mutation from cytosine to thymine was identified on the resistant biotype. The mutation causes a change of one amino acid, but is in agreement with the glyphosate insensitivity of EPSPS (Simarmata and Penner 2008). This undoubtedly points to target site resistance in rigid ryegrass.

Italian ryegrass (*Lolium perenne ssp. multiflorum*)

Glyphosate resistance in Italian ryegrass was first reported in 2001 in Chile. Since then, every year since 2014 have yielded new cases in several different countries and continents and even multiple resistance has reared its head. Amongst these countries are several states in the USA, Brazil, Spain, Argentina, Italy, Japan, Switzerland and New Zealand (Heap 2009).

Weed control in fruit orchard systems in central Chile has a history of 10 years of intense glyphosate dependence (Vila-Aiub et al. 2008). This led to annual ryegrass populations selecting for glyphosate resistance. Studies examining the cause of resistance in these populations have found no differences in leaf absorption and translocation of glyphosate in susceptible and resistant varieties (Vila-Aiub et al. 2008). The origin of the resistance was found in target-site EPSPS gene mutation.

In Southern Chile, traditional winter crops such as wheat are also teeming with high densities of annual ryegrass that have evolved to become resistant (Vila-Aiub et al. 2008). Annual ryegrass resistant biotypes were documented in apple orchards in Southern Brazil. These populations exhibited 16-fold resistance as compared to a susceptible control. Interestingly enough, reduced translocation was the cause of glyphosate inefficacy (Vila-Aiub et al. 2008).

Perennial ryegrass (*Lolium perenne*)

Glyphosate resistant perennial ryegrass was first reported in 2008 in Argentina with subsequent reports in New Zealand in 2012 and Portugal in 2013 (Heap 2009).

Yanniccari et al. (2012) conducted a trial of glyphosate application on a population of perennial ryegrass from Argentina. They assessed germination, chlorophyll content, shikimic acid concentration and survival of these resistant populations as compared to a susceptible population as control.

The results led to the conclusion that chlorophyll content in resistant and susceptible populations varied greatly. The resistant population only exhibited a slight decrease in chlorophyll content whereas the susceptible populations' content plummeted upon glyphosate application (Yanniccari et al. 2012).

Congruent to results found in the other studies, Yanniccari et al. (2012) also found that accumulation of shikimate post-application are higher in susceptible biotypes than in resistant ones. Figure 2.2 illustrates the survival rates of resistant and susceptible populations in relation to glyphosate dosage.

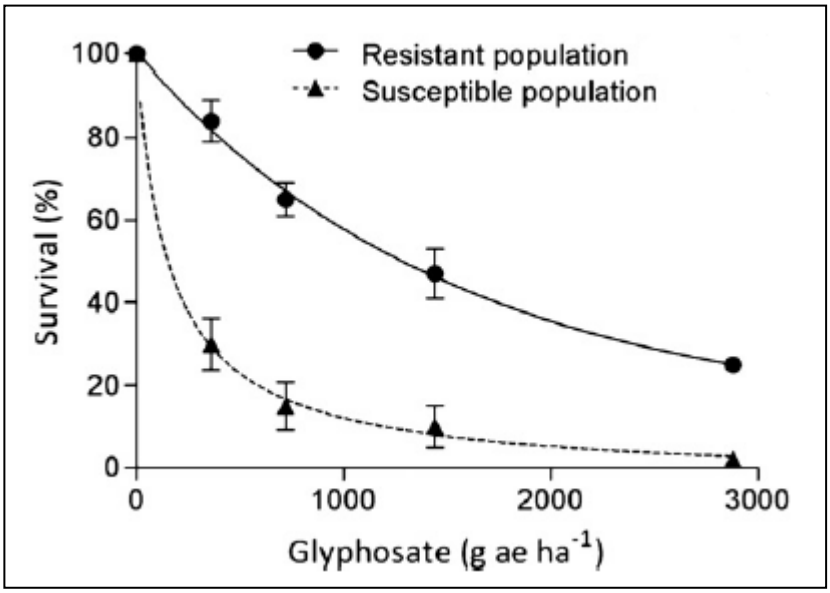

Figure 2.2: Survival rate of susceptible and resistance perennial ryegrass populations, from (Yanniccari et al. 2012)

2.4. Mechanisms of glyphosate resistance in ryegrass

Target site resistance

Evolutionary herbicide resistance can be categorised into target site or non-target site resistance (Powles and Preston 2006). As previously discussed, glyphosate is an extraordinarily effective herbicide to susceptible plant species. Its efficacy is due to the ability of the glyphosate molecule to inhibit a crucial enzyme in the plant (EPSPS) which is the target site enzyme. EPSPS is indispensable to the survival of a plant.

Substitution mutations or point mutations take place as single amino acid substitutions in the target site enzyme (EPSPS) amino acid sequence at proline106 (Pro106) particularly. Substitutions include

substitution of Pro106 to alanine (Ala), leucine (Leu), serine (Ser) and even threonine (Thr) (Powles and Preston 2006; Yu et al. 2007; Heap 2009; Preston et al. 2009; Kaundun et al. 2011). Reported examples include Italian ryegrass from California and Arkansas (USA) as well as rigid ryegrass from Southern and Western Australia with Pro106 to Ala, rigid ryegrass from South Africa as well as rigid ryegrass from Spain with Pro106 to Leu, Italian ryegrass from Argentina as well as rigid ryegrass from Italy with Pro106 to Ser and Italian ryegrass from Japan with Pro106 to Thr (Heap 2009; Kaundun et al. 2011). Pro106 to Thr substitutions have been found specifically in rigid ryegrass biotypes (Wakelin and Preston 2006; Yu et al. 2007) and Pro106 to Ser in Italian ryegrass biotypes (Perez-Jones et al. 2005; Yu et al. 2007).

Yu et al. (2007) addressed the mystery as to why specifically proline gets targeted in this substitution mutation that hinders glyphosate efficacy. Proline is the only amino acid with a cyclic structure. This cyclic structure of proline brings to pass a conformational bend at the specific area where it is present in the structure of the amino acid sequence of the protein. Yu et al. (2007) stated that this happens because proline has one hydrogen bond less than other amino acids and can be characterised as slightly less stable in conformation. In susceptible biotypes, this special conformation makes it possible for EPSPS to be able to bind with the glyphosate molecule. Consequently, the substitution from proline to alanine or other amino acids change the helix conformation and cause glyphosate to be unable to bind to the EPSPS, which ultimately causes resistance to glyphosate (Yu et al. 2007). In supporting literature Powles and Preston (2006) states that this mutational change of amino acid gives rise to changes in the herbicide target site enzyme regarding structure, charge and its affinity for water.

Because of the variety of mutations that is possible at the Pro106 site, the favouritism towards resistance development of the EPSPS binding site is clear (Yu et al. 2007). Yu et al. (2007) decisively concluded that resistance is clearly due to these previously discussed mutations as well as the fact that less glyphosate is translocated to young leaves. They specifically examined multiple resistance mechanisms responsible for resistance to glyphosate along with other well-known herbicides.

Wakelin et al. (2004) cited literature explaining different evolution mechanisms of resistance in rigid ryegrass. A glyphosate resistant rigid ryegrass population examined in a study in California (Simarmata et al. 2003) showed that resistance was ascribed to the EPSPS enzyme that is insensitive to glyphosate. A study by Vila-Aiub et al. (2008) examined resistance in annual ryegrass populations. The origin of the resistance was also found in target-site EPSPS gene mutation.

A consequent study by Simarmata and Penner (2008) on rigid ryegrass revealed that the activity of EPSPS showed more than 90-fold inhibition in the susceptible than in the resistant biotype. It was

concluded that EPSPS insensitivity was once again to blame for the resistance observed in this population of rigid ryegrass.

Powles and Preston (2006) reported that gene mutation that imparts change on the target enzyme results in target site resistance. As previously discussed, this mutational change renders the herbicide ineffective in inhibiting the enzyme function. Thereafter, the mutation develops and progress and manifestation of resistance in larger populations occur.

Physiologically, the EPSPS enzyme must still possess enough of its original functions to not affect plant fitness too adversely for the plant to be able to develop and progress and establish the resistance into populations. Quite a few target site resistance cases aside from glyphosate resistance have been looked into regarding many weed species. Such examples include triazine herbicide resistance, resistance to acetolactate synthase (ALS) inhibiting herbicides as well as resistance to acetyl coenzyme A carboxylase (ACCase) inhibiting herbicides (Powles and Preston 2006).

As Powles and Preston (2006) states, however seemingly small the percentage resistance is that these mutations impart, it will be enriched under selection pressure.

Non-target site resistance

Selection pressure is the pressure applied to a population from an external agent that alters its natural rates of mortality and brings about genetic changes to said population. If a weed is allowed to reproduce under strenuous selection pressure, even a seemingly weak mutation can be successful. Situations of severe selection pressure occur when glyphosate is applied at doses lower than recommended label dosage and when the surviving plants are continuously treated with glyphosate (Shaner et al. 2012).

Interestingly, the evolution of glyphosate resistance has taken longer to manifest than resistance to other popular herbicides. One explanation for this phenomenon is that the plant fitness cost that comes with glyphosate resistance may be higher than the fitness cost for other herbicides (Bradshaw et al. 1997; Powles and Preston 2006; Preston et al. 2009).

Busi and Powles (2009) proved that sub-lethal glyphosate dosage contributes significantly to conferring resistance to glyphosate in rigid ryegrass species. They found that not only major, but also minor genes were evolving as a result of sub-lethal dosage. After only three to four cycles of selection, the previously susceptible populations used in their experiments produced resistant progenies. LD(50) values in the progeny were twice as high as compared to the initial susceptible population.

The study concluded that this was an indication of the continuous change of minor gene traits in order to ensure survival - 33% of the glyphosate resistant progeny in their trial survived after being subjected to the recommended label dosage (Busi and Powles 2009). This percentage might seem small at first glance, but if 33% of the weeds sprayed in a crop situation are not killed, it potentially becomes a major problem the seedbank build-up of the surviving resistant biotype is considered.

Altered translocation is frequently presented as an explanation for resistance development. Resistant plants may exhibit different patterns and quantities of herbicide translocation than susceptible plants (Yu et al. 2007; Preston et al. 2009). In a study conducted by Wakelin et al. (2004), it was found that glyphosate resistant rigid ryegrass populations only translocated about half as much herbicide to the stem meristematic portion of the plant than susceptible plants did.

A big part of what makes glyphosate as effective as it normally is, is the systemic way in which it moves and mobilises through plants. The pattern of translocation closely resembles the manner in which natural photoassimilates translocate and accumulate in sink tissues. Such a broad and expeditious translocation pattern is an important characteristic for systemic herbicide efficiency. This said, Powles and Preston (2006) is of opinion that the manner in which glyphosate translocate or doesn't translocate may confer resistance.

Absorption tends to be similar in resistant and susceptible species. What is interesting about the mentioned Powles and Preston (2006) study, is that the patterns of glyphosate translocation in the respective experiments differed. The difference between susceptible and resistant plants was in the manner in which glyphosate was translocated to the roots. In susceptible plants the tendency was to accumulate glyphosate in the lower part of the plant, but less in the roots. On the other hand, resistant plants accumulated glyphosate in the meristematic tissues of the sprayed leaf with less mobilisation toward the roots (Powles and Preston 2006).

Earlier research by Feng et al. (1999) found that glyphosate translocation in rigid ryegrass at high and low doses were analogous, i.e. the high dose that covered a greater area of the leaf was translocated similarly as the lower dose. They reasoned that this may have been due to the higher dose containing more glyphosate, but also more surfactant. Consequently, this higher surfactant concentration may cause more tissue injury in the leaf that was treated and thus translocation was negatively influenced. The results of their study showed that metabolism, uptake and translocation of glyphosate in resistant and susceptible plants took on similar patterns.

Furthermore, resistant plants reportedly exhibited a greater concentration of phenolics and more shikimate pathway activity than the susceptible plants did (Feng et al. 1999). The general conclusion of the Feng et al. (1999) study was that no significant difference was found between susceptible and resistant plants regarding glyphosate uptake and translocation.

More recent research by Feng et al. (2004) showed that the translocation of glyphosate from shoot to roots in *Conyza canadensis* (horseweed) can be two times higher in susceptible than in resistant biotypes. This result was similar to results of altered cellular transport and decreased glyphosate translocation to roots reported by Lorraine-Colwill et al. (2003) in resistant rigid ryegrass. Consequently, it seems that an alteration in translocation patterns may suggest an association between glyphosate resistance and shoot meristem glyphosate accumulation.

Lorraine-Colwill et al. (2003) conducted experiments showing that glyphosate resistance in rigid ryegrass correlates with an increased translocation of the herbicide to the leaf tips. They concluded that resistance takes place as a result of changes in the cellular transport of glyphosate (Lorraine-Colwill et al. 2003).

Because of the various opinions in literature about the mechanisms of resistance, Wakelin et al. (2004) attempted to establish how many of the rigid ryegrass populations exhibiting resistance could be attributed to an alteration in glyphosate translocation. They found that the resistance in all four of the populations in their study could be associated with changed translocation patterns. They reported less than normal glyphosate accumulation in the shoot meristematic area of resistant plants. This mechanism made it possible for resistant plants to continue growth in contrast with susceptible plants in which leaf growth stopped (Wakelin et al. 2004).

The study of Shaner (2009) correlated with the findings of Lorraine-Colwill et al. (2003) and Wakelin et al. (2004). Shaner (2009) stated that the most important mechanism for resistance is an impaired translocation of glyphosate to the meristematic tissue. He found that glyphosate needs to be translocated to meristematic tissues in order to be effective. Disc assays have revealed that shikimate is mostly found in young, rapidly growing tissue and that the gene encoding for EPSPS is mostly expressed in these meristems. In finding this, Shaner (2009) speculated that there must be some kind of mechanism of inhibition of glyphosate uptake into the phloem of resistant plants.

As for the latest research, Adu-Yeboah et al. (2014) made a conclusion contrasting the finding of the previously mentioned Feng et al. (1999). Their study found that susceptible rigid ryegrass retained much

less herbicide in their leaf blades than resistant plants did. Therefore, resistant plants translocated less herbicide to the untreated leaves than the susceptible plants did.

Ge et al. (2012) presented a theory for the mechanism of glyphosate resistance in ryegrass that was more cell-based. A few years before, Shaner (2009) first mentioned the theory of glyphosate sequestration in the cell, without delving too much into the topic. Ge et al. (2012) conducted an experiment with resistant and susceptible populations of ryegrass lines from four different countries.

It was concluded that susceptible and resistant biotypes take glyphosate up to the same extent, but that the difference occurred when it came down to vacuolar sequestration (Ge et al. 2012). The study proved that sequestration took place in all resistant lines whereas no measurable sequestration took place in the corresponding susceptible lines. The amount of glyphosate vacuolar sequestration was measured and correlated with the degree of glyphosate resistance. Ge et al. (2012) was strongly opinionated that vacuolar sequestration plays a major role in glyphosate resistance in ryegrass.

2.5. Glyphosate and soil

Soil characteristics may play an important role in the efficacy of glyphosate in plants. However, soil types and soil conditions in nature create a significant number of uncontrollable variables simultaneously. Different soil types make some elements more readily available to plants than other and effect plant growth and health incongruously. It is therefore difficult to establish one single scientific method by which to measure the effect of soil conditions on glyphosate efficacy in plants or vice versa.

One such an example is the influence of micro nutrients on glyphosate efficacy and the other way around. Eker et al. (2006) stated that the herbicidal activity of glyphosate is inhibited by the application of iron (Fe) and manganese (Mn) in spray solutions. This is because these micronutrients limit the absorption and translocation of glyphosate in treated leaves by forming complexes with the glyphosate molecule.

Eker et al. (2006) demonstrated in a study with sunflower (*Helianthus annuus*) trials that glyphosate negatively influences the uptake, transport and concentration of Fe and Mn accumulation in tissues in sunflower plants. This was ascribed to glyphosate forming complexes with the metals that are not quite soluble. As Ololade et al. (2014) plainly states; soil metals may decrease glyphosate activity. Earlier, Barrett and McBride (2005) also made a related statement concerning the formation of complexes, especially between glyphosate and Mn. They said that glyphosate herbicidal activity was impaired by divalent manganese (the plant available form).

Tesfamariam et al. (2009) conducted a study investigating the growth inhibition of sunflower seedlings as a reaction to glyphosate. They found that varying soil types play a role in how glyphosate toxicity is expressed. For example, reduced manganese (Mn) nutrition as a result of glyphosate application were more visible from a sandy soil with low buffering capacity (Arenosol) as compared to the result in a well-buffered calcareous subsoil (Tesfamariam et al. 2009).

As previously stated by Eker et al. (2006), glyphosate is known to form complexes with Mn and the resulting complex is poorly soluble and therefore more difficult to translocate. Because of this, soil that is already deficient in available Mn may become even more reduced and plants may suffer Mn deficiency (Tesfamariam et al. 2009).

In a study by Beltrão et al. (2013), it was found that glyphosate application to the soil used in the study had no impact on pH and Fe concentrations, but exhibited an impact on soluble Mn concentrations in the soil as stated by Eker et al. (2006) and Tesfamariam et al. (2009). As glyphosate dosage increased, soluble Mn concentrations decreased in high organic matter soil under flooding (Beltrão et al. 2013). When the same was investigated in low organic matter soil, no distinct trend was found. The study therefore found that the chemical properties of glyphosate may be influenced by soil pH, CEC and the amount of organic matter in the soil (Beltrão et al. 2013).

Barrett and McBride (2005) conducted an experiment investigating the role of Mn oxides (MnO) in the degradation of glyphosate. MnO forms on the plant leaf surfaces from Mn^{2+} salts (Barrett and McBride 2005). It was found that MnO could sorb and degrade glyphosate that has been sprayed with Mn^{2+} glyphosate mixtures (or directly after spraying). The presence of MnO therefore may imply reduction in glyphosate efficiency.

2.6. Conclusion

Glyphosate resistance in agriculture is a monumental problem. Glyphosate resistant ryegrass species leads to impaired yield in orchards, vineyards and cereal crops. Several ryegrass biotypes have developed glyphosate resistance and the problem has become a global one. However, no glyphosate resistance had yet been recorded in the summer rainfall areas of South Africa, with the exception of the possibility of resistance to a range of herbicides in *Palmer amaranth* in the Northern Cape province which is currently under investigation.

Quite a few mechanisms have been investigated as the probable causes for development of resistance. Two broad types of mechanisms have been identified; target site resistance and non-target

site resistance (Ge et al. 2012). Whether a certain mechanism brings to pass a big or small amount of resistance, it still is adding to the big overall herbicide resistance problem which influences a given biotype. Any mechanism that makes survival possible becomes an issue of selection pressure (Powles and Preston 2006).

Target site resistance encompasses the physical and mutational changes that the EPSPS target site undergoes, and the influence that this has on development of resistance. Non-target site resistance seems to be the more favoured category by scientists and researchers when looking at literature. It includes the influence of sub-lethal dosage of glyphosate, altered translocation patterns and vacuolar sequestration.

Altered translocation in ryegrass has been widely researched and many authors have attributed resistance largely to this mechanism of resistance. Resistant plants become able to translocate less glyphosate to the shoot meristematic area of resistant plants, and therefore less of the herbicide is accumulated in the leaves. Altered translocation made it possible for resistant plants to continue growth, unlike susceptible plants (Wakelin et al. 2004).

Vacuolar sequestration of glyphosate is directly correlated with altered translocation and the degree in which glyphosate resistance is observed (Ge et al. 2012). Resistant plants seem to have developed the ability to store glyphosate in vacuoles or other storage organelles, minimizing the amount of glyphosate available for translocation. Less glyphosate reaches the chloroplast where inhibition of the EPSPS takes place. This results in resistant biotypes surviving at a better rate than susceptible biotypes.

External factors outside of the plant body are also very important to look at. Soil composition, fertility, pH, nutrients, moisture and organic matter content are all factors which can play significant roles in the efficiency of glyphosate as it directly influences plant health and vigour. Soil conditions and its integrative effect on plants may still prove to be a contributing factor to glyphosate resistance in ryegrass.

