

**FACTORS INFLUENCING INHIBITION OF GLUTAMINE
SYNTHETASE ENZYME IN GRASS WEEDS BY GLUFOSINATE
AMMONIUM UNDER DIFFERENT TEMPERATURES**

by

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DECLARATION

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SUMMARY

Evolution of weed resistance emphasized the need to implement integrated weed management strategies, however, farmers still immensely rely on chemical weed management. Glufosinate ammonium is an alternative herbicide that can replace or be used in rotations with herbicides such as glyphosate and paraquat, but it poses a problem due to its inconsistencies in controlling weeds. Studies in this dissertation aimed to investigate the influence of temperature on glufosinate ammonium efficacy.

Chapter 3 of this dissertation investigated the influence of temperature on ryegrass cuticle thickness, phenolic acid concentration and calcium accumulation, and subsequently, the effect of the afore-mentioned factors on glufosinate ammonium efficacy. Ryegrass was grown at 10/15, 15/20, 20/25, and 20/30 °C (night/day) temperatures and treated with 0, 1.5, 3, 4.5, 6 and 7.5 L ha⁻¹ glufosinate ammonium dosage rates. The grass was treated six weeks after planting and assessment was done four weeks after glufosinate application. Control of ryegrass decreased with increasing temperature. Results indicated that cuticle thickness and calcium content increased as temperatures increased, probably due to production of phenolic compounds responsible for plant defence mechanisms against herbicide stress, hence resulting in poor control of ryegrass under warmer temperatures.

Chapter 4 investigated ammonia accumulation, glutamine synthetase, glutamate dehydrogenase, nitrate reductase activity and ryegrass photosynthesis in roots and leaves of control (0 L ha⁻¹) and treated ryegrass (4.5 L ha⁻¹) harvested 24 hours after glufosinate ammonium application. There was a significant increase in glutamine synthetase enzyme activity with increasing temperature after glufosinate ammonium application. Better control of ryegrass under cooler temperatures with glufosinate ammonium was mainly attributed to the plants' inability to produce adequate glutamate and α -ketoglutarate, which form the carbon skeleton for transamination processes. Response of glutamine synthetase to glufosinate ammonium was significantly higher at warmer temperatures, such that the use of an alternative glutamate dehydrogenase pathway was not vital. The significant increase in glutamine synthetase activity in ryegrass under warm temperatures was able to circumvent photosynthetic inhibition.

A comparative study to investigate the response of different grass weed species to glufosinate ammonium was conducted on ryegrass (*Lolium* spp.), ripgut brome (*Bromus diandrus* L.) and wild oats (*Avena fatua* L) in **Chapter 5**. The grasses were grown at 10/15, 15/20, 20/25, and 20/30 °C (night/day) temperatures and treated with 0, 1.5, 3, 4.5, 6 and 7.5 L ha⁻¹ glufosinate ammonium dosage rates. The study observed that glufosinate ammonium control differed among weed species. Control of ryegrass increased with decreasing

temperature. Temperature had no effect on wild oats. Control of rigput brome was initially poor at 10/15 °C then increased at 15/20 and 20/25 °C and finally decreased again at 25/30 °C. Such differences in the grass response to glufosinate ammonium, even after being grown under the same conditions, was attributed to their differences in morphological characteristics such as cuticle thickness, calcium accumulation and photosynthesis after herbicide application. Increase in cuticle thickness decreased mortality of all grasses. The study perceived that negative effects of calcium on mortality can only be noticed if the cytosolic and mitochondrial calcium is mobile and active, thus, allowing it to react with glufosinate ammonium.

A possible solution to mitigate problems arising from calcium level, cuticle thickness and phenolic compounds was investigated in **Chapter 6**. The study investigated the role of adjuvants in increasing glufosinate ammonium efficacy. Ryegrass was grown at 20/25 °C and treated with 0, 1, 2, 3 and 4 L ha⁻¹ glufosinate ammonium. Glufosinate ammonium was applied solo and in tank mixtures with Velocity Super™ (ammonium sulfate, L 9603), Summit Super (nitrogen solution/non-ionic surfactant, L 8539) and Class act NG™ (ammonium sulfate plus a non-ionic surfactant, L 10477). Better control of ryegrass was observed when treated with glufosinate ammonium in a tank mixture with Class act NG™ and Velocity Super™ than its solo application as well as in a tank mixture with Summit Super. Ammonium sulfate exhibits surfactant and humectant properties and it facilitates movement of glufosinate ammonium into the plant while non-ionic surfactants aim to reduce water surface tension only. This explains better control observed with glufosinate ammonium in tank mixture with adjuvants containing ammonium sulfate than with Summit Super. The study suggests that adjuvant Class act NG™ and Velocity Super™ can be used to mitigate the defensive response of phenolic compounds after glufosinate ammonium application, hence, increasing its efficacy.

The practical relevance of glasshouse observations in Chapter 3, 4, 5 and 6 was confirmed in **Chapter 7**. The study was conducted under rainfed conditions at Langgewens and Roodebloem farms in 2018 and 2019. Glufosinate ammonium was applied at different times of the day (8:00 am, 12:00 pm and 5:00 pm). The dosage rates applied were 0, 2.5, 5 and 7.5 L ha⁻¹. The study observed that morning (8:00 am) and evening (5:00 pm) applications showed better control of ryegrass than mid-day application provided relative humidity during application time was greater than 75%. Application at mid-day (when temperatures were higher than morning temperatures) showed good control only if relative humidity was recorded above 80%, however, higher dosage rates of 5 or 7.5 L ha⁻¹ were required to achieve greater than 90% control.

OPSOMMING

Die ontwikkeling van onkruidwererstand het die belang van geïntegreerde onkruidbeheermaatreëls beklemtoon maar produsente is nog steeds afhanklik van chemiese onkruidbestuurstrategieë. Glufosinaat ammonium is 'n alternatiewe onkruidwerder wat glifosaat en parakwat kan vervang of afwissel maar wisselvallige werking veroorsaak probleme. Die hoofdoelwit in hierdie studie was om die invloed van temperatuur op die effektiwiteit van glufosinaat ammonium te ondersoek.

In **Hoofstuk 3** van hierdie proefskrif is die effek van temperatuur op die kutikuladikte, fenoliese suur konsentrasie en kalsiumopeenhoping, en voortspruitend daaruit, effektiwiteit van glufosinaat ammonium op raaigras ondersoek. Raaigras is gekweek in glashuise wat by temperature van 10/15, 15/20, 20/25 en 20/30 °C (nag/dag) gestel was en die raaigras is behandel met glufosinaat ammonium dosisse van 0, 1.5, 3, 4.5, 6 en 7.5 L ha⁻¹. Die gras is ses weke na plant behandel en evaluasie is vier weke na toediening van glufosinaat ammonium gedoen. Beheer van raaigras het verlaag met verhoogde temperature. Resultate het aangedui dat kutikuladikte en kalsiuminhoud verhoog het met verhoogde temperature, waarskynlik as gevolg van produksie van fenoliese verbindings wat verantwoordelik is vir beveiliging van die plant teen onkruidwerderstremming en dus veroorsaak het dat swak beheer van raaigras verkry is onder warmer toestande.

In **Hoofstuk 4** is opeenhoping van ammoniak, die aktiwiteit van glutamien sintetase, glutamaat dehidrogenase en nitraatreduktase asook fotosintese in wortels en blare van kontrole raaigras (0 L ha⁻¹) en behandelde raaigras (4.5 L ha⁻¹) wat 24 uur na glufosinaat ammonium toediening ge-oes is, bepaal. Daar was 'n beduidende toename in aktiwiteit van die glutamien sintetase ensiem met toenemende temperatuur na toediening van glufosinaat ammonium. Beter beheer van raaigras met glufosinaat ammonium by koeler temperature is toegeskryf aan die plant se vermoë om genoeg glutamaat en α -ketoglutaaraat, wat die koolstofskelet vir die transaminasieproses vorm, te produseer. Reaksie van glutamien sintetase teenoor glufosinaat ammonium was beduidend hoër by hoër temperature, tot so 'n mate dat die gebruik van 'n alternatiewe glutamaat dehidrogenase weg nie noodsaaklik was nie. Die beduidende toename in glutamien sintetase aktiwiteit in raaigras in warmer toestande het fotosintetiese onderdrukking omseil.

'n Vergelykende studie om die reaksie van verskillende grasspesies op die toediening van glufosinaat ammonium te ondersoek is in **Hoofstuk 5** uitgevoer op raaigras (*Lolium* spp.), predikantsluis (*Bromus diandrus* L.) en wildehawer (*Avena fatua* L.). Die grasse is in glashuise by 10/15, 15/20, 20/25 en 20/30 °C (nag/dag) temperature laat groei en behandel met dosisse van 0, 1.5, 3, 4.5, 6 en 7.5 L ha⁻¹ glufosinaat ammonium. Die studie het aangetoon dat die

beheer van glufosinaat ammonium verskil het tussen spesies. Beheer van raaigras het toegeneem met verlaagde temperature. Temperatuur het geen effek gehad op die beheer van wildehawer nie. Beheer van predikantsluis was swak by 10/15 °C waarna dit toegeneem het by 15/20 en 20/25 °C en toe weer afgeneem het by 25/30 °C. Die verskille tussen die grasspesies se reaksie op glufosinaat ammonium, selfs nadat hulle onder dieselfde omstandighede gegroei het, was toegeskryf aan verskille in morfologiese eienskappe soos kutikuladikte, kalsium opeenhoping en fotosintese na toediening van die onkruiddoder. Toename in kutikuladikte het mortaliteit van die grasse verlaag. Die studie het ook aangetoon dat die negatiewe effek van kalsium op mortaliteit slegs waargeneem kan word as die sitosoliese en mitochondriale kalsium aktief en mobiel is en dit dus met glufosinaat ammonium reageer.

Moontlike oplossings vir die probleme wat deur kalsiumvlakke, kutikuladikte en fenoliese verbindings veroorsaak is, is in Hoofstuk 6 ondersoek. Die studie het die rol wat bymiddels kan speel om effektiwiteit van glufosinaat ammonium te verbeter, ondersoek. Raaigras is laat groei by temperature van 20/25 °C en behandel met 0, 1, 2, 3 and 4 L ha⁻¹ glufosinaat ammonium. Glufosinaat ammonium is alleen en in tenkmengsels met Velocity Super™ (ammonium sulfaat, L 9603), Summit Super (stikstof oplossing/nie-ioniese benatter, L 8539) en Class act NG™ (ammonium sulfaat plus 'n nie-ioniese benatter, L 10477) toegedien. Beter beheer van raaigras is waargeneem waar glufosinaat ammonium in 'n tenkmengsel met Class act NG™ en Velocity Super™ toegedien is as wanneer dit alleen of in 'n tenkmengsel met Summit Super toegedien is. Ammonium sulfaat vertoon benattings en **humectant** eienskappe en fasiliteer absorpsie van glufosinaat ammonium deur die plant terwyl nie-ioniese benatters ten doel het om net wateroppervlakspanning te verminder. Dit verklaar die beter beheer wat glufosinaat ammonium behaal in tenkmengsels met bymiddels wat ammonium sulfaat bevat as in tenkmengsels met Summit Super. Die studie impliseer dat die bymiddels Class act NG™ en Velocity Super™ gebruik kan word om die verdedigingsreaksie van fenoliese verbindings teen te werk na toediening van glufosinaat ammonium en sodoende sy effektiwiteit kan verbeter.

Die praktiese belang van die glashuis bevindings in Hoofstukke 3, 4, 5 en 6 is bevestig in **Hoofstuk 7**. Die studie is uitgevoer onder droëlandtoestande op die Langgewens en Roodebloem proefplase in 2018 en 2019. Glufosinaat ammonium is op verskillende tye van die dag (8:00 vm, 12:00 nm and 5:00 nm) toegedien op raaigras. Die dosisse wat toegedien is, was 0, 2.5, 5 and 7.5 L ha⁻¹. Die studie het getoon dat oggend (8:00 vm) en aand (5:00 nm) toedienings beter beheer van raaigras as middag toedienings tot gevolg gehad het, mits humiditeit tydens toediening hoër as 75% was. Middag toedienings (wanneer temperature hoër was as in die oggende of aande) het goeie beheer verkry slegs as relatiewe humiditeit bo 80% was, maar hoër dosisse van 5 of 7.5 L ha⁻¹ was nodig om meer as 90% beheer te verkry.

DEDICATION

This dissertation is dedicated to my almighty, ever-present God (Jahweh). 1st Corinthians 10:31
– “Whatever you do, do it for the glory of God.”

I also dedicate this dissertation to my mother (Grace Mucheri), my smile keeper and my role
model.

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PREFACE

This dissertation is presented as a compilation of eight (8) chapters. Each chapter is introduced separately. Chapter 1 gives a brief background information of the study, justifies the reason for conducting the study and outlines the objectives of the study. A review of literature that is relevant to the study is given in Chapter 2. Chapters 3 to 7 consist of experiments presented in complete paper format with an introduction, specific objectives, materials, method, results, discussion and conclusion. Chapter 8 summarizes the findings from the experiments as well as providing recommendations. All the references cited in the study are found in the reference list at the end of each chapter. An appendices section containing outputs of statistical analyses of data presented in the paper is placed at the end of this dissertation.

- Chapter 1** General Introduction and objectives.
- Chapter 2** Glufosinate ammonium: A candidate for future chemical weed management.
- Chapter 3** Investigating response of ryegrass (*Lolium* spp.) post glufosinate ammonium application under different temperatures.
- Chapter 4** Plant biochemical factors influencing ryegrass (*Lolium* spp.) response to glufosinate ammonium under different temperature regimes.
- Chapter 5** Efficacy of glufosinate ammonium on three selected grass species as influenced by different temperatures.
- Chapter 6** The effect of selected adjuvants on glufosinate ammonium efficacy in controlling ryegrass (*Lolium* spp.).
- Chapter 7** Influence of glufosinate ammonium time of application on its efficacy in controlling ryegrass (*Lolium multiflorum* cv).
- Chapter 8** General conclusions and recommendations.

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LIST OF ABBREVIATIONS AND UNITS

Abbreviations/ Units

[Ca ²⁺] _{cyt/mit}	Cytosolic or mitochondrial calcium
µl	Microlitres
µm	Micrometers
µmol	Micromoles
µmol (CO ₂) m ⁻² s ⁻¹	Net assimilation rate
µmol g ⁻¹ FW	µmol per gram fresh weight sample
ADP	Adenosine diphosphate
AMR	Ammonium transporters
AS	Asparagine synthetase
ATP	Adenosine triphosphate
BSTFA	O-Bis(trimethylsilyl)trifluoroacetamide
Ca ²⁺	Calcium ions
CO ₂	Carbon dioxide
WDG	Water-dispersible granules
DTT	Dithiothreitol
EC	Emulsifiable concentrates
F	molar flow rate
FAD	Flavin Adenine Dinucleotide
g	Grams
g a.i. ⁻¹	Grams per active ingredient
G	Granules
GCMS	Gas chromatography mass spectrometry
GDH	Glutamate dehydrogenase
GOGAT	Glutamate synthase
GS	Glutamine synthetase
H ⁺	Hydrogen ions
HAA	Hours after application
HBA	Hour before application
HLB	Hydrophilic-lipophilic balance
HMDS	Hexamethyldisilane
HR	Herbicide resistant
h	Hours
ICDH	Isocitrate dehydrogenase
IRGA	Infra-red Gas Analyser

IWM	Integrated Weed Management
L ha ⁻¹	Litres per hectare
WDL	Water-dispersible liquid
M ⁻¹ cm ⁻¹	Molar extinction coefficient
MeOH	Methanol
Mg ²⁺	Magnesium ions
MgSO ₄	Magnesium sulfate
mg kg ⁻¹	Milligrams per kilogram
min	Minutes
ml	Milliliters
mM	Millimolar
Mr	Molar mass
Na ⁺	Sodium ion
NADH	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reducing form of nicotinamide adenine dinucleotide phosphate
NH ₄ Cl	Ammonium chloride
NiR	Nitrite reductase
NR	Nitrate reductase
NRT	Nitrate transporters
°C	Degrees Celsius
PAR	Photosynthetically active radiation
PFA	Paraformaldehyde
ppm	Parts per million
PVPP	Polyvinylpyrrolidone
PXRD	Powder X-ray diffraction
rpm	Revolutions per minute
S, SL	Soluble liquid
SEM	Scanning electron microscope
WSG	Soluble granules
TMCS	Trimethylchlorosilane
TOA	Time of application
v/v	Concentration volume by volume
WDG	Wettable powders
xg (RCF)	Relative centrifugal force

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Evolution of weed resistance has resulted in dire need for implementation of integrated weed management strategies (Llewellyn et al. 2004). The ability of a plant to survive herbicide application dosages that originally were lethal to the susceptible population is generally accepted to be indicative of herbicide resistance (Vencill et al. 2012). Herbicide resistant (HR) plants occur naturally within a population and the phenomenon is considered to pose serious threats to food security (Powles and Yu 2010). Despite the availability of a variety of weed management strategies, modern herbicides have outperformed biological and mechanical weed management strategies due to the former method's superior practicality (Powles and Yu 2010).

Lately, more attention has been given to conservation agriculture, and hence, mechanical weed control has been substituted, virtually totally, by chemical control (Ahmadi et al. 1980). Additionally, modern agricultural developments such as HR crops has led to adoption of simple, low risk systems that are less dependent on tillage but require the use of herbicides (Green and Owen 2011; Vencill et al. 2012). Excessive use of herbicides with the same mechanism of action exerts high selection pressure that promotes evolution of herbicide resistance (Pieterse 2010). Prevention or delay of herbicide resistance can be achieved by practicing integrated weed management (IWM), planting clean seed, accurate record keeping of herbicide applications, crop rotation, use of short-residual herbicides and herbicide rotation (Campbell et al. 2011). The aim of such practices is to reduce selection of HR weed biotypes.

Dynamics and impact of herbicide resistance evolution in a weed population is influenced by genetics, biology of weed species and herbicide factors (Powles and Yu 2010). Several studies done on glyphosate have reported that most instances of glyphosate-HR are a result of persistent reliance on glyphosate (Lorraine-Colwill et al. 2002; Chahal and Johnson 2012). There is an opportunity of using glufosinate ammonium as an alternative to glyphosate, paraquat and other commonly used herbicides in South Africa. No cases of herbicide resistance to glufosinate ammonium have been confirmed in South Africa yet (Heap 2019). Resistance has, however, been reported on *Eleusine coracana* and *Lolium perenne* in some parts of Oregon, California, Greece, Malaysia and New Zealand (Jalaludin et al. 2010; Seng et al. 2010; Avila-Garcia and Mallory-Smith 2011; Heap 2019).

Glufosinate ammonium is a non-selective, post-emergence herbicide. It is used under brand names such as Basta®, Bound®, Finale®, Rely®, and Liberty® by companies such as

Bayer Crop Science, Villa Crop Protection and BASF among others. The parent acid in glufosinate ammonium is a natural microbial phytotoxin extracted from *Streptomyces viridochromogenes* and *S. hydroscopicus* (Jansen et al. 2000). It is the only herbicide in group H according to the HRAC herbicide classification criteria. Group H consists of phosphinic acids that bind irreversibly to glutamine synthetase (GS, EC 6.3.1.2), the central enzyme in ammonium metabolism, therefore inhibiting its metabolic reactions (Sellers et al. 2004; Palou et al. 2008). Efficacy of glufosinate ammonium is dependent on the environmental and plant physiological conditions since it is classified as a contact herbicide (Steckel et al. 1997).

Previous own research to investigate the influence of temperature on glufosinate ammonium in controlling ryegrass observed that glufosinate ammonium efficacy increased as temperature decreased (Mucheri 2016). Molefe (2015) does concur with this finding but several studies have shown that efficacy of glufosinate ammonium increases with increasing temperature (Pline et al. 1999; Kumaratilake and Preston 2005; Everman 2008). Mucheri (2016) represented unsubstantiated theory with regards to calcium and wax accumulation in leaves as temperature increases. Accumulation of plant wax is speculated to reduce both absorption and translocation of glufosinate ammonium. Calcium readily reacts with glufosinate ions in solution to form a less soluble compound, thus reducing the herbicide's absorption into the plant (Pratt et al. 2003). Mucheri (2016) proposed further investigation on the possible role of calcium in leaf tissue in the herbicide's efficacy, as well as the roles of adjuvants in mitigating the calcium effects. This study, therefore, aimed to investigate possible factors affecting glufosinate ammonium efficacy under different temperatures in addition to the suggested calcium and plant wax.

1.2 RESEARCH OBJECTIVES

The overall objective of the study was to determine temperature regimes which favour glufosinate ammonium activity and further investigate how temperature influences efficacy of glufosinate ammonium on ryegrass.

The study addressed the following research questions:

- a) How does temperature influence efficacy of glufosinate ammonium?
- b) Do the temperature effects in a) differ among weed species?
- c) What is the role of adjuvants in increasing glufosinate ammonium efficacy?
- d) Does time of application in the field influence efficacy of glufosinate ammonium?

Therefore, specific objectives of the study were to:

- a) investigate factors that affect efficacy of glufosinate ammonium on ryegrass grown under different temperatures,

- b) compare response of three temperate grass weed species to glufosinate ammonium. The weed species included ryegrass (*Lolium* spp.), ripgut brome (*Bromus diandrus* L.) and wild oats (*Avena fatua* L.),
- c) investigate the influence of selected adjuvants on glufosinate ammonium efficacy for the control of ryegrass, and
- d) determine if time of application in relation to temperature influences efficacy of glufosinate ammonium in controlling ryegrass.

The main hypotheses of the respective objectives state that:

- a) i. warmer temperatures do not result in more accumulation of calcium, accumulation of phenolic compounds and increase in cuticle thickness of ryegrass than at cooler temperatures.

ii. glutamine synthetase activity does not increase more at warmer temperatures post glufosinate ammonium application than at cooler temperatures.
- b) the effect of temperature on glufosinate ammonium efficacy is similar on ryegrass, wild oats and ripgut brome grass species,
- c) adjuvants does not mitigate the negative effects of phenolic compounds on glufosinate ammonium efficacy on ryegrass under warmer temperatures,
- d) glufosinate ammonium application at different times of the day does not influence efficacy in controlling ryegrass in the field.

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CHAPTER 2

LITERATURE REVIEW

Glufosinate ammonium: A candidate for future chemical weed management

2.1 POSSIBILITIES OF GLUFOSINATE AMMONIUM

There has been widespread development of herbicide resistance in the Western Cape to a wide range of selective herbicides which farmers once relied on (Pieterse 2010; Ferreira et al. 2015). Since evolution of herbicide resistance (HR) in weeds is an ever-present problem, its threat to food security is a perpetual problem. There are several factors that influence the evolution of herbicide resistance in weeds (**Table 2.1**). Various extension programs that support Integrated Weed Management (IWM) strategies for mitigation of herbicide resistance have been implemented worldwide (Llewellyn et al. 2007). Despite all the efforts, chemical weed control is still prominent. Farmers understand the significance of herbicide resistance but have not grasped the long-term value of IWM control strategies and the declining number of new herbicide patents (Llewellyn et al. 2007).

Table 2.1: Factors influencing evolution of herbicide weed resistance

1. Genetic	<ul style="list-style-type: none"> • Frequency of resistance genes • Number of resistance genes • Dominance of resistance genes • Fitness cost of resistance genes
2. Biology of weeds	<ul style="list-style-type: none"> • Cross-pollination versus self-pollination • Seed production capacity • Seed longevity in soil seedbank • Seed/pollen movement capacity
3. Herbicides	<ul style="list-style-type: none"> • Chemical structure • Site of action • Residual activity
4. Operational	<ul style="list-style-type: none"> • Herbicide dose • Skills of the operator (treatment machinery, timing, etc.) • Agro-ecosystem factors (non-herbicide weed control practices, crop rotation, agronomy, etc.)

Adapted from (Powles and Yu 2010).

Farmers normally start to consider adopting IWM practices only after they notice herbicide resistance evolving (Beckie 2006). The effect of herbicides on weeds in the field is called selection pressure, and is a factor that selects for proliferation of resistant biotypes in a population (Palou et al. 2008). Palou et al. (2008) noted that herbicides that result in resistance after few years of use have high selection pressure or have targets that enable selection of mutations for resistance that do not affect the activity of the enzyme. **Figure 2.1** shows the top 15 herbicide actives with the highest number of weed species that have developed resistance. Severe selection pressure exerted on weed species by glyphosate

has been observed (Aulakh and Jhala 2015). To date, at least 43 and 31 weed species have been confirmed to have evolved glyphosate and paraquat resistance in 30 and 19 different countries respectively (Heap 2019).

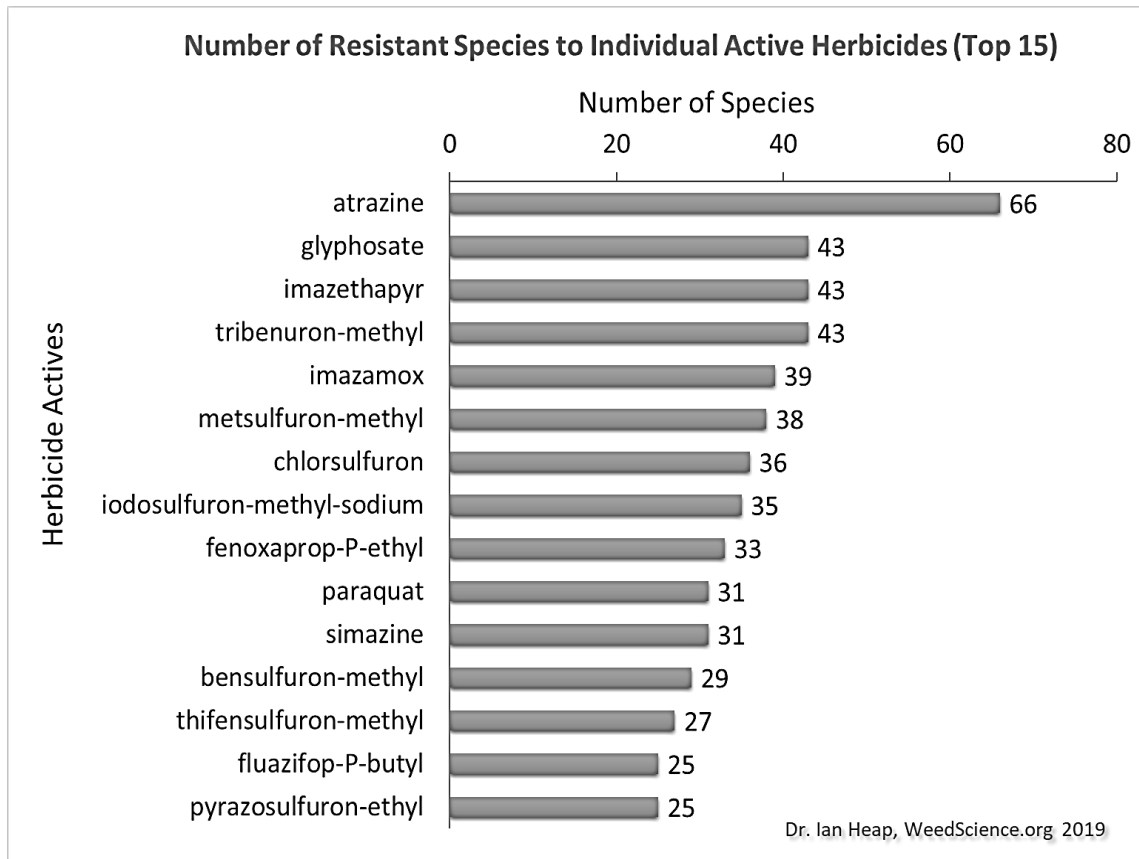


Figure 2.1: Number of resistant species to the top 15 individual active herbicides. Taken from <http://www.weedscience.org>. (Heap 2019).

Glyphosate is by far the most extensively used and important herbicide in the world due to its favourable environmental profile (Baylis 2000; Powles and Yu, 2010; Okumu et al. 2019). It is a non-selective, systematic post-emergence herbicide which inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) hence controls a very broad spectrum of weeds (Baylis 2000). Even though glyphosate exhibits poor rain fastness, antagonism in tank mixtures, slow speed of action, and requires higher rates to control more tolerant weeds, growers still prefer to use it since it provides simple, cheap, flexible, effective and environmentally friendly control (Baylis 2000; Woodburn 2000). Over-usage of the herbicide has played a role in the evolution of glyphosate-resistant weeds (Yuan et al. 2007; Pieterse 2010). Additional reports of weed HR to commonly used herbicides such as paraquat, imazamox, and simazine among others in South Africa have also been confirmed (Pieterse 2010). Therefore, there is need to intensify research in an attempt to delay herbicide resistance from impacting on the utility of existing herbicides. Glufosinate ammonium is one of the best alternatives to glyphosate and paraquat as well as selective herbicides for pre-plant weed control.

With more attention being diverted to conservation agriculture, glufosinate ammonium is very useful in no-till systems since it is a non-selective herbicide (Llewellyn et al. 2004). Glufosinate ammonium is effective against a wide range of monocotyledonous, dicotyledonous and perennial weeds. In addition to its significance in conservation agriculture, glufosinate ammonium application has considerable advantages in the environment. These benefits include no volatilization, low octanol-water partition constant (K_{OW}) thereby making it hydrophilic, rapid degradation in soil, low leaching potential, and little bioaccumulation (Jansen et al. 2000). Palou et al. (2008) reported that glufosinate ammonium exerts low selection pressure. Resistance to glufosinate ammonium has not been confirmed in South Africa according to the International Survey of Herbicide Resistant Weeds by Heap (2019) on the website <http://www.weedscience.org> (**Figure 2.2**). This makes it one of the best candidates to stand in for glyphosate or to be used in herbicide rotations.

Weeds Resistant to Glutamine synthase inhibitors (H/10) by species and country			
#	Species	Country	First Year
1	<i>Eleusine indica</i> Goosegrass	2009 - Malaysia *Multiple - 2 SOA's 2009 - Malaysia *Multiple - 4 SOA's	2009
2	<i>Lolium perenne</i> Perennial Ryegrass	2015 - New Zealand *Multiple - 3 SOA's	2015
3	<i>Lolium perenne ssp. multiflorum</i> Italian Ryegrass	2010 - United States (Oregon) *Multiple - 2 SOA's 2015 - New Zealand *Multiple - 3 SOA's 2015 - United States (California)	2010
4	<i>Lolium rigidum</i> Rigid Ryegrass	2017 - Greece	2017

Figure 2.2: Species and country of weeds that have evolved resistance to glutamine synthetase inhibitors. Table from <http://www.weedscience.org/Summary/MOA.aspx> (Heap 2019).

2.2 GLUFOSINATE AMMONIUM MODE OF ACTION

Glufosinate ammonium is a natural microbial phytotoxin produced by *Streptomyces viridochromogenes* and *S. hygroscopicus* as glufosinate and the herbicide is applied as glufosinate ammonium salt (Jansen et al. 2000). The salt belongs to aminoalkylphosphonic acids family (Forlani et al. 2006). Formation of glutamine in plants involves an initial reaction after γ -glutamyl phosphate synthesis followed by the formation of a tetrahedral intermediate resulting from replacement of the phosphate group by ammonia (Berlicki et al. 2005). This process is adenosine triphosphate (ATP) dependent (Coetzer et al. 2001). The intermediate group resembles the tetrahedral transition state of several enzymatic reactions. The structure of the primary glufosinate acid and glufosinate ammonium salt is presented in **Figure 2.3**. Enzymes are unable to distinguish the size, shape (between flat CO_2H and tetrahedral PO_3H_2) and acidity (pK difference of at least 3 units) of the replaced phosphinic group or related moiety in the herbicide to the carboxylic group.

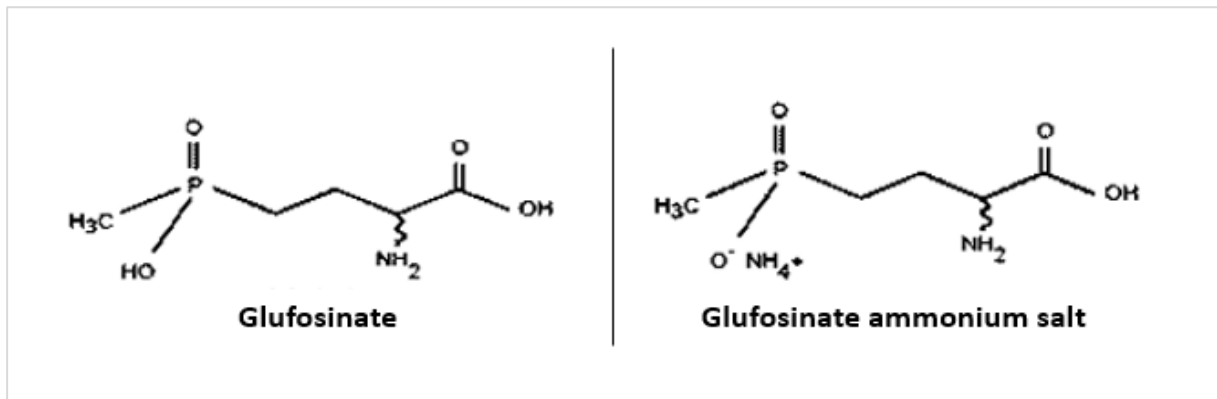


Figure 2.3: Structure of glufosinate and glufosinate ammonium (Cox 1996).

Mode of action of a herbicide describes a biological process in which the herbicide interrupts metabolism and development of the plant (Palou et al. 2008). Glufosinate ammonium acts by inhibiting glutamine synthetase (Forlani et al. 2006). Its mode of action involves three steps: (i) inhibition of protein biosynthesis occurs due to lack of glutamine production (ii) a toxic accumulation of glyoxylate which inhibits RuBP-carboxylate and carbon dioxide fixation and (iii) an interruption of photorespiration that results from deficiency of intermediates of the Calvin cycle (Pline et al. 1999). Plant death is also contributed to depletion of carbon skeletons such as glutamate (sometimes referred to as glutamic acid), thus there is indirect inhibition of photorespiration and photosynthesis. Glutamine synthetase/ glutamine 2-oxo-glutarate aminotransferase (GS/GOGAT) is the most effective pathway in plants which is responsible in detoxifying ammonia released during amino acid degradation, nitrate reduction and photorespiration (Coetzer et al. 2001). Takano et al. (2019) observed that the light-dependent reactive oxides (ROS) are the main causes of cell death resulting from lipid peroxidation of the cell membranes. The effects of glufosinate ammonium after application are presented in **Figure 2.5**.

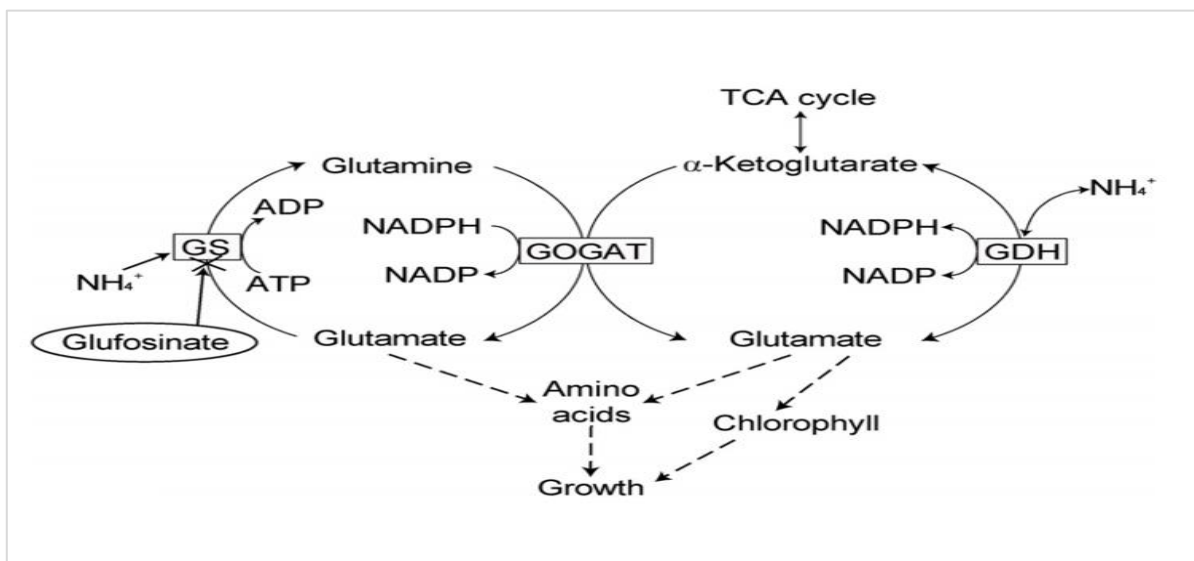


Figure 2.4: Glutamine synthetase/ Glutamine oxoglutarate aminotransferase (GS/GOGAT) plant metabolic pathway in which glufosinate ammonium inhibit glutamine synthetase (Nolte et al. 2017).

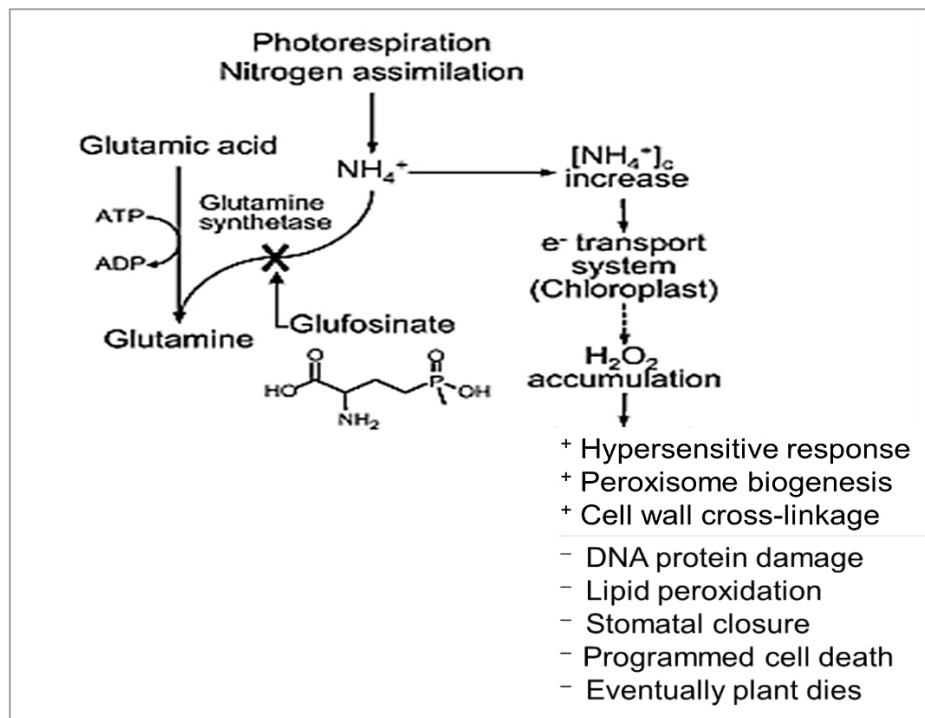
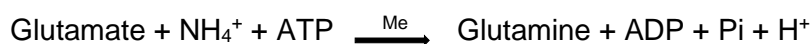


Figure 2.5: Effects of glufosinate ammonium to plant metabolism (modified from Ahn 2007). Symbols + and - represent positive and negative effects to plants, respectively.