Further investigating the mechanisms by which resistance develops and which factors affect glyphosate efficiency is important for possibly discovering ways to mend or control the situation of glyphosate resistance in the future. Future researchers would have to focus on integrated solutions.

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Chapter 3

The effect of applied plant available manganese levels in soil on glyphosate efficiency in ryegrass (*Lolium spp.*)

3.1. Introduction

Manganese (Mn) is a transition metal very similar to zinc and iron and is involved in redox reactions in plant cells (Dučić and Polle 2005). Because of its tetravalent structure, it will generally form salt complexes with specific herbicides and especially an insoluble molecule such as glyphosate (Bailey et al. 2002). Heavy metals like Mn are generally present in all soil types and naturally occur in soil in concentrations of 40-900 mg Mn kg^{-1} on average, but can reach much higher concentrations due to mining activities (Barceloux 1999). Plant availability of Mn greatly depends on soil pH levels (Dučić and Polle 2005). For the purpose of this study, manganese concentrations have to be regarded in context of plant availability.

Glyphosate is a molecule which contains phosphonate and carboxylate (also known as active groups) which exhibit a high probability to bind with metals (Eker et al. 2006). Glyphosate translocation takes place very rapidly and mostly accumulates in meristematic tissues such as growing roots and shoots (Eker et al. 2006).

It is a widely known fact that antagonism exists between glyphosate and certain micro-nutrients, notably Mn (Bernards et al 2005a, Bernards et al. 2005b). This antagonism manifests through a complexation effect where Mn binds with glyphosate. For example, glyphosate easily interact and undergo this complexation with Mn in tank mixtures (Bernards et al. 2005a).

From previous findings by Bernards et al. (2005a), it was stated that it is important for glyphosate to be applied at the full recommended label dosage in accordance with the weeds to be eliminated. In turn, the amount of applied Mn shouldn't exceed to amounts more than the nutritional needs of the specific crop (0.1 to 0.2 kg Mn ha^{-1}), as the situation of an excessive nutrient status increases the chance that effective weed control may be compromised (Bernards et al. 2005a).

In a study by Bailey et al. (2002) on common lambsquarters, a highly significant effect was obtained when the effect that Mn exerts on glyphosate efficacy was evaluated. It was shown that up to 94% weed control was obtained through glyphosate when no forms of Mn were present in the application of glyphosate (Bailey et al. 2002). The moment Mn was added, weed control subsequently decreased to

69% and 56% with two different Mn formulations (manganese lignin and manganese chelate respectively) added to glyphosate (Bailey et al. 2002).

As mentioned, the practical and logistical aspect of nutrient application in combination with herbicide application is a crucial factor which in practice, has to be taken into consideration. In the example of glyphosate resistant soybean crops, Mn foliar application often coincides with the optimum time to spray glyphosate (Soltani et al. 2011). From a logistical point of view, it makes sense for a farmer to practice co-application as to reduce the risk of soil compaction, damage to the crop, fuel costs, labour costs and machinery (Soltani et al. 2011).

Manganese amongst other metal cations is known to reduce the efficacy of glyphosate within hard water carriers (Bailey et al. 2002, Bernards et al. 2005a, Bernards et al. 2005b). Metal cations in combination with glyphosate forms complexes which cause the formation of salts. These salts are not as easily absorbed as free glyphosate. As a result, glyphosate efficacy is impaired (Hartzler 2011).

Observations that glyphosate resistance occur more commonly in areas in the Western Cape where high Mn levels are prevalent (Pers. Com. Prof ALP Cairns, Heap 2009), lead to the question whether high soil Mn levels could possibly play a role in the efficacy of glyphosate in *Lolium spp*. High levels of Mn in agricultural soil is widely present in the Western Cape.

The studies mentioned above report on antagonism of Mn and glyphosate in tank mixtures. The aim of this trial is to test whether high Mn concentrations do exert an influence on glyphosate efficacy in ryegrass.

3.2. Materials and methods

3.2.1. Experimental site

This trial was run under controlled glasshouse conditions at the Department of Agronomy on the Welgevallen Experimental Farm of Stellenbosch University, Western Cape, South Africa.

3.2.2. Experimental design and layout

These experiments were carried out in order to establish whether plant available soil Mn levels affect glyphosate efficiency. The experiment was carried out on four ryegrass biotypes, all grown in the same river sand medium (Table 3.1). The four biotypes were S1 (a commercial glyphosate susceptible variety cv. Mach 1 obtained from Agricol Seed Company), S2 (a local glyphosate susceptible biotype obtained

from Rondebosch Common (33.9561° S, 18.4825° E) in Cape Town, South Africa), R1 (a moderately glyphosate resistant biotype obtained from a wheat field near Malmesbury (33.4655° S, 18.7185° E) in the Western Cape Province of South Africa) and R2 (a strongly glyphosate resistant biotype obtained from Welgevallen Experimental Farm in Stellenbosch (33.9321° S, 18.8602° E).

The experimental procedures involving the four biotypes were considered as four separate experiments and were separately analysed. The procedure for all four of these experiments was identical with the exception of glyphosate dosage rates applied which were altered according to the degree of resistance of the different biotypes.

During 2014, a pot trial was carried out under shade nets and in a glasshouse under 25/20 °C day/night-controlled temperatures. Small pots of 8 cm x 8 cm were filled with river sand as growing medium and four seedlings were transplanted into each pot. One pot represented an experimental unit and was replicated four times per Mn treatment. Using a completely randomized block design, the 6x5 factorial combinations comprised Mn treatments (0, 1.5, 3.0, 4.5, 6.0 and 7.5 mM $MnSO_4$) where each treatment was sprayed with five glyphosate dosage rates. Dosage rates varied between biotypes in correlation with the previously determined susceptibility of the specific biotype as illustrated in Table 3.2.

Table 3.1: Soil characteristics of the river sand medium used in the pots

Parameter	River sand medium	Unit
pH(KCl)	5.3	
Texture	Sand	
Resistance	6760	Ohms
Acidity	0.21	cmol+ kg^{-1}
Calcium (Ca)	0.41	cmol+ kg^{-1}
Magnesium (Mg)	0.09	cmol+ kg^{-1}
Total cations	0.77	cmol+ kg^{-1}
Potassium (K)	11	mg kg^{-1}
Sodium (Na)	8	mg kg^{-1}
Phosphorus (P)	22	mg kg^{-1}
Copper (Cu)	0.10	mg kg^{-1}
Zinc (Zn)	0.18	mg kg^{-1}
Manganese (Mn)	5.69	mg kg^{-1}
Boron (B)	0.02	mg kg^{-1}
Sulphur (S)	3.30	mg kg^{-1}
Carbon (C)	0.03	%

Table 3.2: Glyphosate dosage rates applied to four different ryegrass biotypes with varying levels of susceptibility to glyphosate

Biotypes	**Glyphosate** (360 g a.i. L^{-1} formulation) **dosage rates in L ha^{-1}**				
	1	**2**	**3**	**4**	**5**
Susceptible commercial **biotype S1**	0.0	0.5	1.0	1.5	2.0
Susceptible weedy **biotype S2**	0.0	0.5	1.0	1.5	2.0
Resistant weedy **biotype R1**	0.0	0.8	1.6	2.4	3.2
Resistant weedy **biotype R2**	0.0	2.0	4.0	6.0	8.0

3.2.3. Experimental procedure

Seeds of the S1, S2, R1 and R2 ryegrass biotypes were germinated in petri dishes in an incubation chamber at a constant temperature of 20 °C. Once germinated, seedlings were transplanted into 8 cm x 8 cm size pots filled with previously mentioned river sand medium. Four ryegrass seedlings were transplanted into each pot. The seedlings were grown under shade nets up until eight leaf stage. Once the seedlings matured to the eight-leaf stage, they were moved into a glasshouse of controlled temperature conditions (25/20°C day/night).

Throughout life stages of seedling to eight leaf stage, seedlings were adequately watered with a balanced nutrient solution (Table 3.3). Added Mn ($MnSO_4$) treatment solutions of different concentrations were applied to the pots three times per week. The concentrations of the Mn treatment solutions additionally applied to the seedlings were 0 mM (normal balanced nutrient solution containing 0.55 mg L^{-1} $MnSO_4$ serving as control treatment), 1.5, 3.0, 4.5, 6.0 and 7.5 mM $MnSO_4$. On treatment days, the pots were watered with the Mn treatment solution alone. This was done to enhance optimal uptake of the Mn solution without possible leaching from the soil medium.

An extra replicate (second control) of the Mn treatments were grown from the start of the experiment and harvested on the day of glyphosate application. This leaf material was reserved to be dried and analysed for Mn content (Table 3.4) by the Elsenburg soil laboratory of the Western Cape Department of Agriculture.

Each one of the six Mn treatments were treated with five glyphosate dosage rates (Table 3.2). The recommended label dosage of glyphosate for ryegrass in field situation is 1.5 L ha^{-1}. Glyphosate was applied with a pneumatic pot-spraying apparatus operating at a spraying pressure of 2 bars and a water delivery rate of 108 L ha^{-1}. Irrigation was not applied to pots for 24 hours after treatment to allow for glyphosate to be adequately absorbed by the ryegrass.

Table 3.3: The nutrient concentrations of the feeding solution used in the glasshouse trials

EC = 2.0			
Element	**Concentration**	**Fertiliser**	**Concentration**
(Macro)	**mg L^{-1}**		**g 1000L^{-1}**
K^{+}	237.7	$KN0_3$	303
Ca^{++}	180	K_2S0_4	261
Mg^{++}	48.6	$Ca(N0_3)_2.2H_20$	900
$N0_3^{-}$	661.33	$MgS0_4.7H_20$	492
H_2P0_4	116.4	KH_2P0_4	136
$S0_4$	390.4		
(Micro)	**mg L^{-1}**		
Fe: Libfer (Fe EDTA)	0.85		6.54
Mn: Manganese sulphate	0.55		2.23
Zn: Zinc sulphate	0.30		1.33
B: Solubor	0.30		1.46
Cu: Copper Sulphate	0.05		0.20
Mo: Sodium Molibdate	0.02		0.13

Table 3.4: The Mn content (mg Mn kg^{-1}) of the ryegrass seedlings on the day of glyphosate application

Mn content (mg Mn kg^{-1}) in leaf material at time of glyphosate application				
Mn concentration applied per treatment	***Lolium spp*. biotype**			
	S1	**S2**	**R1**	**R2**
0.0 mM	112.20	206.10	202.00	207.00
1.5 mM	643.10	315.80	310.10	622.20
3.0 mM	1194.0	1186.0	774.80	1439.0
4.5 mM	1212.0	1211.0	658.00	1754.0
6.0 mM	1924.0	2791.0	2347.0	2331.0
7.5 mM	2690.0	1907.0	2446.0	2173.0

3.2.4. Evaluation and data analysis

Six weeks after glyphosate application, the mortality rate of each experimental unit was evaluated and percentage survival was calculated per treatment combination. A single plant was evaluated and recorded as controlled by the herbicide when the youngest leaf was necrotic and could easily be pulled from the stem. After evaluation, all plants per pot were harvested close to the pot soil surface and dried at 80°C for 48 hours. Thereafter dry mass was weighed and total dry mass per pot was calculated.

Survival (%) and dry matter (g) data was subjected to analysis of variance (ANOVA) using the STATISTICA 12® software program. The least significant difference (LSD) values of the Bonferroni post hoc test were calculated at the 5% probability level to expedite comparison between treatment means of main effects and interactions.

3.3. Results

Biotype S1 (commercial susceptible population)

Percentage survival of population S1 exhibited a significant interaction ($p<0.05$) between the main factors (Mn concentration and glyphosate dosage rate) as illustrated in Figure 3.1. At the lowest sub-lethal dosage rate of 0.5 L ha^{-1}, the survival of this biotype proved to be significantly higher at Mn treatment levels 4.5 to 7.5 mM Mn than at lower Mn treatment levels.

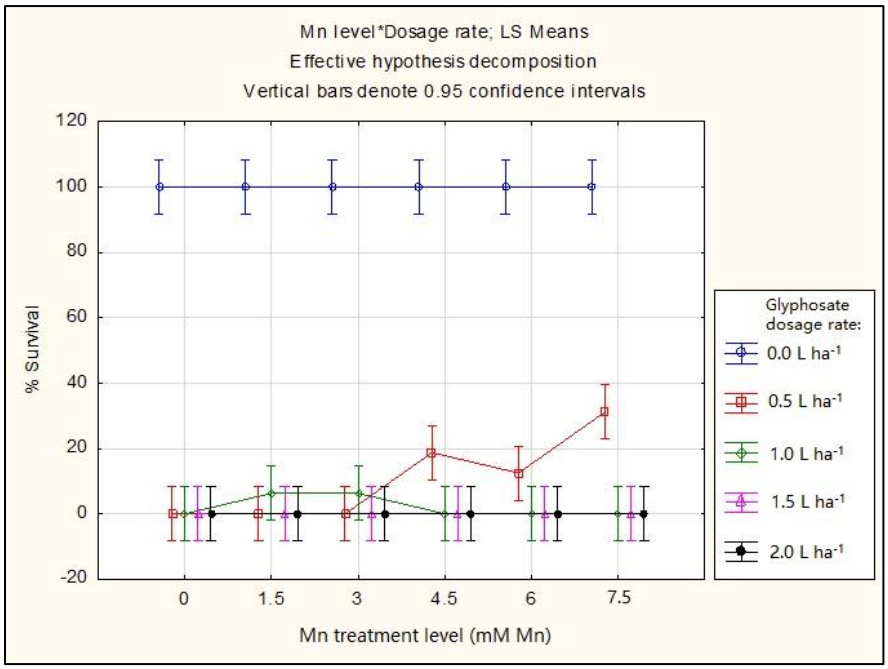

Figure 3.1: The interaction between Mn treatment concentration and glyphosate (360 g a.i. L^{-1}) dosage rate pertaining to percentage survival of ryegrass biotype S1. Vertical bars denote 0.95 confidence intervals

A corresponding significant interaction ($p<0.05$) between the main factors (Mn treatment level and glyphosate dosage rate) was observed in dry matter production as illustrated in Figure 3.2. However, no clear trend could be discerned. At treatment 3.0 mM Mn of the control glyphosate dosage treatment specifically, some disparity between control and treatment concentrations seems to have influenced the results obtained.

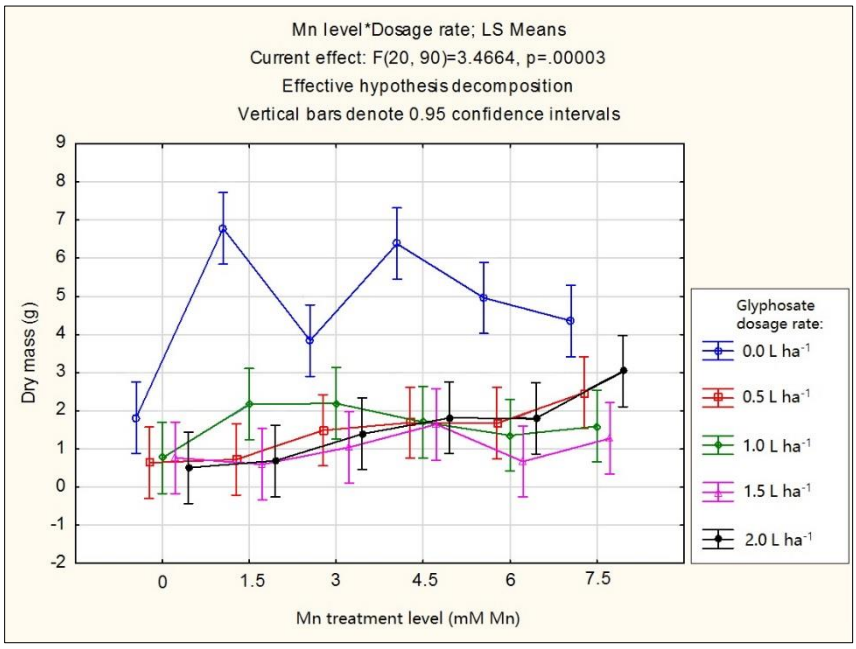

Figure 3.2: The interaction between Mn treatment concentration and glyphosate (360 g a.i. L^{-1}) dosage rate pertaining to dry mass production of ryegrass biotype S1. Vertical bars denote 0.95 confidence intervals

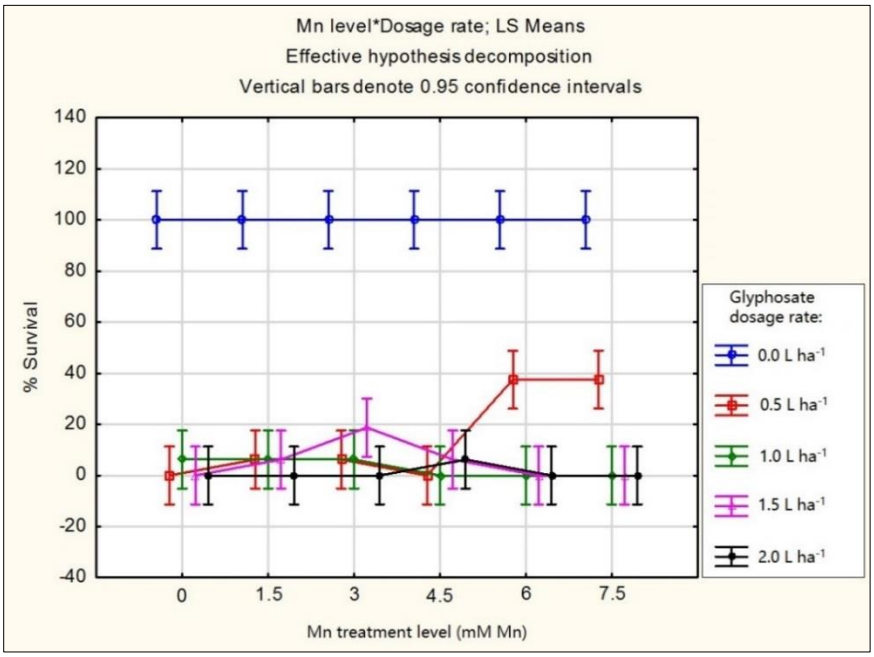

Figure 3.3: The interaction between Mn treatment concentration and glyphosate (360 g a.i. L^{-1}) dosage rate pertaining to percentage survival of ryegrass biotype S2. Vertical bars denote 0.95 confidence intervals

Biotype S2 (susceptible weedy population)

Similar to the S1 population, a significant interaction ($p<0.05$) occurred for survival data between the main factors of Mn concentration and glyphosate dosage rate as illustrated in Figure 3.3. Once again it is evident that within the sub-lethal glyphosate dosage rate of 0.5 L ha^{-1}, the survival of the biotype is significantly higher at the higher Mn concentrations (6.0 and 7.5 mM Mn) than at the lower Mn concentrations.

No significant interactions between treatments and also no significant differences between Mn concentrations were found in the analysis of dry matter production. The only significant differences that were found, was between the different glyphosate dosage rates (Table 3.5). This result is most likely due to the fact that the unsprayed control produced significantly more dry mass than the sprayed plants.

Table 3.5: The significant effect of glyphosate dosage rate on dry matter production of ryegrass biotype S2

Glyphosate dosage rate (L ha^{-1})	**0.0**	**0. 5**	**1.0**	**1. 5**	**2.0**
Dry matter (g)	6.28a	1.79b	2.45b	1.79b	1.57b

Biotype R1 (resistant weedy population)

No significant interaction ($p>0.05$) occurred between main factors Mn of concentration and glyphosate dosage rate regarding mortality. The effect of the main factors is illustrated in Table 3.6 and 3.7. These results do not necessarily correlate with the trend seen in the two susceptible populations. However, a significant increase in percentage survival as Mn levels increase, is evident (Table 3.7).

No significant interaction ($p>0.05$) was observed regarding dry mass production in this population (R1) but significant differences between the respective main effects of glyphosate dosage rates as well as Mn concentrations was evident (Table 3.8 and 3.9). The significance observed within glyphosate dosage rates could mainly be ascribed to the considerable difference between the unsprayed control and the sprayed treatments.