Formation of glutamate and ammonium from glutamine is catalysed by the enzyme GS, whilst GOGAT transfers the amide group of glutamine to α -ketoglutaric acid. The enzyme GS is an oligomer formed by 12 polypeptide chains ($M_r \sim 50000$) (Lei et al. 1979). The active site of GS is a bifunnel with ATP and glutamate entrance (top and bottom respectively) (refer to **Figure 2.6**). Two metal ions (n_1 and n_2) are located at the neck of the bifunnel (Eisenberg et al. 2000). Its important characteristic is its high affinity to ammonia and thus displaying an ability to effectively in-cooperate ammonia into organic combinations. Glutamine synthetase catalyses the reaction;



Where Me is either magnesium or manganese (Eisenberg et al. 2000).

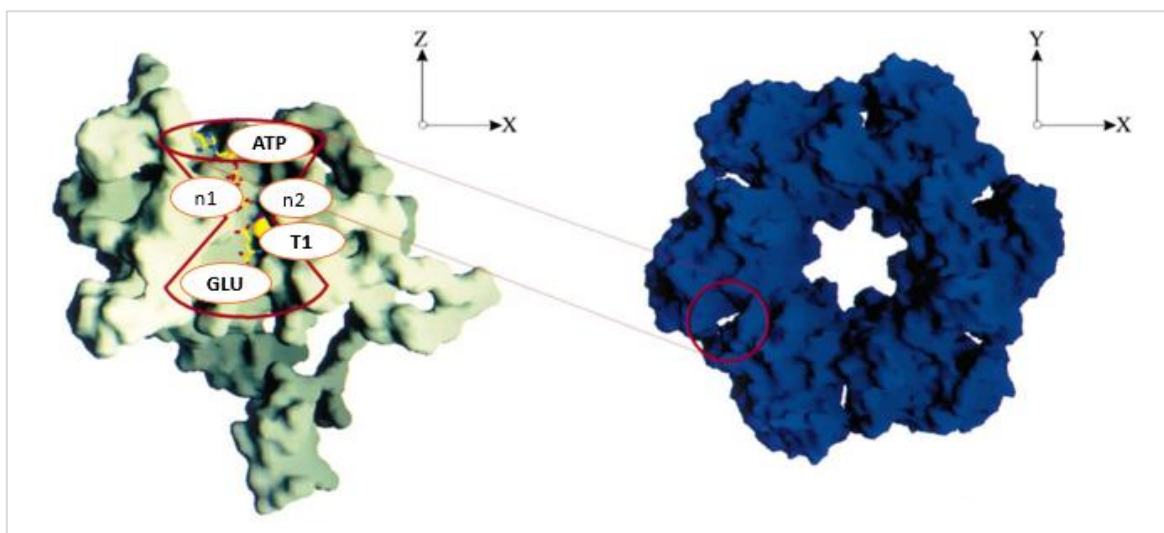


Figure 2.6: Structure of bacterial glutamine synthetase (Eisenberg et al. 2000). The structure is a deodecamer having 622 symmetry. The deodecamer has 12 active sites formed between two neighbouring subunits within a ring (circled in red).

A promoter analysis study of the GS3 A gene of pea suggests that GS is preferentially expressed in the vascular tissue of the leaves, however, further studies have confirmed that a plastidic form of GS is widely distributed in the chloroplast (Miflin 2002). The two-step biosynthetic model reaction of GS in the plant involves i) the formation of γ -glutamyl phosphate and ii) production of glutamine through attacking the intermediate and release of phosphate (Liaw and Eisenberg 1994; Eisenberg et al. 2000). Glufosinate ammonium takes advantage of the transition stage by mimicking the tetrahedral intermediate, thus inhibiting enzyme activity. Computations of glufosinate ammonium bond to GS and the proteins surface surrounding the bond are shown in **Figure 2.7**.

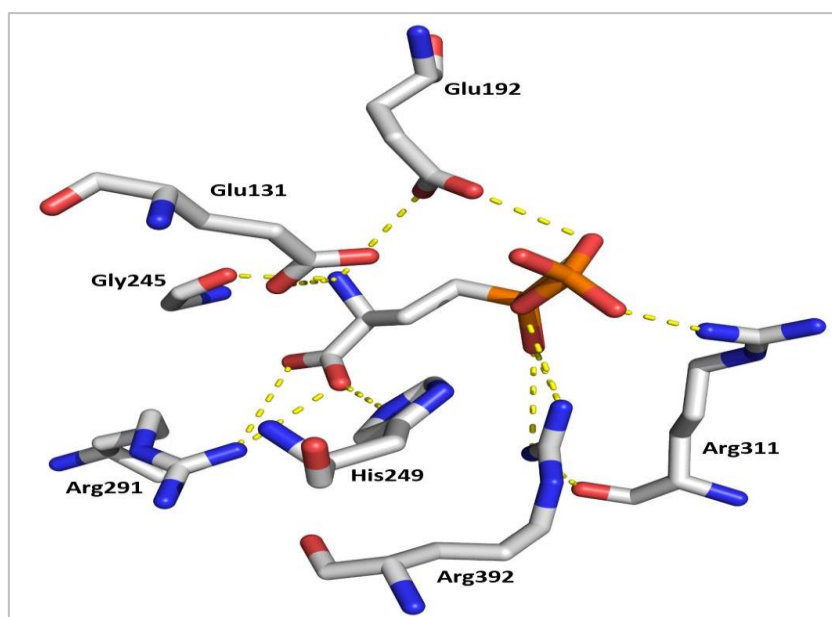


Figure 2.7: Binding pattern of glufosinate to glutamine synthetase enzyme. The carboxy group Glufosinate interact with His249 and Arg291, and the amine interacts with Glu131, glu192, Gly245. The phosphate interacts with glu192, Arg332 and Arg311 (Personal communication with Professor Dayan based on structure of Unno et al 2006).

2.3 FACTORS INFLUENCING EFFICACY OF GLUFOSINATE AMMONIUM

Efficacy of post-emergence foliage-applied herbicides is affected by environmental and plant physiological factors (Mersey et al. 1990; Coetzer et al. 2001). Phytotoxicity of weeds occurs in a four-step process (Palou et al. 2008). These steps include interception of herbicide by weeds, absorbance, translocation and inhibition of metabolic process at the site of action. Factors that can enhance or impair these steps have influence on the efficacy of glufosinate ammonium (Steckel et al. 1997a). Different responses of weeds to glufosinate ammonium has been highly attributed to humidity, temperature, species, weed growth stage, application times and variations in absorption and translocation (Everman 2008). Variations in absorption and translocation arise from inter-related factors that include cuticle thickness and retention, and biochemical and biophysical reactions in the target weed (Stagnari 2007). Isolation of one factor to describe herbicide efficacy in its entirety is, therefore, obscure since all these factors are inter-related.

2.3.1 Preventable factors

Development of effective herbicide weed management programmes requires detailed information of all factors that determine herbicide efficacy (Everman 2008). Some factors can be categorised as preventable, meaning a farmer has control over such factors. These factors include technicalities such as cleaning of implements, constant sprayer inspection, preparation and calibration. This includes tank or nozzle clogging, nozzle leakage, pump working order, boom height, nozzle spacing and direction and ground speed of the sprayer (Jamal 2011).

In addition to technical factors, environmental factors like rain and wind are controllable since applications under such adverse conditions can be avoided. Glufosinate ammonium poses an advantage in this regard since it has a definite rain-free period of 4 h. Additionally, application can be withheld on days with unsuitable wind speed greater than force 2 or 7-11 km per hour on a Beaufort wind force scale (Britt et al. 2003). The present study acknowledges such factors, however, a fairly comprehensive review in **Section 2.3.2 – 2.3.4** shall be reserved for unpreventable factors such as temperature, humidity, and weed species among others.

2.3.2 Role of environmental factors on efficacy of glufosinate ammonium

a. Temperature

In previous studies, it was shown that glufosinate ammonium controls weeds more effectively with increasing temperature (Pline et al. 1999; Everman 2008). Penner (2015) also noted that within a temperature range of 10 °C to 30 °C, an increase in temperature will enhance the phytotoxicity of herbicides. Contrary to these studies, Mucheri (2016) observed that lower temperature of 10/15 °C resulted in higher control of ryegrass compared to 15/20, 20/25 and

25/30 °C (night/day) temperatures. Conclusions drawn from the study done by Molefe (2015) also suggested that enhanced efficacy of glufosinate ammonium in controlling ryegrass is achieved under cooler temperatures.

Enhanced efficacy of herbicides at higher temperatures is attributed to increased herbicide uptake and translocation in plants but decreased efficacy can be attributed to volatilization of the herbicide at higher temperatures (Penner 2015). However, glufosinate ammonium shows no volatilisation (Jansen et al. 2000). A transient increase of cytoplasmic calcium ion concentration and waxy cuticle is observed with increasing environmental temperature (Gong et al. 1998; Jamal 2011). Pratt et al. (2003) noted that an increase in calcium concentrations decreases efficacy of glufosinate ammonium since it forms a less soluble compound with glufosinate ammonium. Ammonium sulfate supposedly mitigates these effects and enhances efficacy of glufosinate ammonium (Mucheri 2016). Mucheri (2016) suggested that studies to confirm the hypothesis need to be conducted.

Additionally, regulation of GS is also influenced by the environment (Mifflin 2002). Mifflin and Habash (2002) also noted that even though reaction and forms of GS differ between organs, metabolism of GS also significantly differs according to tissue, cell and sub-cellular compartments. The nature of metabolism occurring via GS varies with tissues and over the course of the day (Stitt et al. 2002). Even though the bond between glufosinate ammonium and GS is considered to be strong, structural perturbations caused by increase in temperature might reverse it (Gill and Eisenberg 2001).

The catalytic action of the enzyme GS is the widely accepted pathway of assimilating ammonium in plants but an alternative pathway involves a putative glutamate dehydrogenase (GDH) pathway (Lasa et al. 2002; De-Bashan et al. 2008). It is speculated that when the plant is subjected to high concentrations of ammonia, the enzyme glutamate dehydrogenase detoxifies it by assimilating the ammonium into organic compounds (Stewart et al. 1995; Frechilla et al. 2002; Glevarec et al. 2004). Further studies can be conducted based on the literature to investigate how differences in temperature can influence ammonium accumulation. Additionally, influence of temperature on concentration and response of glutamate dehydrogenase can be analysed.

b. Relative humidity

Systematic scientific studies focus more on temperature than they do on humidity, mainly because experimental designs that hold air water content constant are difficult to set up (Stagnari 2007). However, there is a strong relationship between temperature and humidity since transportation of spray water in lipophilic pathways and aqueous pores is highly dependent on the two parameters (Kerstiens 2006). Low relative humidity prior to, during and post application of herbicides can cause dehydration of the cuticle (Steckel et al. 1997a).

Dehydration periods result in increased wax coverage (Schuster 2016). Reduction of weight loss and chlorophyll leaching is observed after dehydration periods. Chlorophyll leaching is caused by disturbance in the structure and function of chloroplasts resulting in photosynthetic pigment degradation (Ma et al. 2019). Absorption of water-soluble herbicides such as glufosinate ammonium is reduced under such conditions (Steckel et al. 1997b). Control of green foxtail and barley has been proven to be more effective at a relative humidity of 95% than at 40%.

c. Light

In an experiment to investigate the influence of the effect of application timing on glufosinate ammonium activity, there was reduced absorption, enzyme inhibition and less ammonia accumulation in plants in darkness compared to those in light (Sellers et al. 2004; Takano et al. 2019). According to Berlicki et al. (2005), glufosinate ammonium undergoes phosphorylation in the presence of ATP when it binds to GS, therefore, ammonia accumulation is greatly enhanced in the presence of light (Steckel et al. 1997b). In addition to ATP, activity of GS is also regulated by Mg^{2+} and H^+ concentration in the stroma which are light dependent (Sellers et al. 2003). As a result, light is essential for optimum glufosinate ammonium control of a variety of weed species (Sellers et al. 2004).

Although literature highlights the importance of light in glufosinate ammonium efficacy, studies conducted by Petersen and Hurlle (2000) on *Galium aparine* and Kumaratilake and Preston (2005), on wild radish (*Raphanus raphanistrum*), provide fairly contrasting evidence. Petersen and Hurlle (2000) noted that ammonia accumulation (a process which only occurs in light) at lower light intensity level was reduced, consequently, reducing the efficacy of glufosinate ammonium on *Galium aparine* L. On the other hand, Kumaratilake and Preston (2005) noted that light intensity had no effect on glufosinate ammonium under lower temperatures ranging around 5/10 °C. The same study proved that light becomes an influencing factor at higher temperatures of 20/25 °C. These observations suggest that the complex interaction of temperature, light and relative humidity in influencing glufosinate ammonium cannot be ignored.

d. Soil moisture and soil temperature

Soil moisture and temperature indirectly influence efficacy of foliar applied herbicides (Varanasi et al. 2016). The relation is accounted to the effects on capillary movement of solutes in the phloem and xylem, and thus, influences absorption, translocation and metabolism processes in the plant. Soil moisture also affects leaf interception since plants grown in moisture-stressed soils minimize water loss from leaves by developing upright oriented leaves which in turn reduces their capabilities of retaining spray droplets (Kells et al. 1984; Varanasi et al. 2016). Additionally, soil moisture influences on physiological factors

such as stomatal closure, leaf thickening and tissue dehydration affects herbicide diffusion, absorption and translocation.

Application of 100 g ha⁻¹ of glufosinate ammonium on barley (*Hordeum vulgare* L. cv) and green foxtail (*Setaria viridis* L.) subjected to soil moisture levels of 18%, 50% and saturation provided contrasting results (Anderson et al. 1993). Amongst the three soil moisture levels, efficacy of glufosinate ammonium on barley was highest at 18% soil moisture while on green foxtail glufosinate ammonium showed the least efficacy at 18% soil moisture content. Such contrasting findings from literature displayed by glufosinate ammonium intensifies the need to study why it shows high variability of results (Petersen and Hurle 2000).

2.3.3 Role of plant factors on efficacy of glufosinate ammonium

a. Growth stage of weed

Younger plants are generally easier to control as compared to mature plants (Ahmadi et al. 1980). A study done to investigate efficacy of glyphosate on horseweed (*Coryza canadensis*) at different growth stages observed that the regression model predicted 40-58% control for weeds at a height above 15 cm and declines to 15-39% when the height increased to 45 cm (Mellendorf et al. 2013). The same experiment found that plant height that ranged from 5 cm to 15 cm gave control of glyphosate greater than 94%. Glasshouse experiments have shown that growth stage does not have significant effect on efficacy of glufosinate ammonium while field studies have shown otherwise (Molefe 2015; Mucheri 2016).

b. Herbicide tolerance and weed species

Selectivity of the herbicide is greatly influenced by weed internal factors among species and climatic factors that incite herbicide molecules. Contrasting results of glufosinate ammonium activity might be due to differences in metabolism of plants (Kumaratilake and Preston 2005). Rapid synthesis of GS after application of glufosinate ammonium may dilute the effects of ammonia accumulation in the plant (Mersey et al. 1990). Weeds that portray such behaviour are naturally herbicide tolerant and require application of higher dosage rates than recommended. The extent of weed tolerance to herbicides is determined by the plant anatomy, succulence, physiology and morphology (Jamal 2011).

c. Plant morphology

Differences in above ground morphology of weeds affects herbicide spray retention. Leaf arrangements on the stem differs amongst weed species. Other morphological factors that influence herbicide efficacy include presence or absence of cambium, physiological and biological activities, root distribution, surface area, capacity and efficiency of plants in absorbing and translocating the herbicide (Jamal 2011). Trichomes are also directly involved

in foliar herbicide absorption (Varshney and Sondhia no date). They provide a microclimate which alters drying time, hence, influencing epidermal exposure time to herbicides.

Additionally, plants form mechanical barriers against external forces by maintaining the structural integrity, stability and flexibility of the cuticle (Schuster 2016). **Figure 2.8** shows a simplified cross sectional structural a plant cuticle and the major chemical components of the different layers. The continuous extracellular wax layer covering the epidermis of all aerial plant organs including the leaves is called the plant cuticle (Schuster 2016). Development of cuticle and deposition of the waxy layer on leaves of different weed species influences herbicide retention and subsequently their absorption rate. Two hydrophobic layers, the insoluble polymer cutin and the solvent-soluble cuticular waxes, make up the plant cuticle. The nature of these layers impede penetration and absorption of hydrophilic herbicides such as glufosinate ammonium (Hess and Chester 2000).

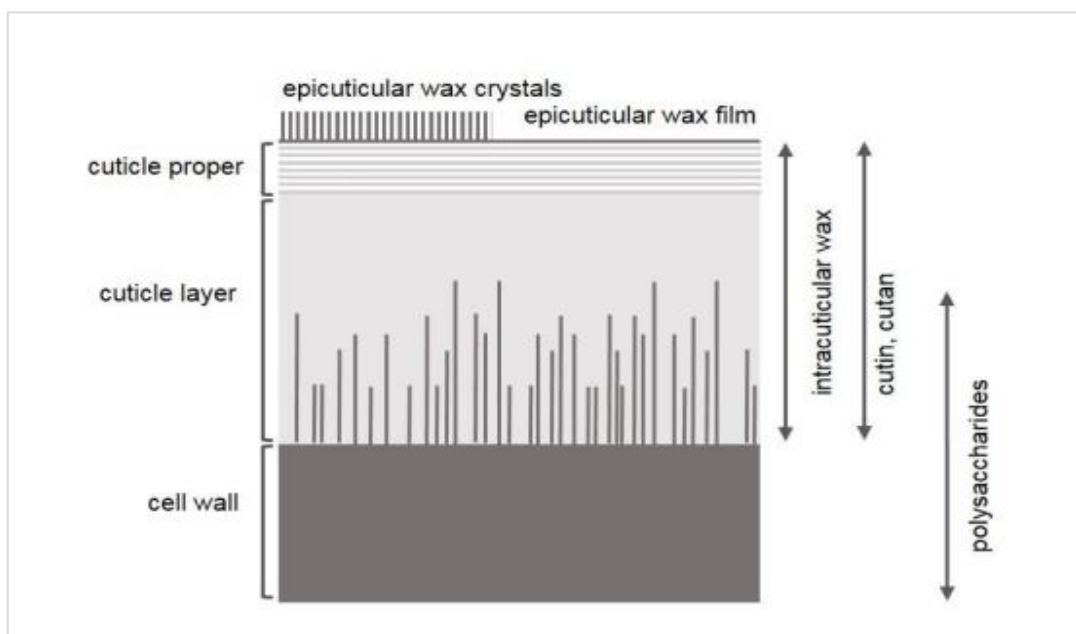


Figure 2.8: Simplified structural features of the cross section of a plant cuticle and the major chemical components of the different layers (Schuster 2016).

2.3.4 Roles of herbicide formulations and added adjuvants on efficacy of glufosinate ammonium

Packaging of herbicides is done in plastic containers varying from 1 to 50 L volume. Referring to **Figure 2.9**, only 200 g of glufosinate ammonium is found in the final herbicide formulation. Inert ingredients added to active ingredients (component responsible for herbicide phytotoxicity) allow for dilution, application and stability of herbicides (Gašić et al. 2015). Primarily, herbicide formulations optimise their biological activity and improves their safety and handiness for use. Different kinds of formulations include liquid water soluble (SL), water-dispersible liquids (WDL), soluble granules (WSG), emulsifiable concentrates (EC), wettable powders (WDG), water-dispersible granules (WDG), granules (G) and encapsulation (Jamal 2011).

<p><i>A non-selective soluble concentrate herbicide for the post-emergence control of annual weeds, as indicated.</i></p> <p><i>'n Nie-selektiewe oplosbare konsentraat onkruidodder vir die na-opkomsbeheer van eenjarige onkruid soos aangedui.</i></p>	
<p>ACTIVE INGREDIENT / AKTIEWE BESTANDEEL glufosinate-ammonium 200 g/l glufosinaat-ammonium</p>	
<p>HRAC HERBICIDE GROUP CODE H HRAC ONKRUIDODDER GROEPKODE</p>	
<p>2. COMPOSITION/INFORMATION ON INGREDIENTS</p>	
Common Name:	GLUFOSINATE-AMMONIUM
Chemical Name:	ammonium (S)-2-amino-4-[hydroxy (methyl) phosphinoyl] butyrate; ammonium DL-homoalanin-4-yl (methyl) phosphinate (IUPAC)
CAS N^o.	77182-82-2
Chemical family:	phosphinic acid
Chemical formula:	C ₅ H ₁₅ N ₂ O ₄ P
Molecular weight:	198.2
Use:	For control of annual and perennial weeds and grasses in Glufosinate-tolerant crops
Formulation:	GLUFOSINATE-AMMONIUM: 200 g/l Soluble Liquid
<u>Inert:</u>	<u>% w/v:</u>
GLUFOSINATE-AMMONIUM (95 %)	15.80
Other inerts	4.01

Figure 2.9: Extract picture from Bound® 200 SL label and Material Safety Data Sheet. Active ingredient is glufosinate ammonium. (Bound 200 SL MSDS 2011; Bound 200 SL Label Sheet 2015).

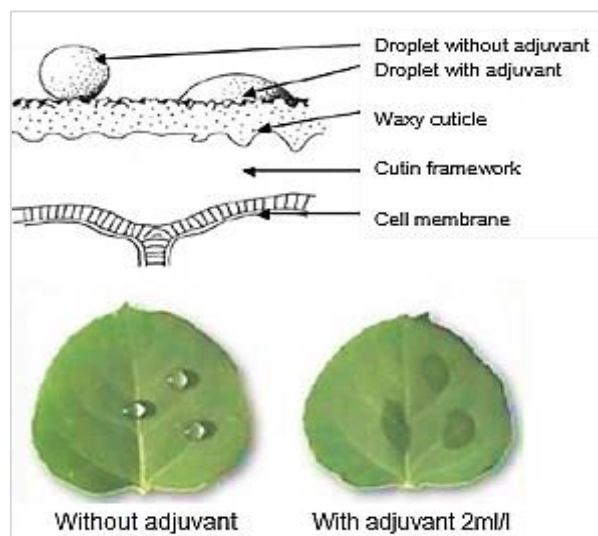
Regardless that herbicide formulations already contain compounds that enhance absorption, solubility or coverage, harsh environmental conditions might require additional adjuvants to eliminate such problems. For instance, low humidity reduces efficacy of water-soluble herbicides by increasing the rate of droplet drying, hence, addition of humectants prolongs the time in which the active ingredient is in soluble form until it can penetrate the cuticle (Ramsey et al. 2006). Adjuvants are spray modifiers or activators that are used to increase the performance of herbicides (Hess 1999).

The greater part of the plant cuticle displays a lipophilic character due to the presence of polymerized hydroxylated fatty acids. These compounds include the cutin, epicuticular wax, embedded wax and pectins (Varshney and Sondhia, no date). Glufosinate ammonium shows low lipophilic characteristics as compared to its hydrophilic characters (MacLachlan 2012). Curran et al. (1999) highlight the importance of adjuvant, herbicide and plant surface interaction in yielding a favourable permeability character to the herbicide. Suitable adjuvants function in achieving optimum specific solubility characteristics of herbicides, which is referred to as hydrophilic-lipophilic balance (HLB) (Curran et al. 1999). **Table 2.2** highlights the role of adjuvants in increasing herbicide efficacy.

Table 2.2: Role of adjuvants in increasing herbicide efficacy (Somervaille et al. 2014)

Surfactants, Oils, Salts and Buffers	
1	Uniform spreading of spray solution and a uniform wetting of the plant.
2	Aiding spray droplets stick to the plant, resulting in less runoff.
3	Assuring that droplets are not suspended on hairs, or other surface projections
4	Preventing crystallization of the active ingredient on the leaf surface
5	Decelerating the drying of spray droplets once on the leaf surface
6	Increasing the water retention on leaf surface
7	Improve penetration of herbicides through the waxy layer
8	Reduces surface tension
9	Reduce problems with hard water
10	Modify and maintain pH of spray solution

Reports confirm that farmers achieve good weed control when they use the adjuvant Ballista® on glufosinate ammonium, however, consultants do not encourage the use of oil since the herbicide formulation has enough oil (Personal communication: Mr Lotter, Bayer South Africa). Studies conducted by Mucheri (2016) to determine the role of Velocity Super™ (ammonium sulfate, L 9603), Summit Super® (nitrogen solution/non-ionic surfactant, L 8539) and Ballista® (methylated vegetable oil with alcohol ethoxylate, L 7442) showed that efficacy of glufosinate ammonium with Ballista® and Summit Super® did not differ significantly from glufosinate ammonium applied without added adjuvants. Ammonium sulfate increased efficacy of glufosinate ammonium (Pratt et al. 2003; Mucheri 2016). Glufosinate ammonium is foliar applied. **Figure 2.10** illustrates how adjuvants influence foliar applied herbicides.

**Figure 2.10:** Influence of adjuvants on foliar applied herbicides (Somervaille et al. 2014).

2.4 CONCLUSION

Introduction of post-emergence weed management by non-selective herbicides was a relief in the agricultural sector, however, it led to over-use of herbicides such as paraquat and glyphosate (Tharp et al. 1999; Pieterse 2010). Inherently, evolution of herbicide resistance to such herbicides has become a problem in arable lands. Glufosinate ammonium is a better alternative to glyphosate or paraquat since there is no weed resistance reported to it yet in South Africa (Mucheri 2016; Heap 2019). However, the herbicides' control of weeds is inconsistent, although it is reported to be very effective against a broad spectrum of weeds (Petersen and Hurle 2000; Coetzer et al. 2001). Own previous study speculated that at different temperature regimes, accumulation of plant calcium and wax decreases efficacy of glufosinate ammonium at warmer temperatures (Mucheri 2016). This study, therefore, aimed to confirm this conjecture.

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CHAPTER 3

Investigating response of ryegrass (*Lolium* spp.) post glufosinate ammonium application at different temperatures

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ABSTRACT

An experiment to investigate why glufosinate ammonium control of ryegrass is better at cooler temperatures than warmer temperatures was conducted at Welgevallen Experimental Farm. Wild ryegrass (*Lolium* spp.) was grown at 10/15, 15/20, 20/25 and 25/30 °C (night and day) temperature regimes and treated with glufosinate ammonium at 0, 1.5, 3, 4.5 and 6 L ha⁻¹, six weeks after planting. Control of ryegrass decreased with increasing temperature. Results from elemental analysis, scanning electron microscopy (SEM), fluorescence imaging and gas chromatography mass spectrometry (GCMS) studies attributed these findings to calcium accumulation, cuticle thickness and plant phenolic compounds. Either cuticle thickness or calcium accumulation increase as temperatures increases, probably due to promotion of plant defence mechanisms induced by herbicide stress, hence resulting in poor control of ryegrass under warmer temperatures.

Key words: auto-fluorescence, cuticle thickness, glufosinate ammonium, phenolic compounds, ryegrass, temperature.

3.1 INTRODUCTION

Evolution of weed resistance to herbicides has become a major problem to agricultural systems, and eventually, global food security (Karn et al. 2018). These authors noted that ryegrass (*Lolium perenne* and *Lolium rigidum*) exhibits high genetic variation within populations and necessitates outcrossing, which increases occurrence of probabilities of herbicide resistance allele traits within and among populations in arable fields. In addition to its remarkable genetic variation, ryegrass exhibits traits that impart competitive advantages compared to many crop species. These characteristics include production of multiple tillers that can grow over 1 m height protruding above wheat canopies, aggressive root growth and seed development (Grey and Newsom 2017).

There have been numerous reports in South Africa on development of resistance in ryegrass weeds to paraquat and glyphosate (Pieterse 2010; Ferreira et al. 2015). Resistance of ryegrass to glufosinate ammonium has been reported in New Zealand and United States. However, herbicide resistance of ryegrass to glufosinate ammonium in South Africa has not been documented yet (Heap 2019). According to Everman (2008), research has shown remarkable control of a variety of weed species with glufosinate ammonium, which makes it a possible alternative or rotational herbicide with other non-selective post-emergence herbicides.

Glufosinate ammonium is a contact, non-selective aminoalkylphosphonic acid. It inhibits glutamine synthetase (GS, EC 6.3.1.2) and is very effective in controlling a wide range of weed species (Green and Owen 2011). However, glufosinate ammonium poses a great challenge because it shows variable results under certain field conditions (Petersen and Hurle, 2000; Pratt et al. 2003). Details of particular field conditions that cause such variability are yet to be discovered. However, environmental factors such as temperature, light intensity and humidity have a great influence on efficacy of this foliar applied herbicide before, during and after application (Steckel et al. 1997; Petersen and Hurle 2000; Coetzer and Al-Khatib 2001). These factors have significant direct and indirect influence on absorption and translocation of glufosinate ammonium.

Temperature has modifying influences on plant factors like growth stage, cuticle thickness, and waxy accumulation, and thus, indirectly influences efficacy of glufosinate ammonium (Ramsey et al. 2006). Some studies on glufosinate ammonium have reported decreasing control of ryegrass with increasing temperatures (Molefe 2015; Mucheri 2016). Contrary to these results, preceding studies have shown that control of weeds with glufosinate ammonium is effective with increasing temperature (Pline et al. 2000; Kumaratilake and Preston 2005; Everman 2008; Everman et al. 2009). A different finding

from the afore-mentioned contradictory findings was reported by Coetzer et al. (2001). They reported better control of weeds (*Amaranthus rudis*, *A. palmer* and *A. retroflex*) grown at 21/26 night/day temperatures than weeds grown at 16/21 and 26/31 °C night/day temperature regimes.

Several studies have been conducted to investigate the effect of temperature, but the results vary, and no conclusive observation can be drawn from the studies. An underlying fact is that the effect of temperature on efficacy of glufosinate ammonium is inevitable and should be studied in detail in order to provide definite results. The aim of this study was to investigate factors that affect efficacy of glufosinate ammonium on ryegrass grown at different temperatures.

3.2 MATERIALS AND METHODS

3.2.1 Experimental design and setup

The experiment was conducted at Welgevallen Experimental farm, Stellenbosch University (33° 56'33" S and 18° 51'56" E at an altitude of 136 m above sea level). A susceptible species of wild ryegrass was sown and trans-planted after 2 weeks in 8 × 8 cm² pots filled with river sand as growth medium. Glasshouses were set at 10/15 °C, 15/20 °C, 20/25 °C and 25/30 °C (12/12 h night/day) temperature regimes. The plants were thinned to four seedlings per pot two weeks after emergence. Irrigation was done with a balanced nutrient solution shown in **Table 3.1**. Irrigation was scheduled at 8:00 am, 12:00 pm, 2:00 pm and 4:00 pm for 2 min per irrigation. A single foliar application of glufosinate ammonium was done three weeks after trans-planting at dosage rates of 0, 1.5, 3, 4.5 and 6 L ha⁻¹. Experimental design was a completely randomized block design (RCBD) arranged as a 4 × 5 factorial with four temperature regimes and five dosage rates. The treatment combinations were replicated six times. Relative humidity ranged from 75% to 90%. The herbicide was applied by means of a pneumatic pot sprayer at a pressure of 2 bar in 200 L ha⁻¹ of water. The pots were immediately returned into their respective glasshouses after spraying and irrigation was switched off for 24 h. Assessment on mortality of ryegrass was done four weeks after glufosinate ammonium application. The equation used to determine percentage mortality per pot was:

$$\text{Percentage mortality} = \frac{\text{number of dead plants}}{\text{total number of plants per pot (4)}} \times 100$$

The experiments were repeated twice and the mean mortality rate values between the two experiments were used for statistical analysis. Statistical analysis of mortality rate was done using STATISTICA 13 (developed by Dell Software). Homogeneity of variance evaluation indicated no significant treatment by experiment interaction, therefore, data from

the experiments were combined and mean differences were separated using Tukey's honest significant difference (HSD) test at 95% confidence interval.

Table 3.1: Composition of the nutrient solution used to fertilize the plants

EC = 2.0			
Element	Concentration (mg L ⁻¹)	Fertilizer	Concentration (g 1000L)
(Macro)			
K ⁺	237.7	KN ₃	303
Ca ⁺⁺	180	K ₂ SO ₄	261
Mg ⁺⁺	48.6	Ca (NO ₃) ₂ · 2H ₂ O	900
NO ₃ ⁻	661.33	MgSO ₄ · 7H ₂ O	492
H ₂ PO ₄	116.4	KH ₂ PO ₄	136
SO ₄	390.4		
(Micro)			
Fe:	0.85	Libfer (Fe EDTA)	6.54
Mn	0.55	Manganese sulfate	2.23
Zn	0.30	Zinc sulfate	1.33
B	0.30	Solubor	1.46
Cu	0.05	Copper Sulfate	0.20
Mo	0.02	Sodium Molybdate	0.13

3.2.2 Confocal fluorescence imaging study

Confocal images of treated and control ryegrass plants were obtained using a Carl ZEISS confocal microscope (Central Analytical Facility, Fluorescence Department, Stellenbosch University). Treated leaves used for the analysis were sprayed with a dosage rate of 4.5 L ha⁻¹ glufosinate ammonium. Three 0.25 cm² segments from the youngest leaf of a plant were excised from the plants and mounted upside down on the slide. To this sample 50 µl of water was added before placing the coverslip. A region of 830 µm × 830 µm was scanned with a vertical resolution of 12.316 µm. Analysis of images and auto-fluorescence intensities was done using IMAGE J software (developed at the University of Wisconsin). Statistical analysis of variance of fluorescence intensities was done using the STATISTICA 13 statistical program and mean differences were separated using Tukey's HSD test at 95% confidence interval.

3.2.3 Gas chromatography mass spectrometry study

A further study was conducted to confirm if concentration of phenolic compounds correlates with the intensities of the blue auto-fluorescence. The following standard operating procedure describes the analysis method of phenolic acids in treated and control ryegrass plants. These tests were done on control samples and ryegrass treated with 4.5 L ha⁻¹ dosage rate across all temperatures.

Reagents preparation: 70% (v/v) methanol (MeOH) reagent was prepared by mixing 70 ml MeOH with 30 ml distilled water.

Procedure for sample preparation: Approximately 10 mg of each sample was weighed into a 1.5 ml Eppendorf tube. Then 1 ml of ready prepared 70% methanol (MeOH) was added to the sample. After that 100 μ l ribitol and naphthol internal standard (10 ppm in 70% MeOH) was added and samples were vortexed for 30 s. Samples were placed in an oven at 60 °C for 3-4 h and 250 μ l of each sample extract was transferred into another set of Eppendorf tubes. Samples were dried completely using a SpeedVac at room temperature overnight.

Derivatization for phenolic acids: O-Bis(trimethylsilyl)trifluoroacetamide + trimethylchlorosilane (30 μ l BSTFA+TMCS, 99:1) was added to the dried samples after adding 100 μ l acetonitrile. The samples were vortexed and placed in the oven at 80 °C for 60 min. After 60 min the samples were vortexed before transferring them into the gas chromatography vial with insert and cap. Samples were then analysed for phenolic compounds using ThermoFisher (TS8000) gas chromatography mass spectrometry equipment at the Central Analytical Facility, Mass spectrometry Department, Stellenbosch University.

Concentration of phenolic compounds detected was subjected to ANOVA using the STATISTICA 13 statistical program and means were separated using Tukey's HSD test at 95% confidence interval.

3.2.4 Elemental (calcium) analysis

Three randomly selected control pots were selected from each glasshouse (10/15, 15/20, 20/25 and 25/30 °C temperature regimes) and harvested during spraying time. The plants were dried for 48 h in an 80 °C oven. Samples were ground using a Bate-85588 (10 20 mesh) milling machine and sent for plant analysis at the Institute of Plant Sciences, Western Cape Agricultural Department, Elsenburg. The samples were analysed for nitrogen, phosphorus, potassium, calcium and magnesium. The elements of interest were calcium and magnesium. Statistical analysis of the elements was done using STATISTICA 13. A one-way ANOVA was used to test for statistical differences and means were separated using Tukey's HSD.

3.2.5 Powder X-ray diffraction analysis

Three randomly selected control and treated pots with 4.5 L ha⁻¹ glufosinate ammonium were selected from each glasshouse (10/15, 15/20, 20/25 and 25/30 °C temperature regimes) and harvested 24 h after spraying. The samples were frozen in liquid nitrogen soon after

harvesting and then dried using a Martin Christ® BETA 1-8 LDplus freeze dryer at the Horticulture Department, Stellenbosch University. The samples were then ground to a powder using a mortar and pestle. Powder X-ray diffraction (PXRD) patterns from the ground ryegrass was then measured using a Bruker D2 Phaser diffractometer with (Bragg–Brentano geometry) using Cu K α radiation ($\lambda = 1.5418 \text{ \AA}$) at 30 kV and 10 mA. Intensity data were captured with a Lynxeye detector with 20 scans performed in the range 4–50° with a 0.020° step size. Samples were spun at 30 rpm. Match Crystal Impact version 3.8 software (Bonn, Germany) was used for plotting and crystalline analysis of the results. The samples were matched with all possible compounds which include Ca, N, O, H and P elements.

3.2.6 Structural analysis

Three segments of 2 cm² were cut from actively growing upper, middle and lower leaf sections of control ryegrass grown at 10/15, 15/20, 25/30 and 25/30 °C temperature regimes. The samples were fixed in 4% (v/v) paraformaldehyde (PFA) with 2% (v/v) glutaraldehyde in 0.1 M buffer, pH 7.4 overnight. The samples were subjected to dehydration in increasing ethanol concentrations (30, 50, 70, 90, 95 and 100% (v/v) for 10 min each). Dehydration in 100% ethanol was repeated for 10 min before placing them in hexamethyldisilazane (HMDS). Samples in HMDS were then left to dry overnight. The dry samples were coated with gold before mounting on a Zeiss MERLIN field scan electron microscope (FESEM, Gemini 2) at the Central Analytical Facility, Scanning Electron Microscope Department, Stellenbosch University. A morphological analysis of the cross-sectional view of leaves was done to determine the cuticle layer thickness. The thickness of epicuticular wax film plus cutin was determined by a low magnification calibration ruler embedded in the scanning electron microscope. The total thickness of epicuticular wax film and cutin is referred to as the cuticle thickness in the text.