Table 3.6: The significant effect of glyphosate dosage rate on percentage survival of ryegrass biotype R1

Glyphosate dosage rate (L ha^{-1})	**0.0**	**0.8**	**1.6**	**2.4**	**3.2**
Survival (%)	100a	70.9b	27.1c	33.4c	27.1c

Table 3.7: The significant effect of Mn level on percentage survival of ryegrass biotype R1

Mn level (mM $MnSO_4$)	**0.0**	**1.5**	**3.0**	**4.5**	**6.0**	**7.5**
Survival (%)	33.8e	46.3d	57.5c	66.3ab	62.5b	68.8a

Table 3.8: The significant effect of glyphosate dosage rate on dry matter production of ryegrass biotype R1

Glyphosate dosage rate (L ha^{-1})	**0.0**	**0.8**	**1.6**	**2.4**	**3.2**
Dry matter (g)	6.94a	3.30b	1.53c	2.32bc	2.17bc

Table 3.9: The significant effect of Mn level on dry matter production of ryegrass biotype R1

Mn level (mM $MnSO_4$)	**0.0**	**1.5**	**3.0**	**4.5**	**6.0**	**7.5**
Dry matter (g)	1.95b	3.59a	3.49a	3.58a	4.12a	2.78ab

Biotype R2 (resistant weedy population)

Mortality in this population yielded no significant interactions between the main factors of glyphosate dosage rate and Mn concentration. Percentage survival also showed no significant differences between different Mn concentration treatments. However, trends could be observed of slightly higher percentage survival exhibited in higher Mn concentrations at glyphosate dosage rates 4.0 to 8.0 L ha^{-1} in comparison to lower Mn concentrations at the same glyphosate dosage rates (Results not shown). Table 3.10 denotes the significant effect of glyphosate dosage rate on survival of biotype R2.

Table 3.10: The significant effect of glyphosate dosage rate on percentage survival of ryegrass biotype R2

Glyphosate dosage rate (L ha^{-1})	**0.0**	**2.0**	**4.0**	**6.0**	**8.0**
Survival (%)	100a	55.21b	21.87c	19.79c	9.37c

An analogous result of no statistically significant interaction was observed regarding dry mass production. Only the main factor of glyphosate dosage rate alone resulted in significant differences in dry mass production between treatments (Table 3.11). A distinctly decreasing amount of dry mass was produced at increasing glyphosate dosage rates as could be expected.

Table 3.11: The significant effect of glyphosate dosage rate on dry matter production of ryegrass biotype R2

Glyphosate dosage rate (L ha^{-1})	**0.0**	**2.0**	**4.0**	**6.0**	**8.0**
Dry matter (g)	3.70a	2.06b	1.52bc	1.33c	0.84c

3.4. Discussion and conclusion

The two susceptible biotypes (S1 and S2) both yielded a significantly increased survival rate under the conditions of highest Mn concentration treatments at the sub-lethal (and lowest) applied glyphosate

dosage rate of 0.5 L ha^{-1}. Glyphosate concentrations higher than 0.5 L ha^{-1} generally resulted in 100% control (mortality) of the two susceptible biotypes tested in this trial, regardless of concentration of Mn treatment applied.

In contrast with the two susceptible biotypes, resistant biotype R1 showed no interaction between main factors. However, this biotype exhibited a survival rate of about 40% after being sprayed with 3.2 L ha^{-1} glyphosate only when treated with the three highest concentrations Mn; 4.5, 6.0 and 7.5 mM $MnSO_4$. Furthermore, the only significant differences exhibited by resistant biotype R2 was at glyphosate dosage rates of 4.0 L ha^{-1} where 80% control was observed and 8.0 L ha^{-1} where 90% control was observed in comparison to 45% control at a dosage rate of 2.0 L ha^{-1}.

In summary, significant interactions between main factors were solely observed in susceptible biotypes (S1 and S2). Significant differences were found in both individual main factors in the resistant biotype R1 as well as in dosage response of the resistant biotype R2.

It seems to be more difficult to discern clear interactions and trends in resistant populations. This might be due to the inclusion of additional variance added to the experiment: the genetic trait of resistance. It may therefore be preferable to investigate principles such as those studied in this trial by using only susceptible weed biotypes. However, plausible explanations in the form of non-target site resistance mechanisms as well as the effect that Mn and glyphosate exerts on one another's performance, may be derived from available literature and linked to the results obtained in this trial.

When plants are subjected to repeated and continuous sublethal dosage rates of herbicides such as glyphosate, it is likely that those plants will develop non-target site resistance due to severe selection pressure (Shaner et al. 2012). Busi and Powles (2009) showed that sublethal dosage rates of glyphosate are capable to induce resistance over merely four generations of continuous selection in a normal susceptible population of rigid ryegrass.

As seen from this trial, percentage survival of the S1 and S2 populations are significantly higher at the increased Mn concentrations (from 4.5 to 7.5 mM Mn) at a sublethal dosage of 0.5 L ha^{-1} glyphosate (Figure 3.1 and 3.3). This may be due to Mn/glyphosate antagonism taking place inside the plant or at absorption interfaces. Reduced glyphosate efficacy has frequently been attributed to complexes formed between glyphosate and metal cations which consequently leads to salt formation that is not as easily absorbed by the plant as free glyphosate is (Soltani et al. 2011).

Complexation of glyphosate with Mn more readily takes place at high Mn-glyphosate ratios. When the Mn-glyphosate ratio within the plant is high, Mn which is readily available may form complexes with the smaller amount of glyphosate present due to the lower dosage rate. Whilst the study of Harris et al. (2012) contradicts this, it may be worth considering that a decreased amount of glyphosate molecules (assuming any at all is indeed present) may then be available for translocation due to the Mn-complexation effect and thus glyphosate efficacy is impaired.

In the case of foliar Mn application, situations exist where glyphosate and Mn^{2+} may form complexes whilst penetrating the cuticle instead of before penetrating the cuticle (Bernards et al. 2005b). Hartzler (2011) stated that glyphosate accumulates in meristematic tissues at concentrations that would lead to the molecule readily forming complexes with metal cations such as Mn^{2+}. Eventually, any free Mn^{2+} in the cytoplasm could affect the efficacy and translocation of glyphosate by reacting with the glyphosate molecules before the molecule may have the chance to exert any function (Bernards et al. 2005b).

Ancillary to the study by Bernards et al. (2005b), Harris et al. (2012) conducted a comprehensive simulation study regarding the interaction of glyphosate and metal ions in the phloem of plants at various soil pH ranges. Amongst many interesting observations, it was found that Mn^{2+} seemed to be more loosely bound in the phloem than other metals such as Fe^{3+}, Cu^{2+}, Zn^{2+} and Fe^{2+}. However, from said calculations only relatively small increases in glyphosate binding was deduced and consequently mostly ineffective competition of glyphosate with the biological chelating agents within the phloem (Harris et al. 2012). Still, reduced absorption and translocation of glyphosate due to complexation has been one of the prevalent explanations regarding reduced glyphosate efficiency (Bernards et al. 2005). It has also been said that this complexation can be eliminated by the addition of other chelation agents in tank mixtures, which will prevent complex formation with glyphosate (Bernards et al. 2005a, Soltani et al. 2011).

The amount of plant available Mn freely available in soil may be a factor to be reckoned with. Rosas et al. (2007) reported a higher Mn concentration in the roots of plants at a pH of 4.8 as opposed to pH of 6.0, but reported no significant differences of Mn concentration in shoots at the two pH levels. However, because of the fact that Mn becomes more plant available at lower soil pH, toxicity levels of this element may still directly or indirectly affect uptake and translocation (Dučić and Polle 2005). Based on the abovementioned literature, one might be able to reason that if the toxicity effect causes impaired translocation, it may also be the cause of limited glyphosate uptake and translocation.

An excess of a transition element such as Mn can easily cause a multitude of negative effects. Examples of such negative effects are; binding to the wrong functional groups or exchanging important ions from enzyme active centres (Bailey et al. 2002). Higher plants exhibit complex responses to heavy metal toxicity. The complexation of metal ions (which occur between glyphosate and Mn) lessens the uptake of said metals and increases antioxidant production. These antioxidants detoxifies oxidative reactive species which serve as a reverberation to toxic metals (Dučić and Polle 2005).

Millaleo et al. (2010) stated that plants can exhibit the ability to survive via resistance mechanisms in soil environments with high levels of metal contamination. This may be achieved via tolerance or avoidance. Avoidance enables the plant to prevent metal ions from entering the cytoplasm of plant cells. The mechanism of tolerance on the other hand entails a detoxification process after these metals have entered across biomembranes or plasmalemmas (Millaleo et al. 2010).

The role of environmental factors in glyphosate efficacy and Mn availability cannot be overlooked. Sub-optimal conditions may exist during glyphosate application due to poor climatic conditions (precipitation, extreme heat etc.). This can result in a sub-lethal dosage rate. In combination with a sub-lethal dosage rate, high Mn levels may then play a role to increase survival rate to some extent by forming complexes (Bernards et al. 2005b) with a large portion of the remaining glyphosate molecules as previously discussed.

According to Eker et al. (2006) a large amount of glyphosate which is applied to target plant leaves, inevitably reaches the soil due to wash off, direct contact and root exudation. Up to 90% of glyphosate residues are said to be present in the top soil level. Complexation of glyphosate with plant available nutrients in the soil can therefore also take place.

A mechanism exists which facilitates efflux from cells and can prevent heavy metal toxicity. Mn will in this instance be transported into the Golgi apparatus and thereafter exported via the secretory pathway of cell vesicles which delivers the metal to the cell surface (Dučić and Polle 2005, Millaleo et al. 2010). This statement raises the question whether the same fate may be bestowed upon glyphosate in the form of a glyphosate-Mn complex under Mn toxic conditions.

Another factor which might be at play in what we know as glyphosate resistance, is a so-called “kickback effect”. This effect works as a self-limiting glyphosate toxicity in plants treated at lethal dosages (Nandula 2010). When glyphosate induce cytotoxic damage, sugar metabolism and photosynthesis are negatively influenced, directly limiting any translocation (Geiger and Bestman 1990, Feng et al. 2004, Nandula 2010). Glyphosate translocation to target tissues are therefore limited at least

for a period of time – essentially as a direct result of the damaged incurred by glyphosate itself. The self-limitation effect may be an explanation as to why plants survive at glyphosate dosage rates above the recommended label dosage.

From this trial, it became clear that interaction between glyphosate dosage rate and Mn treatments were more evident in susceptible than in resistant biotypes. In other words, the effect that higher levels of Mn exert on glyphosate efficacy seems to be more pronounced in susceptible biotypes. As a main factor, Mn levels increased survival rate at higher concentrations in the resistant biotype R1, but no such effect was observed in the resistant biotype R2 used in this trial.

In conclusion, factors responsible for results such as these should be attributed to non-target site resistance in general, but the effects of Mn concentration cannot be overlooked. General trends are discerned where less control (%) was achieved at higher Mn treatment concentrations, notably at sublethal dosage rates of 0.5 L ha^{-1} in the susceptible biotypes.

The general observation may be made that plants growing under high Mn conditions might be at least more predisposed to the development of non-target site resistance to glyphosate than plants growing under conditions of lower plant available Mn.

3.5. References

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Chapter 4

The effect of the natural manganese soil status on glyphosate efficiency in established ryegrass (*Lolium spp.*) populations

4.1. Introduction

Antagonism exists between glyphosate and some micro-nutrients, and notably so, manganese (Mn) (Bailey et al. 2002, Bernards et al. 2005, Hartzler 2010, Soltani et al. 2011). Soil factors can never be overlooked in agricultural trials as plant growth are integrally dependent on soil type, soil health and soil structure (to name but a few characteristics). Different soil types and conditions exert effects on many plant physiological characteristics and the interactions are infinite.

Soil characteristics may therefore play a more important role in the efficacy of glyphosate in plants than initially anticipated. A stated above, soil characteristics and composition create significant amounts of simultaneous uncontrollable variables and it is already an established fact that different soil types sorb glyphosate more strongly than other (Albers et al. 2009).

The different soil types, environmental conditions and agricultural practices glyphosate molecules are exposed to, in essence create environments where some elements become more readily available to plants than other. In a study conducted by Tesfamariam et al. (2009), it was found that at 0 days waiting time, glyphosate present in a specific soil type impaired the growth and biomass production in sunflower seedlings. The seedlings also took 1 to 2 weeks to recover. This is said to occur because shikimate (a biochemical metabolite in plants) accumulated in the root material of the seedlings, which indicates a toxicity effect affected by glyphosate (Tesfamariam et al. 2009).

Tesfamariam et al. (2009) observed that reduced Mn nutrition as a result of glyphosate application were more visible in a sandy soil with low buffering capacity (Arenosol) as compared to the result in a well-buffered calcareous subsoil (Tesfamariam et al. 2009). This underscores the importance of bearing in mind that different soils may influence glyphosate toxicity to different extents.

Micronutrients such as Mn specifically, exert an effect on glyphosate efficacy and vice versa as Mn can directly limit the absorption and translocation of glyphosate in treated leaves by forming complexes with glyphosate molecules (Eker et al. 2006). Eker et al. (2006) stated that the herbicidal activity of glyphosate is inhibited by the application of iron (Fe) and Mn in spray solutions. Ololade et al. (2014) confirmed that the presence of metal oxides (Mn oxides for example) may decrease glyphosate activity

in soil solution. Barrett and McBride (2005) also stated that complexation occur between glyphosate and manganese in particular. The study concluded that glyphosate herbicidal action could well be impaired in a situation where Mn oxides form on plant leaf areas. However, in an environment where microbial activity in the soil is minimal such as in subsoils, glyphosate degradation mediated by Mn oxides are most probable (Barrett and McBride 2005).

Interestingly, Beltrão et al. (2013) found that glyphosate application to tropical wetland type soil had no impact on pH and iron concentrations in soil, but did exhibit an impact on soluble manganese concentrations in the soil (Eker et al. 2006; Tesfamariam et al. 2009). As glyphosate dosage increased, soluble manganese concentrations also decreased in high organic matter soil under flooding (Beltrão et al. 2013). However, when the same was investigated in low organic matter soil, no clear trend was observed. In essence, the study found that the chemical properties of glyphosate may be influenced by soil pH, CEC and the amount of organic matter in the soil (Beltrão et al. 2013).

According to the available literature, it would seem plausible that interesting results may be obtained from examining the glyphosate dosage response under various soil conditions, which is the purpose of this study. The effect soil types (with varying Mn levels) exert on glyphosate efficacy in ryegrass were investigated. Ryegrass (*Lolium spp.*) exhibiting varying levels of glyphosate resistance, were used.

4.2. Materials and methods

Experimental site

These trials were run under controlled glasshouse conditions at the Department of Agronomy on the Welgevallen Experimental Farm of Stellenbosch University, Western Cape, South Africa.

Experimental design and layout

Trial 1

The experiments in this trial were carried out in order to establish whether the inherent plant available manganese (Mn^{2+}) content of three different soils affect glyphosate efficiency in ryegrass. The experiment was carried out on four ryegrass biotypes. The four biotypes included S1 (a commercial glyphosate susceptible variety cv. Mach 1 obtained from Agricol Seed Company), S2 (a local glyphosate susceptible biotype obtained from Rondebosch Common (33.9561° S, 18.4825° E) in Cape Town, South Africa), R1 (a local moderately glyphosate resistant biotype obtained from a wheat field near Malmesbury (33.4655° S, 18.7185° E) in the Western Cape Province of South Africa) and R2 (a strongly

glyphosate resistant biotype obtained from Welgevallen Experimental Farm (33.9321° S, 18.8602° E) in Stellenbosch).

Soils used in this trial were collected from Altona commercial farm (33.40˚ S, 18.35˚ E) near Durbanville (soil MN1), Welgevallen Experimental Farm (33.9321° S, 18.8602° E) in Stellenbosch (soil MN2), and Langgewens Experimental Farm (33.17˚ S; 18.42˚ E) near Malmesbury (soil MN3). The soil characteristics of these soils are compiled in Table 4.1 and special note should be taken of the manganese content of each individual soil. The four biotypes used in this trial were individually tested in each soil and analysed accordingly. The procedure for testing all four of the biotypes within each of the three of the soils was identical, with the exception of the alteration of glyphosate dosage rates applied according to the degree of resistance of the different biotypes.

During 2014, this pot trial was conducted in a glasshouse under 25/20 °C day/night controlled temperatures. Small pots of 8 cm x 8 cm were utilised and four seedlings were transplanted into each pot. Four separate experiments were completed (one for each of the four ryegrass biotypes: S1, S2, R1 and R2). Each experiment within this trial was conducted as a completely randomized block design. The 3x6 factorial combination therefore comprised of three different soil types (Table 4.1) and six glyphosate dosage rates (Table 4.2) and was replicated four times. Replications were done in blocks in order to minimize the effects of possible temperature and/or light gradients within the glasshouse.

Table 4.5: Soil characteristics of the soils used in the pots in this trial.

Parameter	Altona (MN1)	Welgevallen (MN2)	Langgewens (MN3)	Unit
pH (KCl)	6.9	5.7	5.5	
Texture	Sandy loam	Sandy loam	Sandy loam	
Resistance	750	2690	890	Ohms
Calcium (Ca)	6.04	3.80	5.40	cmol+ kg^{-1}
Magnesium (Mg)	0.89	0.54	1.24	cmol+ kg^{-1}
Total cations	7.42	4.83	7.74	cmol+ kg^{-1}
Potassium (K)	90	151	396	mg kg^{-1}
Sodium (Na)	57	22	17	mg kg^{-1}
P (citric acid)	98	156	222	mg kg^{-1}
Copper (Cu)	1.21	2.67	0.86	mg kg^{-1}
Zinc (Zn)	1.32	2.69	5.61	mg kg^{-1}
Manganese (Mn)	**5.26**	**65.98**	**147.3**	**mg kg^{-1}**
Boron (B)	0.32	0.14	0.58	mg kg^{-1}
Sulphur (S)	9.70	2.30	5.5	mg kg^{-1}
Iron (Fe)	264.6	166.10	109.0	mg kg^{-1}
Carbon (C)	0.91	0.78	2.11	%

Table 4.6: Glyphosate dosage rates applied to four different ryegrass biotypes with varying levels of susceptibility to glyphosate

Biotypes	**Glyphosate (360 g a.i. L^{-1} formulation) dosage rates in L ha^{-1}**					
	1	**2**	**3**	**4**	**5**	**6**
Susceptible commercial **biotype S1**	0.0	0.25	0.5	0.75	1.0	1.25
Susceptible weedy **biotype S2**	0.0	0.4	0.8	1.2	1.6	2.0
Resistant weedy **biotype R1**	0.0	1.0	2.0	3.0	4.0	5.0
Resistant weedy **biotype R2**	0.0	2.0	4.0	6.0	8.0	10.0

Trial 2

Experiment 1

This experiment was carried out to establish whether inherent soil Mn content affects glyphosate efficiency in two putative susceptible ryegrass populations. The experiment was carried out on two ryegrass biotypes. Each biotype was grown in two soil types of different manganese content. Topsoil were collected from Altona commercial farm near Durbanville (MN1) and Langgewens experimental farm near Malmesbury (MN3) (see Table 4.1 for soil characteristics). At Welgevallen experimental farm, the topsoil was spread out on plastic sheets, kept moist and resulting germinated seedlings from both sources were transplanted into small pots containing either soil MN1 or MN3. The seedlings obtained from the Altona (MN1) and Langgewens (MN3) soils were both putative susceptible biotypes (S3 and S4 respectively).

During 2014, this pot trial was carried out in a glasshouse under 25/20 °C day/night controlled temperatures. Small pots of 8 cm x 8 cm were used and four seedlings were transplanted into each pot. One single pot represented an experimental unit. The two ryegrass biotypes were treated as two separate sub-experiments. Each sub-experiment was completed using a completely randomized block design. Factorial combination for each ryegrass biotype was two different soil types (MN1 and MN3 – see Table 4.1) and six glyphosate dosage rates (Table 4.4) replicated four times (thus 2x6). The experimental layout is illustrated in Fig. 4.1.

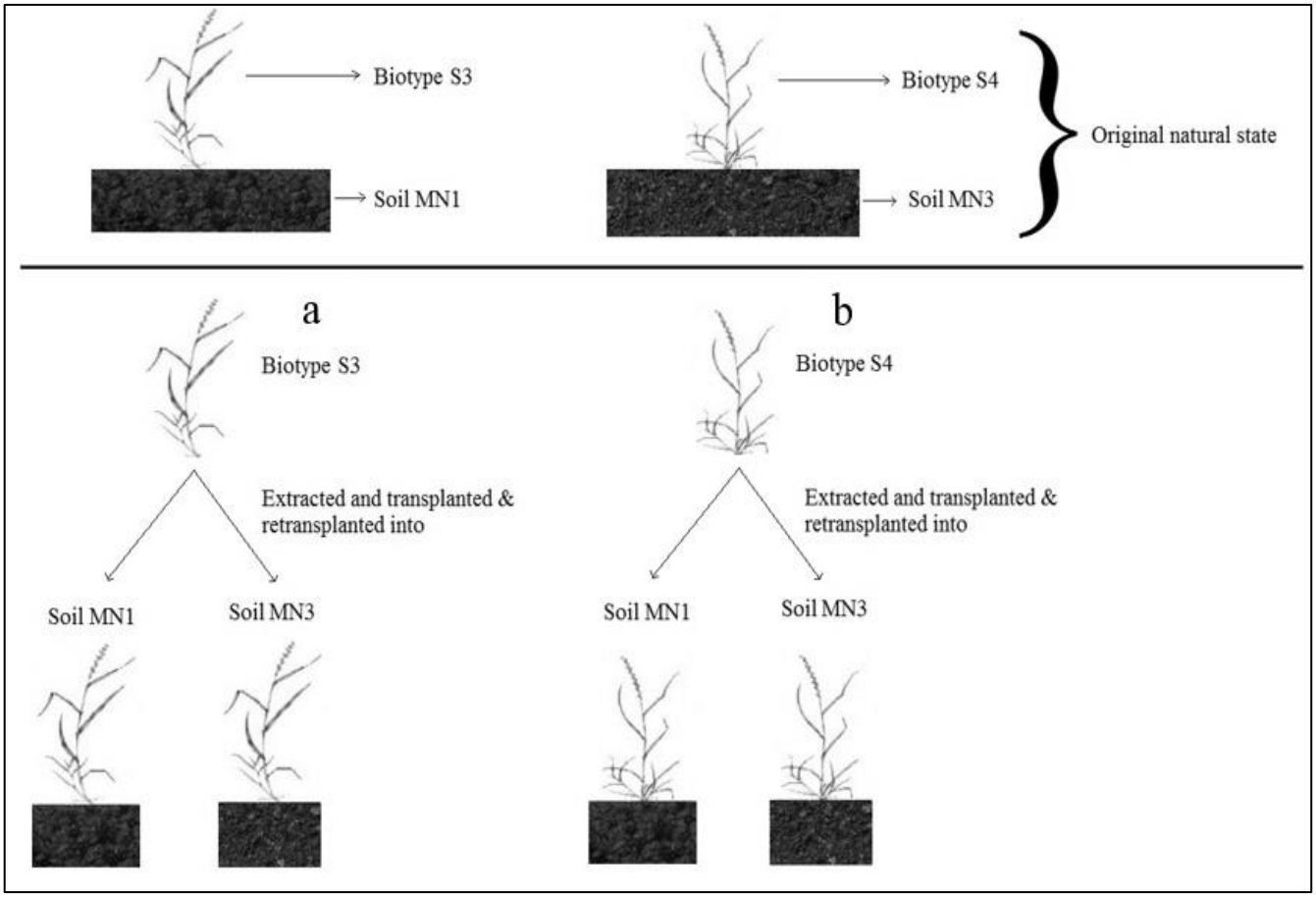

Figure 4.1: Schematic illustration for Trial 2, Experiment 1a and b

Experiment 2

This experiment was conducted to establish whether inherent plant available Mn content in soil affects glyphosate efficiency in two ryegrass biotypes that was previously found to be susceptible (S5) and resistant (R3). The experiment was carried out on two ryegrass biotypes in two soil types of varying inherent manganese content. The ryegrass and soils were collected from Murludi fruit farm in Tulbagh district of the Western Cape (33°20'S, 19°10'E). The soil was collected from two sites separated by a small river and the sites where about 200 m apart.

The first soil sample was a soil with high Mn content (MN4) which was taken from a peach orchard established on a hard and stony slope. The second soil of low Mn content (MN5) was taken from a sandy, flat area. Soil characteristics are compiled in Table 4.3. Ryegrass seedlings from each area were

collected. Seedlings from soil MN4 were a putative resistant biotype (R3) and seedlings from soil MN5 were a putative susceptible biotype (S5).

During 2014, this pot trial was carried out in a glasshouse under 25/20 °C day/night controlled temperatures. Small pots of 8 cm x 8 cm were used and four seedlings were transplanted into each pot. Each pot represented an experimental unit. The two ryegrass biotypes were tested in two separate sub-experiments. The experimental layout is illustrated in Fig. 4.2.

The sub-experiments were completed using a completely randomized block design. Factorial combination of each sub-experiment therefore comprised of two soil types, (Table 4.3) and six glyphosate dosage rates (Table 4.4), thus a 2 x 6 factorial combination replicated four times. Dosage rates varied between biotypes in correlation with the previously determined susceptibility of the specific biotype as summarized in Table 4.3.

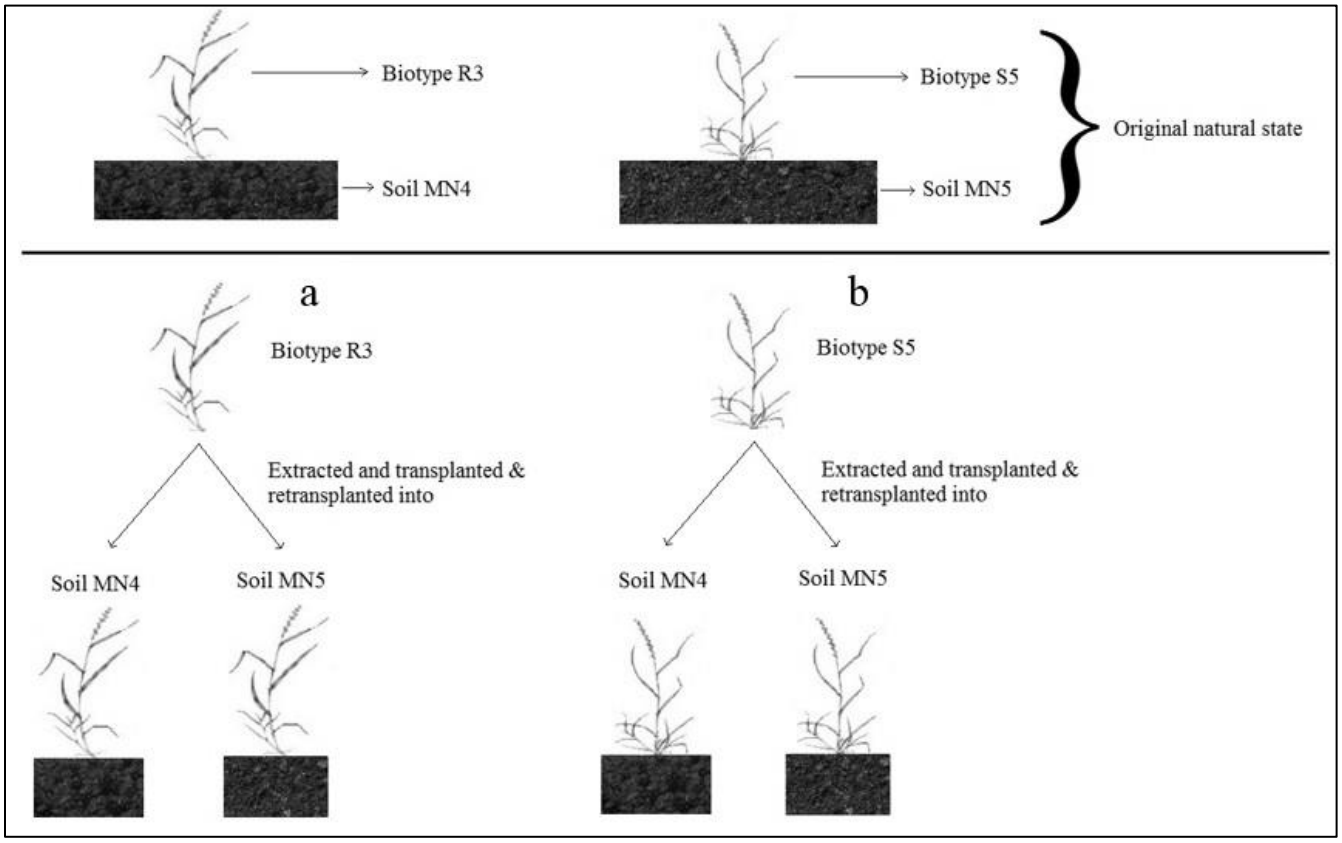

Figure 4.2: Schematic illustration for Trial 2, Experiment 2a and b

Table 4.3: Soil characteristics of the soils with high and low Mn content used in this experiment

Parameter	**High Mn soil (MN4)**	**Low Mn soil (MN5)**	**Unit**
pH (KCl)	6.5	6.3	
Texture	Sandy loam	Sand	
Resistance	510	890	Ohms
Calcium (Ca)	8.09	4.74	cmol+ kg^{-1}
Magnesium (Mg)	6.03	1.82	cmol+ kg^{-1}
Total cations	14.53	7.06	cmol+ kg^{-1}
Potassium (P)	104	171	mg kg^{-1}
Sodium (Na)	30	12	mg kg^{-1}
P (citric acid)	58	65	mg kg^{-1}
Copper (Cu)	21.78	31.42	mg kg^{-1}
Zinc (Zn)	7.75	7.33	mg kg^{-1}
Manganese (Mn)	**215.20**	**13.70**	**mg kg^{-1}**
Boron (B)	1.00	0.25	mg kg^{-1}
Sulphur (S)	6.80	3.70	mg kg^{-1}
Iron (Fe)	197.6	104.6	mg kg^{-1}
Carbon (C)	2.36	1.13	%

Table 4.4: Glyphosate dosage rates applied to four different ryegrass biotypes with varying levels of susceptibility to glyphosate (Experiments 1 and 2)

Biotypes	**Glyphosate** (360 g a.i. L^{-1} formulation) **dosage rates in L ha^{-1}**					
	1	**2**	**3**	**4**	**5**	**6**
Putative susceptible **biotype S3**	0.0	0.5	1.0	1.5	2.0	2.5
Putative susceptible **biotype S4**	0.0	0.5	1.0	1.5	2.0	2.5
Putative susceptible **biotype S5**	0.0	0.5	1.0	1.5	2.0	2.5
Putative resistant **biotype R3**	0.0	1.0	2.0	4.0	8.0	16.0

Experimental procedure

For Trial 1, seeds of S1, S2, R1 and R2 ryegrass biotypes were sown in plastic containers filled with a sandy potting soil and irrigated with tap water. When seedlings were at the approximate two-leaf stage, they were transplanted into plastic trays containing 8 cm x 8 cm pots. Each pot was filled with the particular soil of the specific experiment at hand. Each tray contained 24 of these 8 cm x 8 cm pots and four of the ryegrass seedlings were transplanted into each of the pots and grown in a glasshouse of controlled temperature conditions (25/20 °C day/night).

Throughout the course of the experiment, seedlings were adequately watered. Glyphosate was applied when the plants reached six-leaf stage, approximately four weeks after transplanting. The recommended label dosage of glyphosate for ryegrass in field situation is 1.5 L ha^{-1}. Glyphosate was applied with a pneumatic pot-spraying apparatus operating at a spraying pressure of 2 bars and a water delivery rate of 108 L ha^{-1}. Irrigation was not applied to pots for 24 hours after treatments in order to allow for glyphosate to be adequately absorbed by the ryegrass.

A sample of ryegrass from each soil treatment was taken on the day of glyphosate application. The samples were cut close to the pot soil surface and dried at 80 °C for 48 hours. After the samples were dried, it was analysed for Mn content by the Elsenburg soil laboratory of the Western Cape Department of Agriculture.

For Trial 2, Experiment 1, topsoil was collected from the two respective sites during the dry, late summer months. The topsoil was spread open on a plastic sheet and irrigated to facilitate seed germination. When the seedlings were at the two to three-leaf stage, it was transplanted into small pots and the same procedure as described for Trial 1 was followed.

For Trial 2, Experiment 2, young, two to three leaf plant samples of Biotypes S3 and S4 were collected from their respective sites of origin after the first winter rains stimulated germination in the field. The seedlings were cut back to 2 cm height before being planted into the respective soils described above to facilitate better transplanting success. The procedure followed subsequently was similar to the one followed for Trial 1.

Evaluation and data analysis

Six weeks after glyphosate application, the experimental units were evaluated in terms of mortality and the percentage survival of each treatment combination was calculated. A plant was considered dead when the youngest leaf on the plant was necrotic and could easily be pulled from the stem. After

evaluation, all the plants in the pot were harvested close to the pot soil surface. Harvested material was dried at 80 °C for 48 hours. Thereafter, dry mass per pot was recorded.

Data was subjected to analysis of variance (ANOVA) using the STATISTICA 12® software program. The least significant difference (LSD) values of the Bonferroni post hoc test were calculated at the 5% probability level to expedite comparison between treatment means of main effects and interactions.

4.3. Results

Trial 1

At the time of glyphosate application, a sample of leaf material from each biotype growing in its allocated soil type were cut, dried and sent to the Elsenburg soil laboratory of the Western Cape Department of Agriculture. The results of the Mn content of the samples from each biotype are compiled and illustrated in Figure 4.3.

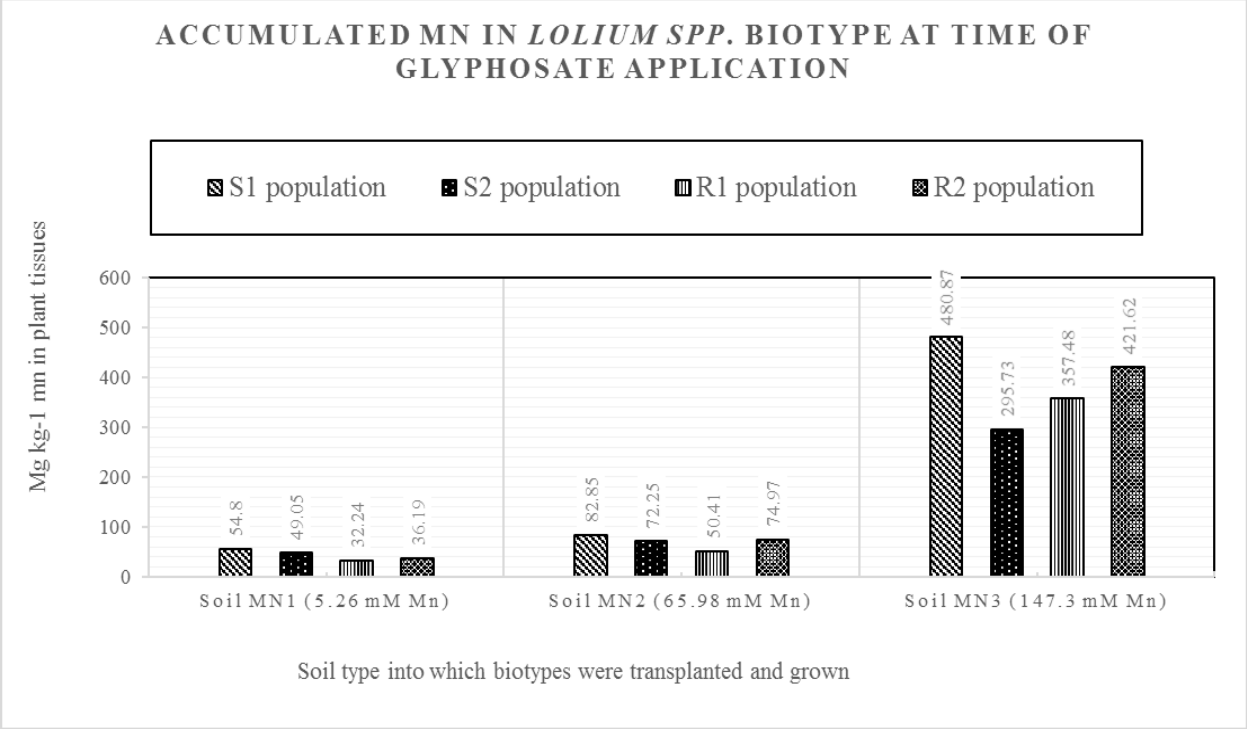

Figure 4.3: Mn accumulation (mg kg^{-1}) at time of glyphosate application obtained from foliar tissue of ryegrass biotypes reared in soils of high, medium and low Mn plant available content

Ryegrass biotype S1

The percentage survival of this biotype resulted in a significant interaction ($p<0.05$) between the main factors of soil type and glyphosate dosage rate (Figure 4.4). At a low, sublethal dosage rate of 0.5 L ha^{-1}, this biotype yielded a significantly higher survival rate (about 82%) from soil MN3 than from soil MN2 (32%). Soil MN2 in turn yielded a significantly higher survival rate than soil MN1 (8%).

No significant interaction ($p>0.05$) between the main factors in terms of dry matter production was observed. The three individual soils had diverse and significant effects on the amount of dry mass that this biotype produced. Soil MN3 yielded a significantly higher dry mass than soils MN1 and MN2 did (Table 4.5). As expected, increased glyphosate dosage rates had a negative effect on the dry mass production of the population (Table 4.6).

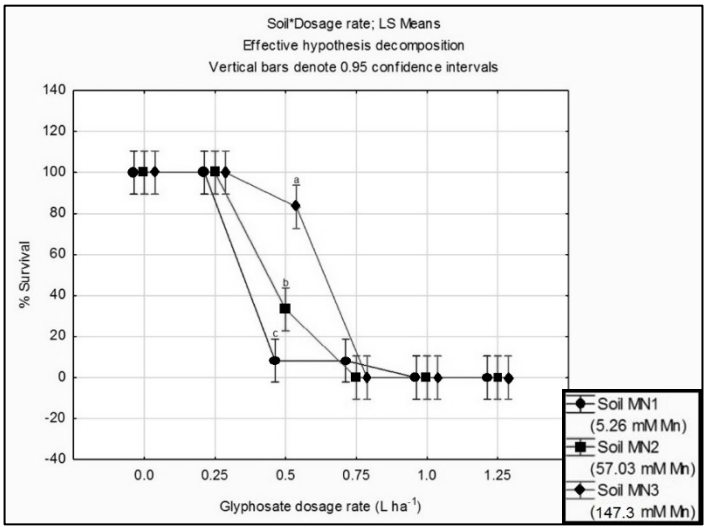

Figure 4.4: The interaction between soil Mn content and glyphosate dosage rate on the survival of ryegrass biotype S1 evaluated in three different Mn content soils. Glyphosate (360 g a.i. L^{-1}) was applied at dosage rates 0, 0.25, 0.5, 0.75, 1.0 and 1.25 L ha^{-1}. Vertical bars denote 0.95 confidence intervals

Table 4.5: The significant effect of soil type on dry matter production of ryegrass biotype S1

Soil type	**MN1**	**MN2**	**MN3**
Dry matter (g)	0.23b	0.15b	0.39a

Table 4.6: The significant effect of glyphosate dosage rate on dry matter production of ryegrass biotype S1

Glyphosate dosage rate (L ha^{-1})	**0.0**	**0.25**	**0.5**	**0.75**	**1.0**	**1.25**
Dry matter (g)	0.53a	0.44a	0.21b	0.10b	0.11b	0.16b

Ryegrass biotype S2

A significant interaction ($p<0.05$) was found between the main factors of soil type and glyphosate dosage rate, indicated at dosage rate 0.4 L ha-1 (Figure 4.5) in this population. Once again, soil MN3 yielded the highest survival rate which was significantly higher than that which were found in soil MN2. However, the survival rate within soil MN3 was not significantly higher than in soil MN1.