3.3 RESULTS

3.3.1 Mortality rate

Interaction of glufosinate ammonium dosage rate and temperature on mortality of ryegrass was significant ($p < 0.05$). Ryegrass grown in glasshouses at 10/15 and 15/20 °C showed significantly greater mortality when compared to ryegrass grown at 20/25 and 25/30 °C (**Figure 3.1**). This shows that cooler temperatures increase efficacy of glufosinate ammonium. The highest mortality rates were 80% and 85.7% at 20/25 and 25/30 °C using a dosage rate of 6 L ha⁻¹ whereas 100% mortality was recorded at 10/15 and 15/20 °C with 3 L ha⁻¹ dosage rate. There were no significant differences between mortality rates of ryegrass grown at 10/15 and 15/20 °C across all dosage rates. No significant differences were

observed on mortality rates of ryegrass at 20/25 and 25/30 °C except when glufosinate ammonium was applied at 1.5 L ha⁻¹ dosage rate.

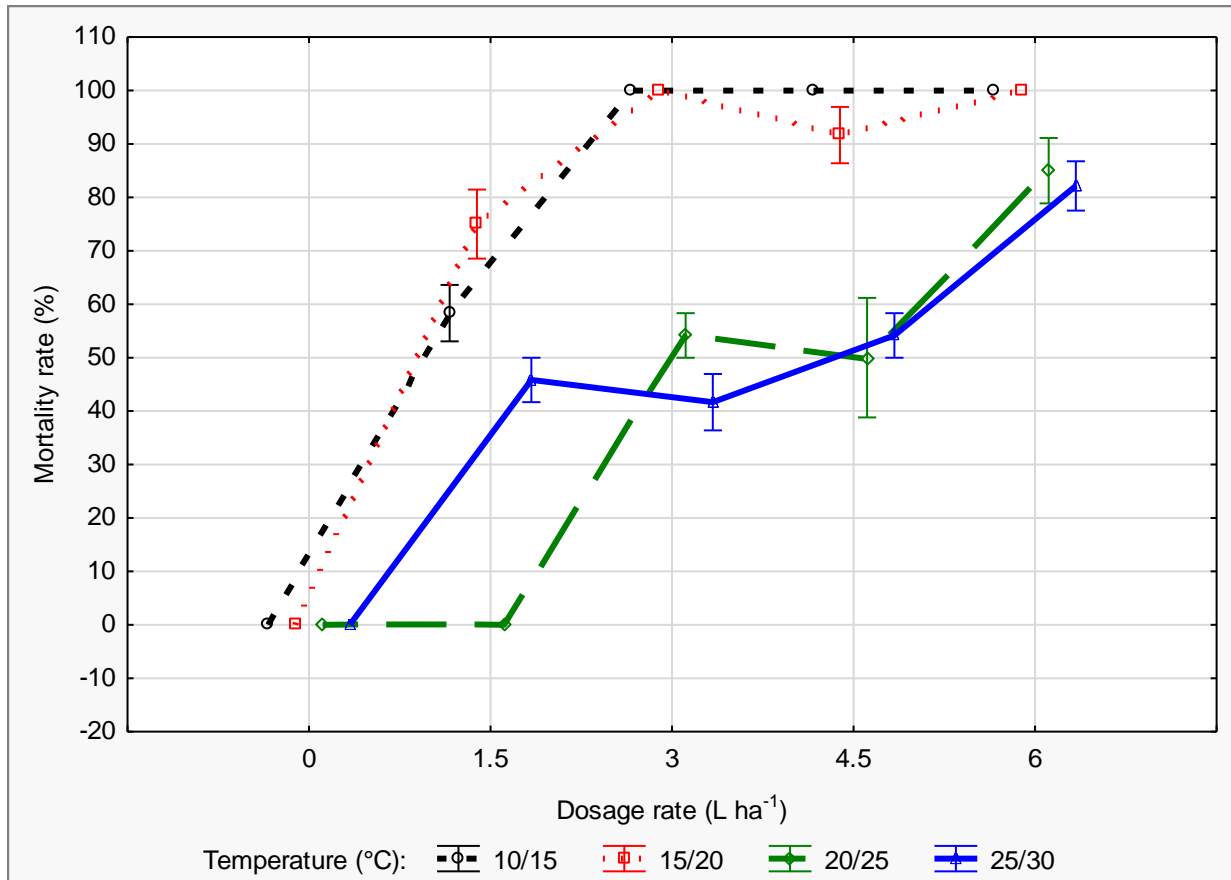


Figure 3.1: Effect of temperature on efficacy of glufosinate ammonium sprayed at different dosage rates for the control of ryegrass. Results are means of two experiments with six replications each (n = 6). Vertical bars denote \pm standard error of the mean.

3.3.2 Calcium and powder X-ray diffraction analysis

Percentage calcium concentration was significantly different between all temperature treatments (**Figure 3.2**). The warmer temperatures, 20/25 °C and 25/30 °C showed significantly higher calcium concentrations than the cooler temperatures. There was a significant reduction in calcium concentration as temperatures increased from 20/25 to 25/30 °C as well as when temperatures increase from 10/15 °C to 15/20 °C. The highest calcium concentration was observed at 20/25 °C temperature regime.

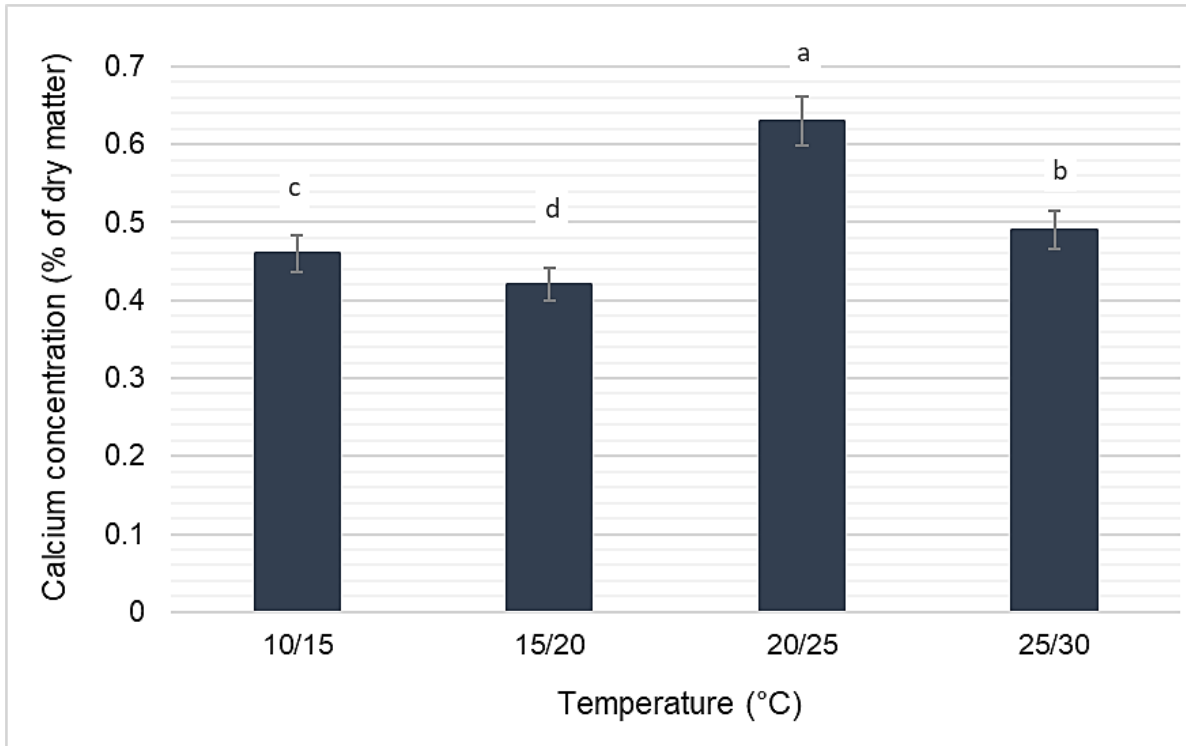


Figure 3.2: Calcium concentration as percentage of dry matter of control (untreated) ryegrass samples grown at different temperatures at spraying time. Letters denote statistically significant differences between treatments as determined by ANOVA followed by Tukey's HSD test ($p < 0.05$). Error bars represent \pm standard error of the mean of three biological replications ($n = 3$).

Phase identification results from powder diffraction data are presented in **Figure 3.3**. The diffraction pattern obtained for the control and treated grasses was matched with all possible compounds that include Ca, N, H, O and P elements mainly because no literature has documented the end product of glufosinate plus calcium reaction. All control grasses showed no diffraction peaks that matched with the selection criteria; hence, quantification was not applicable with percentage quantity of 0. No diffraction peaks were also observed for treated ryegrass grown at 10/15 and 15/20 °C. A total of 5 peaks matching the above-mentioned selection criteria were observed for treated ryegrass grown at 20/25 °C while the treated grass ryegrass grown at 25/30 °C showed 9 matching peaks (**Figure 3.4**) (refer to **Table 3.2** and **3.3**).

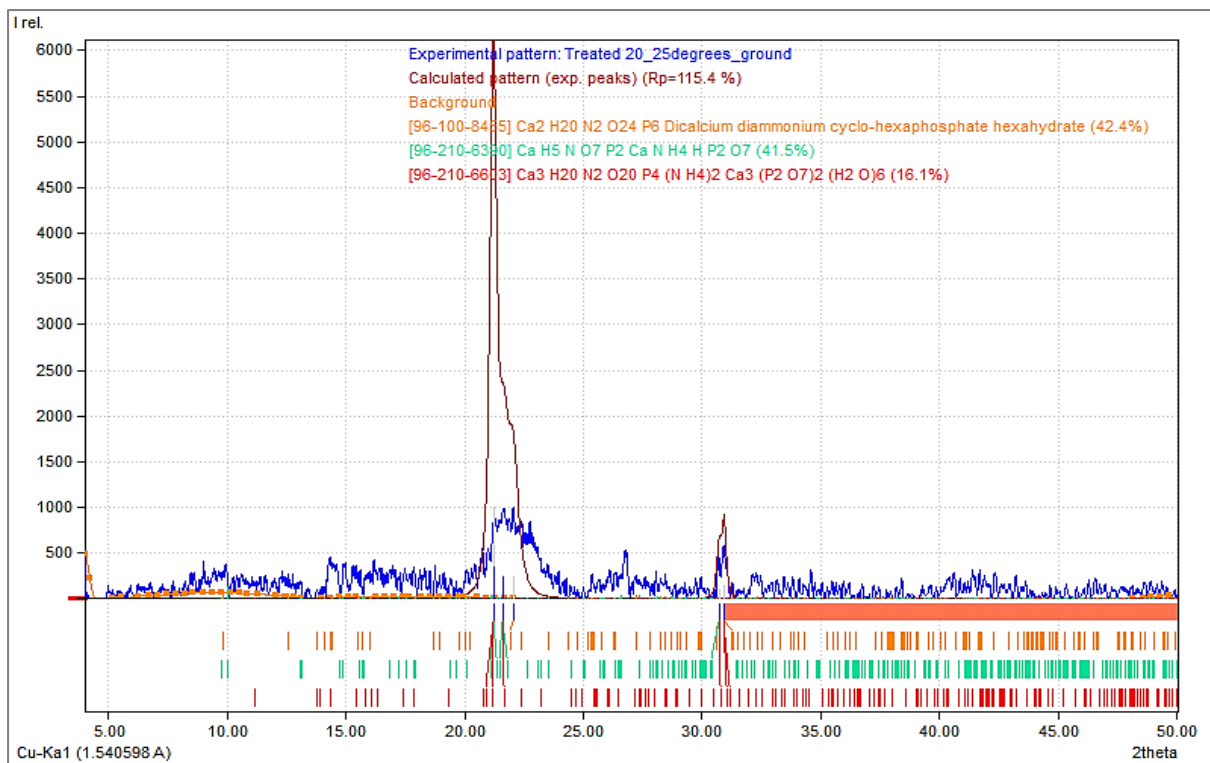


Figure 3.3: Diffraction pattern graphics of matches between treated ryegrass samples grown at 20/25 °C against possible compounds containing Ca, N, O, P and H elements.

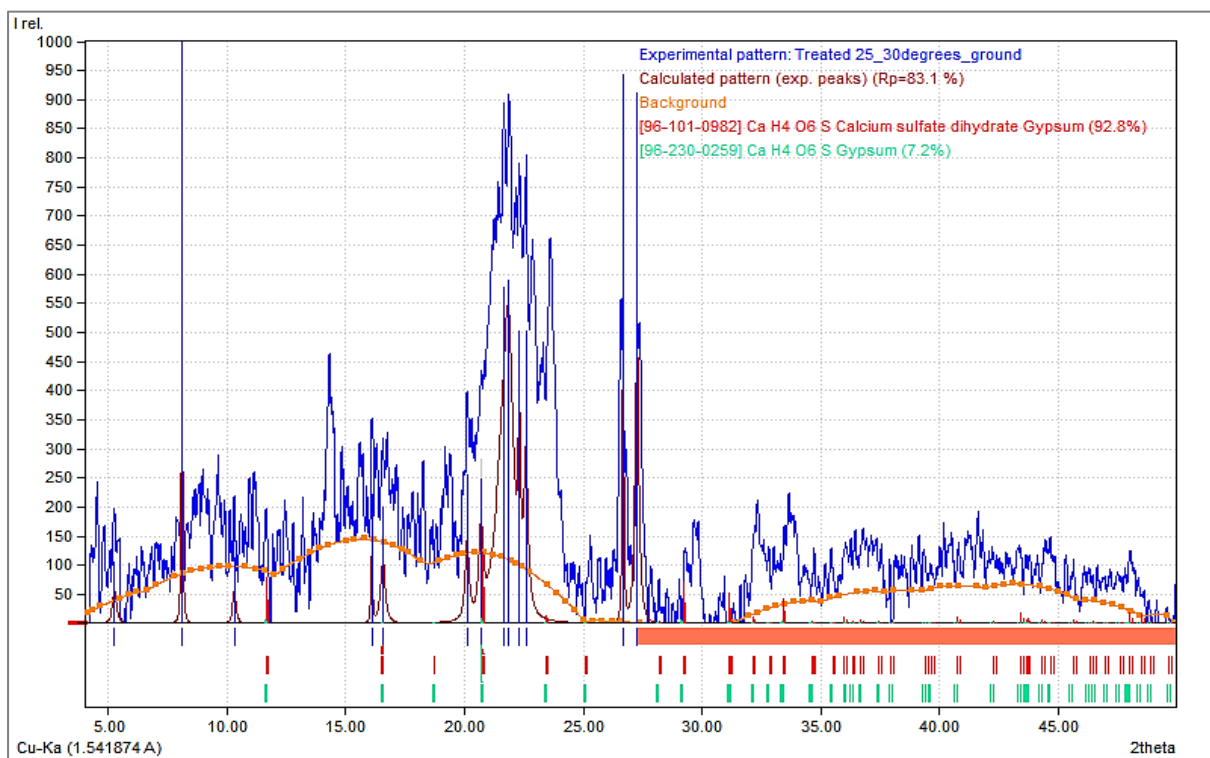


Figure 3.4: Diffraction pattern graphics of matches between treated ryegrass samples grown at 25/30 °C against possible compounds containing Ca, N, O, P and H elements.

Table 3.2: Peak list of treated ryegrass samples grown at 20/25 °C matched against possible compounds containing Ca, N, O, P and H elements in Match Crystal Impact

Number	2theta [°]	Matched
1	21.22	B,C
2	21.65	B,C
3	22.05	A
4	30.73	A,B,C
5	30.94	A,C

Where; amount, name or formula sum of A, B and C are:

A - 42%, Dicalcium diammonium cyclo-hexaphosphate hexahydrate, $\text{Ca}_2\text{H}_{20}\text{N}_2\text{O}_{24}\text{P}_6$.

B - 42%, Ca N H4 H P2 O7, $\text{CaH}_5\text{NO}_7\text{P}_2$.

C - 16%, (N H4)2 Ca3 (P2 O7)2 (H2 O)6, $\text{Ca}_3\text{H}_{20}\text{N}_2\text{O}_{20}\text{P}_4$.

Table 3.3: Peak list of treated ryegrass samples grown at 25/30 °C matched against possible compounds containing Ca, N, O, P and H elements in Match Crystal Impact

Number	2theta [°]	Matched
1	15.08	
2	21.32	A
3	21.90	A,B
4	22.33	A,B
5	22.52	A
6	22.73	
7	23.01	A
8	23.37	B
9	23.70	B

Where; amount (percentage weight), name or formula sum of A and B are:

A - 95.4%, (N H4)2 Ca3 (P2 O7)2 (H2 O)6, $\text{Ca}_3\text{H}_{20}\text{N}_2\text{O}_{20}\text{P}_4$.

B - 3.2%, Dicalcium diammonium cyclo-hexaphosphate hexahydrate, $\text{Ca}_2\text{H}_{20}\text{N}_2\text{O}_{24}\text{P}_6$.

3.3.3 Structural analysis

A cross sectional view of ryegrass leaves grown at different temperature regimes is shown in **Figure 3.5**. Mean thickness of the plant cuticle layers across all temperatures is given in **Table 3.4**.

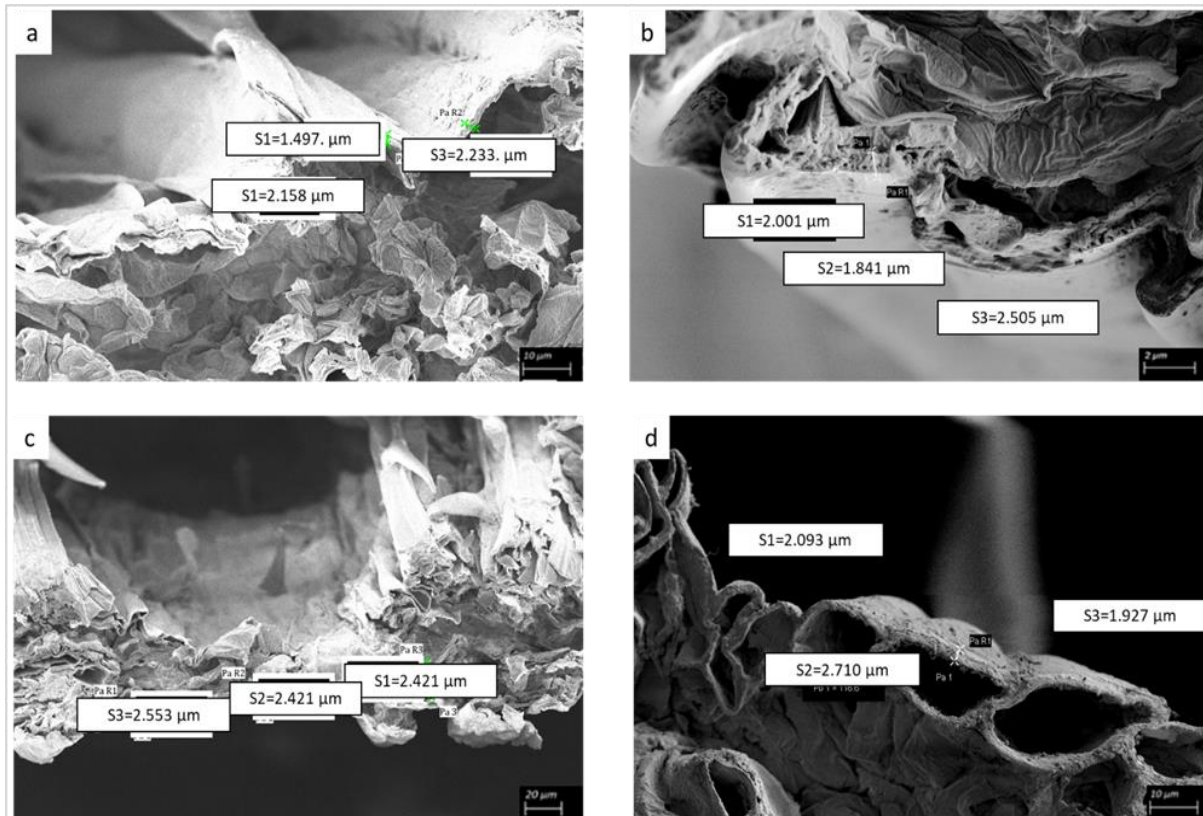


Figure 3.5: Cross-sectional SEM micrographs of ryegrass grown at different temperatures to measure cuticle thickness. Images include ryegrass grown at; a) 10/15 °C (magnification= 1000 X and bar: 10 μm) b) 15/20 °C (magnification = 2 840 X and bar: 2 μm) c) 20/25 °C (magnification= 346 X and bar: 20 μm) and d) 25/30 °C (magnification= 921 X and bar: 10 μm).

A general increase in mean cuticle thickness was shown with increasing temperature up to the 20/25 °C level. However, a decline of 0.222 μm was observed from 2.465 μm at 20/25 °C to 2.243 μm at 25/30 °C. The greatest mean difference in cuticle thickness was 0.349 μm and it was between 15/20 and 20/25 °C.

Table 3.4: Average thickness of ryegrass cuticles grown at different temperatures

Temperature regime night/day (°C)	Average thickness (μm)
10/15	1.962 ^d
15/20	2.116 ^c
20/25	2.465 ^a
25/30	2.243 ^b

Letters denote statistically significant differences between treatments as determined by ANOVA followed by Tukey's HSD test ($p < 0.05$).

3.3.4 Confocal laser scanning microscopy study

Visual observations from confocal microscope images showed that all plants exhibit a blue auto-fluorescence (**Figure 3.6**). However, the intensity differed across all temperature regimes and treatments. Comparison of visual observations between treated plants and

controls show that the blue intensity is greater for treated plants than controls per temperature regime. Increase in temperature increased the intensity of blue auto-fluorescence for both control and treated plants.

Figure 3.7 shows the representation of blue auto-fluorescence as percentage area of the images taken under a confocal microscopy. The highest percentage area of 2.29% blue auto-fluorescence was observed on treated ryegrass at 25/30 °C and was significantly different from the subsequent percentage area of 2.128% shown at 20/25 °C for treated ryegrass. The lowest auto-fluorescence percentage area of treated ryegrass was observed at 10/15 and 15/20 °C and these were not significantly different from each other. Control plants showed the same trend as treated plants whereas warmer temperatures showed a significantly higher percentage area of auto - fluorescence than ryegrass at cooler temperatures. The percentage area auto-fluorescence of treated ryegrass was significantly different from the controls across all temperatures, however, the difference between control and treated plants at 20/25 and 25/30 °C was more prominent.

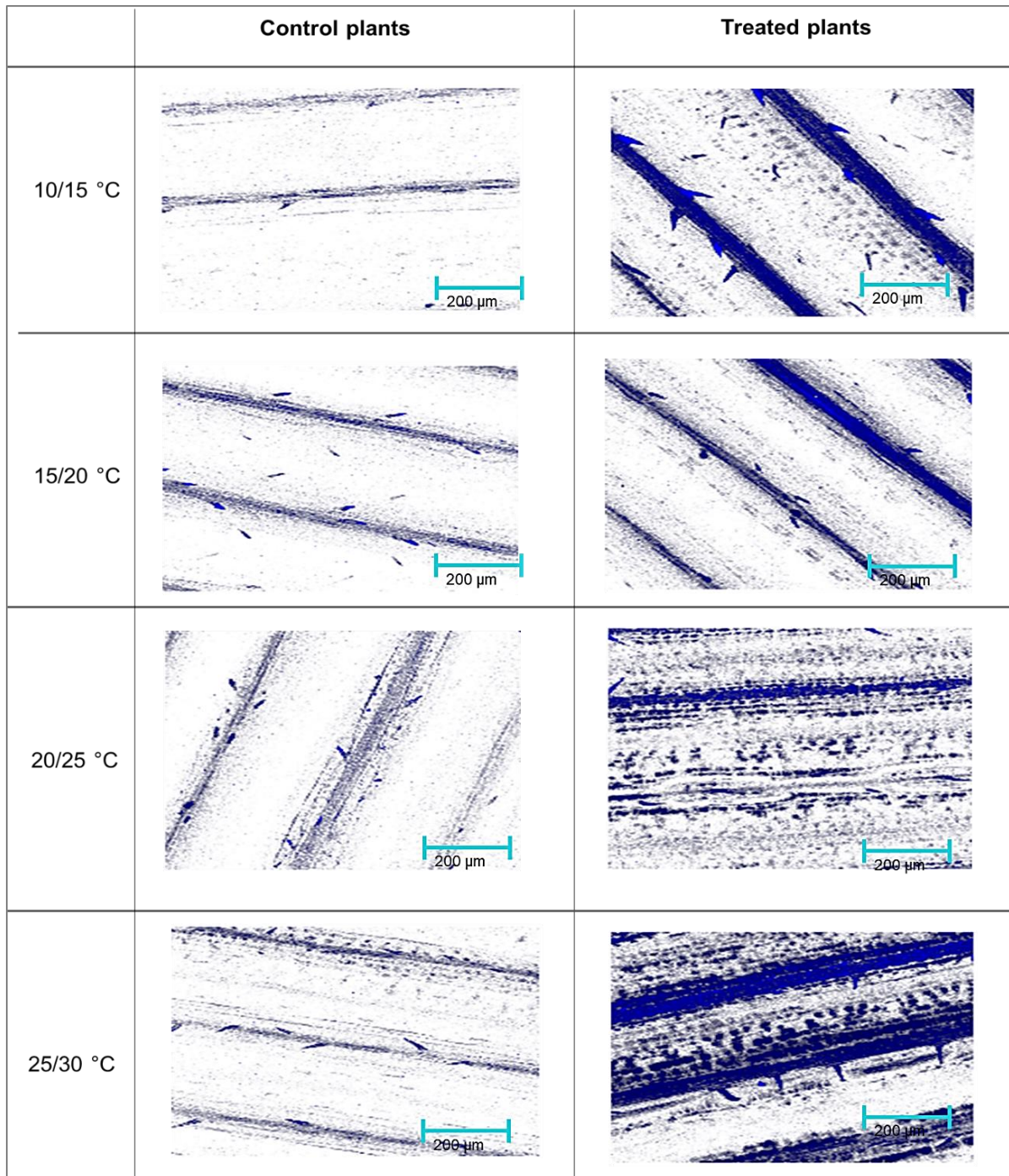


Figure 3.6: Images of ryegrass control specimens under a confocal microscopy compared to treated specimens. The bars in photos represent 200 µm.

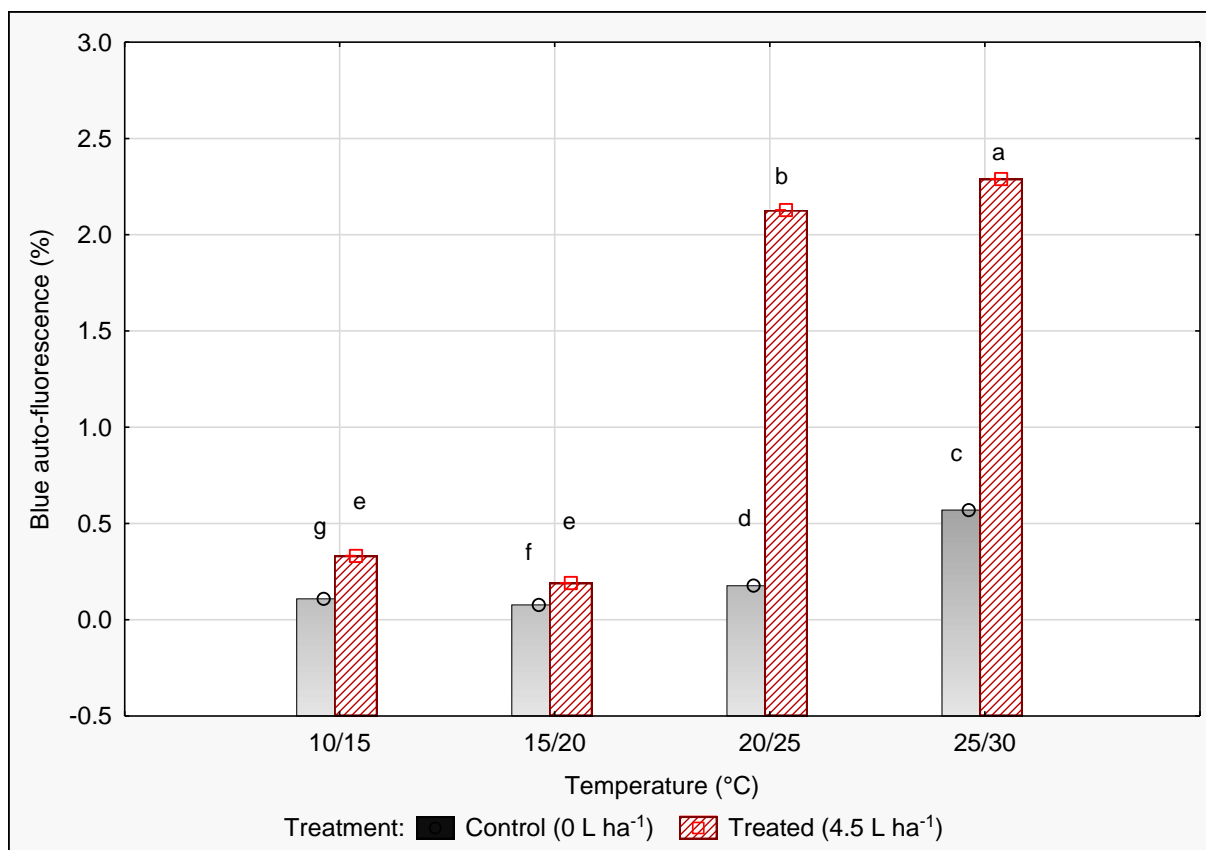


Figure 3.7: Percentage area covered by blue auto-fluorescence in confocal images of ryegrass grown under different temperatures. Letters denote statistically significant differences between treatments as determined by ANOVA followed by Tukey's test ($p < 0.05$). The results represent mean of three replications ($n = 3$).

3.3.5 Gas chromatography mass spectrometry study

Figure 3.8 shows the relationship between detected phenolic acids and influence of temperature on control and treated ryegrass. The study detected protocatechuic, *m*-coumaric, syringic, *p*-coumaric, gallic and ferulic acids. All phenolic acid concentrations showed significant differences between treated ryegrass and controls except for gallic acid with p -value = 0.087557 ($p > 0.05$) (refer to **Appendix 3.3 - 3.8**). All of the acids showed a general concentration increase with increasing temperature for both control plants and treated plants. A distinct trend is shown by ferulic acid and syringic acid (**Figure 3.8a and 3.8d**). The concentration of *p*-coumaric acid only significantly differed between treated and control plants at 10/15 °C (**Figure 3.8b**). Concentration of protocatechuic acid was not significantly different between control and treated plants for ryegrass at 20/25 °C. A significantly drastic increase of protocatechuic acid concentration was observed in treated ryegrass at 25/30 °C.

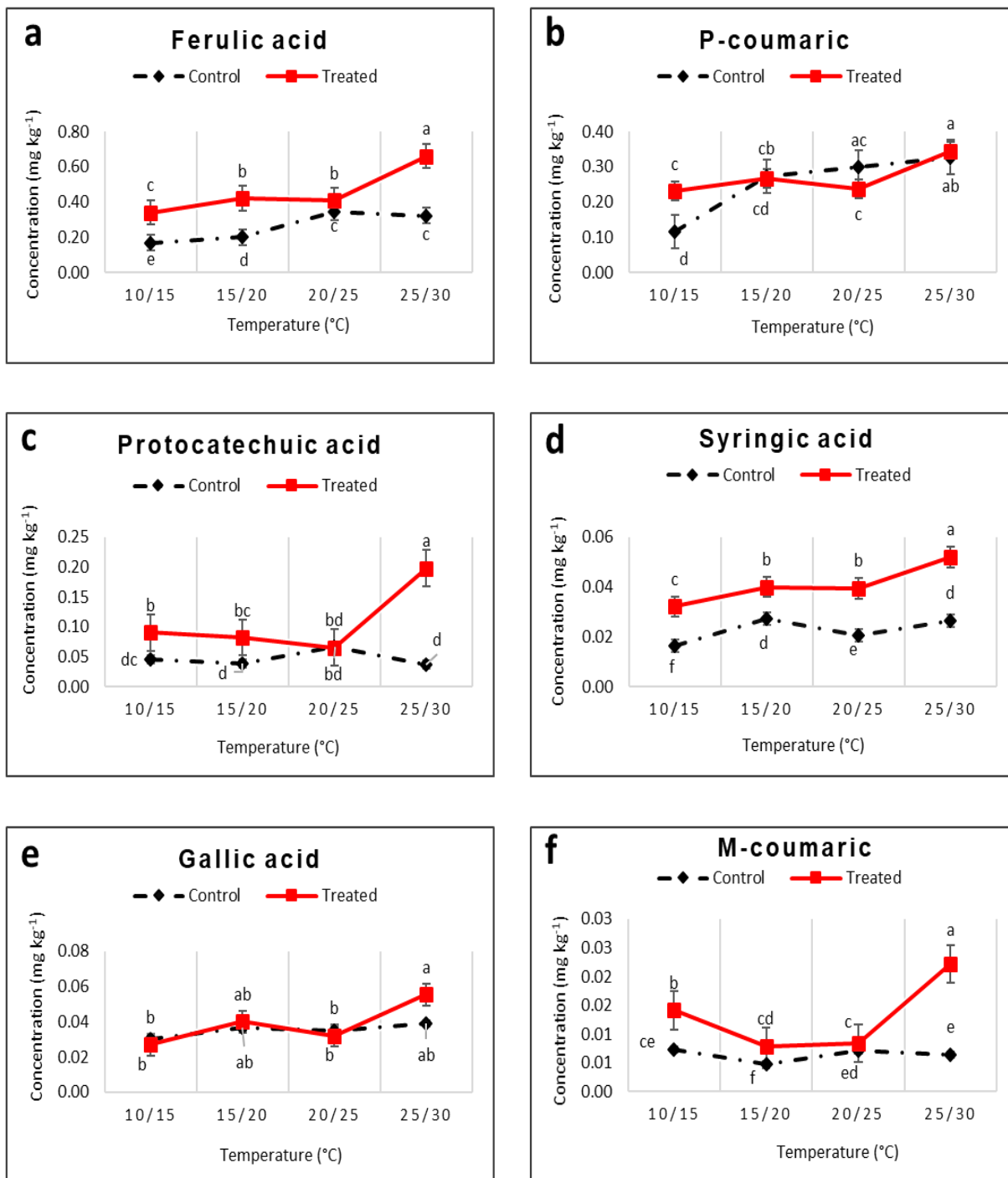


Figure 3.8: Phenolic compounds detected in control and treated ryegrass using the GCMS technique. Treated ryegrass was sprayed with 4.5 L ha⁻¹ glufosinate ammonium dosage rate. Letters denote statistically significant differences between treatments as determined by ANOVA followed by Tukey's test ($p < 0.05$). Error bars represent \pm standard error of the mean of four replications ($n = 4$).

An analysis of all phenolic acids detected is presented in **Figure 3.8**. The figure shows that the most dominant phenolic acids are ferulic acid and *p*-coumaric acid for all temperatures in treated and control plants. However, warmer temperatures show a significantly higher concentration of these phenolic acids than cooler temperatures. The lowest concentration of phenolic acids detected was *m*-coumaric acid which ranged from 0.0047 to 0.0073 mg kg⁻¹ for controls and 0.0079 and 0.0221 mg kg⁻¹ for treated plants across all temperatures.

3.4 DISCUSSION

Control of ryegrass was significantly higher at cooler temperatures (10/15 and 15/20 °C) than warmer temperatures (20/25 and 25/30 °C). These results concur with a study conducted by Mucheri (2016). However, several studies have observed an increase in weed control as temperatures increase (Pline et al. 1999; Coetzer et al. 2001). Mucheri (2016) suspected that calcium and waxy cuticle accumulation at warm temperatures results in poor control of ryegrass because accumulation of cytoplasmic calcium and plant wax increases as environmental temperature increases (Gong et al. 1998; Knight 2000; Knight and Knight 2001; Jamal 2011). Calcium, reportedly, readily binds to glufosinate, and thus, reduces the herbicides absorption into plants (Pratt et al. 2003). An analysis on mineral and nutritive value of ryegrass provided with sufficient nutrients during its growth reported calcium concentration of 0.42% (percentage of dry matter) in *Lolium perenne* (Harrington et al. 2006). Compared to results from the Harrington et al. (2006) study, ryegrass grown at 15/20 °C showed exactly the same concentration of calcium (0.42%). Using calcium concentration of ryegrass at 15/20 °C as a standard for this current study, calcium concentration detected in ryegrass at 10/15, 20/25 and 25/30 °C was significantly different from ryegrass at 15/20 °C with ryegrass at 20/25 °C showing the highest concentration of 0.63% (elemental analysis results, **Figure 3.2**). These observations pose a difficulty to attribute higher efficacy of glufosinate ammonium at cooler temperatures to calcium even though the results did show significantly higher calcium concentration at 20/25 and 25/30 °C than 10/15 and 15/20 °C. This is because, instead of an increase in calcium concentration of ryegrass at 25/30 °C, as would have been expected, calcium concentration significantly decreased from 0.63% at 20/25 °C to 0.49% at 25/30 °C temperature regime. It would then be necessary to distinguish if the differences in calcium concentration influence binding of calcium with glufosinate ammonium and if temperature has an influence on this reaction.

The use of the elemental analysis method to analyse calcium concentration provided a foundation to determine if temperature influences accumulation of calcium but does not provide sufficient evidence to determine if there are any possibilities of any chemical interactions between the cytoplasm calcium and glufosinate ammonium. A powder X-ray

diffraction method was used to further investigate this possibility. The attempt to use powder X-ray diffraction method was not so successful mainly because grass is a nanocrystalline material with smaller crystallite size, hence, the diffraction peaks were broad (Ju et al. 2015). However, the study provided some insight on calcium reaction with glufosinate ammonium. A definite end product of the reaction could not be determined, hence all possible compounds formed by Ca, N, H, P and O were analysed. Diffraction of these compounds were only noticed on treated ryegrass grown at 20/25 and 25/30 °C. No compounds containing formula sums of Ca, N, H, P and O were detected in the control plants for ryegrass grown at the same temperatures. Given that elemental analysis detected calcium (in other forms) in control plants (refer to **Figure 3.3** and **3.4**), it can be concluded that the calcium present was responsible for reactions that resulted in compounds; Dicalcium diammonium cyclo-hexaphosphate hexahydrate (**A**), $\text{CaH}_5\text{NO}_7\text{P}_2$ (**B**) and $\text{Ca}_3\text{H}_{20}\text{N}_2\text{O}_{20}\text{P}_4$ (**C**). Most interestingly, diffraction peaks for compound **A**, **B** and **C**, or any other compounds formed from Ca, N, H, P and O were not observed for treated ryegrass at 10/15 and 15/20 °C. This is evidence that calcium accumulation in ryegrass at 20/25 and 25/30 °C was significantly higher, such that it reacted with glufosinate ammonium within 24 hour after application. An improved wide-angle X-ray scattering (WAXS) based method XRD method is, however, recommended for advanced studies on the role of calcium in influencing glufosinate ammonium efficacy (Ju et al. 2015).

Influence of temperature on cuticle thickness was also investigated. A general increase in cuticle thickness as temperatures increased was noted in the experiments. The cuticle is composed of a framework of polymeric cutins with embedded cuticular and soluble waxes that act as barriers for herbicide penetration (Basi 2013). A clear relationship between cuticle thickness and temperature was shown, which might account for the greater control of ryegrass at lower temperatures than higher temperatures. Although Varanasi et al. (2016) noted that warmer temperatures tend to increase herbicide diffusion into the plants (e.g. glyphosate on *Desmodium tortuosum* and flumiclorac on *Chenopodium album*) by increasing viscosity of cuticle waxes, findings from the current study were contrary to the account given by Varanasi et al. (2016). Huggins et al. (2018) noted that the plants' ability to reduce the amount of irradiation penetrating into the leaf is a survival tactic that reduces transpiration when exposed to high temperatures. Ryegrass is classified under temperate grasses and is characterized by being more cold tolerant and heat sensitive (Bell et al. 2011). Continuous exposure to warmer temperatures like 20/25 and 25/30 °C might have stressed the temperate plant and resulted in the ryegrass developing thicker waxy cuticles to reduce transpiration. This might account for the results shown in **Table 3.4** where there was a general increase in cuticle thickness of ryegrass as temperatures increased. The increase in

cuticle thickness might have decreased herbicide absorption in plants exposed to warmer temperatures hence resulting in poor control.

It is a well-accepted concept that stressed weeds are usually difficult to control (Molefe 2015). Many reasons have been reported to support this claim. In addition to plant morphological changes that result in poor herbicide penetration, some studies have shown that stressed plants have decreased metabolic reactions, hence herbicide absorption and translocation decreases, resulting in poor efficacy of herbicides (Ahmadi et al. 1980). This study, however, provides a different account towards the difficulty of controlling stressed weeds. **Figure 3.6** presenting different confocal microscopy images showed an increase of blue auto-fluorescence as temperatures increased. The increase was observed for both control plants and treated plants. The study also noted that treated ryegrass showed a more intense blue auto - fluorescence than in control plants. The blue fluorescence is an emission of plant phenolic compounds and cinnamic acids (Buschmann et al. 2000). Phenolic compounds are simple phenylpropanoid compounds that play a role in plant stress responses (Ramakrishna and Ravishankar 2011). They are secondary metabolites which are derived from hydroxylation, methylation and dehydrogenation of cinnamic acid (Solecka 1997). In response to stress, phenolic compounds harden the cell wall, hence making it more compact, tighter and less permeable (Gall et al. 2015). Additionally, continuous exposure to stress results in cell wall lignification, a complex process in which several phenolic substrates and enzymes are involved in forming lignin and filling it in vascular and support tissues. Gall et al. (2015) attributed these cell wall modifications to resistance of plants to drought. Herbicide absorption into target sites may also be hindered by the thick cell walls. The observed increase of blue auto-fluorescence with increasing temperatures, therefore, supports that the ryegrass at warmer temperatures (20/25 and 25/30 °C) were stressed. Consequently, control of ryegrass with glufosinate ammonium at warmer temperatures was poor since the plants had already developed defence mechanisms.

A significant increase in the blue auto-fluorescence after glufosinate ammonium application across all temperature regimes shows the responsive activity of phenolic acids. This shows that chemical (glufosinate ammonium) stress applied to ryegrass resulted in the plants producing more phenolic compounds for support and survival. Even though the plants under cooler temperatures show an increase in auto-fluorescence after glufosinate ammonium application, the increase was significantly lower than that of plants under warmer temperatures. It can be noted that ryegrass exposed to warmer temperatures had already adapted to stress by producing more phenolic acids from the shikimate-phenylpropanoid biosynthetic pathway. Production of phenolic compounds after glufosinate ammonium application would have been easier in ryegrass plants which were already producing them in

abundance. Therefore, the plants' ability to produce more phenolics provided them with support and defence to combat applied glufosinate ammonium. A recent study conducted by Takano et al. (2019) established that accumulation of reactive oxygen species (ROS) mainly accounts for the death of plants after glufosinate ammonium application. This is because ROS denatures the cell membrane integrity through lipid peroxidation. Phenolic compounds have been reported to a) chelate and scavenge free radicals, b) inhibit ROS from modifying essential food components such as amino acids and proteins and c) defend the cell membrane against lipid-oxidation damage (Zamora and Hidalgo 2016). This study provides evidence that involvement of phenolic compounds in providing support to the cell wall suppresses lipid peroxidation. This type of defence mechanism may account for poor control of ryegrass with glufosinate ammonium under warmer temperatures.

There are many chemical substrates that fall under the phenolic compounds family. The most abundant of phenolic compounds as shown by **Figure 3.8** were ferulic acid and *p*-coumaric acid. Ryegrass responses to both environmental and glufosinate ammonium stress can be hugely attributed to the presence of ferulic acid and *p*-coumaric acid. This finding concurs with Buschmann et al. (2000) who noted that the blue auto-fluorescence emitted in fluorescence imaging consists of ferulic acid as the major cinnamic acid. Furthermore, both ferulic and *p*-coumaric acids are cross-linked to the type II primary cell walls which consists of glucuronoarabinoxylans (GAX) and β -glucan as the main matrix (Gall et al. 2015). The cross-linkage also includes the secondary wall which causes the matrix to stiffen and hence ensuring support of the plant.

Although application of fluorescence imaging has extensively increased in research and screening in biotechnology, no work has been presented on how it can be used to investigate the efficacy of herbicides. The ability of fluorescence imaging to reveal a wide range of internal plant characteristics makes it a powerful tool to investigate local effects of abiotic stresses such as high or low temperatures and drought (Nedbal and Whitmarsh 2004). The technology is widely accepted since it provides a non-destructive approach to investigate plant disease and stress (Buschmann et al. 2000). Fluorescence imaging is a costly technology to be adopted by farmers for use in their daily activities. However, this study provides a deeper understanding of why farmers need to uphold good agricultural practices before spraying weeds. In addition to reduced plant metabolism rates, stress induces defence mechanisms in plants, hence resulting in poor control of weeds after application. In addition, further studies can be conducted to employ this method in improving herbicide weed management systems in farming, for instance, inducing phenolic compounds in crops to provide more herbicide resistant crops. The example provided is premature

evidence that the phenolic study can be employed in diverse sectors of the agricultural industry.

3.5 CONCLUSION AND RECOMMENDATIONS

In contrast to the hypothesis, the study showed that temperature affects calcium concentration, cuticle thickness and phenolic compounds of ryegrass. These components account for the poor control of ryegrass with glufosinate ammonium at warmer temperatures, 20/25 and 25/30 °C. The study provided evidence that proved that calcium accumulated at warmer temperatures (20/25 and 25/30 °C) reacts with glufosinate ammonium. Even though the general X-ray diffraction method used in this study managed to show some peaks, particularly, for warmer temperatures, a much more improved wide-angle X-ray scattering (WAXS) based method is recommended for further studies on the efficacy of glufosinate ammonium as influenced by calcium. Additionally, the study provides a novel approach to herbicide efficacy studies in the research sector by using phenolic compounds intensities as indicators. The study observed that increasing intensity of blue auto-fluorescence under a confocal microscope related to higher levels of phenolic compounds such as ferulic acid and *p*-coumaric acid among others. These compounds are responsible for the support system in response to stress environments. The study found that the higher the concentration of phenolic compounds observed, the poorer the efficacy of glufosinate ammonium. Further advances into this technology could provide cheaper and easier ways of investigating herbicide efficacy. The ability of phenolic compounds to increase tolerance of plants post herbicide application can be exploited in developing natural herbicide resistant crops in the crop breeding industry. Further investigation into these opportunities would be required.

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CHAPTER 4

Plant biochemical factors influencing ryegrass (*Lolium* spp.) response to glufosinate ammonium at different temperatures

Tendai Mucheri, Petrus J Pieterse, Carl F Reinhardt, Aleysia Kleinert

ABSTRACT

Plant biochemical factors influencing glufosinate ammonium activity in ryegrass at different temperatures were investigated. Ryegrass (*Lolium* spp.) was grown at 10/15, 15/20, 20/25 and 25/30 °C (night/day) temperature regimes. The plants were treated with glufosinate ammonium (foliar application) at 0, 1.5, 3, 4.5, and 6 L ha⁻¹ (0, 300, 600, 900, 1200 g a.i ha⁻¹) dosage rates. The roots and leaves of control plants (0 L ha⁻¹) and treated plants (4.5 L ha⁻¹) were harvested 24 hours after application (HAA), and analysed for ammonia accumulation, glutamine synthetase, glutamate dehydrogenase and nitrate reductase activity. Photosynthesis studies were also conducted on all treatments 24, 48 and 72 HAA using an Infra-red Gas Analyser (IRGA). An increase in glutamine synthetase enzyme activity with increasing temperature after glufosinate ammonium application. Glutamine dehydrogenase enzyme activity decreased with increasing temperature in treated plants. Although a significant increase in ammonia concentration was observed in treated plants, it is postulated that accumulation of ammonia was not the main cause of death. Better control of ryegrass with glufosinate ammonium at cooler temperatures was mainly attributed to the plants' inability to produce adequate glutamate and α -ketoglutarate which form the carbon skeletons for transamination processes. The significant increase in glutamine synthetase activity in ryegrass under warm temperatures provides evidence that glutamate produced was able to circumvent photosynthetic inhibition.

Key words: ammonia accumulation, glufosinate ammonium, glutamate, glutamate dehydrogenase, glutamine, ryegrass, temperature.

4.1 INTRODUCTION

Glufosinate ammonium functions by inhibiting the enzyme glutamine synthetase (GS, L-glutamate:ammonia-ligase; EC 6.3.1.2). This enzyme catalyses the conversion of glutamate and ammonia to glutamine through two steps which are catalysed by either Mg^{2+} or Mn^{2+} (Pline et al. 1999; Green and Owen 2011; Murray 2013). Glufosinate ammonium is a non-selective, post-emergence applied herbicide since all plant species depend on the enzyme GS for nitrogen metabolism (Green and Owen 2011). The action of glufosinate ammonium is rapid and significant injury on treated plants can be observed within 24 to 48 h. Death of the plant is a result of a build-up of ammonia in plant cells and depletion of crucial amino acids (Mersey et al. 1990; Pline et al. 1999). However, various studies have argued that accumulation of ammonia is not the main cause of photosynthesis inhibition post glufosinate ammonium application (Wild et al. 1987; Takano et al. 2019). Although a rapid phytotoxic action is observed with glufosinate ammonium, plant factors and environmental conditions have been reported to influence the herbicide's efficacy. Plant processes affected range from herbicide absorption to its mechanism-of-action at the target enzyme, mainly because plant morphology and physiology are greatly influenced by environmental factors such as temperature, humidity and light (Steckel et al. 1997).

Plant factors influencing efficacy of glufosinate ammonium include physical and biochemical characteristics. Death of susceptible plants is a result of sequential biochemical processes that are activated following penetration of glufosinate ammonium into the plant. The two-step GS enzymatic reaction involves i) the γ -phosphorus of adenosine triphosphate (ATP) is attacked by the ϵ -oxygen of the substrate glutamate to form a γ -glutamyl hydroxamate intermediate and ii) the γ -glutamyl hydroxamate intermediate reacts with ammonia formed by the deprotonation of an ammonium ion (Murray et al. 2013). Analysis of the intermediate product of this process, γ -glutamyl hydroxamate, is therefore, the most direct method of quantifying glufosinate ammonium activity in plants (Seabra et al. 2013; Dayan et al. 2015). An indirect method involves analysing accumulation of ammonia assuming production of ammonia is directly related to the dysfunction of enzyme GS (Petersen and Hurlle 2000). There have been divided positions on the role of glutamate dehydrogenase (GDH, L-glutamate:NAD⁺-oxidoreductase; EC 1.4.1.2) in ammonium assimilation as an alternative pathway when GS is inactive, however, various studies have confirmed an increase in GDH activity with increasing ammonium concentration (Stewart et al. 1995). A decrease or increase in NADH is directly proportional to the activity of GDH. Glutamate is oxidatively deaminated by GDH, yielding NH_4^+ and regenerating α -ketoglutarate (Cooper and Jeitner 2016). The process is reversible with the enzyme GDH catalysing amination of α -ketoglutarate to glutamate. **Table 4.1** shows the biosynthetic

enzyme reactions catalysed during ammonium metabolism. A simplified illustration of the nitrogen pathway and enzymes involved is shown in **Figure 4.1**.

Table 4.1: Biosynthetic reactions catalysed by glutamate dehydrogenase (GDH), glutamine synthetase (GS), glutamate dehydrogenase (GOGAT) and glutamine synthetase/glutamate dehydrogenase (GS/GOGAT)

Enzyme	Biosynthetic reaction
GDH	$\text{NH}_4^+ + 2\text{-oxoglutarate} + \text{NADH} \rightarrow \text{glutamate} + \text{NADP}^+$
GS	$\text{NH}_4^+ + \text{glutamate} + \text{ATP} \rightarrow \text{glutamine} + \text{ADP} + \text{Pi}$
GOGAT	$\text{Glutamate} + 2\text{-oxoglutarate} + \text{NADH} \rightarrow 2\text{-glutamate} + \text{NADP}^+$
GS/GOGAT	$\text{NH}_4^+ + 2\text{-oxoglutarate} + \text{ATP} + \text{NADH} \rightarrow \text{glutamate} + \text{ADP} + \text{Pi} + \text{NADP}^+$

Table adapted from (Heeswijk et al. 2013).

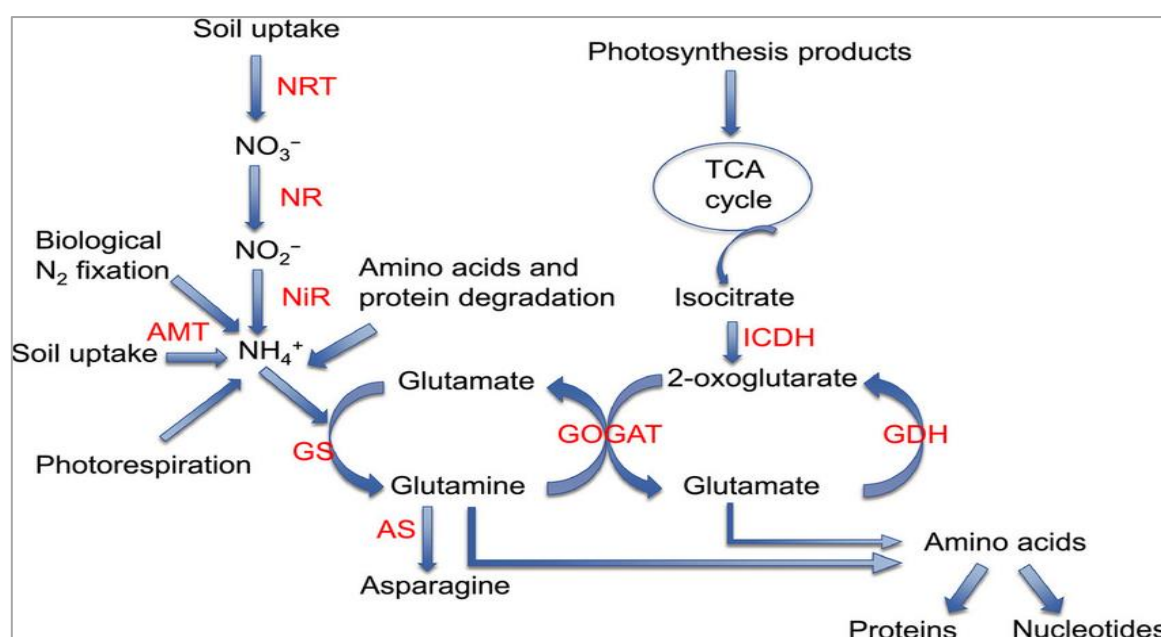


Figure 4.1: Nitrogen-assimilation pathway in higher plants. Enzymes involved include; nitrate transporters (NRT), nitrate reductase (NR), nitrite reductase (NiR), ammonium transporters (AMT), glutamine synthetase (GS), glutamate synthase (GOGAT), asparagine synthetase (AS), glutamate dehydrogenase (GDH), and isocitrate dehydrogenase (ICDH). Adapted from Lu et al. (2016).

Chapter 3 of this dissertation reported significantly improved efficacy of glufosinate ammonium in controlling ryegrass (*Lolium* spp.) at cooler temperatures. That chapter described how plant physical factors contribute to better control of ryegrass at cooler temperatures (10/15 and 15/20 °C). The present investigation further investigated the influence of the above-mentioned biochemical pathways on glufosinate ammonium efficacy in ryegrass at different temperatures. The aim was to determine if temperature influences metabolic reactions after herbicide application, and hence, to deduce if such effects either increase or decrease herbicide efficacy under the different temperature regimes.

4.2 MATERIALS AND METHODS

Glufosinate susceptible wild ryegrass (*Lolium* spp.) seed was harvested on Welgevallen Experimental Farm, dried and four seeds were planted per 0.8 × 0.8 cm pot filled with river sand as growing medium. The pot represented one treatment replicate. Experiments were conducted at 10/15, 15/20, 20/25 and 25/30 °C (12/12 h night/day) temperature regimes in glasshouses at Welgevallen Experimental Farm, Stellenbosch University, in the Department of Agronomy (33° 56'33" S and 18° 51'56" E). Ryegrass plants were fertigated four times a day to prevent nutrient and drought stress (refer to **Table 3.1**). Irrigation was adjusted according to plants' requirements as they grew. Application of glufosinate ammonium was done when the ryegrass was at the 4- to 6-leaf stage. A single foliar application of glufosinate ammonium (200 g L⁻¹, glufosinate ammonium, SL) was applied at 0, 1.5, 3, 4.5 and 6 L ha⁻¹ (0, 300, 600, 900, 1200 g a.i ha⁻¹) dosage rates. Relative humidity in the glasshouses ranged from 75% to 90%. For enzyme assays (GS, aminating/deaminating GDH and nitrate reductase) and ammonia accumulation, four pots from two treatments under each temperature regime were randomly chosen 24 hours after application (HAA), i.e. control plants and plants treated with 4.5 L ha⁻¹ glufosinate ammonium. A dosage rate of 4.5 L ha⁻¹ was chosen because the effect of different temperature regimes on ryegrass was more significant when treated with 4.5 L ha⁻¹ (Mucherri 2016). The plants were then sampled by carefully extracting them from the soil and rinsing the root system to get rid of soil. The roots and above-ground parts of plants were placed in separate storage bags. The separated plants sections were quickly placed in liquid nitrogen and stored in a freezer at -80 °C.

4.2.1 Glutamine synthetase enzyme activity assay

Crude protein extraction: Frozen fresh ground roots and leaves (0.5 g) material was homogenized in 2.0 ml extraction buffer. The homogenate was then centrifuged at 13 000 xg (RCF) for 20 min at 4 °C and the supernatant was removed. The supernatant was used as crude protein extract for the GS enzyme assay.

Synthetase (GSs) assay: A total volume of 250 µl per well in plate was made by adding 30 µl crude protein extract to 220 µl synthetase assay buffer. Blank reactions included: water, assay buffer with no adenosine triphosphate (ATP) and assay buffer with no glutamate. Absorbance of reactions was measured at 500 nm using a BioTek PowerWave™ XS Microplate Spectrophotometer continuously for 5 min at 30 °C. Compound measured;

Reaction intermediate- γ -glutamyl hydroxamate - extinction coefficient= 850 M⁻¹ cm⁻¹.

Glutamine synthetase was expressed as μmol γ -glutamyl hydroxamate per gram root fresh weight sample ($\mu\text{mol g}^{-1}$ FW).

Extraction buffer: 10 mM Tris (pH 7.5), 5 mM sodium glutamate, 10 mM MgSO_4 , 1 mM DTT, 10% (v/v) glycerol, 0.05% (v/v) Triton X-100 and Roche protease inhibitor tablet.

Synthetase assay buffer: 100 mM Tris (pH 7.8), 100 mM glutamate, 8 mM ATP, 8 mM hydroxylamine and 16 mM MgSO_4 .

4.2.2 Aminating and deaminating glutamate dehydrogenase activity assays

Crude protein extraction: Crude enzyme extraction for aminating and deaminating GDH activity assays was done using the same protocol (section 4.2.1). Plant leaves and roots were ground in liquid nitrogen and homogenized in 50 mM KH_2PO_4 buffer (pH 7.5). The ground material (150 mg) was added to 1.5 ml extraction buffer. The homogenate was then centrifuged at (RCF) 3 000 xg for 5 min at 4 °C. The supernatant (1 ml) was collected and centrifuged at (RCF) 15 000 xg for 40 min at 4 °C. The extracted crude protein was used for aminating and deaminating GDH enzyme assays.

Crude extraction buffer: 2 mM EDTA, 1.5% (w/v) soluble casein, 2 mM dithiothreitol (DTT), 1% (w/v) insoluble polyvinylpyrrolidone (PVP), one complete protease inhibitor cocktail tablet (Roche) per 50 ml.

a) Aminating glutamate dehydrogenase assay buffer

The assay buffer was prepared as explained by Glevarec et al. (2004). Crude protein (30 μl) was added to 100 mM Tris-HCl buffer (pH 8) to make a total volume of 250 μl . Oxidation of NADH was measured by reading absorbance at 340 nm for 5 min. A BioTek PowerWave™ XS Microplate Spectrophotometer was used to measure absorbance. Aminating GDH was expressed as NADH $\mu\text{mol g}^{-1}$ FW.

Crude extraction buffer: 100 mM Tris-HCl buffer (pH 8.0), 1 mM CaCl_2 (pH 8.0), 13 mM 2-oxoglutarate (α -Ketoglutaric Acid), 50 mM $(\text{NH}_4)_2\text{SO}_4$ and 0.25 mM NADH.

b) Deaminating glutamate dehydrogenase assay buffer

The assay buffer was prepared as explained by Glevarec et al. (2004). Crude protein (30 μl) was added to 100 mM Tris-HCl buffer (pH 9) to make a total volume of 250 μl . Reduction of NADH (an increase in NADH) was measured by reading absorbance at 340 nm for 5 min. A BioTek PowerWave™ XS Microplate Spectrophotometer was used to measure absorbance. Deaminating GDH was expressed as NADH $\mu\text{mol g}^{-1}$ FW.

Crude extraction buffer: 100 mM Tris-HCl buffer (pH 9.0), 1 mM CaCl₂ (pH 9.0), 33 mM glutamate and 0.25 mM NAD⁺.

c) Relative aminating/deaminating GDH activity model

A model to determine the most significant enzyme reaction between aminating and deaminating GDH was designed as follows;

$$\text{Relative NADH per fresh gram sample, } Z = \frac{\text{Aminating GDH (NADH } \mu\text{mol g}^{-1}\text{ FW)}}{\text{Deaminating GDH (NADH } \mu\text{mol g}^{-1}\text{ FW)}}$$

Where, if $Z > 1$ then aminating GDH activity is significantly higher than deaminating GDH, if $Z < 1$ then deaminating GDH activity is significantly higher than aminating GDH and if $Z = 1$ then aminating and deaminating GDH processes are at equilibrium.

4.2.3 Ammonium accumulation

Determination of ammonia accumulation was done using methods explained by D'Halluin et al. (1992), Zhou and Wang (2006), and Avila-Garcia and Mallory-Smith (2011). Freshly ground leaves and roots (250 mg) were ground in liquid nitrogen. Thereafter, 1 ml polyvinylpolypyrrolidone (PVPP) solution (50 mg Insoluble PVPP in 1 ml per sample) was added and centrifuged at 12 000 xg (RCF) for 5 min at 4 °C. The supernatant (300 µl) was then diluted with 700 µl distilled water. The diluted supernatant (100 µl) was added to 1.5 ml **Reagent A** and solution was mixed. **Reagent B** was added to the diluted solution plus Reagent A and incubated for 15 min at 37 °C. A total of 250 µl final solution of each treatment was added per well in the microplates. An end point absorbance was measured at 625 nm against a set up standard with 0.004 – 0.4 mg ammonium chloride (NH₄Cl: Mr = 53.49 g/mol) using a BioTek PowerWave™ XS Microplate Spectrophotometer.

Preparation of **Reagent A and B**;

Reagent A: 5 g phenol with 25 mg sodium nitroprusside in 500 ml distilled water (1.5 ml per sample).

Reagent B: 2.5 g sodium hydroxide with 1.6 ml sodium hypochlorite (new bottle) in 500 ml distilled water.

4.2.4 Nitrate reductase activity analysis

Crude protein extraction was done by homogenizing ground leaves and roots in extraction buffer (4 ml extraction buffer per gram tissue). Homogenate was centrifuged at 16 000 xg at 4 °C for 5 min and 100 µl supernatant was incubated with 900 µl reaction buffer at 30 °C for

30 min. The reaction medium of total volume 300 μ l was then incubated with 1 ml absorbance buffer at room temperature for 15 min. Absorbance was read at 540 nm to determine product (NO_2) formed at room temperature using a BioTek PowerWave™ XS Microplate Spectrophotometer.

Extraction buffer: 100 mM HEPES-KOH pH 7.6 (N -2-hydroxyethylpiperanzine- N -2-ethanesulfonic acid), 3 mM DTT (dithiothreitol), 10 μ M FAD (flavin adenine dinucleotide), 2 mM EDTA, 10% (v/v) glycerol, 2% (w/v) Casein, 2.5% (w/v) PVPP (polyvinylpolypyrrolidone), 1 μ M sodium molybdate, 1 complete protease inhibitor cocktail tablet (Roche) per 50 ml buffer.

Reaction buffer: 100 mM HEPES-KOH pH 7.6 (N -2-hydroxyethylpiperanzine- N -2-ethanesulfonic acid;), 1 mM DTT (dithiothreitol), 10 μ M FAD (flavin adenine dinucleotide), 20 mM KNO_3 , 200 μ M NADH (β -nicotinamide adenine dinucleotide).

Absorbance buffer: 1 sulphanilamide: 1 NED

(1% (w/v) sulphanilamide in 1.5 M HCl): (0.01 % (w/v) NED (N-(1-naphthyl) ethylenediamine hydrochloride).

4.2.5 Photosynthetic measurements

Spot measurements were done to measure photosynthesis (net assimilation rate, $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) 24, 48 and 72 hours after glufosinate ammonium application (HAA) using an IRGA (Infra-red Gas Analyser- LI 6400). The measurements were done on a randomly selected plant for each treatment. The reference CO_2 was set at 400 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ air, temperature was set at 25 °C, molar flow rate (F) of air entering the leaf chamber was set at 500 $\mu\text{mol s}^{-1}$ and photosynthetically active radiation (PAR) was set at 1500 PAR. Negative photosynthesis recorded for some treated plants denote that the plants were respiring more than photosynthesizing thus negative net assimilation rate represent reduced photosynthesis to less than 0% (Haworth et al. 2018).

4.2.6 Statistical analysis

The data for all the analyses was subjected to ANOVA using the STATISTICA statistical program and means were separated using Tukey's honest significant difference (HSD).

4.3 RESULTS

4.3.1 Glutamine synthase enzyme activity assay

Glutamine synthetase activity was determined by quantification of glutamyl hydroxamate produced. An increased production of γ -glutamyl hydroxamate indicates increased GS activity, and *vice versa*. According to **Figure 4.2**, activity of GS in leaves decreased as

temperatures increased for control plants. An opposite trend was observed with glufosinate ammonium treated plants; an increase in GS activity was observed with increasing temperatures. For control leaves, activity of GS enzyme at 10/15 and 15/20 °C was not significantly different. The activity at 10/15 and 15/20 °C was, however, significantly higher from GS activity at 20/25 and 25/30 °C. No significant differences in GS activity were observed in ryegrass between temperature regimes of 20/25 and 25/30 °C. For treated leaves, the highest GS activity was observed at 25/30 °C. The activity was significantly different from that observed for the rest of the temperatures. There were no significant differences shown by GS activity in glufosinate ammonium treated leaves at 10/15, 15/20 and 20/25 °C.

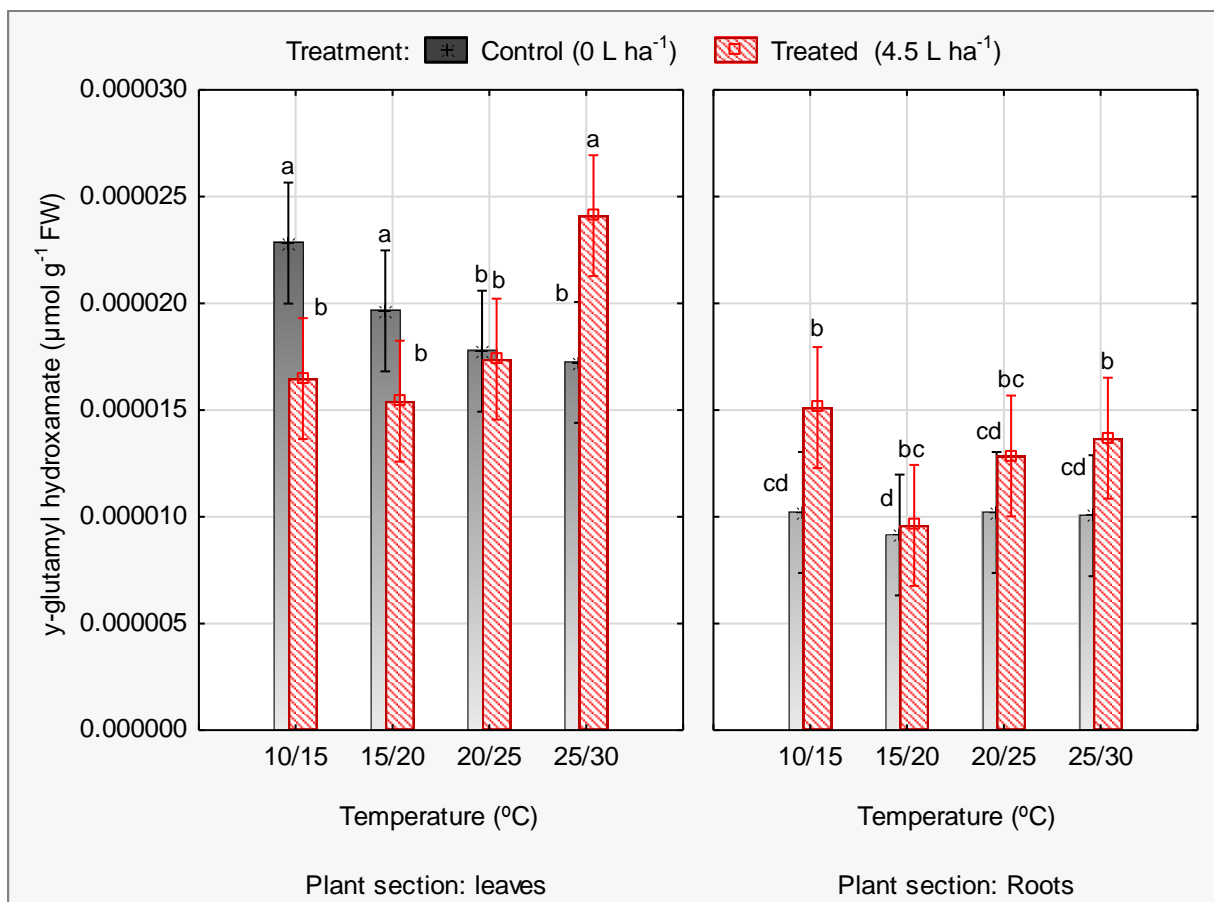


Figure 4.2: Weight of intermediate γ -glutamyl hydroxamate per gram of fresh leaves and roots (μmol) in control and treated ryegrass plants harvested 24 hours after glufosinate ammonium application. Error bars represent \pm standard error of the mean of four replications ($n = 4$).

Generally, activity of GS in control plant roots was significantly lower than in the leaves. Glutamine synthetase activity in roots did not show any significant differences between control and treated plants at 15/20 and 20/25 °C temperatures. Glufosinate ammonium application significantly influenced the activity of GS in roots at 10/15 and 25/30 °C, but not at 15/30 and 20/25 °C.

4.3.2 Aminating and deaminating glutamate dehydrogenase activity assays

The activity of GDH was determined by either the oxidation of NADH or reduction of NAD⁺. Oxidation of NADH yielded NAD⁺ while the reduction process yielded NADH. The graphs in **Figure 4.3** depict the activity of GDH in ryegrass leaves before and after glufosinate ammonium application.

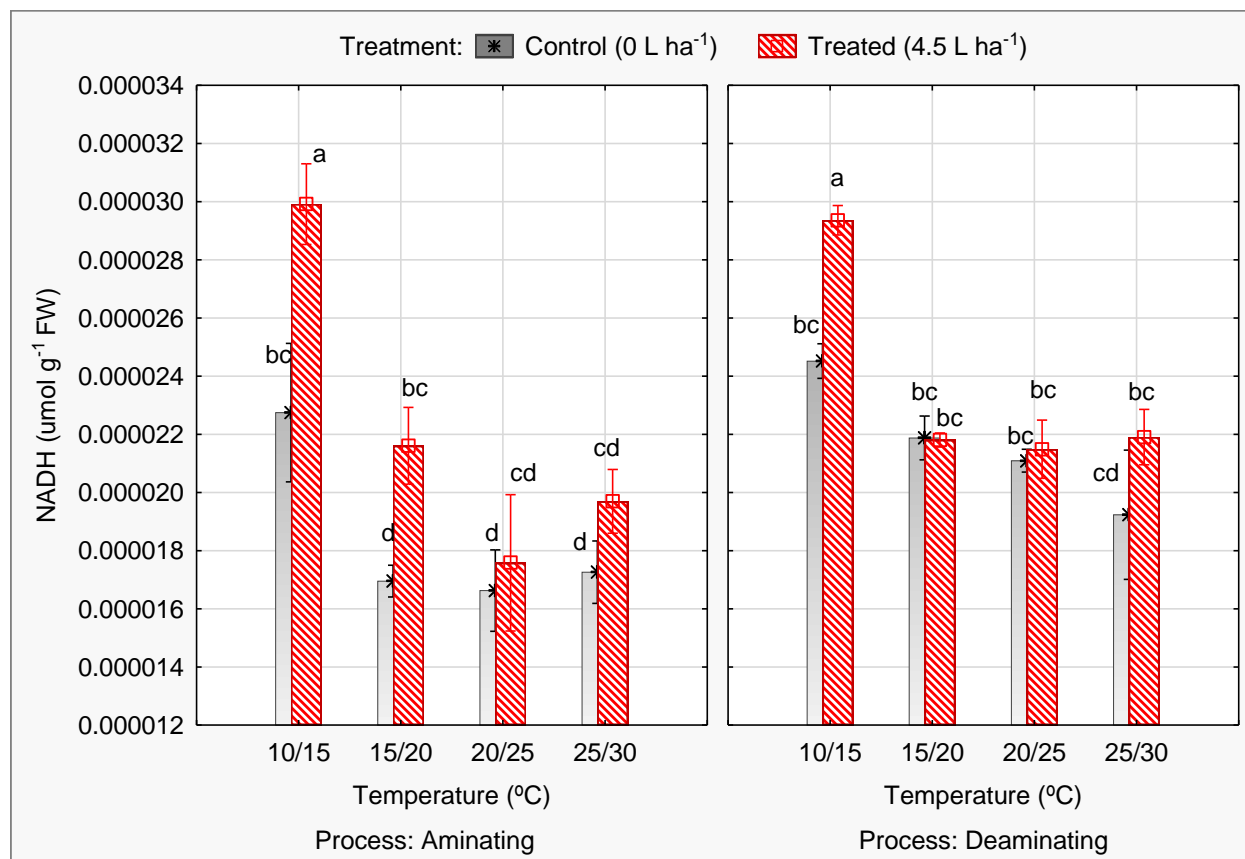


Figure 4.3: Weight of intermediate NADH per gram of fresh leaves (μmol) in control and treated ryegrass at different temperatures harvested 24 hours after glufosinate ammonium application. Error bars represent \pm standard error of the mean of four replications ($n = 4$).

For control plants, amination of α -ketoglutarate was significantly higher at 10/15 °C than the rest of the temperature regimes. The amination process was not significantly different at 15/20, 20/25 and 25/30 °C. Treatment with glufosinate ammonium resulted in a significant increase in aminating GDH activity at 10/15 and 15/20 °C temperatures. Activity of aminating GDH was not significantly different between control and treated leaves at 20/25 and 25/30 °C. There were no significant differences shown by control plants in the deamination process of glutamate to α -ketoglutarate at 10/15, 15/20 and 20/25 °C. The lowest activity was noted at 25/30 °C and it was significantly different to the rest of the temperatures. Treatment of plants by glufosinate ammonium did not influence the activity of deaminating GDH at 15/20, 20/25 and 25/30 °C, hence, there were no significant differences in the enzyme activity observed in control and treated plants. The activity of GDH of treated plants at 15/20, 20/25

and 25/30 °C did not differ significantly from each other but differed significantly from the activity of plants at 10/15 °C.

Figure 4.4 shows the relationship of aminating and deaminating GDH in leaves after using the comparative model in subsection 4.2.2 c of Chapter 4. Almost all the treatments showed an aminating/deaminating GDH ratio of less than 1, meaning that deaminating GDH activity was greater than the aminating GDH activity (as explained in 4.2.2 c). The only treatment that resulted in a ratio above 1 was the treated ryegrass at 10/15 °C. The ratio of aminating/deaminating GDH in control and treated leaves at 20/25 and 25/30 °C did not show significant differences. Application of glufosinate ammonium had influence on the ratio of aminating/deaminating GDH at 10/15 and 15/20 °C. There was a significant increase in aminating GDH activity after herbicide application. There were no significant differences shown between the aminating/deaminating GDH ratio of control ryegrass plants at 10/15 and 25/30 °C. Control plants at 15/20 and 20/25 °C did not show any significant differences. For treated plants, no significant difference was shown by ryegrass grown at 10/15, 15/20 and 25/30 °C.