No significant interaction ($p>0.05$) between the main factors in terms of dry matter production was found. However, significant ($p<0.05$) differences between treatments within the two main factors were observed. The effect of the soil on dry mass production is illustrated in Table 4.7. The S2 population yielded significantly more dry mass from soil MN3 than from soil MN1. Increased glyphosate dosage rates resulted in decreased dry mass production, as would be expected (Table 4.8).

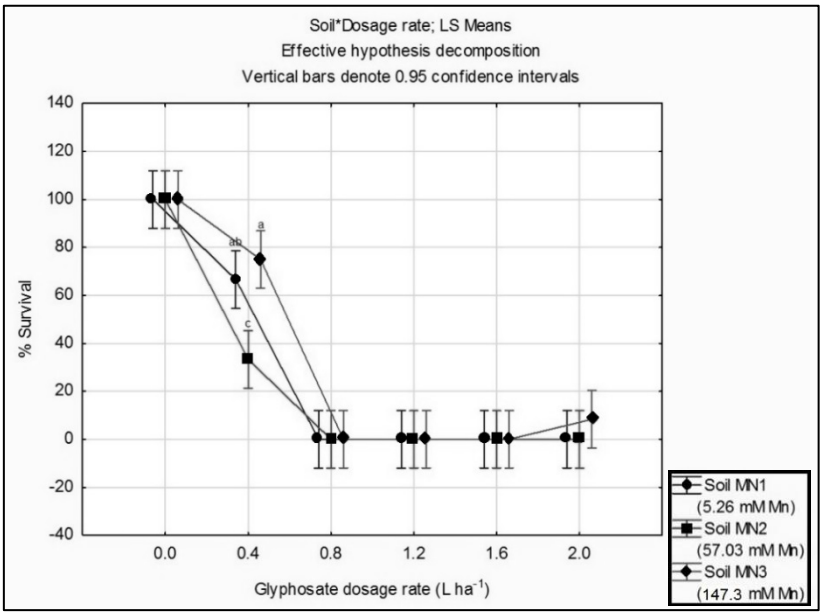

Figure 4.5: The interaction between soil Mn content and glyphosate dosage rate on the survival of ryegrass biotype S2 evaluated in three different Mn content soils. Glyphosate (360 g a.i. L^{-1}) was applied at dosage rates 0, 0.4, 0.8, 1.2, 1.6 and 2.0 L ha^{-1}. Vertical bars denote 0.95 confidence intervals

Table 4.7: The significant effect of soil type on dry matter production of ryegrass biotype S2

Soil type	**MN1**	**MN2**	**MN3**
Dry matter (g)	0.19b	0.22ab	0.36a

Table 4.8: The significant effect of glyphosate dosage rate on dry matter production of ryegrass biotype S2

Glyphosate dosage rate (L ha^{-1})	**0.0**	**0.4**	**0.8**	**1.2**	**1.6**	**2.0**
Dry matter (g)	0.68a	0.42ab	0.09c	0.07c	0.23cb	0.06c

Ryegrass biotype R1

No significant interaction ($p<0.05$) was found between the main factors of soil type and dosage rate for survival data in this biotype. However, a trend resembling the one observed from the results of biotype S2 could be observed (Results not shown). The results of the main factor dosage rate showed a significantly lower survival rate of plants treated with 3.0 L ha-1 in comparison to the control treatment (Table 4.9).

Table 4.9: The significant effect of glyphosate dosage rate on percentage survival of ryegrass biotype R1

Glyphosate dosage rate (L ha-1)	**0.0**	**1.0**	**2.0**	**3.0**	**4.0**	**5.0**
Survival (%)	100a	97a	69ab	33c	25c	17cd

Regarding the yield of dry matter production within this biotype, no significant interaction was detected. This result is analogous to the previous two populations. Significant differences were however found between treatments within the two main factors. This population yielded significantly more dry mass from soil MN3 than it did from soil MN2, for example (Table 4.10). Once again, an increase in dosage rates decreased dry mass production (Table 4.11).

The results of the main factor dosage rate showed a significantly lower survival rate of plants treated with 3.0 L ha-1 in comparison to the control treatment.

Table 4.10: The significant effect of soil type on dry matter production of ryegrass biotype R1

Soil type	**MN1**	**MN2**	**MN3**
Dry matter (g)	0.35a	0.06b	0.43a

Table 4.11: The significant effect of glyphosate dosage rate on dry matter production of ryegrass biotype R1

Glyphosate dosage rate (L ha^{-1})	**0.0**	**1.0**	**2.0**	**3.0**	**4.0**	**5.0**
Dry matter (g)	0.67a	0.30bc	0.32b	0.20bc	0.05c	0.14bc

Ryegrass biotype R2

No significant interaction was found between soil type and glyphosate dosage rate in this particular biotype. Neither was any significant differences found between the respective soils regarding mortality. However, there was a significant ($p<0.05$) effect of glyphosate dosage rate on the mortality of the population (Table 4.12). Only at a dosage rate of 4 L ha-1 a significantly lower survival rate was exhibited compared to the unsprayed control.

Table 4.12: The significant effect of glyphosate dosage rate on percentage survival of ryegrass biotype R2

Glyphosate dosage rate (L ha-1)	**0.0**	**2.0**	**4.0**	**6.0**	**8.0**	**10.0**
Survival (%)	100a	80ab	42c	25cd	22cd	6d

A significant interaction ($p<0.05$) was found between glyphosate dosage rate and soil type in the evaluation of dry mass production (Figure 4.6). The figure illustrates that soil MN3 yielded significantly more dry mass than soil MN2 at the 0 L ha-1 (control) glyphosate dosage rate. Although the differences were not significant, it is interesting to note that soil MN3 (at 2 L ha-1) and soil MN1 (at 0 and 2 L ha-1) appeared to have yielded more dry matter than soil MN2. At higher glyphosate dosage rates, no significant differences were obtained between the three different soils in terms of dry matter production.

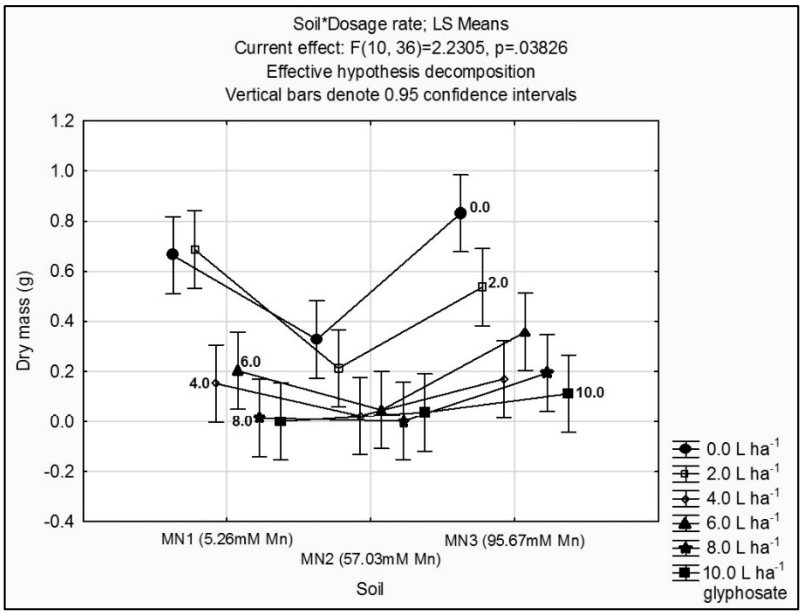

Figure 4.6: The interaction between glyphosate dosage rate and soil type in terms of dry mass production of ryegrass biotype R2 (glyphosate resistant weedy ryegrass population). Vertical bars denote 0.95 confidence intervals

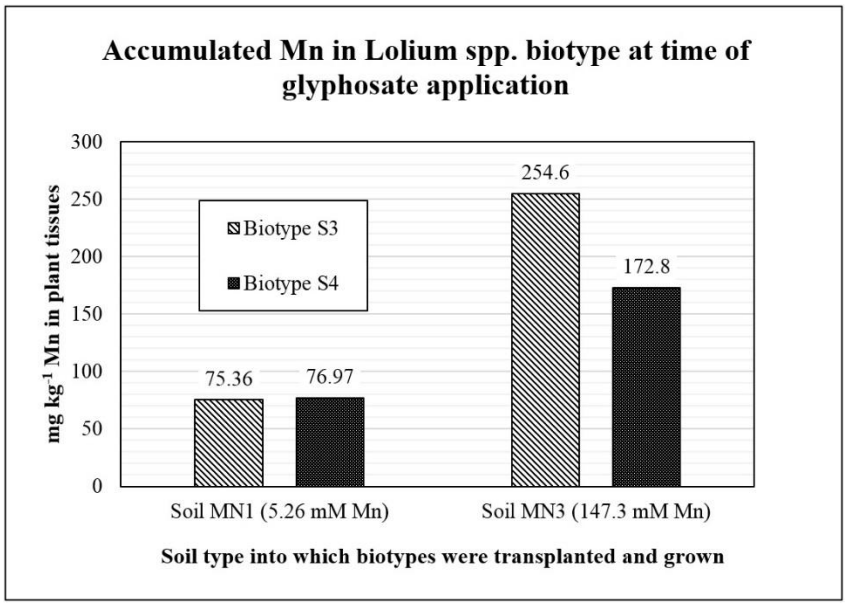

Figure 4.7: Mn accumulation (mg kg^{-1}) at time of glyphosate application obtained from tissues of ryegrass biotypes grown in soils of high and low Mn plant available content. Biotype S3 was collected from the site where Soil MN1 was collected and Biotype S4 was collected from the site where Soil MN3 was collected

Trial 2

Experiment 1

At the time of glyphosate application, a sample of leaf material from each biotype growing in its allocated soil type were cut, dried and sent to the Elsenburg soil laboratory of the Western Cape Department of Agriculture. The results of the Mn content of the samples from each biotype are compiled and illustrated in Figure 4.7.

No significant interaction ($p>0.05$) between the main factors of soil type and glyphosate dosage rate were found in biotype S3 when mortality was evaluated. However, the main factor of glyphosate dosage rate did have a significant impact on the survival rate of biotype S3 (Table 4.13).

Table 4.13: Significant effect of glyphosate dosage rate on percentage survival in S3 ryegrass species

Glyphosate dosage rate (L ha-1)	**0.0**	**0.5**	**1.0**	**1.5**	**2.0**	**2.5**
Survival (%)	100a	53.12bc	18.75bd	6.25dc	3.12d	3.12d

Biotype S4 exhibited a significant interaction between soil type and dosage rate. A better survival rate of 56.25% was obtained at sub-lethal dosage rate of 0.5 L ha-1 in the soil it was planted into (MN1) than in its native soil (MN3) (0% survival rate) in terms of percentage survival (Figure 4.8) although at dosage rates closer to recommended label dosage, results proved to be more even.

Significant interaction ($p<0.05$) was found between the main factors of soil type and glyphosate dosage rate in biotype S3 when dry mass of the surviving live material was evaluated (Figure 4.9). At recommended label dosage (1.5 L ha-1), the difference between total dry matter in the two soils became non-significant.

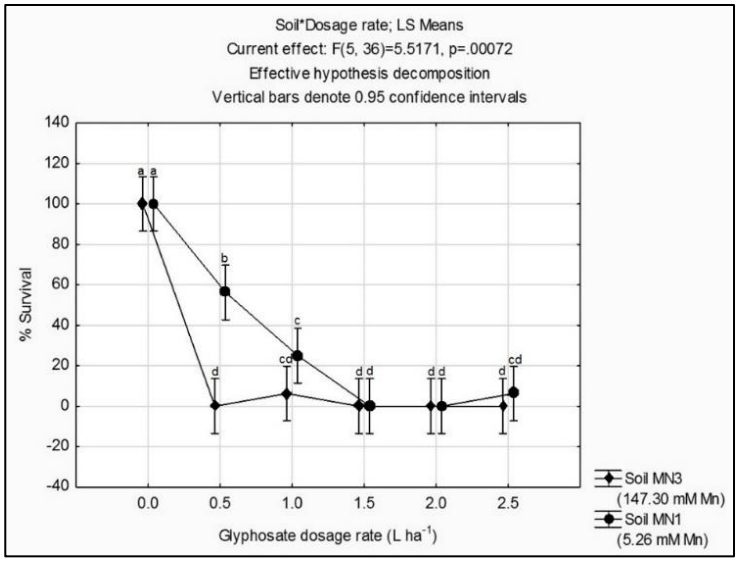

Figure 4.8: The effect of different glyphosate dosage rates on survival rate of ryegrass biotype S4 (glyphosate susceptible population) grown in two soils with different Mn levels. Glyphosate (360 g a.i. L^{-1}) was applied at dosage rates 0, 0.5, 1.0, 1.5, 2.0 and 2.5 L ha^{-1}. Vertical bars denote 0.95 confidence intervals

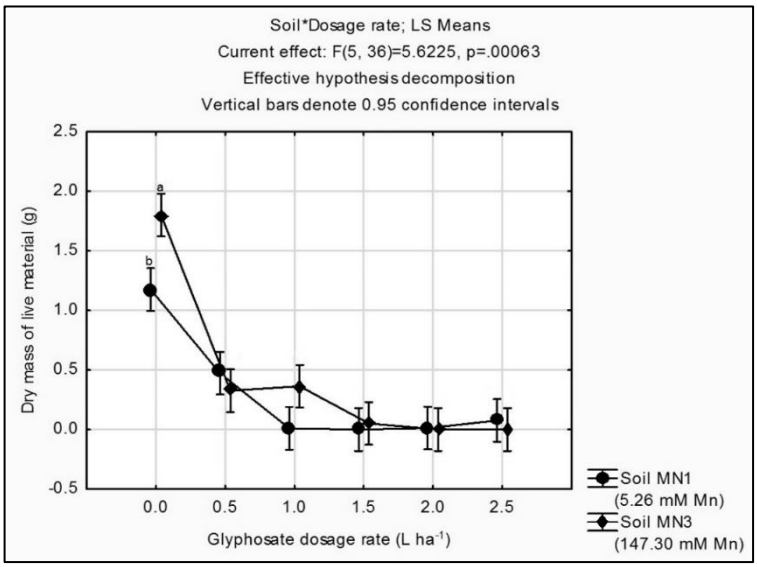

Figure 4.9: The interaction between glyphosate dosage rate and soil type regarding the evaluation of live dry mass production of ryegrass biotype S3 (glyphosate susceptible population). Glyphosate (360 g a.i. L^{-1}) was applied at dosage rates 0, 0.5, 1.0, 1.5, 2.0 and 2.5 L ha^{-1}. Vertical bars denote 0.95 confidence intervals

Biotype S4 exhibited results similar to biotype S3. In biotype S4, native soil MN3 yielded less dry matter than new soil MN1 at dosage rates below recommended label dosage. As seen in Figure 4.10 differed significantly at 0.0 and 0.5 L ha-1 but not at higher dosage rates.

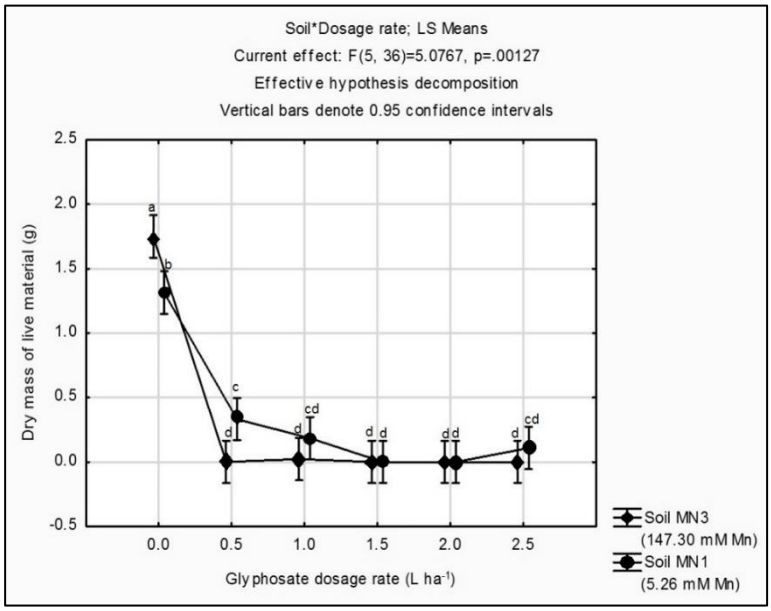

Figure 4.10: The interaction between glyphosate dosage rate and soil type regarding the evaluation of live dry mass production of ryegrass biotype S4 (glyphosate susceptible population). Glyphosate (360 g a.i. L^{-1}) was applied at dosage rates 0, 0.5, 1.0, 1.5, 2.0 and 2.5 L ha^{-1}. Vertical bars denote 0.95 confidence intervals

Experiment 2

At the time of glyphosate application, a sample of leaf material from each biotype growing in its allocated soil type were cut, dried and sent to the Elsenburg soil laboratory of the Western Cape Department of Agriculture. The results of the Mn content of the samples from each biotype are compiled and illustrated in Figure 4.11.

Significant interaction ($p<0.05$) between the main factors of soil type and glyphosate dosage rate was found in biotype S5 (putative glyphosate susceptible). Significant differences in percentage survival were especially clear between the soil types at 0.5 and 1.5 L ha-1 (Figure 4.12). On the other hand, biotype R3 (putative glyphosate resistant) did not exhibit significant interaction ($p>0.05$) between the main factors. The effect of the main factors on their own showed significant differences (Table 4.14 and 4.15).

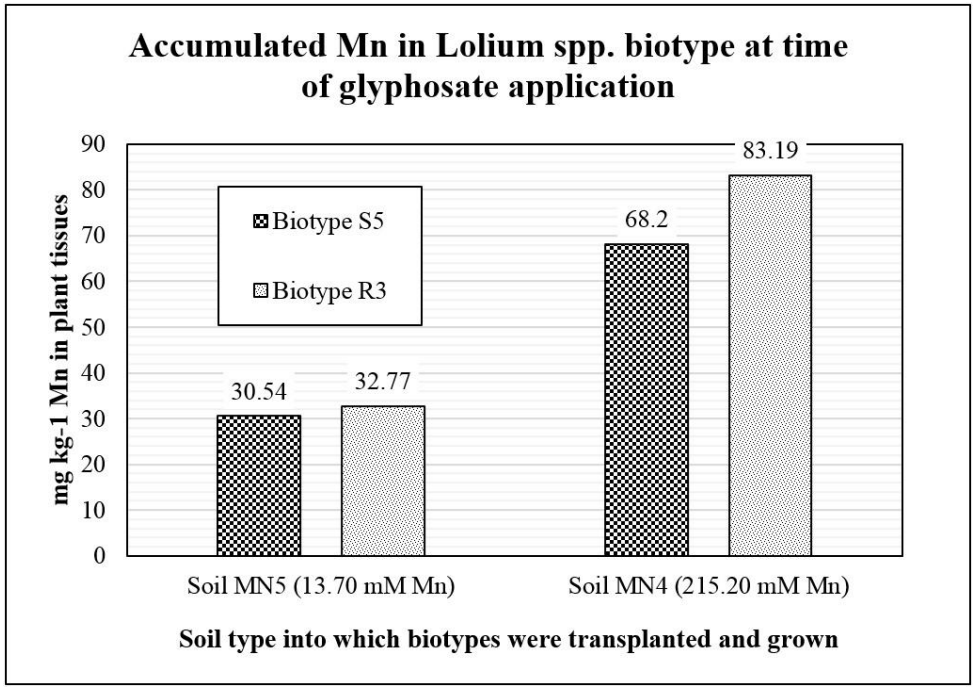

Figure 4.11: Mn accumulation (mg kg^{-1}) at time of glyphosate application obtained from tissues of ryegrass biotypes grown in soils of high and low Mn plant available content

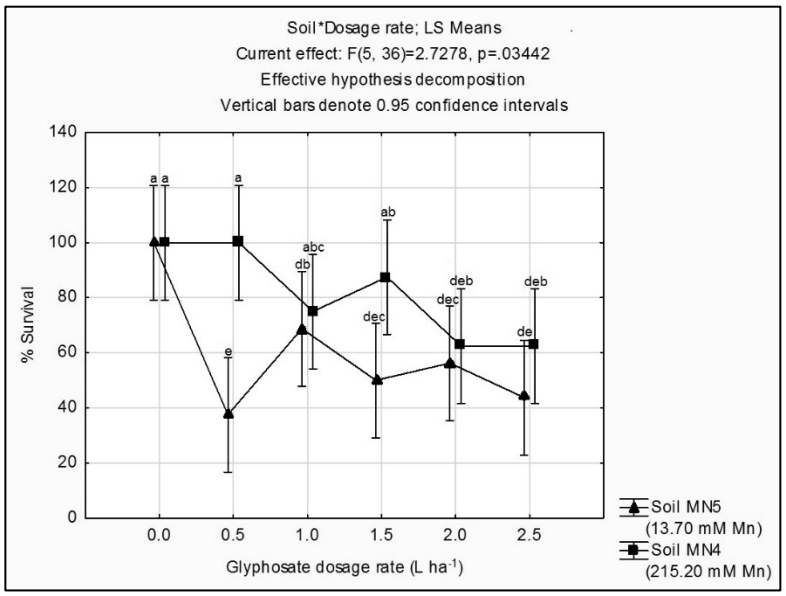

Figure 4.12 The interaction between glyphosate dosage rate and soil type regarding percentage survival of ryegrass biotype S5 (putative glyphosate susceptible population). Glyphosate (360 g a.i. L^{-1}) was applied at dosage rates 0, 0.5, 1.0, 1.5, 2.0 and 2.5 L ha^{-1}. Vertical bars denote 0.95 confidence intervals

Table 4.14: Significant effect of glyphosate dosage rate on percentage survival in R3 ryegrass species.