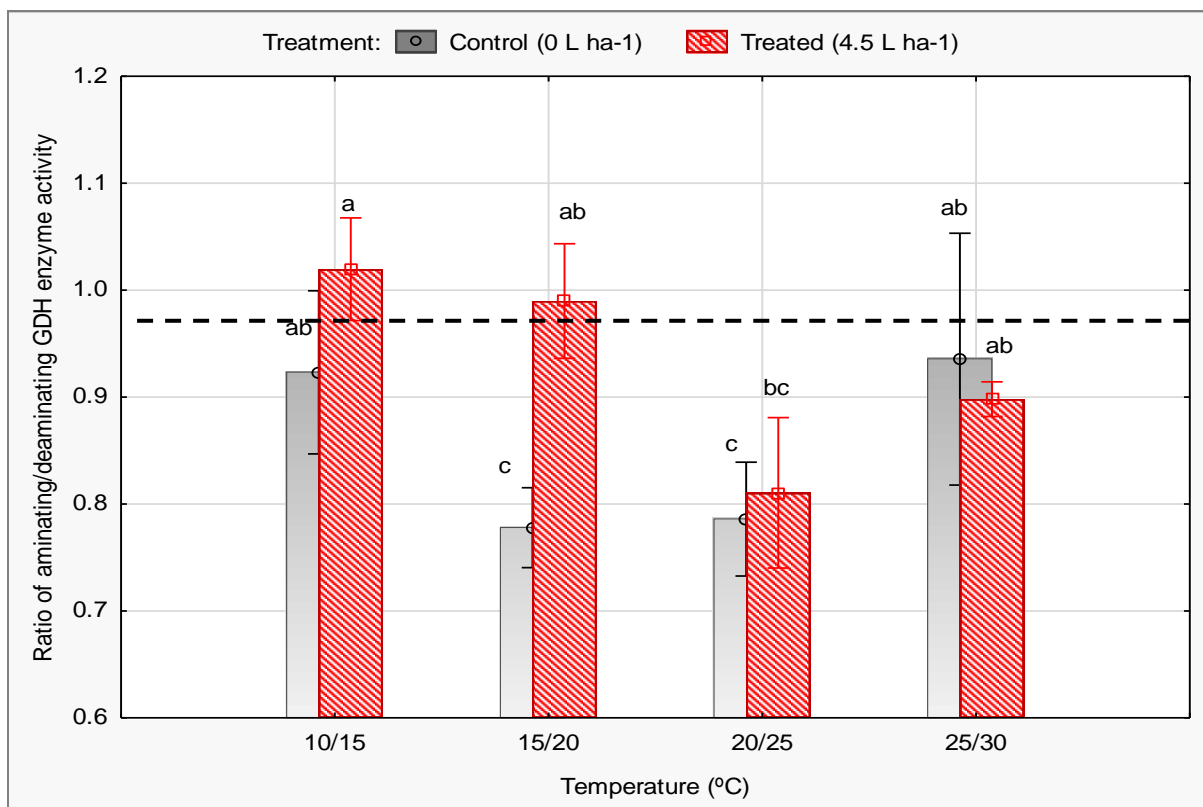


Figure 4.4: Ratio of aminating/deaminating GDH activity in control and treated ryegrass leaves with glufosinate ammonium at different temperatures and harvested 24 hours after glufosinate ammonium application. Error bars represent \pm standard error of the mean of four replications ($n = 4$).

4.3.3 Ammonium accumulation

Figure 4.5 shows the effect of temperature, plant section (leaves or roots) and glufosinate ammonium dosage rate on ammonia accumulation 24 hours after application. The three-way interaction was not significant ($p > 0.05$) (see **Appendix 4.4**).

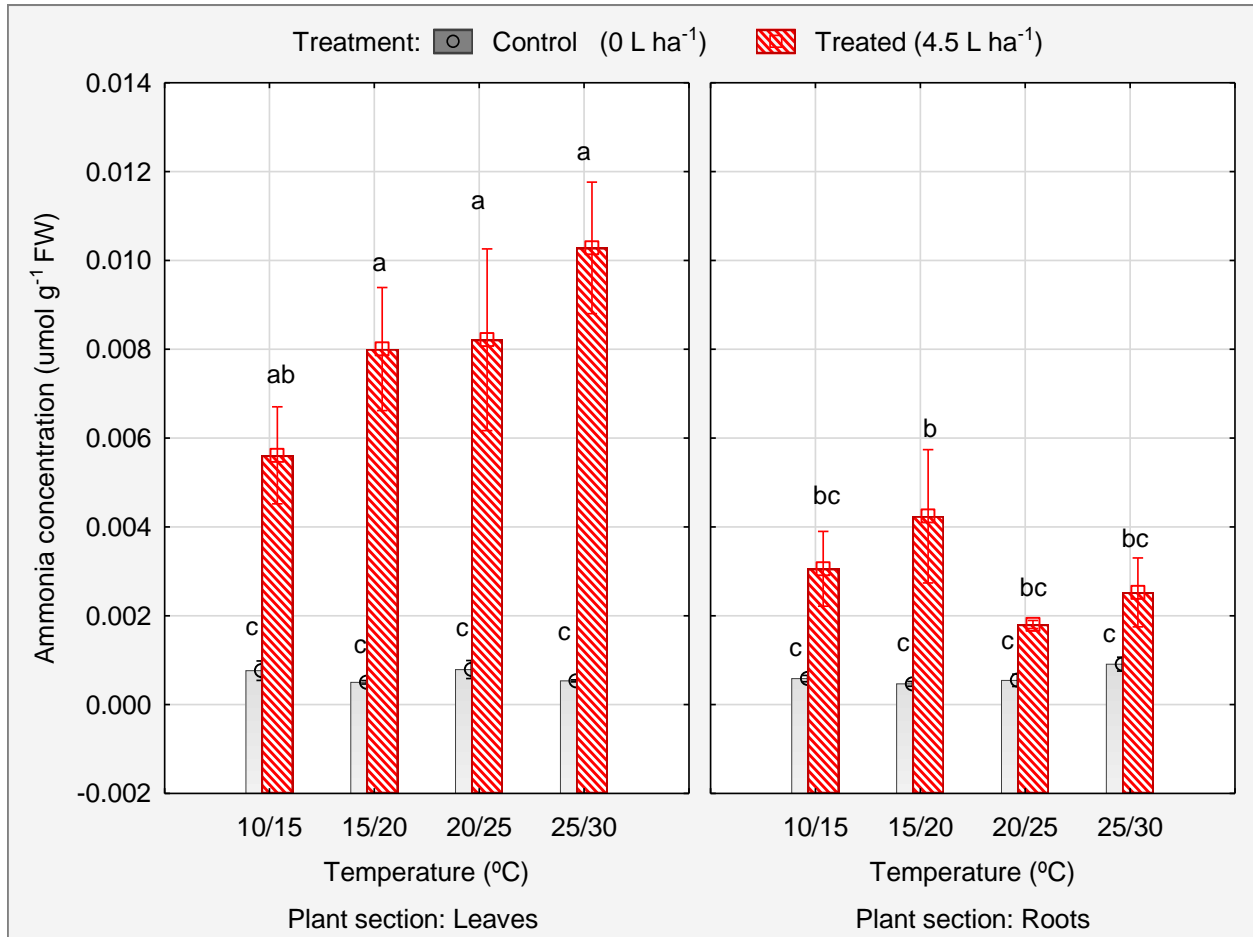


Figure 4.5: Concentration of ammonium ($\mu\text{mol g}^{-1}$ FW) detected in the control and glufosinate ammonium treated ryegrass leaves at different temperatures and harvested 24 hours after application. Error bars represent \pm standard error of the mean of four replications ($n = 4$).

There was a greater and significant increase in ammonia in treated leaves compared to the increase in treated roots. Ammonium concentration in treated leaves showed a general increase with increasing temperature. The lowest ammonium accumulation observed in treated ryegrass leaves was at 10/15 °C and was significantly different from all the other temperatures. No significant differences were shown by ammonia accumulation in ryegrass treated leaves at 15/20, 20/25 and 25/30 °C. Ammonium concentration in the untreated leaves and roots did not show any significant differences across all temperatures. No significant differences were observed in the treated roots

4.3.4 Nitrate reductase activity analysis

Nitrogen assimilation in ryegrass leaves and roots was determined by analysing production of nitrous oxide (NO_2). Production of NO_2 indicated the activity of nitrate reductase enzyme and, hence, nitrogen assimilation (**Figure 4.6**). Nitrogen assimilation was not significantly different between control and treated plant across almost all temperatures in both leaves and roots. Nitrogen assimilation was significantly higher in treated compared to control roots at 10/15 °C. For both control and treated ryegrass, nitrogen assimilation was high at 10/15 °C and significantly different from the rest of the temperatures. There were no significant differences in nitrogen assimilation observed at 15/20, 20/25 and 25/30 °C.

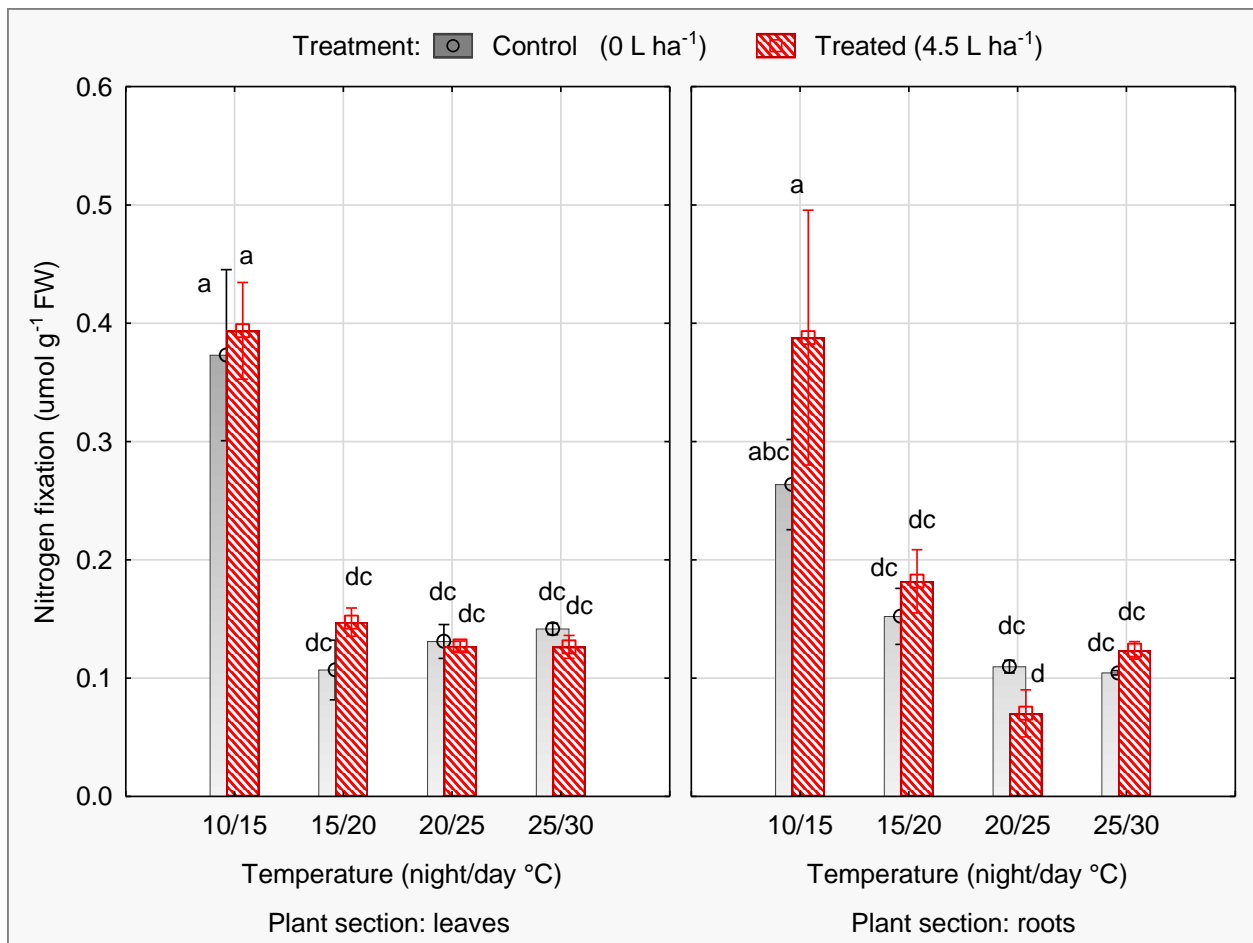


Figure 4.6: Weight of NO_2 ($\mu\text{mol g}^{-1}$ FW) in control and glufosinate ammonium treated ryegrass at different temperatures. Error bars represent \pm standard error of the mean of four replications ($n = 4$).

4.3.5 Photosynthesis study

Measurements of photosynthesis 24, 48 and 72 hours after glufosinate ammonium application are shown in **Figure 4.7**. Results showed a drastic decrease in photosynthesis 24 hours after glufosinate ammonium application across all temperature regimes which resulted in negative net assimilation rate. Positive values were observed for all the temperatures across all dosage rates after 48 and 72 hours. Increase in photosynthesis 48

and 72 HAA was significantly higher at 20/25 and 25/30 °C. For control plants, photosynthesis rate of ryegrass grown at 25/30 °C was significantly lower than the rest of the temperatures.

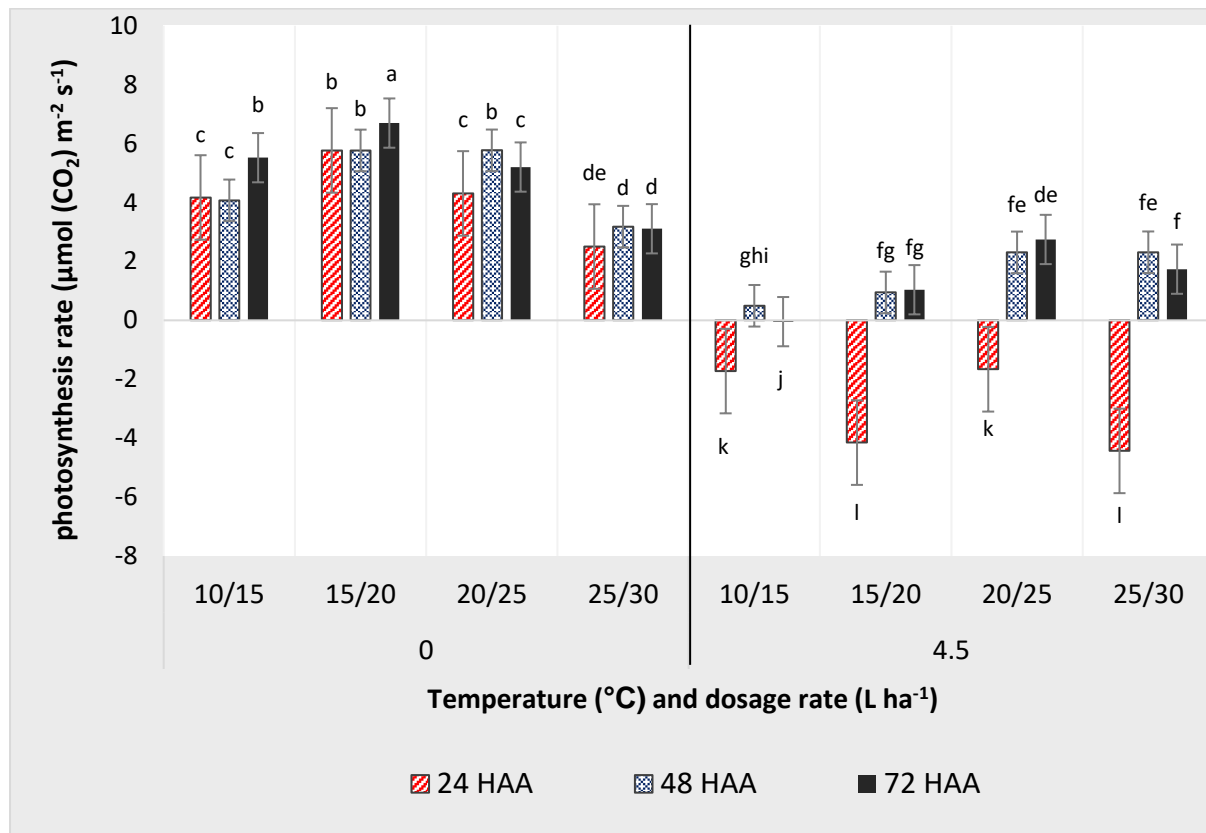


Figure 4.7: Photosynthesis rate ($\mu\text{mol (CO}_2\text{) m}^{-2} \text{s}^{-1}$) of control (untreated) and treated ryegrass at different temperatures 24, 48 and 72 hours after application. Error bars represent \pm standard error of the mean three spot measurement replications ($n = 3$). Values followed by the same letters indicate no significant differences between treatments at $P \leq 0.05$.

4.4 DISCUSSION

Molefe (2015) and Mucheri (2016) found that glufosinate ammonium efficacy on ryegrass decreases with increasing temperature. Those studies were done on ryegrass at 10/15, 15/20, 20/25 and 25/30 °C - better control of ryegrass was observed at 10/15 and 15/20 °C compared to control at 20/25 and 25/30 °C. Chapter 3 of this dissertation reported similar results. Various studies have reported that plant factors and environmental factors influence activity of glufosinate ammonium (Coetzer et al. 2001; Everman 2008). Biochemical factors were, therefore, the focus of investigation in the present study.

The most effective pathway used by higher plants to detoxify ammonia released from amino acid degradation, nitrate reduction and photorespiration is the GS/GOGAT pathway (Coetzer et al. 2001). A correlation was observed between glutamine synthase activity in treated ryegrass and glufosinate ammonium efficacy (refer to **Chapter 3**) under different temperatures. An increase in GS activity after treatment with glufosinate ammonium corresponded with decreased control of ryegrass at warmer temperatures. The study suggests that ryegrass at warmer temperatures was able to tolerate better the effect of the herbicide. A drastic increase of GS enzyme activity in leaves at 25/30 °C after treatment might have increased production of glutamate which occupies the central position in amino acid metabolism (**Figure 4.2**). Steady GS activity observed in leaves at 20/25 °C might also have ensured a steady supply of glutamate. This could explain the decreased efficacy of glufosinate ammonium at 20/25 and 25/30 °C. Contrary to the response of ryegrass under warmer temperatures, leaves at 10/15 and 15/20 °C showed a significant decrease in GS activity after glufosinate ammonium treatment. This effect could be the result of plants lacking glutamate, which is required for amino acid metabolism, and eventually leading to the death of plants.

Amination depends on the GOGAT pathway since aminotransferases use glutamate and α -ketoglutarate for transamination reactions (Lasa et al. 2002; Glevarec et al. 2004). Depletion of such carbon skeletons indirectly inhibits photosynthesis and photorespiration. Glutamate dehydrogenase pathway is an alternative route to metabolise ammonia and, in the process, yield glutamate required for photosynthesis (Temple et al. 1998; Miflin 2002; Miyashita and Good 2008). The present study observed a drastic decrease in photosynthesis 24 HAA at lower temperature regimes, (**Figure 4.7**). Plants across all temperatures, however, showed photosynthetic recovery 48 HAA. Various studies have confirmed that ammonia accumulation interferes with metabolism by being mistaken as K^+ ions, consequently inhibiting photo-dependent activation of ATP synthetase, NADP⁺ reduction, chlorophyll biosynthesis and cell division (Wild et al.1987). Although Mersey et al. (1990) and Avila-Garcia and Mallory-Smith (2011) have observed that ammonia levels are a

direct indication of damage caused by glufosinate ammonium, the present study did not observe a correlation between ammonia accumulation and glufosinate ammonium efficacy (refer to **Chapter 3**). Other studies have indicated that ammonia accumulation interferes with photosynthesis but it is not the main cause of death (Wild et al. 1987; Hall et al. 1999, Takano et al. 2019). These studies have reported that the main cause of photosynthesis inhibition after glufosinate ammonium application is depletion of glutamine and accumulation of glyoxylate. In this study, initial accumulation of ammonia probably explains the drastic decrease in photosynthesis 24 HAA. Other factors, such as excess glutamate production, leading to less glyoxylate accumulation, and phenolic compounds (refer to **Chapter 3**) appear to outweigh the influence of ammonia at warmer temperatures, resulting in poor control of ryegrass at warmer temperature.

There were no significant differences in the nitrogen assimilation across all temperatures after glufosinate ammonium application (**Figure 4.6**). This indicates that glufosinate ammonium application had no effect on nitrate reductase, and hence, nitrate metabolism. Since there was no effect on nitrogen assimilation, the plants continued to supply nitrite. Consequently, unceasing reaction of nitrite reductase resulted in ammonia accumulation due to GS inhibition (Refer to **Figure 4.1**). Ammonia concentration in treated ryegrass at warmer temperatures was significantly higher than in ryegrass at 10/15 °C (**Figure 4.5**). The reason for such an observation may be due to the significant increase in GDH activity shown by ryegrass plants at 10/15 °C (**Figure 4.3**). Various studies have found that an increase in ammonia concentration increases the activity of GDH in plants (Frechilla et al. 2002; Lasa et al. 2002). The present study, therefore, suggests that GDH activity was able to convert a significant concentration of ammonia at cooler temperatures even though GS was inhibited. On the contrary, GDH activity was very low in ryegrass at warmer temperatures. The incomparable response of GS at warmer temperatures might have repressed the activity of GDH. Therefore, low GDH activity in ryegrass at warmer temperatures, coupled with inhibition of GS, resulted in higher accumulation of ammonia compared to ammonia accumulation in ryegrass under cooler temperatures which showed higher GDH activity. According to Mersey et al. (1990) and Avila-Garcia and Mallory-Smith (2011), higher ammonia levels at warmer temperatures would have been expected to result in higher control of ryegrass, however, this was not the case in Chapter 3. As discussed above, this study suggests that factors such as glutamate production and phenolic compounds production after glufosinate ammonium application have more influence on mortality of ryegrass than ammonia accumulation.

Extensive studies have shown that the enzyme GDH is regulated by sugar levels in plants (Miyashita and Good 2008). Increase in sucrose level suppresses the activity of GDH,

and *vice versa*, hence indicating that GDH provides an alternative carbon source to the respiratory pathway during sucrose depletion. The present study showed an increase in aminating GDH activity after glufosinate ammonium application. The increase was significant in plants at 10/15 and 15/20 °C. The same temperatures (10/15 and 15/20 °C) resulted in a significant decrease of GS activity after glufosinate ammonium application. Results show that the increase in aminating GDH was related to the decrease in GS. This suggests that the GDH pathway was used as an alternative route for glutamate metabolism in significantly affected plants (Temple et al. 1998; Frechilla et al. 2002).

GDH reversibly converts glutamate to α -ketoglutarate (deaminating GDH) and α -ketoglutarate to glutamate (aminating GDH) (Temple et al. 1998). A comparative model created to determine the predominate reaction between aminating and deaminating GDH before and after glufosinate ammonium application (refer to **Section 4.2.2 c**) showed that deaminating GDH reaction was greater than the aminating GDH across all treatments, except in treated plants at 10/15 °C (**Figure 4.4**). The significant increase in ratio of aminating/deaminating GDH shown at 10/15 and 15/20 °C after glufosinate ammonium application signifies that there was a significant shift from deaminating GDH to aminating GDH. This reaction yields glutamate which is required for photosynthesis; this suggests that the plants were compensating for the depletion in glutamate which forms the basis of gamma-aminobutyric acid, arginine, and proline synthesis (Forde and Lea 2007). Contrary to the reaction shown by plants at 10/15 and 15/20 °C, ryegrass at warmer temperatures (20/25 and 25/30 °C) did not show any significant differences in GDH activity before and after glufosinate ammonium application. This indicates that the significant increase in GS after glufosinate ammonium application was adequate to the extent that the use of the alternative GDH pathway was not vital.

4.5 CONCLUSIONS AND RECOMMENDATIONS

The main aim of the study was to observe if ryegrass physiological factors under different temperatures affect the efficacy of glufosinate ammonium. The results showed that survival of ryegrass is dependent on the continuous production of glutamate for photosynthesis. A significantly higher GS activity and photosynthetic recovery of ryegrass at warmer temperatures (20/25 and 25/30 °C) ensured the survival of plants after treatment, hence, resulting in poor control of ryegrass at such temperatures. Ammonia accumulation has also been reported to be involved in photosynthesis inhibition after glufosinate ammonium application. However, various studies together with the current study have shown that ammonia accumulation is not the main cause of ryegrass death. Continuous production of glutamate is required to avoid photosynthetic inhibition. The drastic and amicable response shown by GS activity at warmer temperatures might suggest that phenolic compounds

discussed in **Chapter 3** had a role to play in the plants' survival. The use of the GDH alternative pathway was not noticed in ryegrass grown at warmer temperatures, whereas its activity increased at cooler temperatures. This was because response of GS to glufosinate ammonium was significantly higher at warmer temperatures, such that the use of the alternative GDH pathway was not vital.

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CHAPTER 5

Efficacy of glufosinate ammonium on three selected grass species as influenced by different temperatures

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ABSTRACT

A comparative study to investigate the response of different grass weed species to glufosinate ammonium was conducted at Welgevallen Experimental Farm, Stellenbosch University. The study was done on ryegrass (*Lolium* spp.), ripgut brome (*Bromus diandrus* L.) and wild oats (*Avena fatua* L.). The grasses were grown at 10/15, 15/20, 20/25 and 25/30 °C (night/day) temperature regimes and treated with 0, 1.5, 3, 4.5 and 6 L ha⁻¹ glufosinate ammonium dosage rates six weeks after transplanting. Ryegrass control increased with decreasing temperature while no significant temperature effects were shown on wild oats. Control of ripgut brome was very poor at 10/15 °C and increased with increasing temperature, only to slightly decrease at 25/30 °C. Differences in control were mainly due to cuticle thickness of the grass species growing at the different temperatures. A negative correlation between cuticle thickness and weed mortality was found, in which thicker cuticles resulted in poor control of grasses. Accumulation of calcium in ripgut brome and wild oats had no influence on efficacy of glufosinate ammonium. The multiple regression analysis showed that there was no correlation between relative percentage photosynthesis (RPP) and mortality of wild oats, whereas, an increase in RPP of ryegrass and ripgut brome resulted in a decrease in mortality of the grass species.

Key words: glufosinate ammonium efficacy, ripgut brome, ryegrass, temperature, wild oats.

5.1 INTRODUCTION

Understanding the principles of weed management is fundamental in agricultural cropping systems, especially with increasing evolution of resistance to most herbicides (Beckie 2006; Powles and Yu 2010). Locally, glufosinate ammonium has lately been given much attention as a preferred herbicide option since no resistance has been documented in South Africa yet. However, glufosinate ammonium has shown inconsistencies in control of several weed species under different environmental conditions (Petersen and Hurlle 2000). Studies investigating the effect of temperature on glufosinate ammonium efficacy have not provided similar and consistent results. Generally, herbicide efficacy increases with increasing

temperature due to increased plant metabolic activities (Varanasi et al. 2016). Although this evidence is supported by Kumaratilake and Preston (2005), Everman (2008) and Everman et al. (2009), other studies have shown that an increase in control of palmer amaranth (*Amaranthus palmeri* S.) and redroot pigweed (*Amaranthus retroflexus* L.) is noted when temperature increases to 21/26 °C night/day and then decreases with increasing temperature, hence, the trend can be defined as hyperbolic (Coetzer et al. 2001). However, some studies have found that increasing temperatures decrease control of ryegrass with glufosinate ammonium (Molefe 2015; Mucheri 2016).

Regardless of the inconsistent efficacy of glufosinate ammonium, Everman (2008) reported that the herbicide displays remarkable results in controlling a variety of weed species, although the response to glufosinate ammonium among weed species varies (Kumaratilake et al. 2002; Everman, 2008). These different tolerance levels are attributed to variability in herbicide uptake, translocation and metabolic degradation amongst weed species. A preceding comparative study conducted by Mucheri (2016) on ryegrass (C₃ temperate grass) and bahia grass (*Paspalum notatum* cv Pensacola) (C₄ tropical grass), showed that there was a general decrease in control of both weed species with glufosinate ammonium as temperature increased. Although not significant for some dosage rates, ryegrass control was more negatively influenced by the higher temperatures than control of Bahia grass. The study did confirm that tolerance of glufosinate ammonium differs among species, and therefore, serves as foundation for this present study. This present study aimed to compare responses of three temperate grass weed species to glufosinate ammonium at different temperatures. The grass weed species studied were ryegrass (*Lolium* spp.), ripgut brome (*Bromus diandrus* L.) and wild oats (*Avena fatua* L.). These three weeds are amongst the most troublesome grass weeds in the western Cape province of South Africa.

5.2 MATERIALS AND METHODS

5.2.1 Mortality rate

The experiment was conducted in glasshouses at Welgevallen Experimental Farm, Stellenbosch University (33° 56'33" S and 18° 51'56" E at an altitude of 136 m above sea level). Experimental design was a completely randomized block design (RCBD) arranged as a 3 x 4 x 5 factorial experiment with main factors species, temperature and glufosinate ammonium dosage rate and all treatment combinations were replicated six times. The glasshouses were set at 10/15 °C, 15/20 °C, 20/25 °C and 25/30 °C (12/12 hr night/day) temperatures regimes. Seed was harvested from known glufosinate-susceptible populations of wild ryegrass, wild oats and ripgut brome from areas around Welgevallen Farm and sown in 8 cm x 8 cm plastic pots filled with river sand in the respective glasshouses. The plants were thinned to four seedlings per pot two weeks after emergence. Irrigation was done with a balanced nutrient solution shown in **Table 3.1**. The plants were irrigated at 8:00 am, 12:00 pm, 2:00 pm and 4:00 pm for 2 min per irrigation. Relative humidity in glasshouses ranged from 75% to 90%. Glufosinate ammonium was applied at the 4- to 6-leaf stage of the grass species at 0, 1.5, 3, 4.5 and 6 L ha⁻¹ dosage rates by means of a pneumatic pot sprayer operating at a pressure of 2.0 bar and delivering 200 L of water ha⁻¹. Mortality of treated plants was assessed four weeks after glufosinate ammonium application. The equation used to determine percentage mortality per pot was;

$$\text{Percentage Mortality} = \frac{\text{number of dead plants in a pot}}{\text{total number of plants per pot (4)}} \times 100\%$$

5.2.2 Structural analysis

Three segments of 2 cm² were cut from actively growing upper, middle and lower leaf section of control ryegrass grown at 10/15, 15/20, 25/30 and 25/30 °C temperature regimes just before herbicide application was carried out. The samples were fixed overnight in 4% (v/v) paraformaldehyde (PFA) with 2% (v/v) glutaraldehyde in 0.1M Buffer, pH 7.4. Thereafter, the samples were subjected to dehydration in increasing ethanol concentrations (30, 50, 70, 90, 95 and 100% (v/v) for 10 mins each). Dehydration in 100% ethanol was repeated for 10 min before placing them in hexamethyldisilazane (HMDS) for 10 min. Samples in HMDS were then left to dry overnight. The dry samples were coated with gold before mounting on a MERLIN scan electron microscope (SEM). Morphological analysis of the cross-sectional view of leaves was done to determine waxy (cuticle) layer thickness. Thickness of the epicuticular wax film plus cutin was determined by a low magnification calibration ruler embedded in the SEM. The total thickness of epicuticular wax film and cutin is referred to as the cuticle thickness in the text.

5.2.3 Photosynthesis study

Spot measurements were done to measure photosynthesis (net assimilation rate, $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) of ryegrass, wild oats and ripgut brome 24, 48 and 72 h after glufosinate ammonium application (HAA) using an IRGA (Infra-red Gas Analyser- LI 6400). The measurements were done on youngest, mid-laminar leaf region of randomly selected plants for each treatment. The reference CO_2 was set at $400 \mu\text{mol CO}_2 \text{ mol}^{-1}$ air, temperature was set at 25°C , molar flow rate (F) of air entering the leaf chamber was set at $500 \mu\text{mol s}^{-1}$ and photosynthetically active radiation (PAR) was set at 1500 PAR. Negative photosynthesis recorded for some treated plants denote that the plants were respiring more than photosynthesizing (Douthe et al. 2018; Haworth et al. 2018). A negative RPP represent reduced photosynthesis to less than 0%. Chapin and Eviner (2003) noted that photosynthetic capacity differs among plant species even when grown under the same conditions, hence, photosynthesis measurements were calculated as a percentage of the photosynthesis of control plants per species (Relative percentage photosynthesis - RPP). The calculation was done using the following formula:

$$\text{Relative percentage photosynthesis} = \frac{\text{Photosynthesis rate of treated plants}}{\text{Photosynthesis rate of control plants}} \times 100 \%$$

5.2.4 Elemental (calcium) analysis

Calcium content in ryegrass, wild oats and ripgut brome was analysed by harvesting a randomly selected replication of control plants on the day of application. The stems and leaves (above ground plant parts) were dried at a constant temperature of 80°C for 48 h in a drying oven. The samples were then ground using a Bate-85588 (10 20 mesh) milling machine and sent for plant analysis at the Institute of Plant Sciences, Western Cape Agricultural Department, Elsenburg.

5.2.5 Statistical analysis

Statistical analysis of experimental data was carried out using the STATISTICA 13 software. ANOVA was used to test for statistical differences on mortality rate, cuticle thickness, net assimilation rate, and calcium content. A regression analysis was done using a simple multiple regression tool in STATISTICA 13 software. Means were separated by Tukey's honest significant difference (HSD) test at 95% confidence interval.

5.3 RESULTS

5.3.1 Mortality rate

According to **Figure 5.1**, mortality rate was dosage rate dependent for all grass species. Control of all weed species increased with an increase in glufosinate ammonium dosage rate. Influence of temperature on efficacy of glufosinate ammonium differed across the grass species. A three-way interaction of grass species, dosage rate and temperature on mortality of grass species was significant ($p < 0.05$). Ryegrass mortality significantly increased with decreasing temperatures at 1.5 and 3 L ha⁻¹ dosage rates. A dosage rate of 6 L ha⁻¹ glufosinate ammonium was required to control 100% of ryegrass grown at 20/25 and 25/30 °C, while 100% control of ryegrass was reached by a dosage rate of 3 L ha⁻¹ at 10/15 and 15/20 °C. The response of ripgut brome to glufosinate ammonium at different temperatures showed a hyperbolic trend. There was poor control of ripgut brome at 10/15 °C, such that the highest dosage rate applied (6 L ha⁻¹) resulted in less than 60% control of the grass. A significant increase in control of ripgut brome was noticed at 15/20 and 20/25 °C. Mortality of ripgut brome at 15/20 °C reached 100% only when treated with 6 L ha⁻¹ glufosinate ammonium, while 100% control was achieved with 3 L ha⁻¹ at 20/25 °C. Although not significant, an increase in temperature to 25/30 °C showed a decrease in the control of ripgut brome, with control failing to reach 100% even at 6 L ha⁻¹ of glufosinate ammonium. Unlike ryegrass and ripgut brome, mortality of wild oats did not show any significant differences across all temperature regimes. Glufosinate ammonium dosage rate of 3 L ha⁻¹ achieved 100% control of this grass species across all temperature regimes.

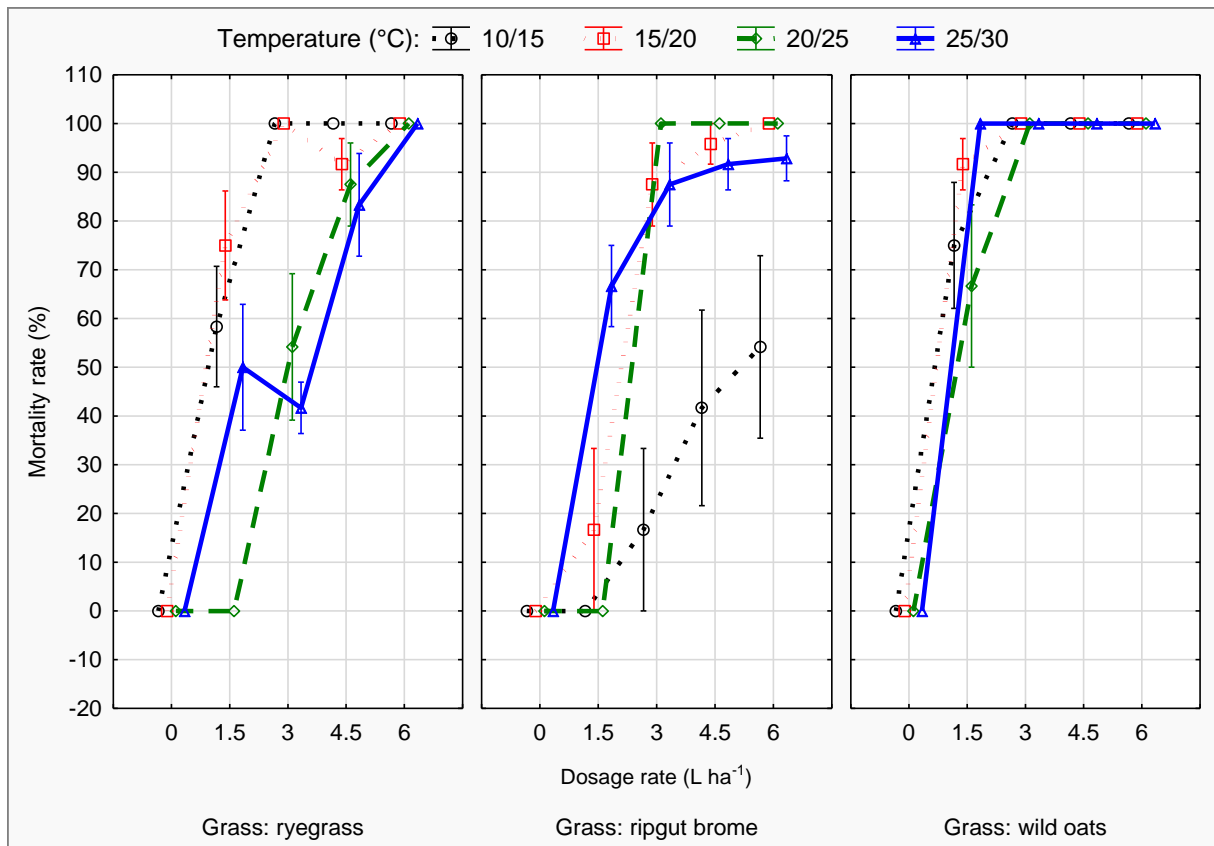


Figure 5.1: The effect of temperature and dosage rate on glufosinate ammonium efficacy on ryegrass, ripgut brome and wild oats. Results are means of two experiments with six replications each ($n = 6$). Vertical bars denote \pm standard error of the mean.

5.3.2 Structural analysis

Figure 5.2 shows cross sectional images of control ryegrass, ripgut brome and wild oats of three segments excised from each grass species obtained from the SEM. A detailed analysis of the grass species cuticle thickness is presented in **Figure 5.3**. Cuticle thickness of ryegrass, ripgut brome and wild oats showed no significant differences ($p > 0.05$) between all temperatures and grass species.