Glyphosate dosage rate (L ha^{-1})	**0.0**	**1.0**	**2.0**	**4.0**	**8.0**	**16.0**
Survival (%)	100a	96.50a	71.88b	56.25bc	43.75dc	31.25d

Table 4.15: Significant effect of soil type on percentage survival in R3 ryegrass species.

Soil type	**MN4 (215.20 mg kg^{-1} Mn)**	**MN5 (13.7 mg kg^{-1} Mn)**
Survival (%)	75a	58b

In the analyses of dry matter, no significant interaction ($p>0.05$) was obtained in dry matter from both of the biotypes tested. Some individual main factors however exhibited significant effects. For both biotypes, soil MN4 which contained a higher amount of plant available Mn yielded a higher dry mass accumulation than soil MN5 did (Table 4.16 and 4.18). Generally, significant differences were seen between higher glyphosate dosage rates and the control.

Table 4.15: Significant effect of glyphosate dosage rate on dry matter production in S5 ryegrass species

Glyphosate dosage rate (L ha^{-1})	**0.0**	**0.5**	**1.0**	**1.5**	**2.0**	**2.5**
Dry matter (g)	3.08a	1.42b	1.41b	1.01b	1.10b	0.90b

Table 4.16: Significant effect of soil type on dry matter production in S5 ryegrass species

Soil type	**MN4 (215.20 mg kg^{-1} Mn)**	**MN5 (13.7 mg kg^{-1} Mn)**
Dry matter (g)	2.02a	0.95b

Table 4.17: Significant effect of glyphosate dosage rate on dry matter production in R3 ryegrass species

Glyphosate dosage rate (L ha^{-1})	**0.0**	**1.0**	**2.0**	**4.0**	**8.0**	**16.0**
Dry matter (g)	0.75a	0.50b	0.52b	0.45bc	0.31c	0.30c

Table 4.18: Significant effect of soil type on dry matter production in R3 ryegrass species

Soil type	**MN4 (215.20 mg kg^{-1} Mn)**	**MN5 (13.7 mg kg^{-1} Mn)**
Dry matter (g)	0.76a	0.18b

4.4. Discussion and conclusion

Interesting trends and observations arose from the trials conducted. However, it seems that a single, clear conclusion remains elusive. Trial one led to the conclusion that a soil comprising of a high Mn content may well decrease the efficacy of glyphosate applied to specific ryegrass biotypes. Mn antagonism does therefore exist in this trial as also observed by Bailey et al. (2002), Bernards et al. (2005), Hartzler (2011) and Soltani et al. (2011). According to some of these authors, the true extent of the Mn antagonism seems to be dependent on a variety of circumstances and factors such as soil pH,

soil structure, the presence of other minerals in the soil, soil moisture content, climatic conditions and general soil type.

Greater differences in survival rates between soil types were observed at sub-lethal dosage rates, especially within susceptible biotypes. These greater survival rates were present within soils comprising of a higher Mn content, although not necessarily at recommended label dosage rate (1.5 L ha-1 glyphosate) and not exclusively within these specific soils. A trend does however seem to emerge from the use of susceptible biotypes when observing the amount of dry mass harvested after glyphosate application in soil higher in Mn content.

Resistant biotype R1 more or less exhibited the same trend of greater survival within soil of higher Mn content, but only at an “elevated” sublethal dosage rate (2.0 L ha-1) as could be expected of resistant biotypes. However, this result was almost reversed on the same curve at the elevated glyphosate dosage rate of 4.0 L ha-1 where soil of lower Mn content yielded greater survival rates. No effect of the sort was observed in biotype R2 – the biotypes’ high level of resistance is seemingly unperturbed by any effect that Mn might have exhibited.

The effects that “native soil” (soil from which the specific biotype was extracted for the use of the trial) versus “new soil” (different soil than that which the specific biotype was extracted from) might exhibit on survival were tested and explored in soils with high and low Mn content (soil MN4 and MN5). It appeared as though susceptible biotypes exhibited good survival at sub-lethal dosage rates in native as well as new soils into which they were transplanted, regardless of Mn content of the specific soil (native or new soil).

Resistant biotype R3 exhibited better survival in the soil into which it was transplanted (soil MN5). This particular soil had a much lower Mn content than its original soil had. Susceptible biotype (S5) exhibited this same trend, but the soil into which it was transplanted had in turn a high Mn content.

According to the leaf analysis at time of spray, biotype S3 seems to have taken up more Mn from soil MN3 than biotype S4 did (MN3 is the native soil of biotype S4). Certain plant species had been known for exhibiting a better tolerance to Mn (Yost 2000). Perhaps the plants of this biotype (S4) comprise of an inherent Mn tolerance selected from being exposed to high levels of Mn for many generations.

As seen from the analyses, biotype S3 had taken up more Mn from soil MN3 as compared to S4. As proven by Busi and Powles (2009), ryegrass is prone to the evolution of glyphosate resistance by selection processes at sub-lethal dosage rates. Perhaps never being subjected to high levels of Mn, this

particular biotype (S3) had not been through a similar type of natural selection process where it had acquired the same kind of tolerance to Mn uptake (Yost 2000) as biotype S4 could possibly have been. That would to some extent explain why a comparison of the percentage survival between the two biotypes results in no correlation.

Furthermore, it would seem that the resistant ryegrass originating from a soil of higher Mn content (biotype R3) as opposed to their counterparts are naturally more inclined to a magnified resistance, regardless of the soil into which it was transplanted.

From the results obtained in these trials, it seems possible that ryegrass subjected to soil of high Mn content had over time evolved a type of non-target site resistance. Perhaps this non-target site resistance was aided or at least favoured in some way by the effect of internal antagonism between Mn and glyphosate molecules we already know to exist (Bailey et al. 2002, Bernards et al. 2005, Hartzler 2011, Soltani et al. 2011) – thus reducing the efficacy of glyphosate over long periods of time.

According to Ololade et al. (2014), the amount of soil organic matter, soil structure, clay content and also iron oxides present in a soil type influences glyphosate sorption to soil particles. They particularly found that the presence of soil metals and organic carbon can decrease the activity of glyphosate (Gerritse et al. 1996; Sørensen et al. 2006). However, in light of the results obtained in this trial, amount of soil organic matter (% C) and possible glyphosate re-uptake does not seem to exert a noticeable effect on percentage survival.

Mineral particles in soil however, may well play a substantial role in glyphosate availability. Fe oxides, Mn oxides and clay minerals are examples of the types of soil minerals which glyphosate has the affinity to sorb to (Gerritse et al. 1996; Borggaard and Gimsing 2008). In essence, sorption sites on soil structure may be blocked by these metals and thereby less sorption of glyphosate to the soil surface takes place. As a consequence, more glyphosate is available for re-uptake by the plant (Ololade et al. 2014).

Be as it may, the fact remains that many different soil conditions (structure, soil organic matter, clay content and metals or minerals such as iron oxides) may well influence possible outcomes and should never be overlooked as it determines the amount and transport rate of glyphosate through plants (Ololade et al. 2014).

Some ryegrass biotypes at least prefer soil of high Mn content and produce more dry mass in comparison to the same biotype reared in different soils sprayed at the same sub-lethal glyphosate dosage rates. It may also be deduced that high levels of Mn in soil might make plants less susceptible

to sub-lethal dosages of glyphosate. After prolonged periods of time, development of non-target site resistance might be developed under the correct set of conditions as compared to soil with low levels of Mn in soil, but more research is needed to confirm this conclusion.

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Chapter 5

The effect of temperature in combination with manganese treatments on glyphosate efficiency in ryegrass (*Lolium spp.*)

5.1. Introduction

Temperature plays a very significant role in respiration and consequently affect optimal growth and metabolism of any given plant species (Waddington et al. 1992; Criddle et al. 1997). Glyphosate resistance in plants may be affected as glyphosate activity is often altered by environmental factors (Cerdeira et al. 2007). Optimum temperature ranges exist by which plants adept to different environments will produce optimal yield or grow considerably better than in sub-optimal temperatures – a simple yet significant principle which have been tested in many plant species for many decades (Went 1957).

Therefore, it is a given that the effect of temperature on any given plant or crop species cannot be overlooked in a scientific study where growth or production might be tested against any given factor. In this particular study, it was decided that special attention should be given to the potential effect that temperature might exert on glyphosate resistance in ryegrass (*Lolium spp*.).

Glyphosate resistance may occur as target site or non-target site resistance. Target site resistance is when a mutation at specific binding sites influence binding of herbicides or if a gene at the target site is amplified (Wiersma et al. 2015). Non target site resistance may include decreased translocation of the specific herbicide (Powles and Preston 2006) or reduced uptake from the leaf surface (Michitte et al. 2007), amongst others.

It is well known that the efficacy of glyphosate withers somewhat at non-ideal temperatures (Vila-Aiub et al. 2013; Ghanizadeh et al. 2015). In the study of Ghanizadeh et al. (2015), it was found that glyphosate was more effective in cooler temperatures as a result of restriction of vacuolar sequestration. On the other hand, transpiration or photosynthesis may decrease under too high temperatures, slowing down the entire metabolism of any given plant and in essence curbing the efficacy of a systemic herbicide such as glyphosate in this particular example (Pline et al. 1999; Nguyen et al. 2016).

Manganese (Mn) likely plays an important contributory role in the issue of glyphosate efficacy (Barrett and McBride 2005; Eker et al. 2006; Tesfamariam et al. 2009). In the context of this study, it makes

sense to further investigate the role that plant available levels of Mn in soil may contribute to the matter of inhibition of glyphosate efficacy.

The effect of temperature on glyphosate resistance have been investigated in a small variety of plants up to date. For instance, Vila-Aiub et al. (2013) tested the level to which glyphosate resistance is dependent on temperature in Johnson grass (*Sorghum halepense*) as well as glyphosate resistant rigid ryegrass (*L. rigidum*) biotypes and Nguyen et al. (2016) tested the same effect in barnyard grass (*Echinochloea crus-galli*). The particular study of Vila-Aiub et al. (2013) had shown that non target site resistance is present in the form of reduced translocation. Previous work had also already demonstrated that glyphosate resistance is inhibited at lower temperatures in resistant *Conyza* biotypes (Ge et al. 2011).

In this present study, the effects of temperature on glyphosate efficacy was tested on two susceptible ryegrass biotypes at four temperature regimes under controlled conditions in combination with manganese soil treatments. In testing susceptible biotypes, the behaviour of glyphosate efficacy under controlled temperatures will be shown when subjected to Mn soil treatments under the assumption that to some degree, Mn possess the potential to inhibit glyphosate efficacy (Bernards et al. 2005).

5.2. Materials and methods

Experimental site

This trial was run under controlled glasshouse conditions at the Department of Agronomy on the Welgevallen Experimental Farm of Stellenbosch University, Western Cape, South Africa.

Experimental design and layout

Using a completely randomized block design, four separate experiments were completed. The pot trials were conducted in four separate glasshouses under controlled day/ night temperatures of 10/15 °C, 15/20 °C, 20/25 °C and 25/30 °C respectively. The four temperature regime experiments in this trial were carried out in order to establish whether certain temperatures in combination with soil manganese content affect glyphosate efficiency in ryegrass.

River sand medium was utilized in these trials. Soil characteristics thereof are compiled in Table 5.1. The trial was carried out on two susceptible ryegrass biotypes (S1 and S2). S1 is a glyphosate susceptible commercial variety called “Mach1” - obtained from the seed company Agricol whereas S2

is also a local glyphosate susceptible biotype obtained from Rondebosch Common (33.9561° S, 18.4825° E) in Cape Town, South Africa.

Table 5.7: Soil characteristics of the river sand medium used in the pots

Parameter	**River sand medium**	**Unit**
pH(KCl)	5.3	
Texture	Sand	
Resistance	6760	Ohms
Acidity	0.21	cmol+ kg^{-1}
Calcium (Ca)	0.41	cmol+ kg^{-1}
Magnesium (Mg)	0.09	cmol+ kg^{-1}
Total cations	0.77	cmol+ kg^{-1}
Potassium (K)	11	mg kg^{-1}
Sodium (Na)	8	mg kg^{-1}
Phosphorus (P)	22	mg kg^{-1}
Copper (Cu)	0.10	mg kg^{-1}
Zinc (Zn)	0.18	mg kg^{-1}
Manganese (Mn)	**5.69**	**mg kg^{-1}**
Boron (B)	0.02	mg kg^{-1}
Sulphur (S)	3.30	mg kg^{-1}
Carbon (C)	0.03	%

Biotypes S1 and S2 were subjected to treatments of varying Mn concentrations (0.0, 2.5, 5.0 and 7.5 mM Mn). At each Mn treatment level (0.0, 2.5, 5.0 and 7.5 mM Mn), the biotype was eventually subjected to glyphosate dosage rates (0.0, 0.4, 0.8, 1.2, 1.6, and 2.0 L ha^{-1}) in order to obtain dosage response data. The same set of Mn concentrations and glyphosate dosage rates were repeated under each temperature regime for each biotype.

The experimental procedures involving the controlled temperatures in this trial constituted four separate experiments and were analysed accordingly. Apart from the controlled temperature applied, the experimental procedure for all four experiments was the same.

Experimental procedure

Seeds of the S1 and S2 ryegrass biotypes were germinated in petri dishes in an incubation chamber at a constant temperature of 20 °C. Once germinated, seedlings were transplanted into 8 cm x 8 cm size pots filled with river sand medium. Four ryegrass seedlings were transplanted into each pot. Seedlings were grown in previously mentioned glasshouse conditions (10/15 °C, 15/20 °C, 20/25 °C and 25/30 °C).

Throughout life stages of seedling to eight leaf stage, seedlings were adequately watered with a balanced nutrient solution (Table 5.2). Mn ($MnSO_4$) treatment solutions of different concentrations were applied to the surface of the pot soil three times per week. The concentrations of the Mn treatment solutions additionally applied to the seedlings were 0 mM (normal balanced nutrient solution containing 0.55 mg L^{-1} $MnSO_4$ serving as control treatment), 2.5, 5.0, and 7.5 mM $MnSO_4$. On treatment days, the pots were watered with the Mn treatment solution alone. This was done to enhance optimal uptake of the Mn solution without possible leaching from the soil medium.

Each one of the four Mn treatments were treated with six glyphosate dosage rates (0.0, 0.4, 0.8, 1.2, 1.6, and 2.0 L ha^{-1}). The glyphosate formulation used contained 360 g L-1 of glyphosate. The recommended label dosage of glyphosate for ryegrass in field situation is 1.5 L ha^{-1}. Glyphosate was applied with a pneumatic pot-spraying apparatus operating at a spraying pressure of 2 bars and a water delivery rate of 108 L ha^{-1}. Irrigation was not applied to pots for 24 hours after treatment to allow for glyphosate to be adequately absorbed by the ryegrass.

Table 5.8: The nutrient concentrations of feeding solution used in the glasshouse trials

EC = 2.0			
Element	Concentration	Fertiliser	Concentration
(Macro)	mg L^{-1}		g $1000L^{-1}$
K^+	237.7	$KN0_3$	303
Ca^{++}	180	K_2S0_4	261
Mg^{++}	48.6	$Ca\ (N0_3)_2.\ 2H_20$	900
$N0_3^-$	661.33	$MgS0_4.7H_20$	492
H_2P0_4	116.4	KH_2P0_4	136
$S0_4$	390.4		
(Micro)	mg L^{-1}		
Fe: Libfer (Fe EDTA)	0.85		6.54
Mn: Manganese sulphate	0.55		2.23
Zn: Zinc sulphate	0.30		1.33
B: Solubor	0.30		1.46
Cu: Copper Sulphate	0.05		0.20
Mo: Sodium Molibdate	0.02		0.13

Evaluation and data analysis

Six weeks after glyphosate application, the mortality rate of each experimental unit was evaluated and percentage survival was calculated per treatment combination. A single plant was evaluated and recorded as controlled by the herbicide when the youngest leaf was necrotic and could easily be pulled from the stem. After evaluation, all plants per pot were harvested close to the pot soil surface and dried at 80°C for 48 hours. Thereafter dry mass was weighed and total dry mass per pot was calculated.

Survival (%) and dry matter (g) data was subjected to analysis of variance (ANOVA) using the STATISTICA 12® software program. The least significant difference (LSD) values of the Bonferroni post hoc test were calculated at the 5% probability level to expedite comparison between treatment means of main effects and interactions.

5.3. Results

10-15°C day/night-controlled temperature

Under this temperature range of 10-15°C day/night-controlled temperature, significant interaction ($p<0.05$) was observed between the main factors of glyphosate dosage rate and Mn level (Figure 5.1). Mn treatments seemed to exhibit better survival rates than the control treatment (0.0 mM Mn). Significant interaction ($p<0.05$) was also observed in percentage survival between ryegrass biotype and glyphosate dosage rate (Figure 5.2).

Significant three-way interaction ($p<0.05$) was also observed in dry matter production results between biotype, dosage rate and Mn level (Figure 5.3). With exception of the control treatment (0.0 L ha-1 at 0.0 mM Mn), Mn treatments 2.5, 5.0 and 7.5 mM Mn generally seemed to produce slightly more dry matter.

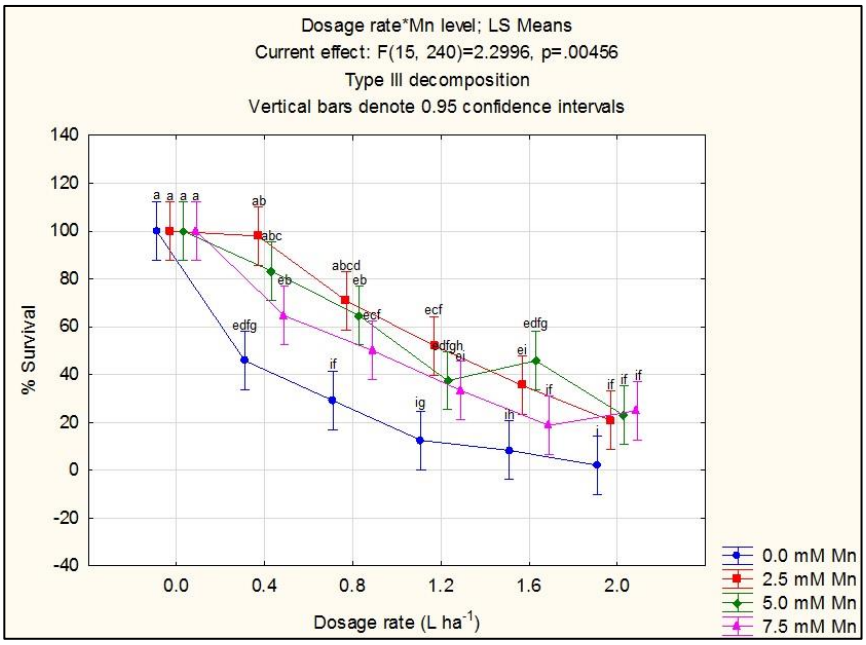

Figure 5.1: The effect of glyphosate dosage rate and Mn treatment level on percentage survival of ryegrass growing under 10-15°C day/night-controlled temperatures. Glyphosate (360 g a.i. L^{-1}) was applied at dosage rates of 0.0, 0.4, 0.8, 1.2, 1.6 and 2.0 L ha^{-1}. Vertical bars denote 0.95 confidence intervals

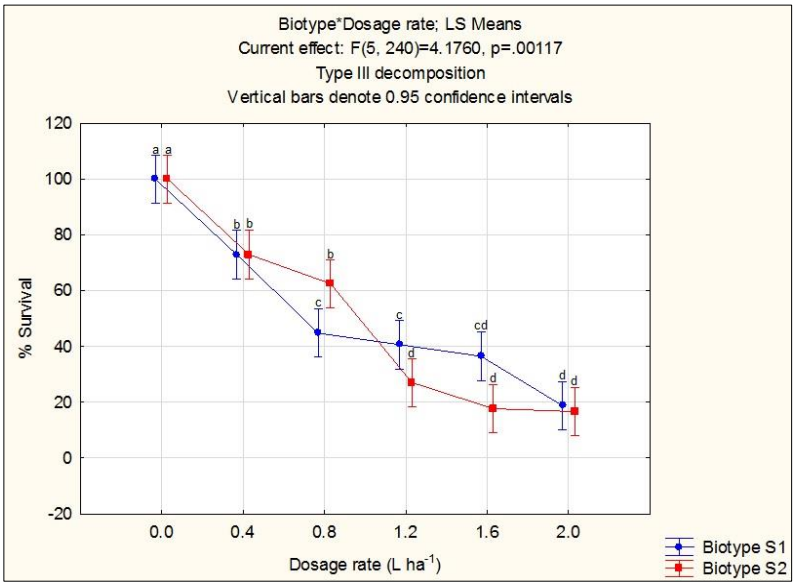

Figure 5.2: The effect of glyphosate dosage rate on percentage survival in ryegrass biotypes S1 and S2 under 10-15°C day/night-controlled temperatures. Glyphosate (360 g a.i. L^{-1}) was applied at dosage rates 0.0, 1.0, 0.4, 0.8, 1.6 and 2.0 L ha^{-1}. Vertical bars denote 0.95 confidence intervals

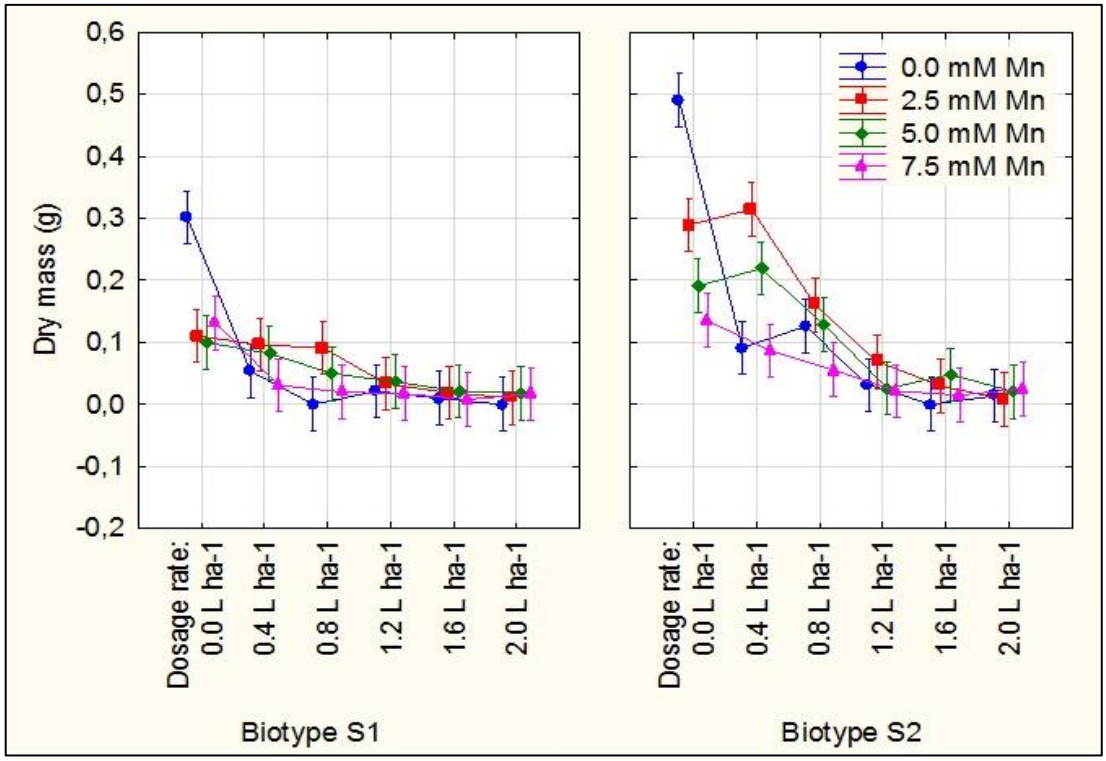

Figure 5.3: The effects of Mn treatment levels and glyphosate dosage rates on dry matter production in two ryegrass biotypes (S1 and S2) under 10-15°C day/night-controlled temperatures. Glyphosate (360 g a.i. L^{-1}) was applied at dosage rates 0.0, 0.4, 0.8, 1.2, 1.2 and 2.0 L ha^{-1}. Vertical bars denote 0.95 confidence intervals

15-20°C day/night-controlled temperature

Under this temperature range, significant interaction ($p<0.05$) was observed between the main factors of glyphosate dosage rate and manganese level (Figure 5.4). At sublethal dosage rates (0.4, 0.8 and 1.2 L ha-1), better survival was exhibited in Mn treatments 2.5, 5.0 and 7.5 mM Mn than in the control treatment (0.0 mM Mn). Significant interaction was also obtained between ryegrass biotype and Mn level (Figure 5.5) in terms of percentage survival. Survival of the two biotypes was exhibited to be significantly better at 2.5, 5.0 and 7.5 mM Mn. This could perhaps indicate involvement of Mn levels in resistance of ryegrass to glyphosate.

Significant interaction ($p<0.05$) between dosage rate and Mn level was also observed in dry matter production results (Figure 5.6). Generally, more dry matter was produced within treatments containing added Mn as opposed to the control treatment (0.0 mM Mn). The interaction between ryegrass biotype and glyphosate dosage rate also proved to be significant in this temperature range (Figure 5.7).

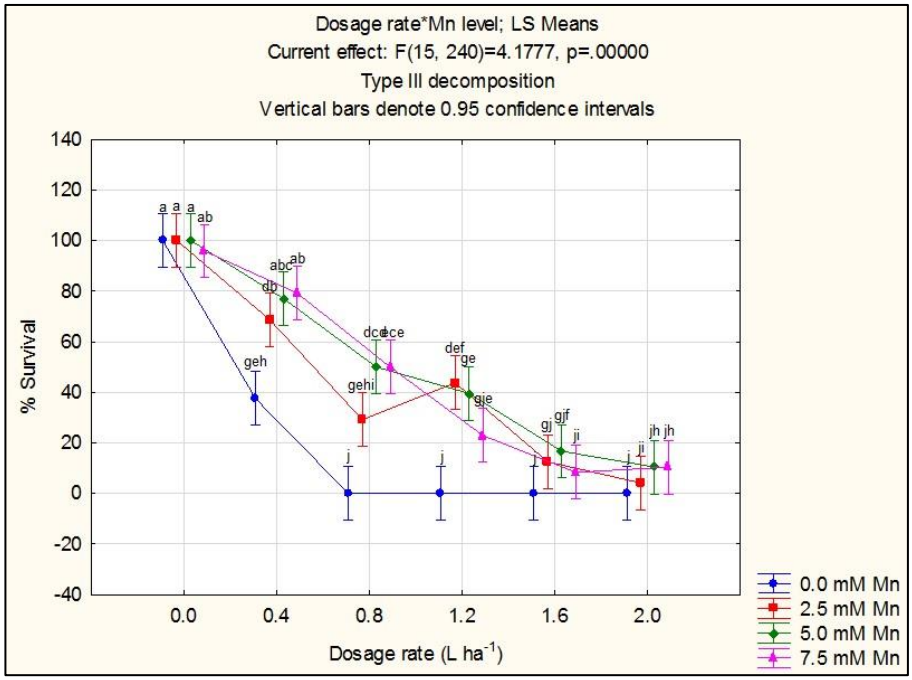

Figure 5.4: The effect of glyphosate dosage rate and Mn treatment level on percentage survival of ryegrass under 15-20°C day/night-controlled temperatures. Glyphosate (360 g a.i. L^{-1}) was applied at dosage rates 0.0, 0.4, 0.8, 1.2, 1.6 and 2.0 L ha^{-1}. Vertical bars denote 0.95 confidence intervals

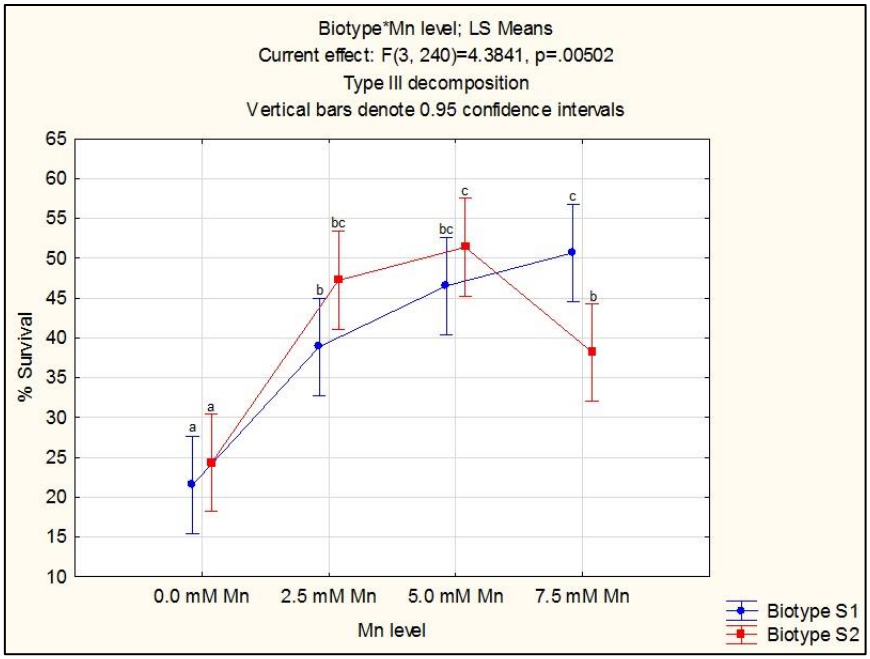

Figure 5.5: The effect of manganese treatment level on percentage survival in ryegrass biotypes S1 and S2 under 15-20°C day/night-controlled temperatures. Manganese treatments were applied at 0.0, 2.5, 5.0 and 7.5 mM MnSO4. Vertical bars denote 0.95 confidence intervals

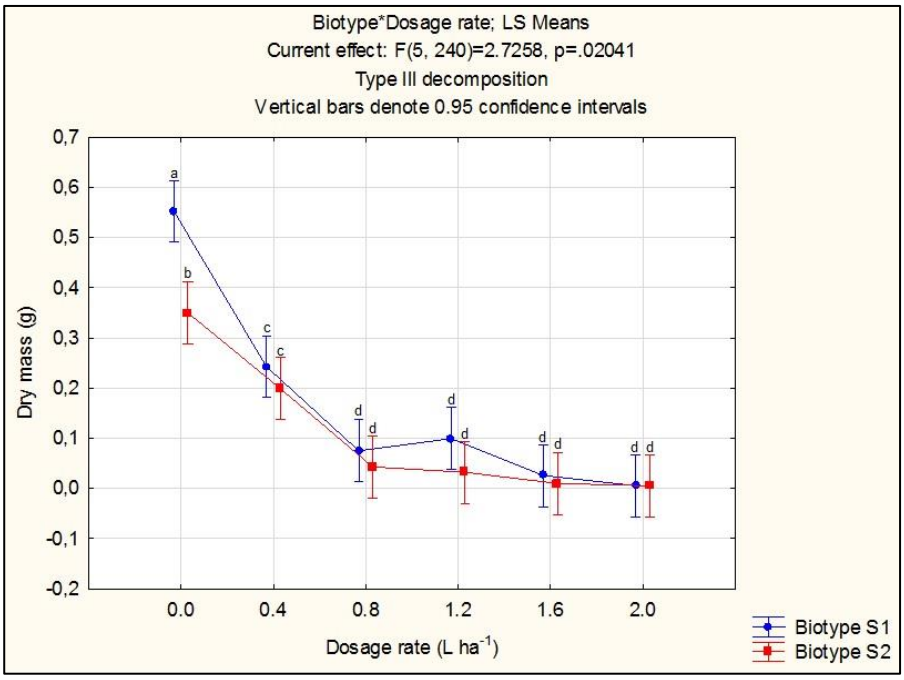

Figure 5.6: The effect of glyphosate dosage rate on dry matter production in ryegrass biotypes S1 and S2 under 15-20°C day/night-controlled temperatures. Glyphosate (360 g a.i. L^{-1}) was applied at dosage rates 0.0, 1.0, 0.4, 0.8, 1.6 and 2.0 L ha^{-1}. Vertical bars denote 0.95 confidence intervals

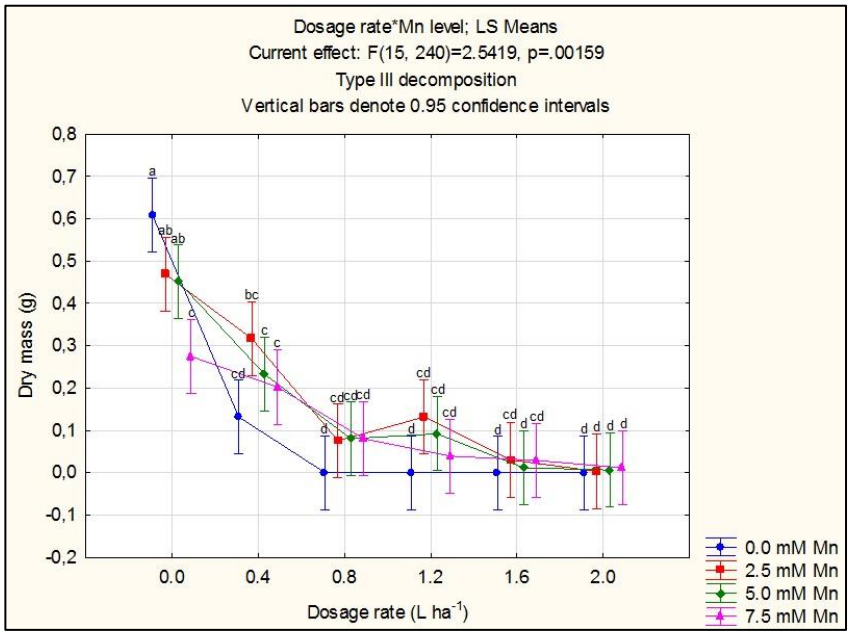

Figure 5.7: The effect of glyphosate dosage rate and Mn treatment level on dry matter production of ryegrass under 15-20°C day/night-controlled temperatures. Glyphosate (360 g a.i. L^{-1}) was applied at dosage rates 0.0, 0.4, 0.8, 1.2, 1.6 and 2.0 L ha^{-1}. Vertical bars denote 0.95 confidence intervals

20-25°C day/night-controlled temperature

Under this temperature range, significant interaction ($p<0.05$) was observed between the main factors of glyphosate dosage rate and Mn level in terms of percentage survival. A distinct difference could be observed between added Mn treatments and the control treatment (0.0 mM Mn) at sublethal dosage rates of 0.4 and 0.8 L ha-1 where Mn treatments exhibited better survival rates by 40 to 60% (Figure 5.8). No significant differences were found with regards to biotype for survival or dry matter analyses. Biotype S1 exhibited 29% survival whereas biotype S2 exhibited 35% survival.

Significant interaction ($p<0.05$) between dosage rate and Mn level was also observed in dry matter production results. This result corroborates the abovementioned percentage survival result as seen in Figure 5.9.

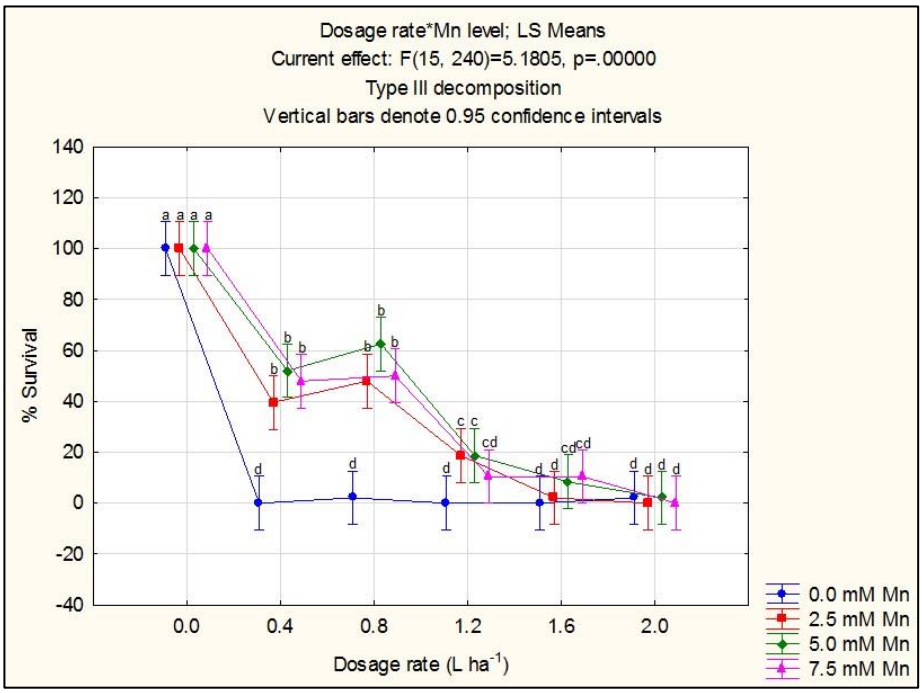

Figure 5.8: The effect of glyphosate dosage rate and Mn treatment level on percentage survival of ryegrass under 20-25°C day/night-controlled temperatures. Glyphosate (360 g a.i. L^{-1}) was applied at dosage rates 0.0, 0.4, 0.8, 1.2, 1.6 and 2.0 L ha^{-1}. Vertical bars denote 0.95 confidence intervals

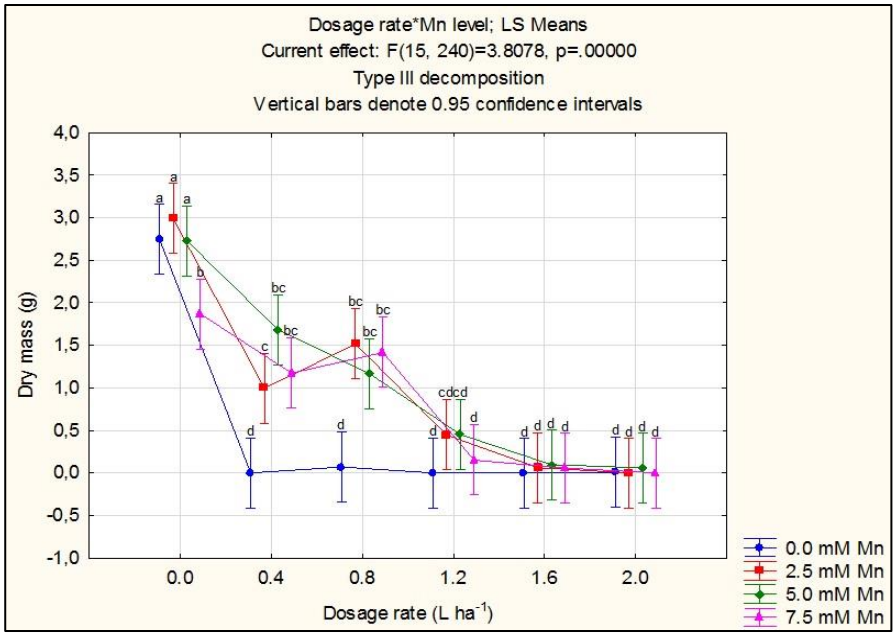

Figure 5.9: The effect of glyphosate dosage rate and Mn treatment level on dry matter production of ryegrass under 20-25°C day/night-controlled temperatures. Glyphosate (360 g a.i. L^{-1}) was applied at dosage rates 0.0, 0.4, 0.8, 1.2, 1.6 and 2.0 L ha^{-1}. Vertical bars denote 0.95 confidence intervals

25-30°C day/night-controlled temperature

Under this temperature range, significant interaction ($p<0.05$) was observed between the main factors of glyphosate dosage rate and manganese level in terms of percentage survival. Interestingly, under this temperature, dosage response curves generally seemed to only start to exhibit a decline from 1.2 L ha-1 glyphosate (Figure 5.10). Mn levels 2.5 and 5.0 mM Mn exhibited the highest survival rates as opposed to 7.5 mM Mn and the control treatment (0.0 mM Mn). No significant differences were found with regards to biotype for survival analysis. Biotype S1 exhibited 75% survival whereas biotype S2 exhibited 73% survival.