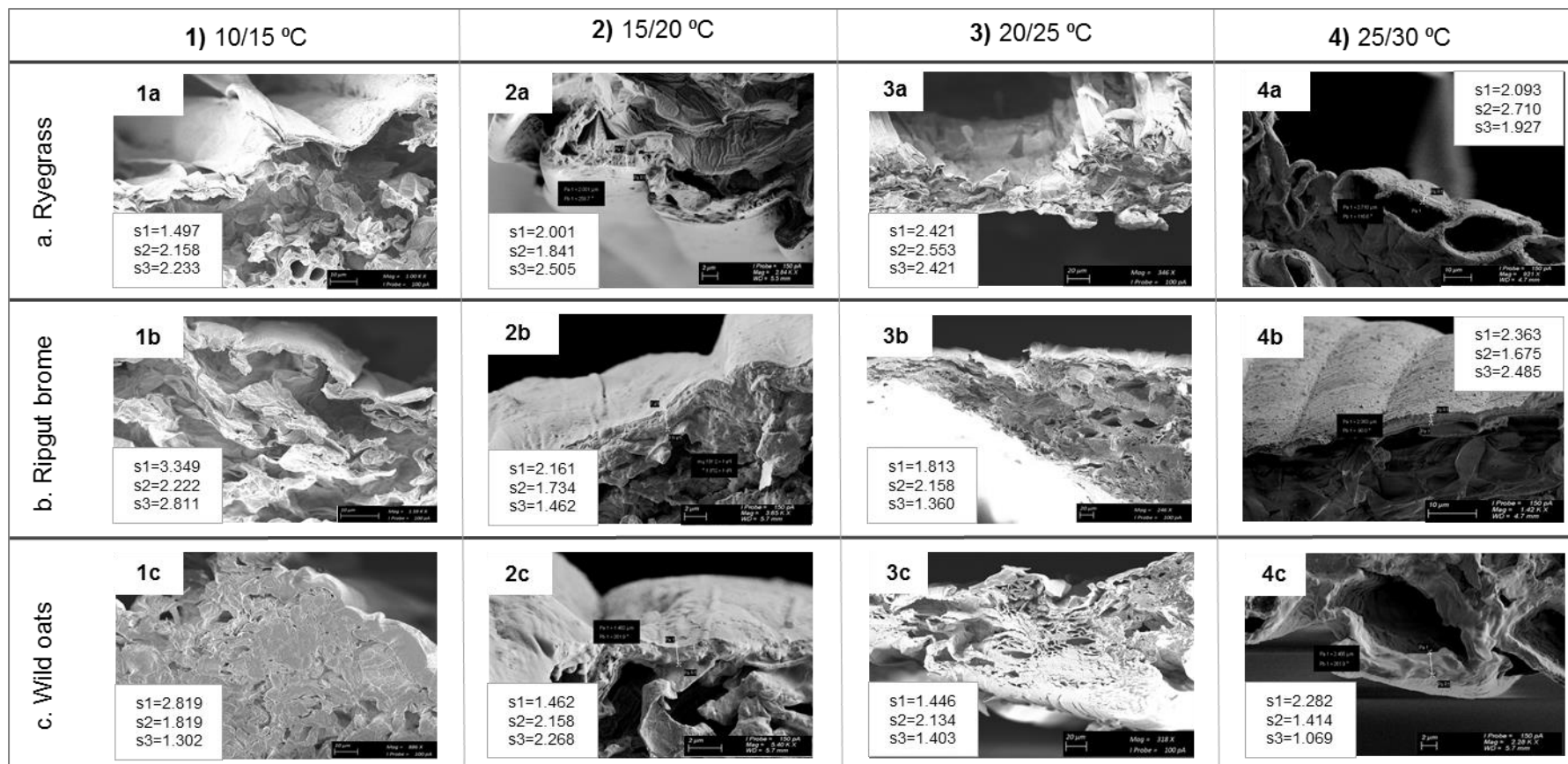


Figure 5.2: Scanning electron microscope images of control treatments of, a) ryegrass, b) ripgut brome and c) wild oats grown at 1) 10/15, 2) 15/20, 3) 20/25 and 4) 25/30 °C, respectively. Cuticle thickness measured from the three segments of each grass species are denoted by s1, s2 or s3.

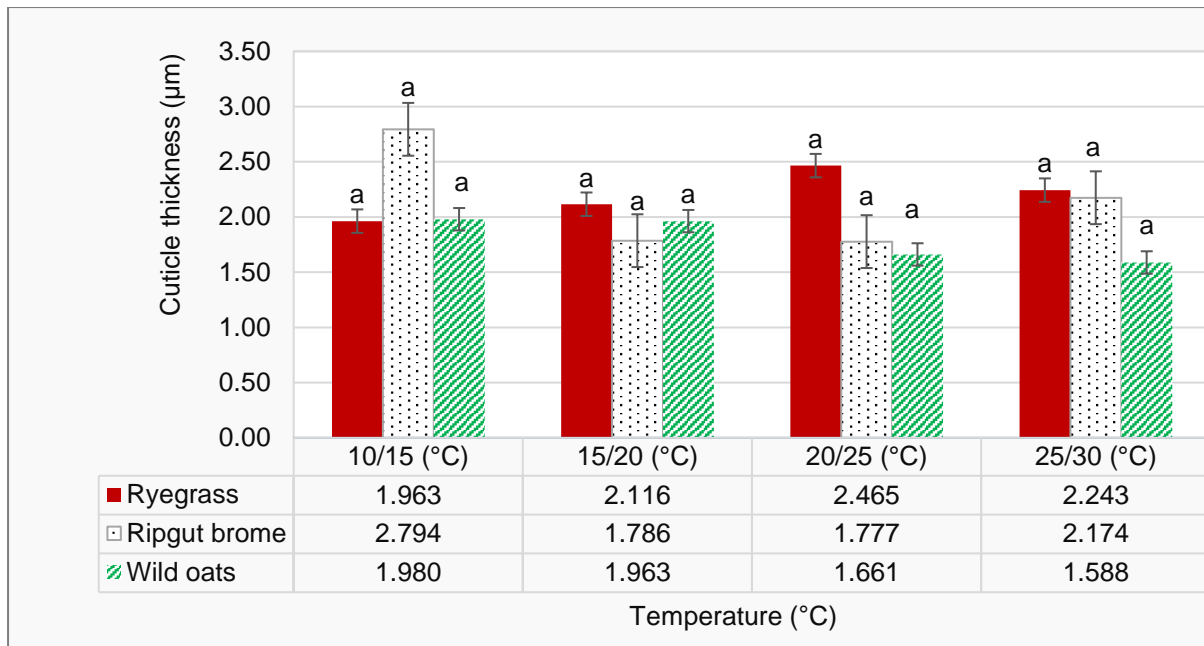


Figure 5.3: The effect of temperature on cuticle thickness of control ryegrass, ripgut brome and wild oats grown at 10/15, 15/20, 20/25 and 25/30 °C. Vertical bars denote \pm standard error of the mean of three replications ($n = 3$).

Table 5.1 further shows the influence of grass species on cuticle thickness among the grass species. Statistically, there were no significant differences of cuticle thickness between the grass species. However, although not statistically significant, ryegrass had the thickest cuticle of 2.197 μm , followed by ripgut brome and finally wild oats. No significant differences were shown by cuticle thickness between temperature regimes (**Table 5.2**).

Table 5.1: Cuticle thickness of grass weeds as influenced by weed species

	Cuticle thickness (μm)		
Factor	Ryegrass	Ripgut brome	Wild oats
Weed species	2.197 ^a	2.133 ^a	1.798 ^a

Values in a row followed by the same letters indicate no significant differences between treatments at $p = 0.05$.

Table 5.2: Cuticle thickness of grass weeds as influenced by different temperature regimes

	Cuticle thickness (μm)			
Factor	10/15 °C	15/20 °C	20/25 °C	25/30 °C
Temperature	2.246 ^a	1.955 ^a	1.968 ^a	2.002 ^a

Values in a row followed by the same letters indicate no significant differences between treatments at $p = 0.05$.

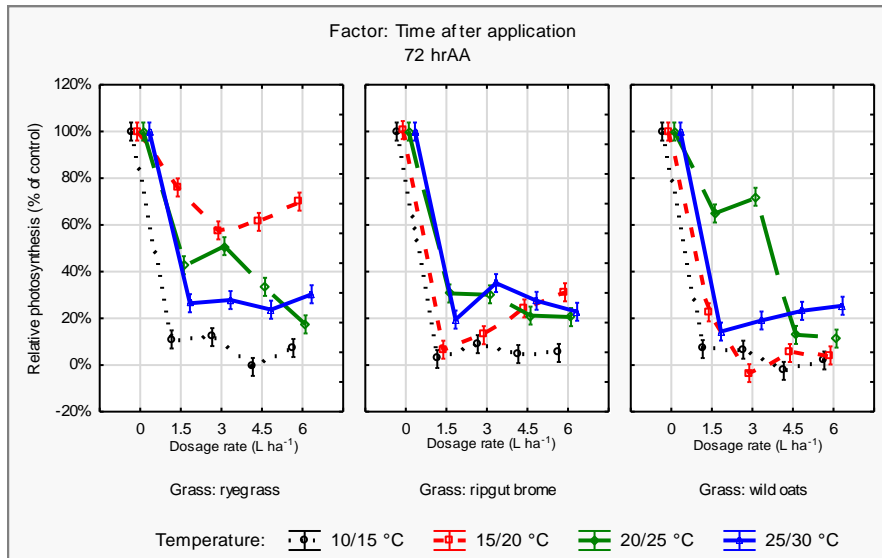
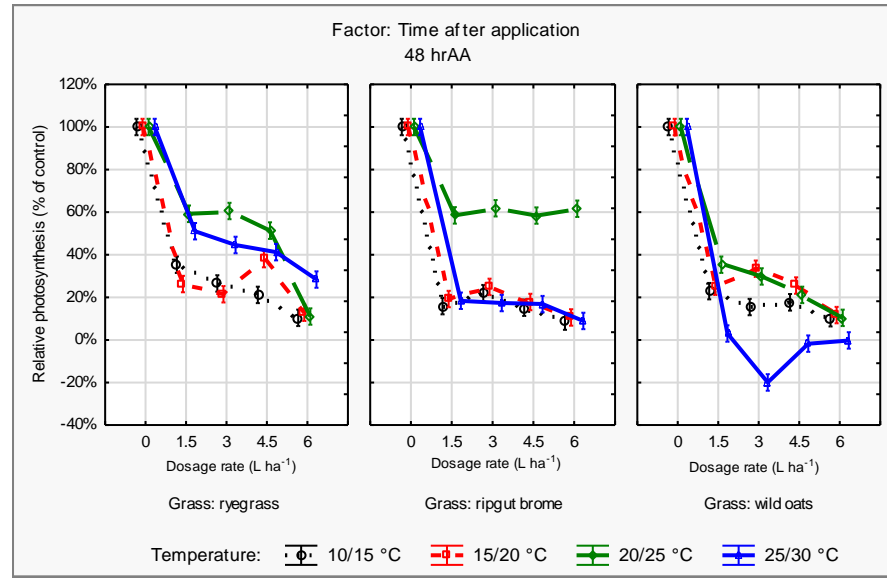
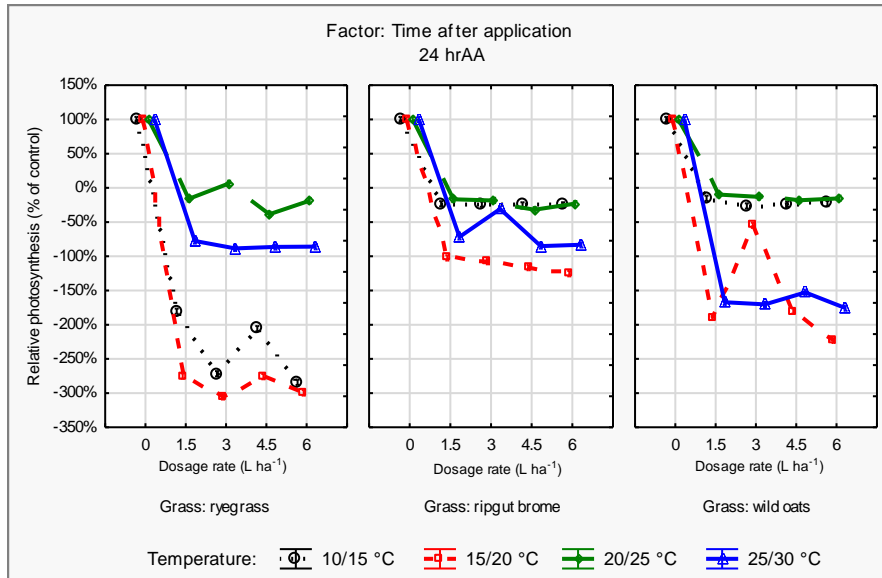
5.3.3 Photosynthesis study

As shown in **Figure 5.4**, there was significant three-way interaction of glufosinate ammonium dosage rate, grass species and temperature across all grass species 24 hours after application (24 HAA). At 24 HAA, relative photosynthetic rates of all grass species, with the exception of ryegrass treated with 3 L ha⁻¹ glufosinate ammonium at 20/25 °C, fell below 0%

across all temperature regimes as well as dosage rates. A significant drop of photosynthesis was observed for ryegrass at 10/15 and 15/20 °C where RPP reached a range of -305 to -180% compared to the other two temperature regimes. Another significant drop in RPP was observed for wild oats at 15/20 and 25/30 °C where RPP was less than -150%. The lowest photosynthetic rates for ripgut brome were observed at 15/20 °C. The lowest RPP for wild oats occurred at 15/20 and 25/30 °C. Increasing glufosinate ammonium dosage rate did not significantly influence RPP except for ryegrass grown at 10/15 °C and treated with 4.5 L ha⁻¹ and wild oats grown at 15/20 °C and treated with 3 L ha⁻¹.

A significant three-way interaction of glufosinate ammonium dosage rate, grass species and temperature across all grass species 48 HAA is shown in **Figure 5.5**. Almost all RPP values for all grasses across all temperatures showed positive values 48 HAA. The only exception in which the RPP was still negative was observed in wild oats grown at 25/30 °C and treated with 3, 4.5 and 6 L ha⁻¹ glufosinate ammonium dosage rates. The highest RPP observed was that of ripgut brome at 20/25 °C. No significant differences in RPP were shown by ripgut brome at 10/15, 15/20 and 25/30 °C across all dosage rates. Ryegrass treated with 6 L ha⁻¹ showed a significant decrease in RPP across all temperatures even though the RPP was still positive. Increase in glufosinate ammonium dosage rate significantly decreased RPP of ryegrass grown across all temperatures, 48 HAA. No significant differences in RPP were observed for ripgut brome with increasing dosage rate. For wild oats, increase in glufosinate ammonium dosage rate significantly decreased RPP for plants grown at 15/20 and 20/25 °C but not at 10/15 °C. A significant decrease in RPP was observed on wild oats grown at 25/30 °C and treated with 3 L ha⁻¹.

A significant three-way interaction of glufosinate ammonium dosage rate, grass species and temperature across all grass species 72 HAA is shown in **Figure 5.6**. All grass species showed a positive RPP 72 HAA except for wild oats grown at 15/20 °C. No significant differences among all grass species was shown at 10/15 °C and 25/30 °C. At 20/25 °C, relative percentage photosynthesis of ryegrass and wild oats were dosage rate dependent in which higher dosage rates significantly decreased photosynthesis of the weed species.



Top left - **Figure 5.4:** The effect of temperature on relative percentage photosynthesis of ryegrass, ripgut brome and wild oats 24 hours after glufosinate ammonium application.

Top right - **Figure 5.5:** The effect of temperature on relative percentage photosynthesis of ryegrass, ripgut brome and wild oats 48 hours after glufosinate ammonium application.

Bottom left - **Figure 5.6:** The effect of temperature on relative percentage photosynthesis of ryegrass, ripgut brome and wild oats 72 hours after glufosinate ammonium application.

Vertical bars denote ± standard error of the mean of three replications 95% confidence intervals (n = 3).

5.3.4 Elemental (calcium) analysis

The interaction effect of temperature and grass species on calcium content of ryegrass, ripgut brome and wild oats was significant ($p < 0.05$) as shown in **Figure 5.7**. Calcium content in ryegrass decreased when temperature increased from 10/15 to 15/20 °C after which a drastic significant increase of calcium content was noticed at 20/25 °C and finally significantly decreasing again at 25/30 °C. A similar trend to that of ryegrass was noticed in ripgut brome. However, calcium content in ripgut brome was significantly higher than the other two grass species, particularly, at 10/15, 20/25 and 25/30 °C. Calcium content in wild oats showed a different trend to that of ryegrass and ripgut brome in that there was an initial increase of calcium content when temperatures increased from 10/15 to 15/20 °C after which a significant decrease was noticed at 20/25 °C and another decrease at 25/30 °C.

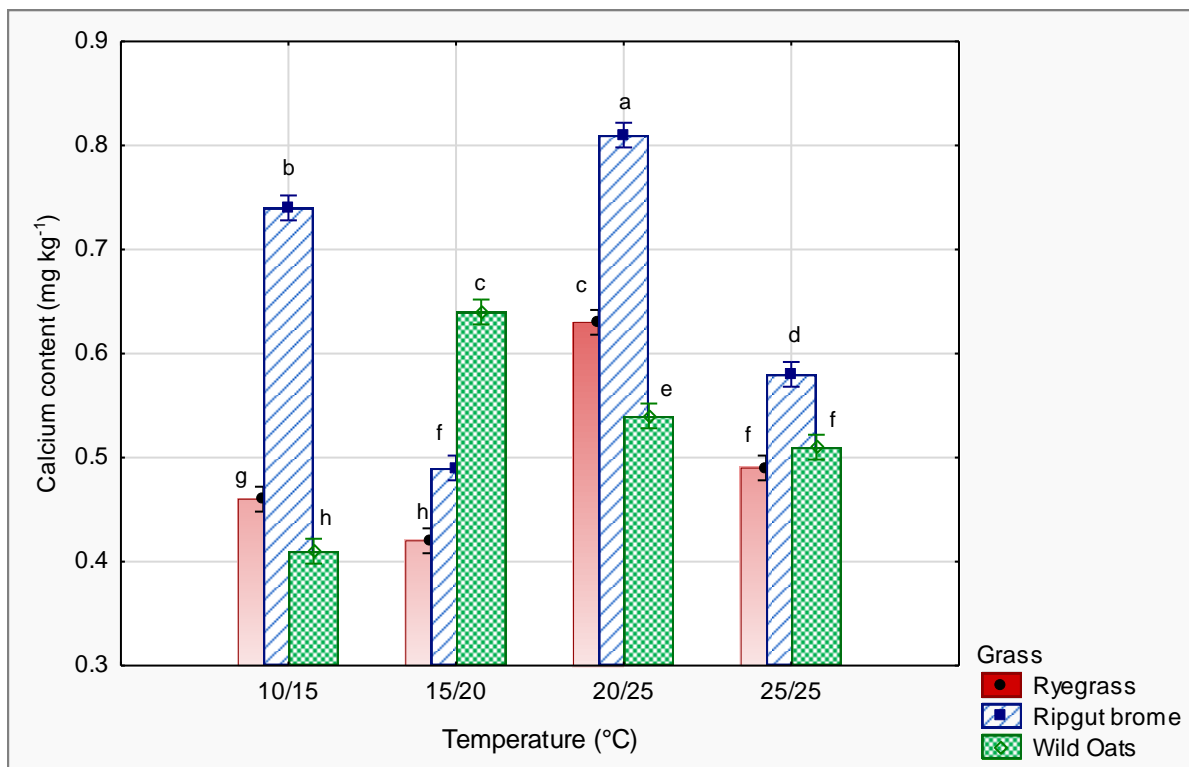


Figure 5.7: Calcium content detected in control (untreated) ryegrass, ripgut brome and wild oats grown at 10/15, 15/20, 20/25 and 25/30 °C at the time of glufosinate ammonium application. Vertical bars denote \pm standard error of the mean of four replications ($n = 4$). Values followed by the same letters indicate no significant differences between treatments at $P \leq 0.05$.

5.3.5 Multiple regression analysis

Regression analysis of calcium concentration, cuticle thickness and RPP 24, 48 and 72 HAA on ryegrass, ripgut brome and wild oats are shown in **Table 5.3**, **5.4** and **5.5**, respectively. There was a negative correlation between all variables tested and mortality of ryegrass and ripgut brome except for calcium accumulation on ripgut brome. There were no significant correlations between all variables and wild oats mortality rate.

Table 5.3: Multiple regression analysis of calcium accumulation, cuticle thickness and RPP (24, 48 and 72 HAA) on mortality of ryegrass

	R= 0.99914649, R ² = 0.99829370 Adjusted R ² = 0.99687179		
	b*	Standard error of b*	p-value
Calcium	-2.47988 *	0.114424 *	0.000001 *
Cuticle thickness	-1.64969 *	0.291762 *	0.001314 *
RPP - 24 HAA	-1.22806 *	0.174539 *	0.000412 *
RPP - 48 HAA	-0.03165	0.059431	0.613492
RPP - 72 HAA	-0.46622 *	0.146181 *	0.018852 *

Value represents a significant correlation ($p < 0.05$). RPP: Relative percentage photosynthesis. HAA: hours after application. b represents standardized coefficient.

Table 5.4: Multiple regression analysis of calcium accumulation, cuticle thickness and RPP (24, 48 and 72 HAA) on mortality of ripgut brome

	R= 0.99999977, R ² = 0.99999955, Adjusted R ² = 0.99999917		
	b*	Standard error of b*	p-value
Calcium	-0.00577	0.010420 *	0.120002
Cuticle thickness	-1.60372 *	0.007411 *	0.000000 *
RPP - 24 HAA	-0.02369 *	0.009620 *	0.048965 *
RPP - 48 HAA	-0.04059 *	0.009606 *	0.005528 *
RPP - 72 HAA	-4.28921 *	1.301648 *	0.016506 *

Value represents a significant correlation ($p < 0.05$). RPP: Relative percentage photosynthesis. HAA: hours after application. b represents standardized coefficient.

Table 5.5: Multiple regression analysis of calcium accumulation, cuticle thickness and RPP (24, 48 and 72 HAA) on mortality of wild oats

	R=0 .95466195, R ² = 0.91137944, Adjusted R ² = 0.83752898		
	b*	Standard error of b*	p-value
Calcium	0.74370	1.066330	0.511632
Cuticle thickness	-2.26812	1.097591	0.084300
RPP - 24 HAA	-0.38286	0.876551	0.677554
RPP - 48 HAA	-1.48906	1.145376	0.241285
RPP - 72 HAA	-0.00186	0.002473	0.481556

Value represents a significant correlation ($p < 0.05$). RPP: Relative percentage photosynthesis. HAA: hours after application. b represents standardized coefficient.

5.4 DISCUSSION

Results presented in **Figure 5.1** show that response of weeds to glufosinate ammonium at different temperatures differs amongst weed species. Although the experiments were conducted on three temperate grass species, no similar trend in mortality rate was observed for any of the species. Control of ryegrass with glufosinate ammonium decreased with increasing temperature while temperature had no significant effect on the response of wild oats to glufosinate ammonium across all dosage rates. For ripgut brome, very poor control was observed at 10/15 °C and control increased with increasing temperature. However, a non-significant decrease was observed at 25/30 °C. A study by Tharp et al. (1999) observed that velvetleaf (*Abutilon theophrasti* L.) and common lambsquarters (*Chenopodium album* L.)

were more sensitive to glufosinate ammonium when compared to barnyard grass (*Echinochloa crus-galli* L.), giant foxtail (*Setaria faberi* L.), large crabgrass (*Digitaria sanguinalis* L.) and fall panicum (*Panicum dichotomiflorum* Michx). Additional studies have noted that absorption, translocation and metabolism largely contribute to herbicide tolerance in weed species (Mersey et al. 1990; Pline et al. 1999; Neto et al. 2000; Everman et al. 1999). Variations in mortality of ryegrass, riggut brome and wild oats might be also attributed to differences in glufosinate ammonium absorption, translocation and metabolism among grass species.

No significant differences in cuticle thickness were observed among weed species, however, the measurements showed that cuticle thickness of ryegrass is greatest followed by riggut brome and finally wild oats. Visual observation of ryegrass, riggut brome and wild oats supports this finding. Ryegrass leaves are mainly identified by their glassy waxy cuticle while riggut brome is characterised by its voluminous hairs (**Figure 5.8**). Wild oats possess neither an obvious glassy waxy cuticle nor hairs. The Grain SA issue (2012) describes wild oats sheaths and leaves as hairless. These prominent morphological and physical characteristics influence the response of grass species to herbicides (Jamal 2011), hence this might account for the exceptional control of wild oats even with a low glufosinate ammonium dosage rate of 3 L ha⁻¹ and greater tolerance of ryegrass and riggut brome at different temperatures. Efficacy of glufosinate ammonium for a particular weed species is therefore dependent on how that species responds to environmental conditions. The differences in morphological and physical characteristics among weed species gives rise to different responses to herbicide application observed in this study, therefore, no general conclusion with regards to the effect of temperature can be drawn for all weed species. Several studies on efficacy of glufosinate ammonium have also found that sensitivity or tolerance of weed species differ (Mersey et al. 1990; Neto et al. 2000; Tharp et al. 1999). A study to determine efficacy of glufosinate ammonium on two weed species observed greater control of sterile oats than rigid ryegrass and attributed the difference to translocation of glufosinate ammonium into the meristematic zone (Kumaratilake et al. 2002). In another study conducted by Neto et al. (2000), they demonstrated that absorption of glufosinate ammonium in common cocklebur (*Xanthium strumarium* L.) was three times greater than tall morningglory (*Ipomoea purpurea* L.) and spreading dayflower (*Commelina diffusa* Burm.).

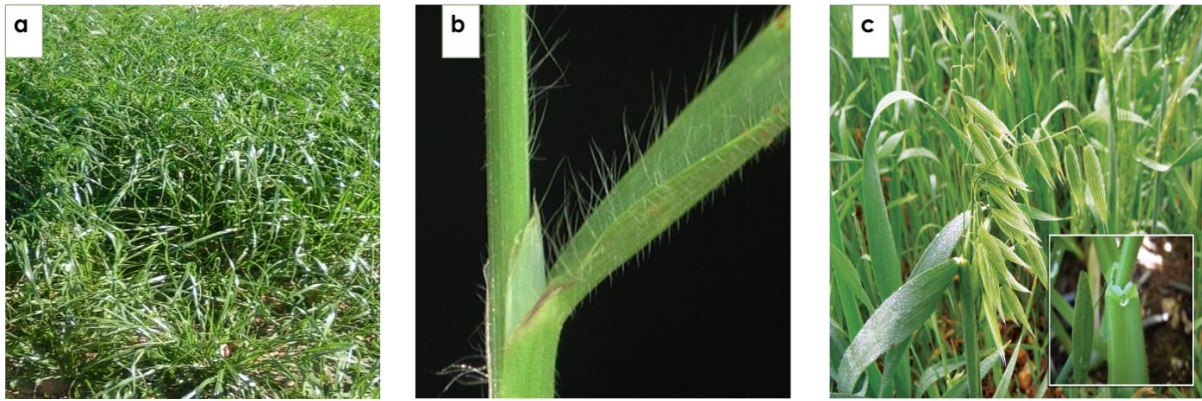


Figure 5.8: Images of a) ryegrass, b) rippgut brome and c) wild oats. (Adapted from rippgut brome: Lezama (2000); wild oats: Grain SA issue (2012); ryegrass: Mucheri (2016)).

The variability in control shown by glufosinate ammonium is a cause of concern in weed management mainly because it results in weed shifts. Weed shift is a change in weed populations or compositions as a consequence of differential efficacy and selection (Wibawa et al. 2009). Several studies have reported that widespread adoption of glyphosate coupled with its extensive use in cropping systems hastened weed shift (Reddy 2001; Hilgenfeld et al. 2004; Culpepper 2006; Owen 2008). A study on weed shift resulting from use of glufosinate ammonium observed that the herbicide is suitable to control mixed weed populations, however, its sole application or in rotation with paraquat resulted in a community coefficient of less than 56% meaning that their similarities in control were poor and resulted in weed shifting from grass to broadleaf weeds (Wibawa et al. 2009). Weed shift from herbicide use is inevitable since no single herbicide controls all weeds equally, hence, this allows inherently tolerant weeds to thrive and proliferate (Owen and Zelaya 2005). Rotation of herbicides and use of tank mixtures are recommended strategies to be implemented in chemical weed control systems in order to manage weed shifts (Culpepper 2006; Owen 2008). Owen (2008) recommends early monitoring of weed population shifts so that research can be directed to the new populations. Existing literature on weed shift focuses less on glufosinate ammonium, therefore, more studies on adaptation of weed populations to glufosinate ammonium need to be conducted.

The study showed a drastic decrease in photosynthesis 24 hours after glufosinate application. Almost all treatments provided positive values 48 and 72 HAA, although it still did not reach pre-application levels. No defined trend was observed for all grass species at different temperatures 24, 48 and 72 HAA. The multiple regression analysis showed that there was no correlation between RPP and mortality of wild oats. The results found a negative correlation between RPP and mortality of ryegrass and rippgut brome – an increase in RPP resulted in a decrease in mortality.

Calcium content negatively influenced mortality of ryegrass but not ripgut brome and wild oats. Studies by Mucheri (2016) suggest that calcium accumulation at warmer temperatures result in poor control of ryegrass. For ripgut brome and wild oats, no correlation between calcium accumulation and mortality rate was noticed. Instead, control of ripgut brome at 20/25 °C when calcium content was significantly high was great, achieving 100% control with 3 L ha⁻¹ glufosinate ammonium. Cytosolic or mitochondrial calcium ($[Ca^{2+}]_{\text{cyt/mit}}$) is bound by proteins and calmodulin, hence it is not mobile (Trewavas and Knight 1994; White and Broadley 2003). Price et al. (1994) noted that a certain amount of endogenously produced hydrogen peroxide after exposure of plant to oxidative stress must penetrate into cells to initiate mobilization of free, reactive $[Ca^{2+}]_{\text{cyt/mit}}$. Varying responses shown by different species to external condition are perceived to influence or determine initialization of calcium mobility. Calcium analysis (refer to **Figure 5.7**) conducted in this study provided total calcium content detected in the plant, regardless of its form. This might suggest calcium detected in ripgut brome was inert, hence, it was not able to react with glufosinate ammonium. Chapter 3 of this dissertation noted that elemental analysis only provides a foundation to calcium study. Improved XRD methods need to be exploited to determine the form in which calcium exists in plants and its reactions with glufosinate ammonium. Studies to monitor reaction of glufosinate ammonium with calcium might be a problem due to technical difficulties in measuring changes in $[Ca^{2+}]_{\text{cyt/mit}}$ (Logan and Knight 2003), however, more research is recommended in this regard.

In Chapter 3 of this dissertation it was postulated that phenolic compounds such as ferulic acid and *p*-coumaric acid amongst others accumulate in plant leaves when the plant is exposed to stressing environmental conditions. Accumulation of phenolic compounds in ryegrass in Chapter 3 was significant at 20/25 and 25/30 °C, and hence the compounds might have provided defence from glufosinate ammonium, thus resulting in poor control under such temperatures. According to Ramakrishna and Ravishankar (2011), plant species tolerance to stress differs. Additionally, plant species inherently differ in production of phenolic compounds (Ramakrishna and Ravishankar 2011). According to Iannucci et al. (2013), wild oats is adaptable to different biological and ecological conditions, hence the grass might have been tolerant to all temperature conditions it was exposed to in the experiments. This could have resulted in less or no accumulation of phenolic compounds and eventually good control of wild oats by glufosinate ammonium. The afore-mentioned explanation suggests that ripgut brome might have been more stressed at 10/15 °C compared to other weed species hence prompting production of phenolic compounds which resulted in its poor control. At this stage, the role of phenolic compounds in ripgut brome and

wild oats represent unsubstantiated speculation hence more research is required to test this hypothesis.

5.5 CONCLUSIONS AND RECOMMENDATIONS

The study observed that control of ryegrass increased with decreasing temperature, whereas, temperature had no significant effect on wild oats. For ripgut brome, very poor control was observed at 10/15 °C and control increased with increasing temperature. However, a non-significant decrease was observed at 25/30 °C. Calcium accumulation and RPP had negative effect on ryegrass mortality when treated with glufosinate ammonium. There was no correlation found between wild oats mortality and cuticle thickness, calcium accumulation and RPP. Calcium had no effect on mortality of ripgut brome even though it was detected at higher concentrations than ryegrass and wild oats. The study perceived that calcium form found in ripgut brome was inactive since activation of $[Ca^{2+}]_{cyt/mit}$ is dependent on the response of the plant. Although difficulties in further research on this conjecture are perceived, the study recommends investigation of this hypothesis. Response of grass species to glufosinate ammonium under different temperatures show inter- and intra-species variation, and this was attributed to the differences in morphological and physical characteristics among weed species, thus, no general conclusion can be drawn for all grass species. This variability in weed control shown by glufosinate ammonium might give rise to weed shifts in cropping systems, hence, early monitoring of weed adaptations and weed shifts is recommended so that research can be directed to the new weed populations.

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CHAPTER 6

The effect of selected adjuvants on glufosinate ammonium efficacy in controlling ryegrass (*Lolium* spp.)

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ABSTRACT

An experiment to investigate the role of adjuvants in increasing glufosinate ammonium efficacy was conducted in a glasshouse at Welgevallen Experimental Farm, Stellenbosch University. The experiment was done on wild ryegrass (*Lolium* spp.) at 20/25 °C (night/day) temperatures. Glufosinate ammonium was sprayed at 1, 2, 3 and 4 L ha⁻¹. In addition to the solo application of glufosinate ammonium, adjuvants added to glufosinate ammonium tank mixtures included Class act NG™ (2%), Summit Super (0.2%) and Velocity Super™ (0.5%). Among the selected adjuvants, the most effective adjuvants were Class act NG™ and Velocity Super™. There was no significant difference between control of ryegrass glufosinate ammonium applied alone and in a tank mixture with Summit Super. Further investigations on phenolic acids observed under a confocal fluorescence microscope as blue auto-fluorescence found a correlation between blue intensity at 24, 48 and 72 h after application and mortality of ryegrass. An increase in the blue auto-fluorescence resulted in poor control of ryegrass and *vice versa*. This was confirmed by the gas chromatography mass spectrometry analysis which observed the same trend with ferulic and *p*-coumaric acid. The study concluded that production of phenolic compounds, particularly ferulic and *p*-coumaric acid, within 48 h after glufosinate ammonium application is a determinant factor of ryegrass mortality or survival.

Key words: adjuvants, glufosinate ammonium, phenolic compounds, ryegrass.

6.1 INTRODUCTION

Factors affecting absorption and translocation have a great influence on foliar applied herbicides (Everman 2008). There is high possibility of losing herbicides that remain on the leaf surface over extended periods to volatilization, wash off or degradation (Everman 2008). Additionally, humidity effects on cuticle hydration, droplet drying or deposit formation greatly influence efficacy of foliar herbicides (Ramsey et al. 2006). Adjuvants are added in tank mixtures to lessen these shortcomings and thus enhance herbicide efficacy (McMullan 2000).

It has been observed that ammonium sulfate remarkably increases efficacy of glufosinate ammonium (Pratt et al. 2003). Ammonium sulfate's role as an adjuvant is to overcome large amounts of antagonistic salt interactions in the spray tank and improve activity of weak herbicides (Pratt et al. 2003; Singh et al. 2011). Pratt et al. (2003) further relates the role of ammonium sulfate in enhancing efficacy of glufosinate ammonium to glyphosate due to their similarities in structure. In addition to decreasing antagonistic effects in the tank, it also serves to react with cations that may antagonise glufosinate ammonium on the leaf surface.

Fluorescence studies presented in Chapter 3 of this dissertation showed that ryegrass treated with glufosinate ammonium emitted a more intense blue auto-fluorescence as compared to that of control plants. This emission exhibits a fluorescence spectrum ranging from 430 nm to 450 nm and it originates from hydroxycinnamic acids (secondary metabolites) in the cell wall and other plant phenolics in the primary epidermal layer of vacuoles (Lichtenthaler and Schweiger 1998). Such compounds are naturally existent in plants and play no fundamental role in metabolic processes. However, they act as support structure and function in adaptation and defence under stress (Ramakrishna and Ravishankar 2011).

Exposure to very low temperatures results in accumulation of anthocyanins in some plants which increase the plant's photosynthetic rate by increasing resistance to photo-inhibition (Solecka 1997). The account given by Solecka (1997) might explain how some plants are able to withstand harsh conditions for a long time. The principle around accumulation of secondary metabolites in relation to the plants' ability to withstand stress can be useful in investigating efficacy of herbicides. Results in Chapter 3 showed that more resilience and defence of ryegrass was shown by plants with higher intensity of secondary metabolites, eventually, resulting in poor control of such plants. The role of adjuvants in suppressing excessive production of phenolic compounds should be investigated.

Mucheri's (2016) study to investigate the influence of selected adjuvants on glufosinate ammonium at different growth stages of ryegrass observed that among the selected adjuvants, Velocity Super™ (ammonium sulfate, L 9603) was the only adjuvant that positively influenced glufosinate ammonium efficacy when compared to Summit Super (nitrogen solution/non-ionic surfactant, L 8539). Velocity Super increases glufosinate ammonium by restricting calcium cations, in either the tank or on the leaf, from binding with the glufosinate molecule (Pratt et al. 2003). The sulfate ion in Velocity Super™ reacts with the calcium cations to form calcium sulfate, thus allowing the ammonium ion to form readily absorbed NH₄-glyphosate molecules. The non-ionic nitrogen solution in Summit Super functions by reducing tension in the tank mixture (McMullan 2000). Better control resulting

from adding Velocity Super™ when compared to Summit Super was attributed to the ability of ammonium sulfate to bind with magnesium or calcium, whereas, the non-ionic Summit Super nitrogen lacks the anion which binds with cations (Mucheri 2016). This study, therefore, investigated glufosinate ammonium efficacy as influenced by adjuvant Class act NG™ (ammonium sulfate plus a non-ionic surfactant, L 10477) which contains both ammonium sulfate and non-ionic surfactant in addition to Velocity Super™ and Summit Super.

This study aimed to:

- a) investigate the influence of selected adjuvants on glufosinate ammonium efficacy for the control of ryegrass (*Lolium* spp.),
- b) determine if addition of adjuvants has an influence on production of secondary metabolites and,
- c) further justify the hypothesis that accumulation of secondary metabolites has a correlation with survival of ryegrass weeds.

6.2 MATERIALS AND METHODS

6.2.1 Trial establishment and management

The study was conducted at Welgevallen Experimental Farm, Stellenbosch University. The site is located at 33° 56'33" S and 18° 51'56" E and at an altitude of 136 m above sea level. Wild ryegrass seeds were sown in plastic containers containing river sand and transplanted a week after emergence into 8 cm × 8 cm plastic pots. Four plants were transplanted per pot and the growth medium was sand. The weeds were fertilized using a standard solution (refer to **Table 3.1**). Irrigation was done thrice a day to ensure that plants were not moisture stressed. Relative humidity in glasshouses ranged from 75% to 90%. Additional weeds observed in the trials were handpicked.

6.2.2 Study design

The experiment was a randomised complete block design with 4 replications. The study was conducted in a glasshouse set at 20/25 °C (12/12 h night/day) temperatures. A single foliar application of glufosinate ammonium was done by means of pneumatic pot sprayer at a pressure of 2 bar in 200 L ha⁻¹ of water. Glufosinate ammonium was applied to ryegrass six weeks after transplanting at 0, 1, 2, 3 and 4 L ha⁻¹. The herbicide was applied solo and in tank mixtures with 0.5% (v/v) Velocity Super™ (ammonium sulfate, L 9603), 0.2% (v/v) Summit Super (nitrogen solution/non-ionic surfactant, L 8539) and 2% (v/v) Class act NG™ (ammonium sulfate plus a non-ionic surfactant, L 10477). The experiment was therefore arranged as a 4 x 5 factorial with main factors Adjuvant treatments and Dosage rate.