Significant interaction ($p<0.05$) between ryegrass biotype and Mn level was also observed in dry matter production results. A significant difference between the two biotypes were observed in 2.5 and 5.0 mM Mn treatments (Figure 5.11) which may be ascribed to a slight difference in adaptability under stress conditions. The significant interaction between ryegrass biotype and dosage rate manifested the same observation as in Figure 5.11. Biotype S1 seemed to be slightly better adapted to growth under the warm conditions of this temperature range, subjected to the specific glyphosate dosage rates (Figure 5.12).

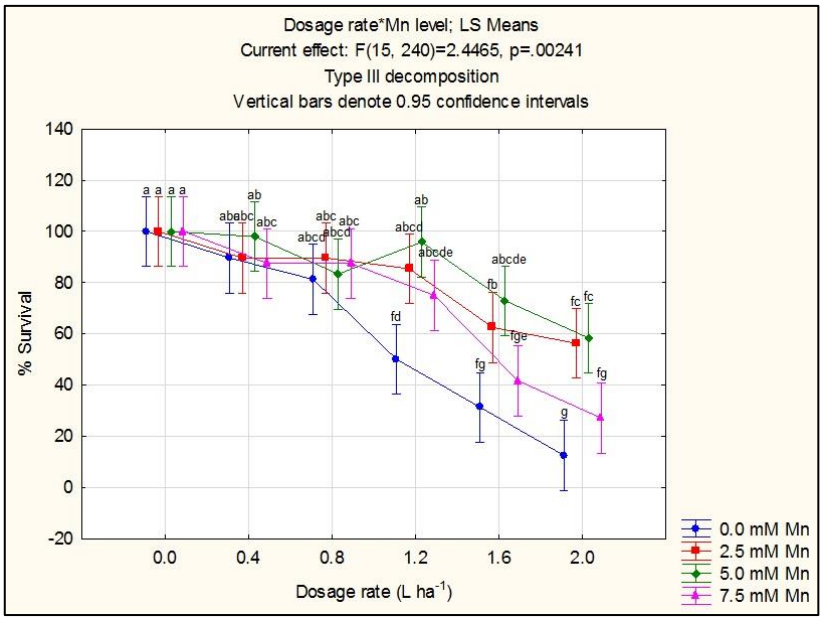

Figure 5.10: The effect of glyphosate dosage rate and Mn treatment level on percentage survival of ryegrass under 25-30°C day/night-controlled temperatures. Glyphosate (360 g a.i. L^{-1}) was applied at dosage rates 0.0, 0.4, 0.8, 1.2, 1.6 and 2.0 L ha^{-1}. Vertical bars denote 0.95 confidence intervals

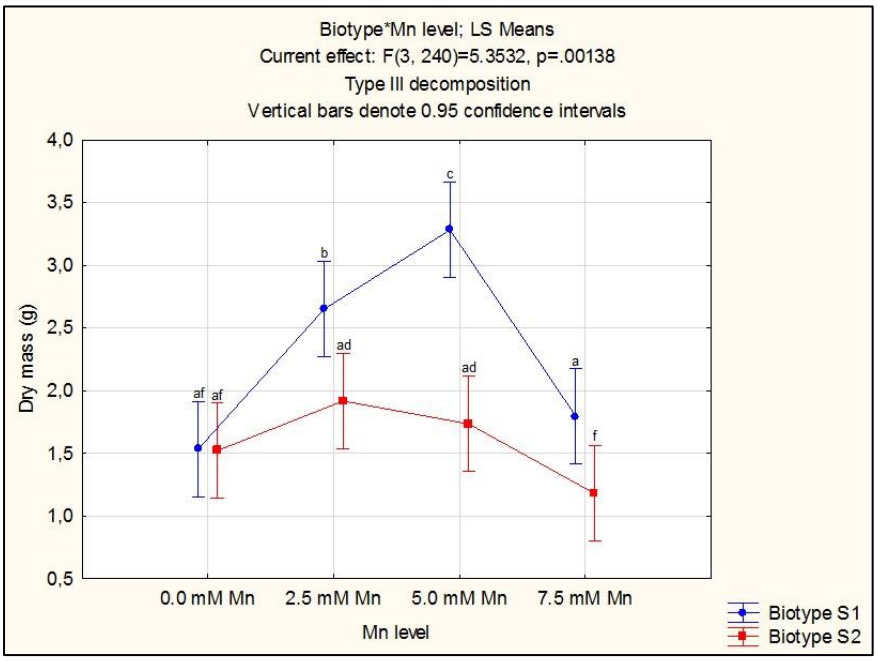

Figure 5.11: The effect of manganese treatment level on dry matter production in ryegrass biotypes S1 and S2 under 20-25°C day/night-controlled temperatures. Manganese treatments were applied at 0.0, 2.5, 5.0 and 7.5 mM $MnSO_4$. Vertical bars denote 0.95 confidence intervals

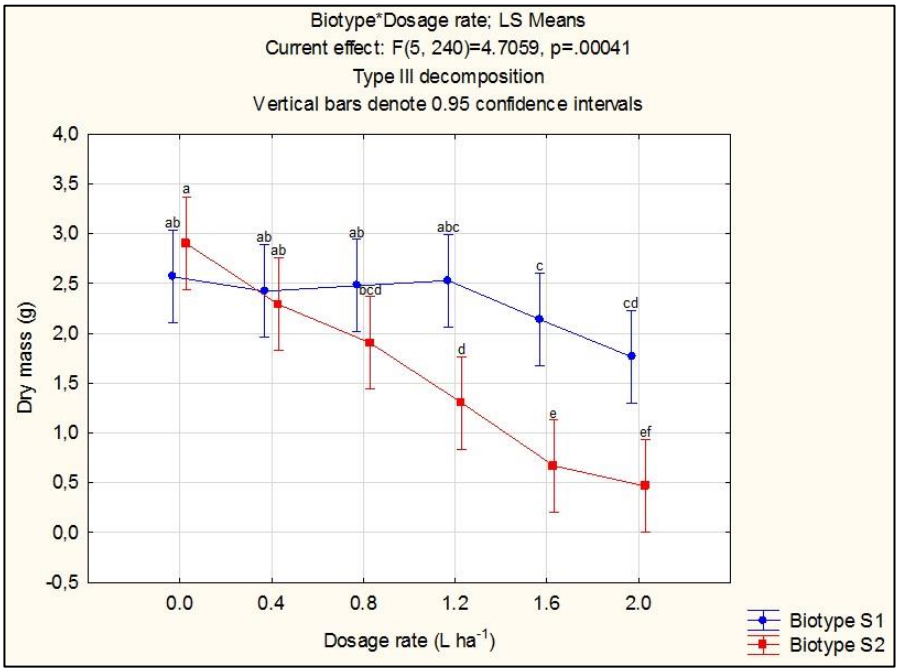

Figure 5.12: The effect of glyphosate dosage rate on dry matter production in ryegrass biotypes S1 and S2 under 20-25°C day/night-controlled temperatures. Glyphosate (360 g a.i. L^{-1}) was applied at dosage rates 0.0, 0.4, 0.8, 1.2, 1.6 and 2.0 L ha^{-1}. Vertical bars denote 0.95 confidence intervals

5.4. Discussion and conclusion

In all four temperature ranges under which this trial was conducted (10-15, 15-20, 20-25 and 25-30 °C), the main factors of glyphosate dosage rate and Mn level exhibited significant interactions. In other words, the percentage survival of the tested biotypes was significantly influenced by the combined effect of glyphosate dosage rate and the Mn level that the biotypes were subjected to. In every temperature range except for 25-30 °C, this result was corroborated by the recorded results of dry matter production.

In the study of Sammons and Gaines (2014), high temperatures negatively influenced the efficacy of glyphosate in *E colona*. Although the ryegrass used in this particular study was not of a resistant nature but rather susceptible, it was evident from the results obtained that higher temperatures affects the efficacy of glyphosate. Survival declined much more gradually in dosage response curves at 25-30°C than in corresponding graphs at lower temperatures (Figures 5.1, 5.4, 5.8 and 5.10).

Sammons and Gaines (2014) proposed that the negative effect at higher temperatures may well be as a result of lower absorption of glyphosate at said temperatures as lower absorption would certainly influence glyphosate efficacy. Despite this observation, Nguyen et al. (2016) measured higher glyphosate activity (shikimate accumulation) at target sites at higher temperatures. This implies the existence of some sort of barrier which limits the amount of glyphosate that reaches chloroplasts under higher temperatures. A barrier such as this may be found at the plasmalemma or chloroplast membrane or may well be due to the mechanism which is vacuolar sequestration (Sammons and Gaines 2014).

Tanpipat et al. (1997) also reported reduced glyphosate efficacy in *E. colona* at increased temperatures. The authors concluded that higher temperatures may well have put stress on the plants, causing higher evapotranspiration. Increased evapotranspiration in turn leads to limited translocation within plants and consequently, reduced glyphosate efficacy. (Ge et al. 2011) also reported more glyphosate resistance in weed species due to a higher level of vacuolar sequestration at higher temperatures. Concomitantly, glyphosate resistance decreased in these same weed species under cooler temperatures. .

Throughout the results of this study, Mn treatment level seemed to influence the efficacy of glyphosate. Mn treatment levels exhibited better survival rates and dry matter production at Mn treatment levels 2.5, 5.0 and 7.5 mM Mn as opposed to the control treatment of 0.0 mM Mn (Figures 5.2, 5.4, 5.8 and 5.10). In Figure 5.1, under 10-15°C Mn treatment levels 2.5 and 5.0 mM Mn exhibited significantly better survival rates than the control treatment at sublethal to lethal glyphosate dosage

rates. Under 15-20°C as well as 20-25°C, Mn treatment levels 2.5, 5.0 and 7.5 mM Mn exhibited better survival rates than the control treatment under sublethal dosage rates (Figures 5.4 and 5.8).

Interestingly, under 25-30°C, Mn treatment levels only started to significantly differ from the control treatment under higher dosage rates of 1.2, 1.6 and 2.0 L ha-1 (Figure 5.10). This is better survival rates at higher dosage rates than the survival rates seen in lower temperature ranges. For example, at the sublethal dosage rate of 1.2 L ha-1, Mn treatment level 5.0 mM Mn exhibited around 40% survival at 10-15°C as well as 15-20°C, around 20% survival at 20-25°C and just below 100% survival at 25-30°C. Correspondingly, at the lethal dosage rate of 1.6 L ha-1 , Mn treatment level 5.0 mM Mn exhibited around 50% survival at 10-15°C, almost 20% survival at 15-20°C, around 15% survival at 20-25°C and around 70% survival at 25-30°C (Figures 5.2, 5.4, 5.8 and 5.10).

However, the trend that elevated levels of plant available Mn influence glyphosate efficacy in a negative way, is evident, regardless of temperature range ryegrass species was subjected to. Barrett and McBride (2005) found that the herbicidal activity of glyphosate impaired by divalent manganese which is the plant available form. This statement could well explain the influence Mn was seen to exert on glyphosate efficacy. Eker et al. (2006) also reported of the complexation of glyphosate with Mn. Such a complex is reportedly poorly soluble and consequently affects translocation of glyphosate negatively.

In conclusion, Mn treatments in general seemed to decrease glyphosate efficacy over all temperature ranges tested when compared to the control treatment. Dosage response results in combination with Mn treatment levels showed shifts in glyphosate efficacy as temperatures increased where the least glyphosate efficacy was seen under extreme growing temperatures (10-15 and 25-30 °C).

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Chapter 6

Summary and general conclusions

In this study, the effect that Mn might exert on glyphosate efficacy was investigated from different angles. Firstly, susceptible and resistant ryegrass biotypes were subjected to six different Mn treatment levels in a sterile sand medium. Generally significant interactions between main factors were solely observed in susceptible biotypes as opposed to glyphosate resistant biotypes where no significant interactions occurred. At sublethal glyphosate dosage rate (0.5 L ha^{-1}) in the susceptible biotypes, significantly better survival was exhibited in high Mn treatment levels 6.0 and 7.5 mM Mn.

Significant interaction seen in this part of the study may be attributed to Mn/glyphosate antagonism. Complexes have been found to form between metal cations and glyphosate. This complexation may hinder absorption and/ or translocation (Soltani et al. 2011). Glyphosate efficacy is most likely impaired due to complexation and consequent reduced absorption (Bernards et al. 2005a of b). This effect of complexation can occur in soil before plant uptake as well as after plant absorption within plant tissues (Eker et al. 2006).

From this trial, it was observed that the interaction between glyphosate dosage rate and Mn treatments were more evident in susceptible than in resistant biotypes. The effect that higher levels of Mn exert on glyphosate efficacy seems to be more evident in susceptible biotypes. Mn levels significantly increased survival rate at higher concentrations (4.5, 6.0 and 7.5 mM Mn) in the resistant biotype R1, but no such effect was observed in the resistant biotype R2. Plants growing under high Mn conditions might well be more likely predisposed to non-target site resistance development to glyphosate than plants growing under conditions of lower plant available Mn.

The next set of conditions this study focused on, was the effect of natural soil Mn status on glyphosate susceptibility of various ryegrass populations. Established ryegrass populations were tested in soils ranging in plant available Mn content from high Mn content to low Mn content. Established populations were also tested in soils they were native to as opposed to soil of different Mn content they were germinated or transplanted into for the purpose of this study. It was observed that soil comprising of a high Mn content may well exert negative effects on the efficacy of glyphosate applied to specific ryegrass biotypes – especially at sublethal glyphosate dosage rates. It can be concluded that Mn antagonism with glyphosate may exist inside the plant as observed in this trial as was also observed, albeit it in a different context, by Bailey et al. (2002), Bernards et al. (2005a of b), Hartzler (2011) and Soltani et al. (2011).

The effects that “native soil” (soil in which the specific biotype at hand was naturally found in) versus “new soil” (different soil than that which the specific biotype was naturally found in) might exhibit on percentage survival were tested and explored in soils with high and low Mn content (soil MN4 and MN5). It was observed that susceptible biotypes exhibited good survival at sub-lethal dosage rates in native as well as new soils into which they were transplanted, regardless of Mn content of the specific soil (native or new soil).

Resistant ryegrass originating from a soil of higher Mn content (biotype R3) as opposed to their counterparts seemed to be naturally more inclined to a magnified resistance, regardless of the soil into which it was transplanted.

When considering the results obtained in these trials, it may be possible to deduce that ryegrass subjected to soil of high Mn content may over time evolve a type of non-target site resistance. This non-target site resistance may be aided or at least favoured in some way by the effect of internal antagonism between Mn and glyphosate molecules we already know to exist (Bailey et al. 2002, Bernards et al. 2005a of b, Hartzler 2011, Soltani et al. 2011) – thus reducing the efficacy of glyphosate over the course of a few generations.

The fact that many different soil conditions (structure, soil organic matter, clay content and metals or minerals such as iron oxides) may well influence possible outcomes should never be overlooked as it greatly influences the amount absorbed and transport rate of glyphosate through plants (Ololade et al. 2014).

Some ryegrass biotypes seem to at least prefer soil of high Mn content and consequently produce more dry matter in comparison to the same biotype reared in different soils sprayed at the same sub-lethal glyphosate dosage rates. Decreased susceptibility to sub-lethal dosages of glyphosate may be influenced by levels of Mn in soil. After a few generations of selection pressure, development of non-target site resistance might rather occur in soils of high Mn content as compared to soil with low levels of plant available Mn in soil.

The last part of the study was dedicated to investigating the role that various temperatures may have on glyphosate efficacy whilst testing the influence of Mn levels in two different susceptible biotypes.

In every one of the four temperature ranges under which this trial was conducted (10-15, 15-20, 20-25 and 25-30 °C), the main factors of glyphosate dosage rate and Mn level exhibited significant

interactions. The percentage survival of the tested biotypes was significantly influenced by the combined effect of glyphosate dosage rate and the Mn level that the biotypes were subjected to.

The results obtained showed that higher temperatures affect the efficacy of glyphosate. Under the warmer temperatures of 25-30°C, percentage survival only started to decline at higher glyphosate dosage rates (1.2 L ha^{-1}) than under lower temperature conditions (0.4 L ha^{-1}) (Figures 5.1, 5.4, 5.8 and 5.10). (Sammons and Gaines 2014) proposed that a result such as the one found in this study may well be due to lower absorption of glyphosate at high temperatures as lower absorption would automatically influence translocation of glyphosate throughout the target plant and consequently curb glyphosate efficacy. (Nguyen et al. 2016)proposed the existence of a barrier of sorts which limits the amount of glyphosate that reaches chloroplasts under higher temperatures. (Ge et al. 2011) also reported more glyphosate resistance in weed species due to a higher level of vacuolar sequestration at higher temperatures. Concomitantly, glyphosate resistance decreased in these same weed species under lower temperature conditions.

Throughout the results of this study, Mn treatment level seemed to influence the efficacy of glyphosate as opposed to the control treatment of 0.0 mM Mn. Mn treatment levels exhibited better survival rates (reduced glyphosate efficacy) and more dry matter production at Mn treatment levels 2.5, 5.0 and 7.5 mM Mn as opposed to the control treatment (Figures 5.2, 5.4, 5.8 and 5.10). The trend that elevated levels of plant available Mn reduces glyphosate efficacy is evident, regardless of temperature range ryegrass species was subjected to. Barrett and McBride (2005) found that the presence of divalent manganese (plant available form) impaired glyphosate herbicidal activity. Eker et al. (2006) also reported complexation of glyphosate with Mn. Such a Mn-glyphosate complex is reportedly poorly soluble and consequently affects translocation of glyphosate negatively.

In conclusion, Mn treatments in general seemed to exert an effect of increased glyphosate resistance in ryegrass. This result was not observed with significant interactions in all the Chapters of this study. However, where significant interactions lack, trends pointing toward the same conclusion was observed at, at least some Mn treatment levels of added plant available Mn. Throughout the results obtained in this study, one could see that some effect must exist where plant available Mn influences the herbicidal activity of glyphosate in a negative manner.

Future research

This study showed definite potential for further investigation into the influence Mn exerts on glyphosate efficacy. My recommendation would be to test the influence of even higher Mn levels on susceptible as

well as resistant ryegrass species more extensively. The added factor of other elements such as Fe in combination with Mn levels on glyphosate efficacy, would also be a valuable exploration. This study being a pot trial, it would be invaluable in field trial setting as well as perhaps on active agricultural soils where resistance have been prevalent for several years in combination with soil analyses proving high micro element concentrations of Mn and Fe for instance. Generational studies of ryegrass biotypes tested under set conditions would most likely generate corroborative data. Finally, a closer look into the physiological pathway of glyphosate uptake and translocation in susceptible and resistant plants could provide valuable insight into non-target site resistance mechanisms.

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