6.2.3 Data collection

Mortality rate assessment was done four weeks after glufosinate ammonium application. A single replication of ryegrass was randomly selected from control plants and ryegrass treated with 3 L ha⁻¹ dosage rate, 24, 48 and 72 h after glufosinate ammonium application (HAA). The plants were sent for fluorescence analysis (confocal imaging) as well as gas chromatography mass spectrometry analysis.

i. Mortality rate evaluation

Evaluation of ryegrass mortality was done four weeks after treatment application. The following calculation was used to determine the percentage mortality;

$$\text{Percentage mortality} = \frac{\text{number of dead plants per pot}}{4 \text{ (plants per pot)}} \times 100\%$$

ii. Confocal fluorescence imaging

Control (untreated) and treated (3 L ha⁻¹) ryegrass leaves were used for the confocal fluorescence imaging. Three 0.25 cm² segments from the youngest leaf of a plant were excised from the plants and mounted upside down on the slide 24, 48 and 72 HAA. Distilled water (50 µl) was added on the sample before placing the coverslip. Confocal images of treated and control ryegrass plants were obtained by using Carl ZEISS Confocal LSM 780 Elyra S1 microscope (Central Analytical Facility, Fluorescence Department). A region of 830 µm × 830 µm was scanned with a vertical resolution of 12.316 µm. The area covered by the blue auto-fluorescence over the total area (688 900 µm²) was determined by IMAGE J software (developed by the University of Winconsin).

iii. Gas chromatography mass spectrometry analysis (GCMS)

Sample preparation: Gas chromatography mass spectrometry analysis was done on control samples and ryegrass treated with 3 L ha⁻¹ dosage rate. Approximately 10 mg of each sample was weighed into a 1.5 ml Eppendorf tube. Ready prepared 70% (v/v) MeOH (1 ml) was then added to the sample. An internal standard (100 µl ribitol and naphthol) was added and samples were vortexed for 30 seconds. Samples were placed in an oven at 60 °C for 3-4 h and 250 µl of each sample extract was transferred into another set of Eppendorf tubes. Samples were dried completely using a Savant SC210A Speed Vac® plus at room temperature overnight.

Derivatization for Phenolic Acids: O-Bis(trimethylsilyl)trifluoroacetamide + trimethylchlorosilane (30µl BSTFA+TMCS, 99:1) was added to the dried samples after adding 100 µl acetonitrile. The samples were vortexed and placed in the oven at 80 °C for

60 min. After 60 min, the samples were vortexed before transferring them into the GC-vial with insert and cap. Samples were then analysed for phenolic compounds using a ThermoFisher (TS8000) GCMS equipment.

6.2.4 Statistical analysis

Analysis of variance of blue auto-fluorescence percentage area, concentration of phenolic compounds and mortality rate was done by STATISTICA 13 statistical tool. Mean separation was done using Tukey's honest significant difference (HSD) test at 95% confidence interval.

6.3 RESULTS

There was a significant two-way interaction between glufosinate ammonium dosage rate and adjuvant treatment (**Appendix 6.1**). According to **Figure 6.1**, addition of Class act NG™ to glufosinate ammonium significantly increased its efficacy such that 1 and 2 L ha⁻¹ dosage rates controlled >90% ryegrass. Control of ryegrass with glufosinate ammonium at 3 L ha⁻¹ dosage rate was 100% when adjuvant Class act NG™ and Velocity Super™ were added to the tank mixture. A solo application of glufosinate ammonium together with a tank mixture including Summit Super could not reach 100% control even after application of 4 L ha⁻¹ dosage rate.

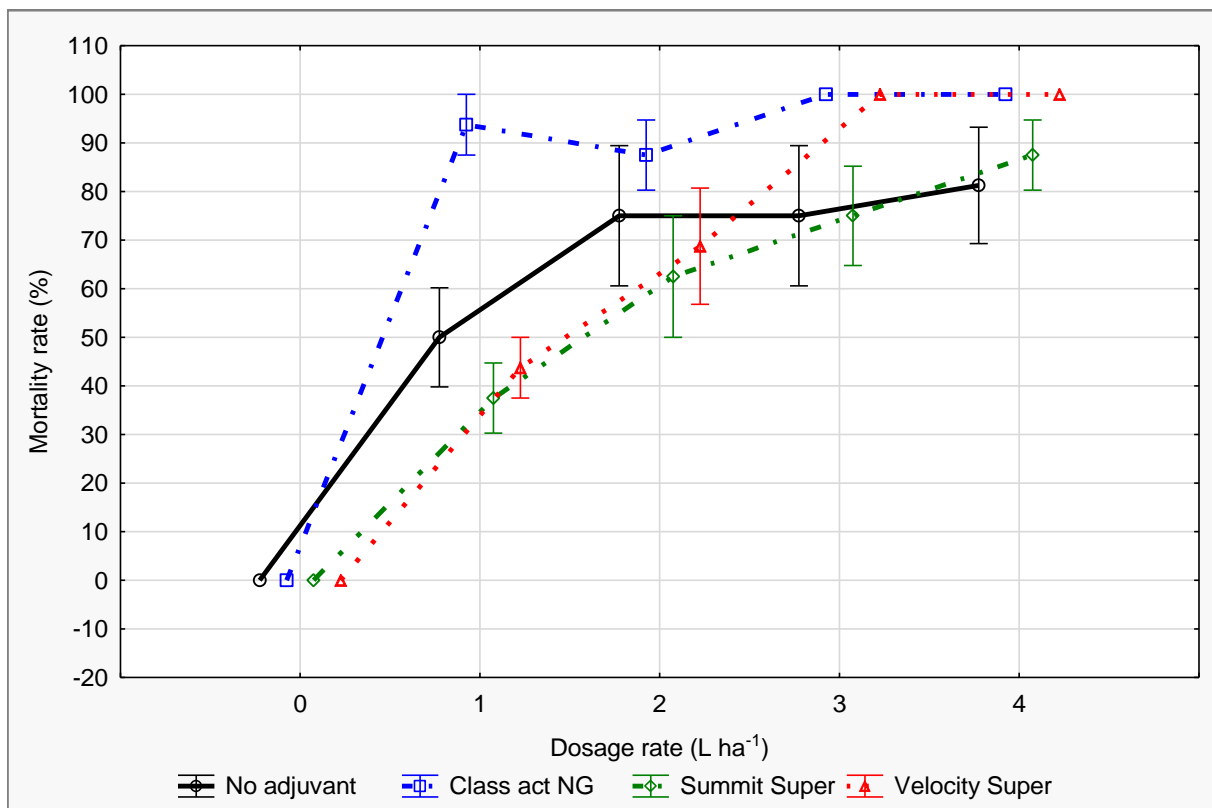


Figure 6.1: Effect of adjuvants and glufosinate ammonium dosage rate on mortality percentage of ryegrass. Results are means of two experiments with six replications each ($n = 6$). Vertical bars denote \pm standard error of the mean.

Figure 6.2 shows the images taken by a fluorescence confocal microscope of ryegrass 24, 48 and 72 hours after glufosinate ammonium application.

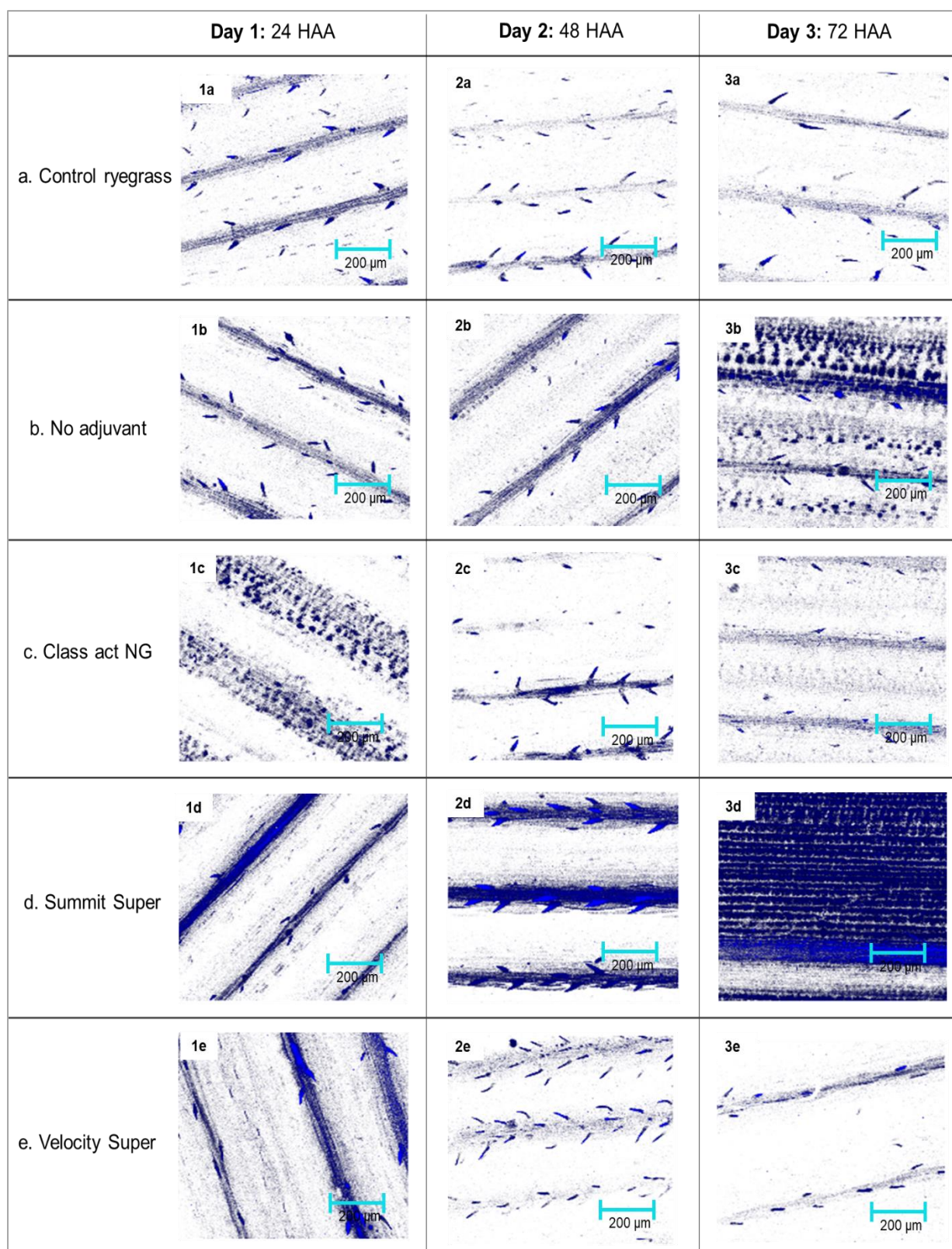


Figure 6.2: Confocal images of ryegrass treated with glufosinate ammonium in tank mixture with various adjuvants, 24, 48 and 72 hours after treatment.

Visual observation of blue intensity showed that ryegrass treated with solo glufosinate ammonium as well as in tank mixture with Summit Super increased as hours after treatment increased, particularly, at 72 HAA. Visually, initial blue intensity of ryegrass treated with glufosinate ammonium in tank mixtures with Class act NG™ and Velocity Super™ increased 24 HAA but then decreased 48 and 72 HAA. Quantification results of the blue auto-fluorescence to percentage area are presented in **Figure 6.3**. There was a significant interaction ($p < 0.05$) between adjuvant treatment and time after application. Percentage area of blue auto-fluorescence on ryegrass treated with glufosinate ammonium (solo) significantly increased 72 HAA. The same trend was observed on ryegrass treated with glufosinate ammonium in a tank mixture with Summit Super. For tank mixtures including Class act NG™ and Velocity Super™, a decrease 48 and 72 HAA was observed on the ryegrass.

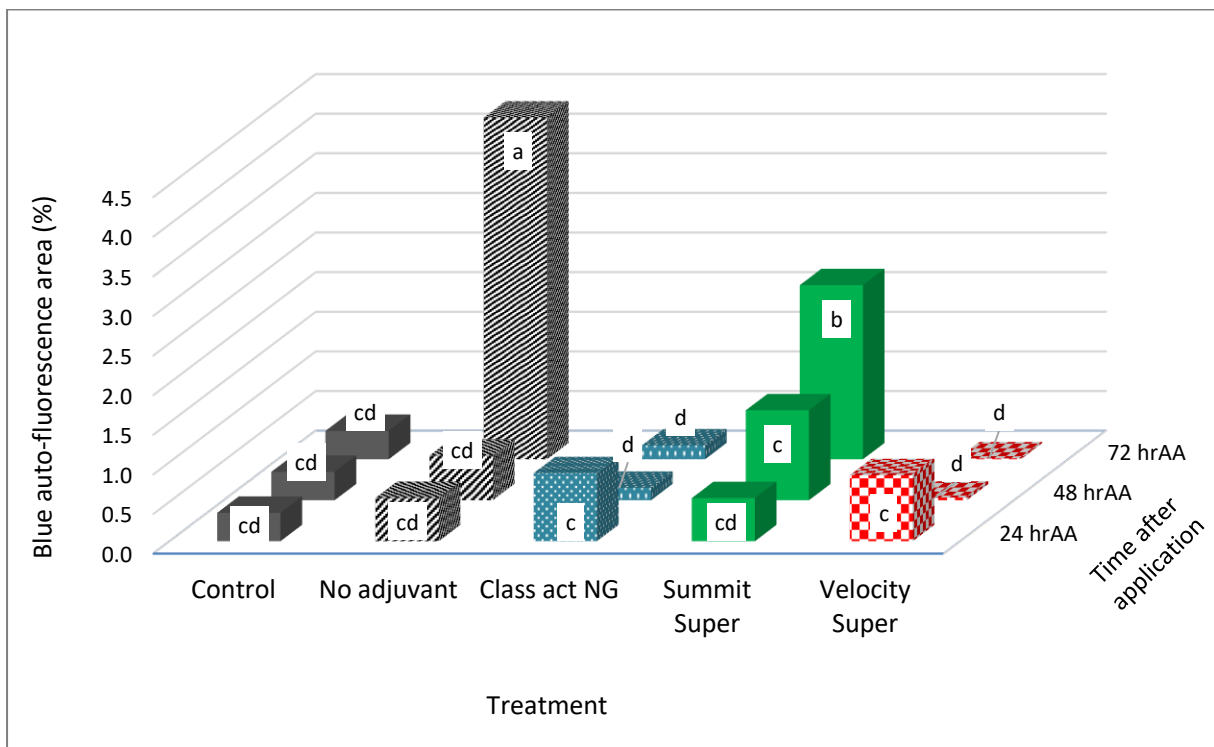


Figure 6.3: Effect of added adjuvants on intensity of ryegrass blue auto-fluorescence after glufosinate ammonium application. Letters denote statistically significant differences ($p \leq 0.05$) between treatments.

An analysis to determine the most influential phenolic acids is shown in **Figure 6.4**. Solo application of glufosinate ammonium significantly increased both ferulic and *p*-coumaric acid 24, 48 and 72 HAA. The same trend was observed on ryegrass treated with glufosinate ammonium in a tank mixture with Summit Super. Ryegrass treated with glufosinate ammonium in a tank mixture with Class act NG™ and Velocity Super™ showed different trends. The contrasting trends showed that concentration of ferulic acid and *p*-coumaric acid decreased with increasing hours after treatment.

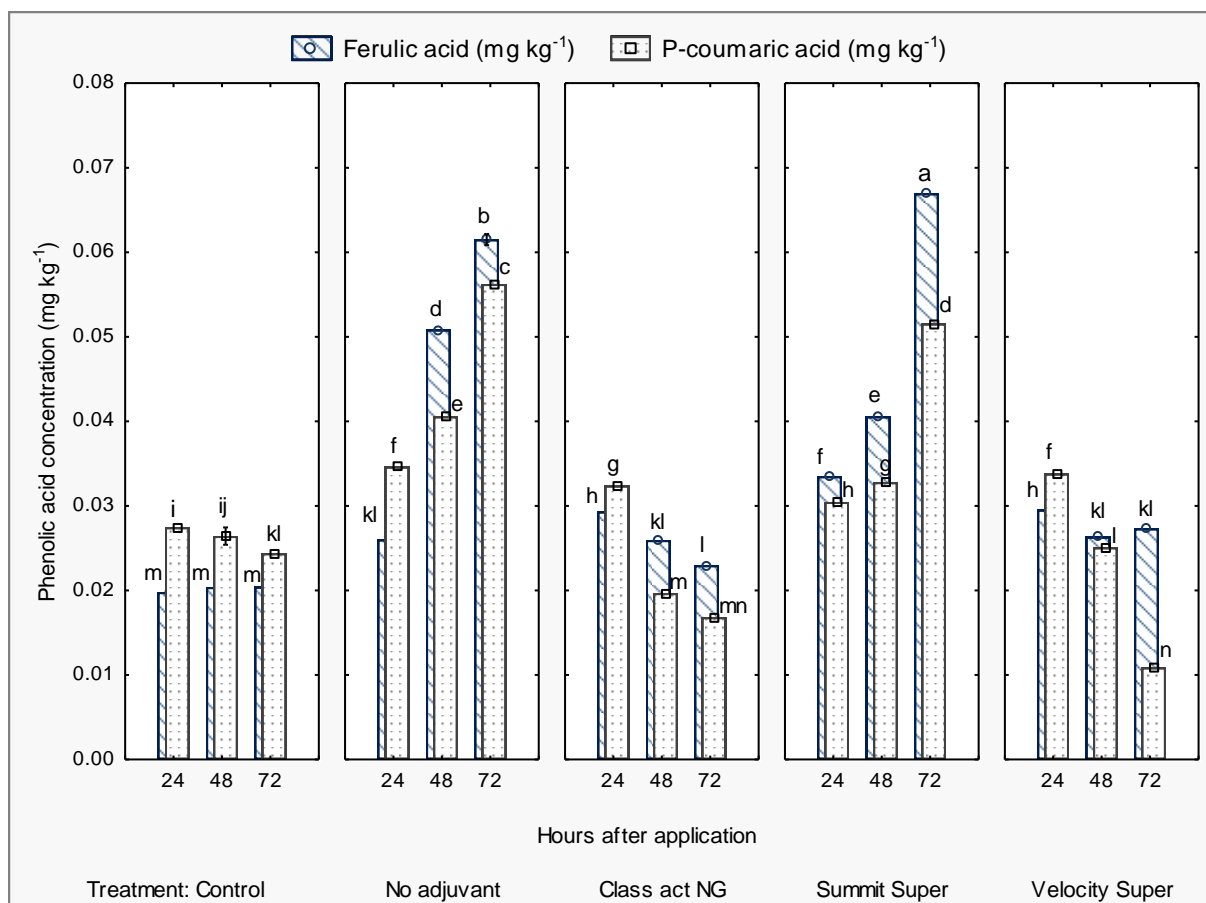


Figure 6.4: Effect of added adjuvants on concentration of ferulic and *p*-coumaric acid 24, 48 and 72 hours after glufosinate ammonium application. Vertical bars denote 95% confidence interval.

6.4 DISCUSSION

Addition of Class act NGTM and Velocity SuperTM significantly increased efficacy of glufosinate ammonium such that 100% ryegrass control was achieved with 3 L ha⁻¹ glufosinate ammonium dosage rate (Refer to **Figure 6.1**). Good control was even shown by glufosinate ammonium in a tank mixture with Class act NGTM at 1 L ha⁻¹ dosage rate were control of ryegrass reached 93.75%. Summit Super did not improve efficacy of glufosinate ammonium, instead, control of glufosinate ammonium in a tank mixture with Summit Super was poorer when compared to that of glufosinate ammonium solo application at 1 and 2 L ha⁻¹ dosage rates. A higher dosage rate of glufosinate ammonium with Summit Super might be required to attain 100% control of ryegrass. Mucheri (2016) suggested that a more pronounced response of ryegrass to glufosinate ammonium in a tank mixture with Velocity Super might be attributed to the ammonium sulfate it contains, which is actively involved in reactions and movement of glufosinate ammonium to the target site while the less pronounced response to glufosinate ammonium in a tank mixture with Summit Super might be accounted to the non-ionic Summit Super which is able to reduce tension only (McMullan 2000; Pratt et al 2003). This study included Class act NGTM which has both the non-ionic

and ammonium sulfate effect. Combination of these two characteristics in Class act NG™ could be accounted for the exceptional control of ryegrass in a tank mixture with glufosinate ammonium even at very a low dosage rate such as 1 L ha⁻¹. The study, therefore, suggests that Class act NG™ is the most effective adjuvant, followed by Velocity Super™ for the control of ryegrass with glufosinate ammonium. No significant difference was shown between glufosinate ammonium solo application and glufosinate ammonium in a tank mixture with Summit Super. Pratt et al (2003) observed that 2% ammonium sulfate and Class Act Next Generation® (ammonium sulfate plus alkyl polyglycosides) were the only adjuvants that consistently enhanced glufosinate ammonium efficacy when compared to Ultra Guard® (NIS plus drift retardant) and Cayuse Plus® (NIS plus ammonium salts). Maschhoff et al. (2000) reported that glufosinate ammonium uptake increased by 23% when applied with ammonium sulfate.

A correlation was observed between response of ryegrass to added adjuvants in glufosinate ammonium and blue auto-fluorescence intensity 48 and 72 HAA. Treatments including the most effective adjuvants (Class act NG™ and Velocity Super) showed decreasing blue auto-fluorescence and eventually provided exceptional control of ryegrass. Ryegrass treated with glufosinate ammonium alone and in a tank mixture with the less effective adjuvant (Summit Super) increased intensity of blue auto-fluorescence as hours after treatment increased. As explained in the introduction section of this chapter, the blue auto-fluorescence is an emission of phenolic compounds (Lichtenthaler and Schweiger 1998). This correlation shows that phenolic compounds had a role to play in the survival or mortality of ryegrass. **Figure 6.4** does show the declines and inclinations in ferulic acid and *p*-coumaric acid when ryegrass was treated with glufosinate ammonium in various tank mixtures. Previous studies have confirmed that phenolic compounds function in providing plants with support and defence under stress (Ramakrishna and Ravishankar 2011; Jacobo-Velázquez et al. 2015). A greater intensity of blue auto-fluorescence depicts that the plant was more resilient towards chemical (glufosinate ammonium) stress, hence the more the resilience the better the defence. This is because ferulic and *p*-coumaric acids are cross-linked to the type II primary cell walls which consist of glucuronoarabinoxylans (GAX) and β -glucan as the main matrix which stiffen the cell walls, and hence, ensures support of the plant (Gall et al. 2015) (also refer to the Chapter 3 discussion section of this dissertation). This might have resulted in survival of ryegrass treated with glufosinate ammonium solo and glufosinate ammonium in a tank mixture with Summit Super. Addition of adjuvants Class act NG™ and Velocity Super™ might have restricted or suppressed the production of phenolic compounds, resulting in poor ryegrass defence of the stress, thereby, the treatments were able to remarkably control the weed species.

Two possible explanations can be extrapolated from this finding. Either the adjuvants increased exposure time of ryegrass leaves to glufosinate ammonium or they enhanced absorption into the plant. Ammonium sulfate has been reported to enhance herbicide activity by a) neutralizing water-borne cations (Ca^{2+} , Mg^{2+} , Na^+ , etc.), b) increasing droplet drying time since it exhibits humectant characteristics and c) donating the H^+ ion during protonation which in turn neutralizes the herbicide molecules (Reinhardt 2018). Wang and Liu (2007) noted that ammonium ions alter the permeability of the plasma membrane and also participate in ion trapping – reducing pH of the medium for weak acid herbicides. Non-ionic surfactants aim to improve spray droplet retention and penetration of active ingredients (Liu 2004). Combined effects of ammonium sulfate and non-ionic surfactant in class act NG™ suggests that the adjuvant reduces alkaline water pH, overcomes complex antagonistic ions in carrier water and enhance the activity of herbicides in addition to spreading properties, hence, it significantly increased efficacy of glufosinate ammonium. Better control of glufosinate ammonium in a tank mixture with Velocity Super™ when compared to Summit Super is attributed to the presence of ammonium sulfate which possesses the aforementioned characteristics, whereas, Summit Super only reduces surface tension. The reported influence of ammonium sulfate on plasma membranes by Wang and Liu (2007) suggests that it might be responsible for suppressing phenolic compounds. It appears that effective adjuvants such as Class act NG™ and Velocity Super™ suppressed the production of phenolic compounds. The inability of Summit Super to suppress phenolic compound production might have resulted in poor control of ryegrass after glufosinate ammonium application.

6.5 CONCLUSIONS AND RECOMMENDATIONS

The study concluded that adjuvants Class act NG™ and Velocity Super™ have positive influence on efficacy of glufosinate ammonium. No significant difference was shown between efficacy of solo glufosinate ammonium and when in a tank mixture with Summit Super. It was confirmed that production of phenolic compounds, mainly ferulic and *p*-coumaric acid, within 48 hours after glufosinate ammonium determines survival or mortality of ryegrass. The study suggests that adjuvant Class act NG™ and Velocity Super™ can be used to mitigate the defensive response of phenolic compounds after glufosinate ammonium application, hence increasing its efficacy. At this juncture it is not evident if the suppression of phenolic compounds is a result of extended glufosinate ammonium exposure to the leaf or increase in absorption and eventually its metabolism. This phenomenon requires further investigation.

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CHAPTER 7

Influence of glufosinate ammonium time of application on its efficacy in controlling ryegrass (*Lolium multiflorum* cv)

Tendai Mucheri, Petrus J Pieterse, Carl F Reinhardt, Aleysia Kleinert

ABSTRACT

Three field experiments were conducted in 2018 and 2019 at Roodebloem and Langgewens farm, South Africa. Each experiment was designed as a randomized complete block with three replications. Ryegrass (*Lolium multiflorum* Lam. cv) was treated with glufosinate ammonium at five- to seven-leaf stage. Glufosinate ammonium was applied at 0, 2.5, 5 and 7.5 L ha⁻¹ (0, 500, 1000 and 1500 g a.i ha⁻¹). The treatments were applied at 8:00 am (morning), 12:00 pm (mid-day) and 5:00 pm (evening). Visual assessments of glufosinate ammonium efficacy on ryegrass were done 21 days after application. The study observed that ryegrass control was significantly higher when treated at 8:00 am and 5:00 pm, particularly, when temperatures were cool (< 15 °C) and relative humidity (RH) was high (> 80%). A decrease in RH resulted in a decrease in ryegrass control even at cooler temperatures. However, increasing dosage rate under such conditions increased control of ryegrass to greater than 80%. Higher RH at temperatures greater than 15 °C increased efficacy of glufosinate ammonium. This showed that RH has more effect on glufosinate ammonium efficacy than temperature.

Keywords: glufosinate ammonium, relative humidity, ryegrass, temperature, time of application.

7.1 INTRODUCTION

Glufosinate ammonium is a non-selective, contact, foliar applied post-emergence herbicide, hence environmental conditions play a critical role in its efficacy (Tharp et al. 1999; Sellers et al. 2004). The herbicide functions by inhibiting the enzyme glutamine synthetase (GS; E.C. 6.3.1.2). Glufosinate ammonium reaction involves its phosphorylation by adenosine triphosphate (ATP) (Berlicki et al. 2005). It has been noted that ammonia accumulation resulting from this reaction is light-dependent, hence its application is recommended during the day (Lacuesta et al. 1990; Coetzer & Al-Khatib 2001). However, several studies have reported that time of application (TOA) during the day has effect on glufosinate ammonium efficacy (Martinson et al. 2002; Hoss et al. 2003; Sellers et al. 2003).

Constant changes in temperature, light intensity and humidity during the day were reported to influence the plants' sensitivity to the environment, diurnal leaf movements and leaf angle (Sellers et al. 2003). This can result in poor herbicide interception and absorption, which sequentially decreases its efficacy. Low relative humidity (RH) dehydrates the cuticle, hence reducing herbicide uptake by plants (Steckel et al. 1997). Kumaratilake and Preston (2005) reported that light intensity effects on glufosinate ammonium controlling wild radish (*Raphanus raphanistrum* L.) were observed under warmer temperatures (20/25 °C night/day). In another study, control of common waterhemp (*Amaranthus rudis* S.), Palmer amaranth (*Amaranthus palmeri* S.) and redroot pigweed (*Amaranthus retroflexus* L.) indicated that RH greatly influences efficacy of glufosinate ammonium when compared to temperature (Coetzer et al. 2001). Kumaratilake and Preston (2005) noted that the contrasting effects on glufosinate ammonium efficacy resulting from light intensity, temperature and relative humidity interaction is complex, with temperature and RH being the dominant variables.

Glasshouse studies conducted by Molefe (2015) and Mucheri (2016) showed that warmer temperatures (20/25 and 25/30 °C night/day) resulted in poor efficacy of glufosinate ammonium when compared to cooler temperatures (10/15 and 15/20 °C). It is important to determine whether such observations apply under field conditions. This study, therefore, aimed to determine if changes in temperature and relative humidity during daytime had influence on glufosinate ammonium efficacy in controlling ryegrass (*Lolium multiflorum* cv) under field conditions.

7.2 MATERIALS AND METHODS

7.2.1 Trial establishment

Ryegrass (*Lolium multiflorum* Lam cv.) seeds were planted on rainfed plots at Roodebloem Experimental Farm (34°9'0" S and 21°49'0" E, 155 m above sea level) near Caledon and Langgewens Experimental Farm near Malmesbury (33°16'0" S and 18°42'0" E, 144 m above sea level), South Africa. The trials on Langgewens Experimental Farm were run from May to July over two years (2018 and 2019). A single trial was conducted in 2019 on Roodebloem Experimental Farm from May to July. The bio-climatic zone of Langgewens farm is Csa and that of Roodebloem farm is Csb, according to Köppen-Geiger zones climatic classification (2019). The experimental design at each site was a randomised block design with three replications.

7.2.2 Treatment application

Ryegrass was treated with glufosinate ammonium (200 g L⁻¹, SL) six weeks after planting (five- to seven-leaf stage). Glufosinate ammonium was applied at 0, 2.5, 5 and 7.5 L ha⁻¹ (0, 500, 1000 and 1500 g a.i ha⁻¹). Application of glufosinate ammonium was made with a manual knapsack sprayer using a 110-degree flat fan nozzle and calibrated to deliver 200 L ha⁻¹ water. Plants were treated with the above-mentioned dosage rates at 8:00 am (morning), 12:00 pm (mid-day) and 5:00 pm (evening). The application dates at Langgewens Experimental Farm in 2018 and 2019 were 29 June 2018 and 4 July 2019, respectively, while the application date at Roodebloem Experimental Farm was 5 July 2019.

7.2.3 Data collection

Mortality rate

Visual phytotoxicity assessments were done 21 days after glufosinate ammonium application by three people. Scoring was done using the Latin American Weed Association (ALAM) visual assessment scale shown in **Table 7.1**. Mortality rate was subjected to ANOVA using STATISTICA 13. The data was analysed separately for each site and year. Means were separated by Tukey's honest significant difference (HSD) test at 95% confidence interval.

Table 7.1: Latin America Weed Association (ALAM) visual assessment Scale

Index	Percentage (%)	Description of the control level
1	0-40	None or poor
2	41-60	Regular
3	61-70	Sufficient
4	71-80	Good
5	81-90	Very good
6	91-100	Excellent

Adapted from Mendes and Rezende (2014).

Weather data

Long term data for the trial period was provided by Weather South Africa upon request. Temperature and RH data recorded during glufosinate ammonium application days was summarized in an Excel spreadsheet. Correlation of temperature and RH before and after (1 h, 3 h and 24 h) glufosinate ammonium application was done using the Product-Moment and Partial Correlations tool in STATISTICA 13 software. Analysis of maximum, minimum and average temperature 21 days before application of glufosinate ammonium was done using STATISTICA 13 statistical tool. Means were separated by Tukey's HSD test at 95% confidence interval.

7.3 RESULTS

7.3.1 Langgewens Experimental Farm (2018)

Interaction of glufosinate ammonium dosage rate and TOA on mortality of ryegrass grown on Langgewens Experimental Farm in 2018 was not significant ($p > 0.05$). Increasing glufosinate ammonium dosage rate from 2.5 L ha⁻¹ to 5 L ha⁻¹ significantly increased mortality of ryegrass from a range of 58 – 63% to a range of 79 – 84% (**Figure 7.1**). Mortality rate of ryegrass treated with 5 L ha⁻¹ was not significantly different from mortality of ryegrass treated with 7.5 L ha⁻¹ glufosinate ammonium. The effect of TOA (morning, mid-day and evening) on ryegrass mortality did not show any significant differences across all glufosinate ammonium dosage rates.

Temperature and relative humidity (RH) summaries for the application day at Langgewens farm in 2018 are shown in **Figure 7.2**. The figure showed that temperatures below 10 °C were experienced during early hours (12:00 am to 7:00 am). From 8:00 am to 23:00 pm the temperature was steady, ranging between 10.2 to 11.6 °C. Relative humidity was greater than 80% except at 2:00 pm, 3:00 pm and 4:00 pm.

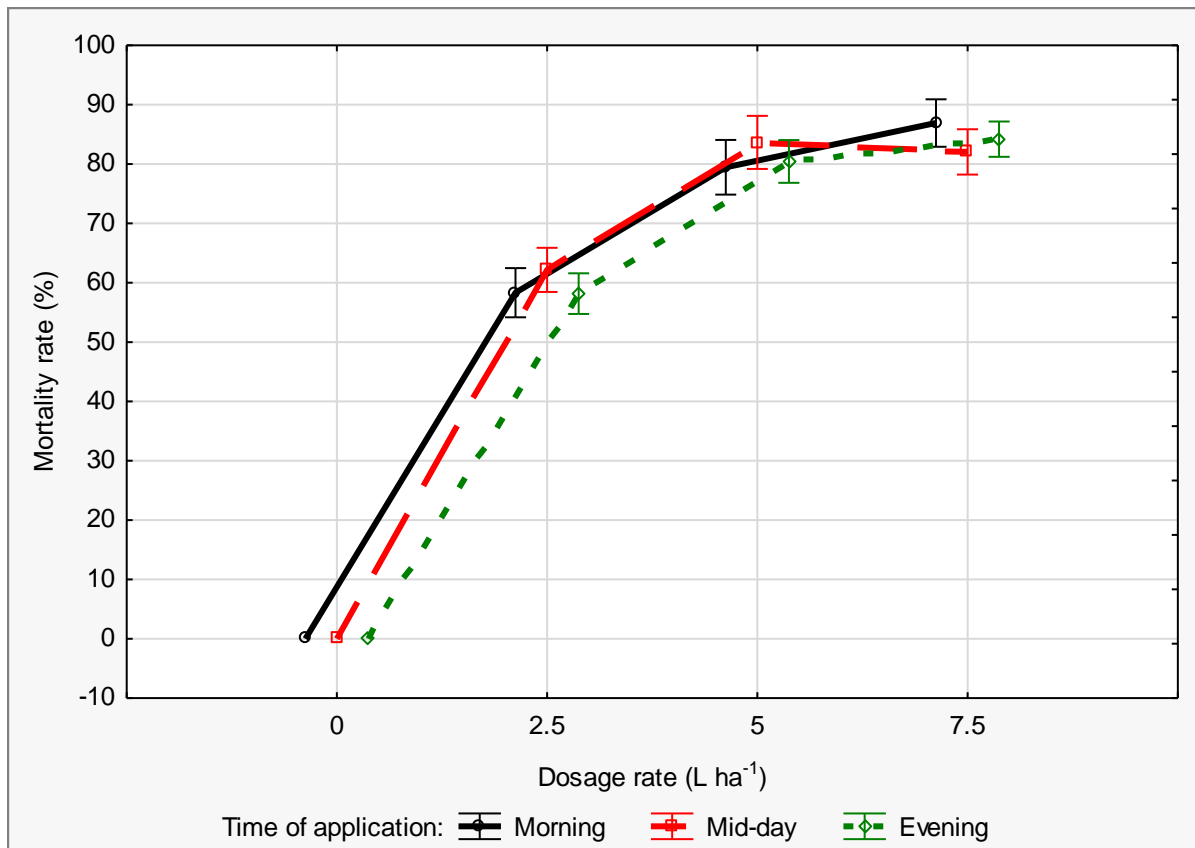


Figure 7.1: Effect of time of day on efficacy of glufosinate ammonium sprayed at different dosage rates for the control of ryegrass at Langgewens farm in 2018. Error bars represent \pm standard error of the mean of three replications ($n = 3$).

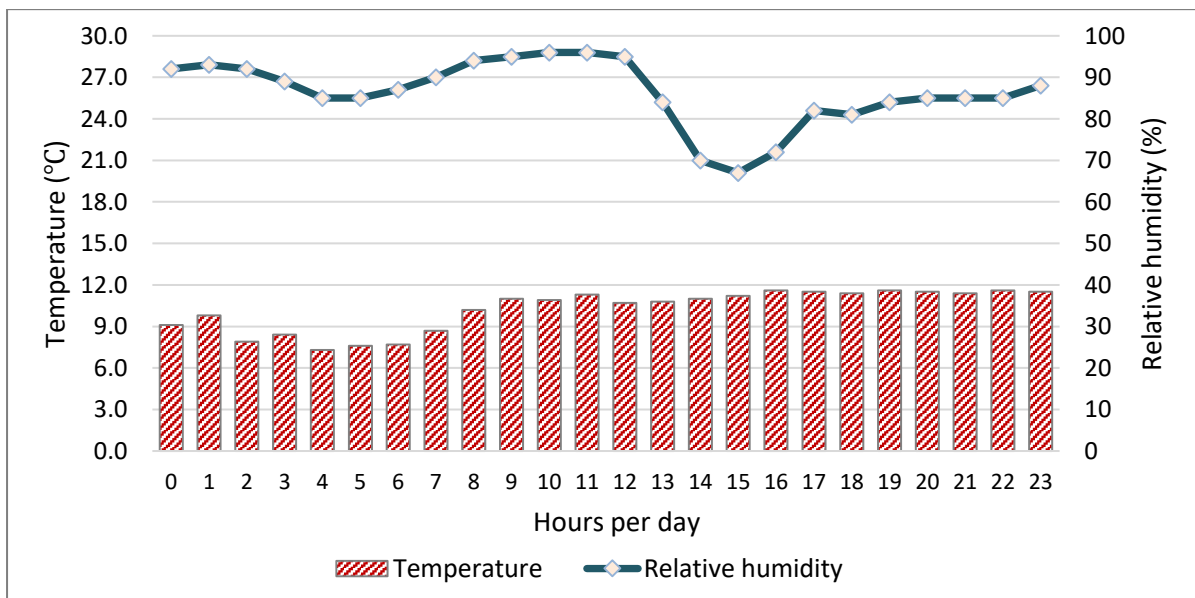


Figure 7.2: Summary of temperatures experienced on the 29th of June 2018 at Langgewens Experimental Farm from 12:00 am to 11:00 pm. The times presented as 0, 1, 2 ... and 23 denotes 00:00, 01:00, 02:00 ... and 23:00 hrs, respectively.

7.3.2 Langgewens Experimental Farm (2019)

The two-way interaction of glufosinate ammonium dosage rate and TOA shown in **Figure 7.3** was significant on mortality of ryegrass on Langgewens Experimental Farm in 2019 ($p < 0.05$). Increase in glufosinate ammonium dosage rate significantly increased efficacy of glufosinate ammonium. No significant differences were shown between mortality rate of ryegrass treated in the mid-day and evening across all dosage rates. Application of glufosinate ammonium in the morning showed significantly lower control of ryegrass, particularly, when treated with 2.5 L ha^{-1} . No significant differences were shown by mortality rate of ryegrass treated in the morning, mid-day and evening with a dosage rate of 5 and 7.5 L ha^{-1} .

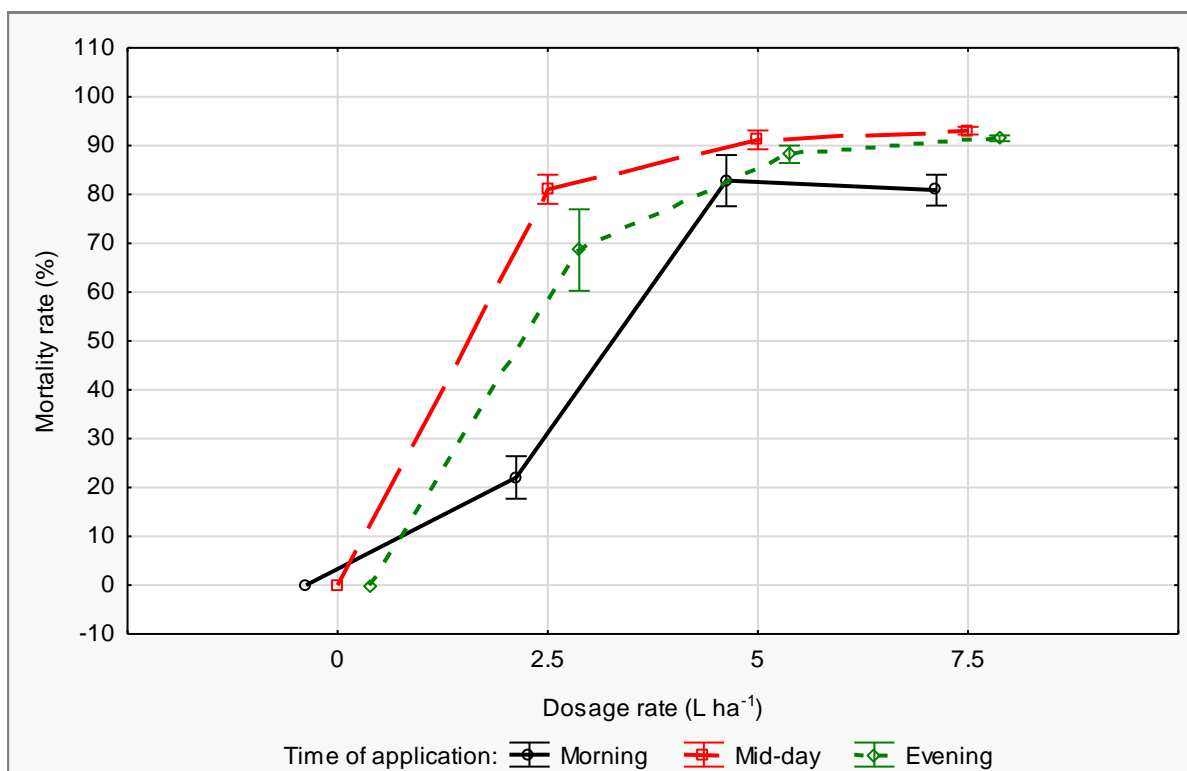


Figure 7.3: Effect of temperature on efficacy of glufosinate ammonium sprayed at different dosage rates for the control of ryegrass at Langgewens farm in 2019. Error bars represent \pm standard error of the mean of three replications ($n = 3$).

Temperature and RH summaries for the application day at Langgewens farm in 2019 are shown in **Figure 7.4**. The results showed that the temperatures below $10 \text{ }^\circ\text{C}$ were experienced from 12:00 am to 8:00 am. During this time, RH recorded was greater than 80% except at 8:00 am when it decreased to 76%. A continuous decrease in RH from 74% at 9:00 am to 70% at 10:00 am was noticed. Relative humidity increased at 11:00 am and continued to gradually increase with time. At 5:00 pm, RH was recorded at 84%. A drastic temperature increase to $13.2 \text{ }^\circ\text{C}$ was recorded at 9:00 am. A range of $14.4 \text{ }^\circ\text{C}$ to $16.3 \text{ }^\circ\text{C}$

was noticed from 10:00 to 5:00 pm. Thereafter, a noticeable decrease of temperature was noted at 6:00 pm and ranged between 11.4 °C to 12.5 °C until 11:00 pm.

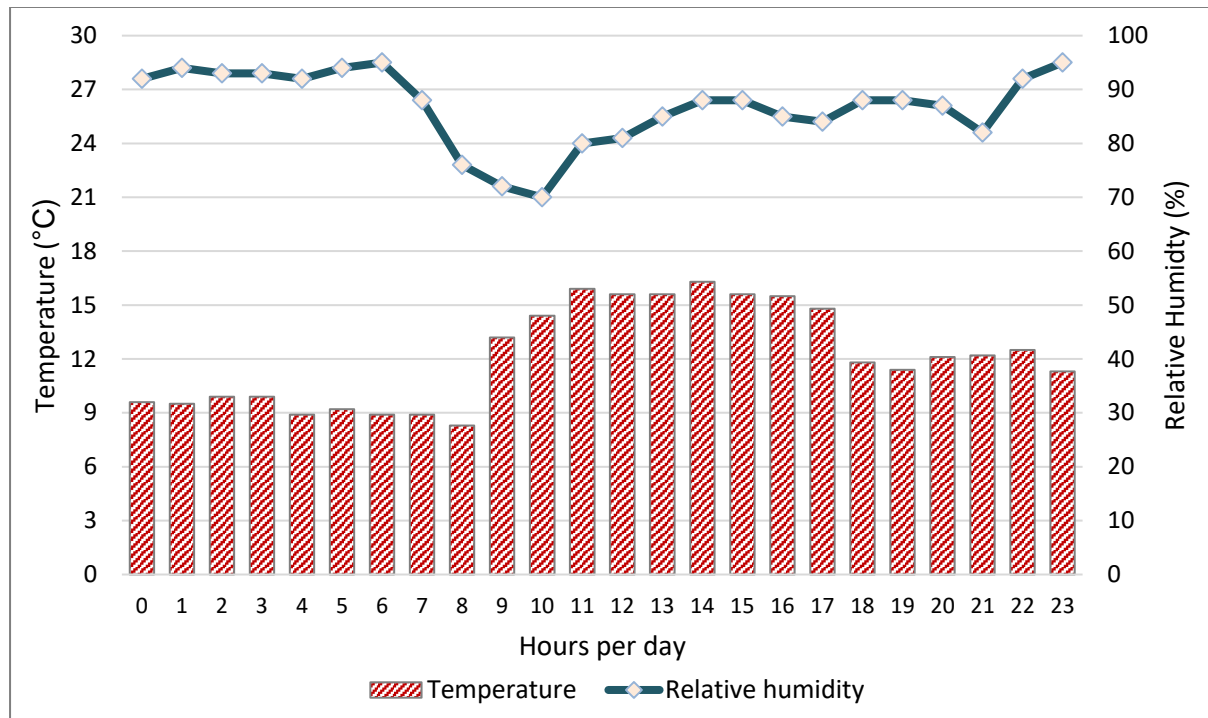


Figure 7.4: Summary of temperatures experienced on the 4th of July 2019 at Langgwens Experimental Farm from 12:00 am to 11:00 pm. The times presented as 0, 1, 2 ... and 23 denotes 00:00, 01:00, 02:00 ... and 23:00 hrs, respectively.

7.3.3 Roodebloem Experimental Farm (2019)

There was a significant interaction of glufosinate ammonium dosage rate and TOA on the trial conducted at Roodebloem Experimental Farm in 2019 (**Figure 7.5**). Mortality of ryegrass was dosage rate dependent – mortality rate of ryegrass increased as dosage rate of glufosinate ammonium increased. Mortality was significantly lower for ryegrass treated at mid-day than ryegrass treated in the morning and evening across all dosage rates, such that less than 75% ryegrass was controlled with a high dosage rate of 7.5 L ha⁻¹. No significant difference in mortality was shown between ryegrass treated in the morning and evening across all glufosinate ammonium dosage rates.

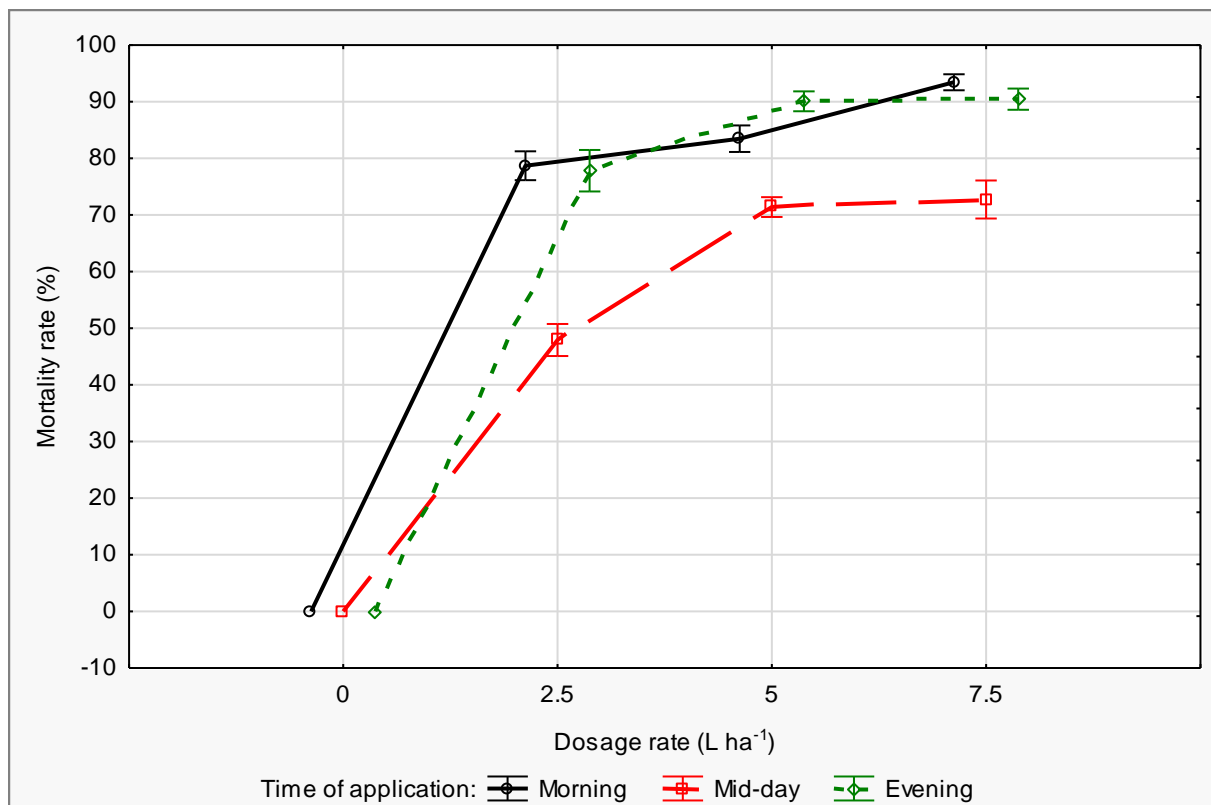


Figure 7.5: Effect of temperature on efficacy of glufosinate ammonium sprayed at different dosage rates for the control of ryegrass at Roodebloem farm in 2019. Error bars represent \pm standard error of the mean of three replications ($n = 3$).

Temperature and RH summaries for the application day at Roodebloem farm in 2019 are shown in **Figure 7.6**. The farm received variable temperatures during the day. A gradual decrease in temperature from 11.2 °C at 12:00 am to 7 °C at 8:00 am was noticed, after which a gradual increase in temperature from 8.7 °C at 9:00 am to 16 °C at 2:00 pm was recorded. The increase in temperature was concurrently noted with a decrease in RH from 96% at 9:00 am to 55% at 2:00 pm. Relative humidity gradually increased from 55% at 2:00 pm to 90% at 11:00 pm. Relative humidity at 5:00 pm was recorded at 76%. After 2:00 pm, temperature gradually decreased to 9.8 °C at 8:00 pm. Temperature ranged from 10.4 °C to 10.5 °C during the last 3 hours of the day (9:00 pm to 11:00 pm).

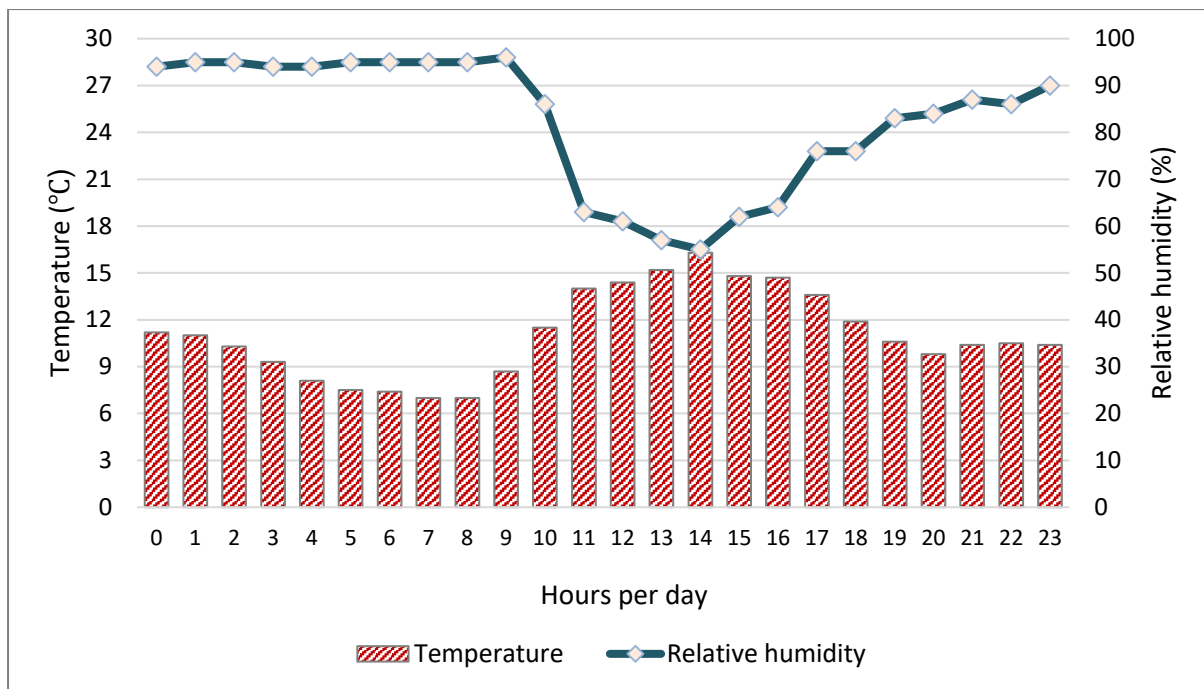


Figure 7.6: Summary of temperatures experienced on the 5th of July 2019 at Roodebloem Experimental Farm from 12:00 am to 11:00 pm. The times presented as 0, 1, 2 ... and 23 denotes 00:00, 01:00, 02:00 ... and 23:00 hrs, respectively.

Correlation results between ryegrass mortality and average temperature and RH is shown in **Table 7.2**. There was no significant correlation observed at Langgewens farm in 2018 between mortality rate and both temperature and RH. Generally, an increase in temperature negatively influenced ryegrass mortality. Negative correlations were observed 1 HBA, 1 HAA and 24 HAA at between mortality and average temperature at Langgewens farm in 2019, however, strong correlations were observed 1 HBA and 1 HAA ($r = -0.751586$ and -0.829161 respectively). Weak, negative correlations between mortality and temperature were observed 1 HAA, 1 HBA and 3 HBA ($r = -0.506693$, -0.684658 and -0.428094 respectively). Significant, positive correlations were observed between mortality rate and RH 1 hour before application (HBA), 1 hour after application (HAA), 3 HBA, 24 HBA and 24 HAA at Langgewens farm in 2019. At Roodebloem farm in 2019, the only significant correlations between mortality and RH were observed 1 HBA and 1 HAA.

Results from analysis of average, maximum and minimum temperature observed in the two farms 21 days prior to glufosinate ammonium application are shown in **Table 7.3**. No significant differences were shown by average and maximum temperature across all farms. Minimum temperature at Roodebloem farm was significantly different from Langgewens farm (2018 and 2019). No significant difference in minimum temperature was observed between the two Langgewens seasons (2018 and 2019).

Table 7.2: Correlation of ryegrass mortality rate with average temperature and humidity recorded 1, 3 and 24 hours before and after glufosinate ammonium application

	Farm and trial year		
	Langgewens 2018	Langgewens 2019	Roodebloem 2019
Average Temperature	Mortality rate		
1 HBA*	0.153775	-0.751586*	-0.506693*
1 HAA*	-0.236549	-0.829161*	-0.684658*
3 HBA	0.115177	-0.066350	-0.428094*
3 HAA	0.039165	0.004672	0.289213
24 HBA	-0.045669	-0.504847*	0.301270
24 HAA	-0.243073	-0.226004	-0.200882
Average relative humidity	Mortality rate		
1 HBA	0.158011	0.878401*	0.595323*
1 HAA	0.214826	0.555586*	0.611861*
3 HBA	-0.137677	0.779007*	0.113566
3 HAA	-0.233240	0.336673	0.271182
24 HBA	0.105930	0.875674*	0.135598
24 HAA	-0.157126	0.849466*	0.110356

Value* represents a significant correlation ($p < 0.05$). HBA*: Hours before application. HAA*: Hours after application.

Table 7.3: Average, maximum and minimum temperature 21 days before glufosinate ammonium application at Langgewens farm (2018 and 2019) and Roodebloem farm (2019)

Temperature (°C)	Langgewens (2018)	Langgewens (2019)	Roodebloem (2019)
Average*	13.8 ^a	13.9 ^a	13.3 ^a
Maximum	18.6 ^a	19.3 ^a	18.2 ^a
Minimum	8.8 ^a	8.5 ^a	6.6 ^b

*Values in a row followed by the same letters indicate no significant differences between treatments at $p = 0.05$.

7.4 DISCUSSION

Molefe (2015) and Mucheri (2016) observed that efficacy of glufosinate ammonium increased with decreasing temperature. Chapter 3 of this dissertation presented the same results – control of ryegrass was greater at 10/15 and 15/20 °C than at 20/25 and 25/30 °C (night/day) temperature regimes. Results found in this study concurred with the results from glasshouse experiments observed in Chapter 3. Trials to investigate efficacy of glufosinate ammonium on ryegrass as influenced by TOA at Langgewens farm in 2019 and Roodebloem farm in 2019 observed that lower temperatures increase efficacy of glufosinate. Results showed that performance of glufosinate ammonium was significantly higher for ryegrass treated when temperatures were below 15 °C, however, dosage rates of 2.5 L ha⁻¹ did not exceed 80% control under the cool temperatures. Increase of dosage rate to 5 L ha⁻¹

resulted in control of over 80% but less than 90% at Langgewens (2019) and Roodebloem (2019) while 7.5 L ha⁻¹ controlled > 90% ryegrass. This high control at lower temperatures was noticed when RH was greater than 80% for all observations. At Langgewens farm in 2019, poor control was noted for ryegrass treated in the morning when the temperature was 8.3 °C (< 15 °C) and RH had decreased to 76%. This suggests that greater control of ryegrass was achieved when glufosinate ammonium was applied in the morning and evening than at mid-day. A similar result on glyphosate observed by Mohr et al. (2007) suggested that better control of barnyardgrass (*Echinochloa crus-galli* L.) and velvetleaf (*Abutilon theophrasti* Medicus ABUTH) occurred when weeds were treated at 8:00 am vs. 2:00 pm is attributed to diurnal changes in leaf movements. An investigation done by Sellers et al. (2004), however, recommends that applications should be done during the light period when glutamine synthetase is catalytically active. The study showed that influence of temperature on efficacy of glufosinate ammonium cannot be isolated from RH. Varanasi et al. (2016) noted that transpiration flow influenced by environmental temperature and RH affect herbicide absorption and translocation.

A different trend was shown by ryegrass mortality at Langgewens farm in 2019 where application at mid-day when temperature was 15.6 °C resulted in significantly higher control than the morning application. Relative humidity recorded at 12:00 pm on that day was 81% which is relatively high. At Roodebloem farm (2019), mid-day application at a temperature of 14.4 °C (< 15 °C) and RH of 61% resulted in very poor control such that dosage rate of 7.5 L ha⁻¹ controlled less than 75% ryegrass. This shows that RH had greater effect on glufosinate ammonium efficacy than temperature. These results concur with a study conducted by (Coetzer et al. 2001) which concluded that control of glufosinate ammonium on common waterhemp (*Amaranthus rudis* S.), Palmer amaranth (*Amaranthus palmeri* S.) and redroot pigweed (*Amaranthus retroflexus* L.) is more influenced by RH than temperature. Petersen and Hurle (2000) noted that low RH which occurs under field conditions accounted for the variable response shown by *Brassica rapa* L. and *Galium aparine* L. to glufosinate ammonium. This might have been the same case for ryegrass in the present study. Studies conducted to investigate the influence of relative humidity and temperature on other post-emergence, foliar applied diphenylether herbicides such as acifluorfen, fomesafen and lactofen found that an increase in RH from 50% to 85% increased efficacy of the herbicides, however, changes in temperature at the same RH did not influence herbicide efficacy (Wichert et al. 1992).

This study observed that efficacy of glufosinate ammonium is influenced by immediate conditions after spraying. This was shown at Langgewens farm in 2018. Mid-day application of glufosinate ammonium on that day was done when RH was 95%. Thereafter, a noticeable

decrease in RH to 67% was observed at 2:00 pm to 4:00 pm. However, this decrease in RH did not decrease efficacy of glufosinate ammonium as shown by **Figure 7.2** suggesting that changes in environmental conditions more than an hour after application had no effect on herbicide activity. **Table 7.2** showed a strong negative influence of temperature and RH on mortality 1 HBA and 1 HAA only at Langgewens and Roodebloem farms in 2019 meaning that environmental conditions observed more than an hour before or after application had little or no effect on glufosinate ammonium efficacy. Ramsey et al. (2006) observed that droplets of glufosinate ammonium on wild oats (*Avena fatua* L.) leaves dried within 10 min at 40% RH but moisture was retained an hour after application at 95% RH. Ramsey et al. (2006) supports that immediate environmental conditions, particularly RH, after application influence glufosinate ammonium efficacy, whereas, Sellers et al. (2004) suggested the 12 hr period after glufosinate ammonium had influence on its translocation but attributed these findings to plant metabolism factors. Sellers et al. (2004) found that glufosinate ammonium applied following dark periods when the chloroplast is not the sink is sequestered into the vacuole, hence the molecules become unavailable.

This present study confirmed that increased temperature immediately after glufosinate ammonium application decreases its efficacy, however, the highest temperatures recorded during the application days at Langgewens 2018, Langgewens 2019 and Roodebloem 2019 were 11.6, 16.3 and 16.4 °C, respectively. According to Molefe (2015) and Mucheri (2016), these temperature were considered as cooler temperatures, hence, an analysis on temperatures experienced 21 days prior to application was done to determine if the ryegrass was exposed to at least greater than 20 °C during the trial period (**Table 7.2**). None of the fields received at least 20 °C or greater temperature and no significant differences in average and maximum temperature was shown between the farms. Previous studies done to investigate the influence of temperature on glufosinate ammonium were done on a wide range of temperatures which include warmer temperatures greater than 20 °C and cool temperatures which are less than 20 °C (Pline et al. 1999; Harker and Dekker 1988; Kumaratilake and Preston 2005). It is therefore, difficult to link the accumulation of calcium and phenolic compounds to variability of glufosinate ammonium efficacy mainly because calcium and phenolic compound accumulation in plants results from exposure to high temperatures or stress (Gong et al. 1998; Knight 2000; Ramakrishna and Ravishankar 2011; Gall et al. 2015). Additional bioclimatic zones which include areas receiving very warm temperatures should be considered for further investigations on the effects of calcium and phenolic compounds on glufosinate ammonium efficacy.

7.5 CONCLUSIONS AND RECOMMENDATIONS

The study concluded that control of ryegrass under field conditions is higher when temperatures are low, and RH is high. More attention has to be given to RH since it results in variability in the results. The study, therefore, recommends that glufosinate ammonium application be done early in the morning or later in the afternoon. Applications done at mid-day or when temperatures are warmer should be done when RH is high (> 80%). The role of calcium and phenolic compounds need to be investigated with additional bio-climatic zones in warmer regions.

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CHAPTER 8

GENERAL CONCLUSIONS AND RECOMMENDATIONS

The ever-increasing herbicide resistance evolution on commonly used post-emergence, non-selective herbicides land poses a great risk to the global food security. Glufosinate ammonium is an alternative herbicide that can replace or be used in rotations with herbicides such as glyphosate and paraquat. Previous studies by Molefe (2015) and Mucheri (2016) observed that glufosinate ammonium increases its efficacy on ryegrass (*Lolium* spp.) with decreasing temperatures. This study confirmed that ryegrass control was better at cooler temperatures (10/15 and 15/20 °C) than at warmer temperatures (20/25 and 25/30 °C). Poor control of ryegrass at warmer temperatures was attributed to accumulation of calcium, thickening of the leaf cuticle and increased production of phenolic compounds. The study provided supporting evidence that calcium reacted with glufosinate ammonium by analysing results from the X-ray diffraction, however, it was difficult to match the broad peaks from treated grasses at cooler temperatures. The use of an improved, wide-angle X-ray scattering (WAXS)-based method is recommended for further studies on the efficacy of glufosinate ammonium as influenced by calcium. Warmer temperatures increased ryegrass cuticle thickness, sequentially resulting in poor efficacy of glufosinate ammonium. The study observed that production of phenolic compounds is influenced by temperature – warmer temperatures increase production of phenolic compounds. These compounds harden the cell wall, hence making it more compact, tighter and less permeable when responding to stress. Therefore, high concentration of phenolic compounds, particularly ferulic and *p*-coumaric acid, at warmer temperatures, resulted in poor control of ryegrass. The use of confocal imaging in herbicide efficacy studies is a novel methodology noted from these findings. Furthermore, the ability of phenolic compounds to increase tolerance of plants to herbicide treatments can be exploited in developing herbicide-tolerant crops.

Phenolic compounds were believed to be responsible for the amicable response shown by glutamine synthetase (GS) at warmer temperatures. This response ensured continuous supply of glutamate which is required for amino acid metabolism. At warmer temperatures, the alternative glutamate dehydrogenase pathway (GDH) was not used because GS was still active, however, ryegrass at cooler temperatures made use of the GDH pathway. The GDH is believed to have not provided sufficient glutamate, hence control was greater at cooler temperatures. Ammonium accumulation was lower at cooler temperatures but there was no relation observed with efficacy of glufosinate ammonium. The study, therefore, suggested that death of ryegrass post glufosinate ammonium application is attributed to the depletion of

glutamate more than accumulation of ammonium. The study of photosynthesis post glufosinate ammonium application showed a drastic decrease in photosynthesis to ($< 0 \mu\text{mol} (\text{CO}_2) \text{ m}^{-2} \text{ s}^{-1}$) 24 HAA, after which photosynthesis became positive 48 and 72 HAA. A significant increase in photosynthesis at 20/25 and 25/30 °C 48 and 72 HAA resulted in better survival of ryegrass.

The study further investigated if response of ryegrass to glufosinate ammonium is similar to some important grass weed species in the western Cape, South Africa. The results observed that control of ryegrass increased with decreasing temperature, whilst temperature had no significant effect on wild oats (*Avena fatua* L.). For ripgut brome (*Bromus diandrus* L.), very poor control was observed at 10/15 °C and control increased with increasing temperature. However, a non-significant decrease was observed at 25/30 °C. High calcium concentration detected in ripgut brome did not influence mortality of the grass species. The study perceived that the calcium form found in ripgut brome was inactive since activation of $[\text{Ca}^{2+}]_{\text{cyt/mit}}$ is dependent on the response of the plant. Variability in weed response to glufosinate ammonium among weed species will inevitably result in weed shift, hence, the study recommends early monitoring of population adaptability so as to channel research into the new weeds.

Decrease in efficacy of glufosinate ammonium under warmer temperature regimes might be a problem, especially in summer cropping areas. The study recommended the use of adjuvants to mitigate phenolic compounds production and calcium reaction with glufosinate ammonium. There was evidence that adjuvants with ammonium sulfate as active ingredient remarkably suppressed production of phenolic compounds. Lower concentration of phenolics detected meant that the plants' defence against chemical (glufosinate ammonium) stress was poor, resulting in higher efficacy of glufosinate ammonium. The most effective adjuvants were Class act NG™ and Velocity Super™. The surfactant properties exhibited by the non-ionic Summit Super adjuvant did not significantly improve glufosinate ammonium efficacy when compared to glufosinate ammonium solo application.

Finally, the study aimed to investigate the practical implications of temperature effects on glufosinate ammonium in the field by treating ryegrass at different day times (8:00 am, 12:00 pm and 5:00 pm). Although there was not much variability in the day temperatures, the study concluded that applications in the mornings and evenings provided better control of ryegrass but only when relative humidity was greater than 75%. Control of ryegrass was more dependent on humidity than on temperature. Applications to be done at mid-day required very high humidity to achieve good control of ryegrass. The study recommends that glufosinate ammonium applications in temperate climates should be done in the morning or

in the evening. Mid-day applications should be done when humidity is very high to achieve good control of ryegrass. Further studies to investigate possibilities of calcium, phenolic compounds and cuticle thickness effects on glufosinate ammonium efficacy in the field are recommended to be done on a wide range of bioclimatic zones representing temperate, tropical and arid cropping areas.

APPENDICES

Appendix 3.1: Analysis of variance- mortality of ryegrass treated with glufosinate ammonium in the glasshouse under different temperature regimes

Effect	Mortality rate				
	SS	Degree. of Freedom	MS	F	p
Intercept	386418.1	1	386418.1	1013.318	0.000000*
Temperature	30707.0	3	10235.7	26.841	0.000000*
Dosage rate	121989.3	4	30497.3	79.974	0.000000*
Temperature*Dosage rate	19195.3	12	1599.6	4.195	0.000025*
Error	38133.9	100	381.3		

Appendix 3.2: Analysis of variance- percentage area of blue auto-fluorescence in ryegrass grown under different temperature regimes

Effect	Fluorescence area				
	SS	Degree. of Freedom	MS	F	p
Intercept	12.95217	1	12.95217	51808693	0.00
Temperature	7.68916	3	2.56305	10252213	0.00
Treatment	6.02703	1	6.02703	24108121	0.00
Temperature*Treatment	4.21493	3	1.40498	5619905	0.00
Error	0.00000	16	0.00000		

Appendix 3.3: Analysis of variance- protocatechuic acid concentration in ryegrass under different temperature regimes

Effect	Protocatechuic acid			
	SS	MS	F	p
Intercept	0.144755	0.144755	737.1832	0.000000
treatment	0.023354	0.023354	118.9311	0.000000
Temperature	0.012743	0.004248	21.6311	0.000007
Treatment*Temperature	0.021569	0.007190	36.6150	0.000000
Error	0.003142	0.000196		
Total	0.060807			

Appendix 3.4: Analysis of variance- *m*-coumaric acid concentration in ryegrass under different temperature regimes

Effect	<i>M</i> -coumaric acid			
	SS	MS	F	p
Intercept	0.002286	0.002286	11419.83	0.000000
Treatment	0.000274	0.000274	1370.38	0.000000
Temperature	0.000226	0.000075	376.83	0.000000
Treatment*Temperature	0.000184	0.000061	306.34	0.000000
Error	0.000003	0.000000		
Total	0.000688			

Appendix 3.5: Analysis of variance- syringic acid concentration in ryegrass under different temperature regimes

Effect	Syringic acid			
	SS	MS	F	p
Intercept	0.024225	0.024225	126414.3	0.000000
Treatment	0.001985	0.001985	10357.8	0.000000
Temperature	0.000708	0.000236	1231.7	0.000000
Treatment*Temperature	0.000133	0.000044	231.5	0.000000
Error	0.000003	0.000000		
Total	0.002829			

Appendix 3.6: Analysis of variance- *p*-coumaric acid concentration in ryegrass under different temperature regimes

Effect	<i>P</i> -coumaric acid			
	SS	MS	F	p
Intercept	1.650631	1.650631	2589.135	0.000000
Treatment	0.001472	0.001472	2.308	0.148200
Temperature	0.080788	0.026929	42.241	0.000000
Treatment*Temperature	0.025073	0.008358	13.109	0.000141
Error	0.010200	0.000638		
Total	0.117533			

Appendix 3.7: Analysis of variance- gallic acid concentration in ryegrass under different temperature regimes

Effect	Gallic acid			
	SS	MS	F	p
Intercept	0.032503	0.032503	675.9926	0.000000
Treatment	0.000071	0.000071	1.4750	0.242184
Temperature	0.001130	0.000377	7.8334	0.001935
Treatment*Temperature	0.000376	0.000125	2.6065	0.087557
Error	0.000769	0.000048		
Total	0.002346			

Appendix 3.8: Analysis of variance- ferulic acid concentration in ryegrass under different temperature regimes

Effect	Ferulic acid			
	SS	MS	F	p
Intercept	3.094989	3.094989	31892.51	0.000000
Treatment	0.234835	0.234835	2419.87	0.000000
Temperature	0.186257	0.062086	639.77	0.000000
Treatment*Temperature	0.056919	0.018973	195.51	0.000000
Error	0.001553	0.000097		
Total	0.479563			

Appendix 4.1: Analysis of variance- glutamate synthetase activity in control and treated ryegrass leaves after glufosinate ammonium application under different temperature regimes

Effect	Degree of Freedom	F	p
Intercept	1	1838,543	0,000000
Temperature	3	3,689	0,018073
Treatment	1	1,798	0,186302
Plant section	1	113,333	0,000000
Temperature*Treatment	3	4,933	0,004571
Temperature*Plant section	3	0,758	0,523396
Treatment*Plant section	1	7,778	0,007562
Temperature*Treatment*Plant section	3	4,477	0,007515
Error	48		

Appendix 4.2: Analysis of variance- glutamate dehydrogenase synthetase activity in control and treated ryegrass leaves after glufosinate ammonium application under different temperature regimes

Effect	Degree of Freedom	F	p
Intercept	1	4267,913	0,000000
Temperature	3	28,112	0,000000
Treatment	1	19,194	0,000064
Process	1	12,891	0,000774
Temperature*Treatment	3	2,906	0,044122
Temperature*Process	3	1,255	0,300420
Treatment*Process	1	1,967	0,167248
Temperature*Treatment*Process	3	0,698	0,558004
Error	48		

Appendix 4.3: Analysis of variance- ammonia accumulation in ryegrass control and treated leaves and roots after glufosinate ammonium application under different temperature regimes

Effect	Degree of Freedom	F	p
Intercept	1	180,8069	0,000000
Temperature (°C)	3	1,0817	0,365811
Treatment	1	113,0820	0,000000
Plant section	1	32,0336	0,000001
Temperature (°C)*Treatment	3	1,2052	0,317961
Temperature (°C)*Plant section	3	1,5034	0,225597
Treatment*Plant section	1	31,5412	0,000001
Temperature (°C)*Treatment*Plant section	3	1,9831	0,129052
Error	48		

Appendix 4.4: Analysis of variance- nitrate reductase activity in control and treated ryegrass leaves and roots after glufosinate ammonium application under different temperature regimes

effect	Degree of Freedom	F	p
Intercept	1	378,9268	0,000000
Temperature (°C)	3	36,9997	0,000000
Treatment	1	1,3470	0,251540
Plant section	1	1,0348	0,314127
Temperature (°C)*Treatment	3	1,1734	0,329695
Temperature (°C)*Plant section	3	1,2535	0,300900
Treatment*Plant section	1	0,3653	0,548422
Temperature (°C)*Treatment*Plant section	3	0,6546	0,584035
Error	48		

Appendix 5.1: Analysis of variance- mortality of ryegrass, rigput brome and wild oats treated with glufosinate ammonium in the glasshouse under different temperature regimes

Effect	SS	Degree of Freedom	MS	F	p
Intercept	1459802	1	1459802	4896.775	0.000000
Temperature (°C)	11285	3	3762	12.618	0.000000
Dosage rate	456275	4	114069	382.633	0.000000
Grass	35280	2	17640	59.173	0.000000
Temperature (°C)*Dosage rate	25664	12	2139	7.174	0.000000
Temperature (°C)*Grass	40580	6	6763	22.687	0.000000
Dosage rate*Grass	29956	8	3744	12.560	0.000000
Temperature (°C)*Dosage rate*Grass	28859	24	1202	4.034	0.000000
Error	89435	300	298		

Appendix 6.1: Analysis of variance- mortality of ryegrass treated with glufosinate ammonium in tank mixtures with selected adjuvants

Effect	SS	Degree of Freedom	MS	F	p
Intercept	306281.3	1	306281.3	1176.120	0.000000
Dosage rate	89109.4	4	22277.3	85.545	0.000000
Adjuvant	6531.3	3	2177.1	8.360	0.000099
Dosage rate*Adjuvant	6203.1	12	516.9	1.985	0.041577
Error	15625.0	60	260.4		

Appendix 7.1: Analysis of variance on ryegrass mortality at Langgewens farm (2018) after glufosinate ammonium application in the morning, mid-day and evening

Effect	SS	Degree of Freedom	MS	F	p
Intercept	304030.1	1	304030.1	1155.619	0.000000
Dosage rate (L ha ⁻¹)	67636.3	3	22545.4	85.695	0.000000
TOA	26.4	2	13.2	0.050	0.951072
TOA*Dosage rate (L ha ⁻¹)	497.7	6	82.9	0.315	0.928305
Error	41831.1	159	263.1		

Appendix 7.2: Analysis of variance on ryegrass mortality at Langgewens farm (2019) after glufosinate ammonium application in the morning, mid-day and evening

Effect	SS	Degree of Freedom	MS	F	p
Intercept	183723.0	1	183723.0	2140.842	0.000000
TOA	7046.1	2	3523.0	41.052	0.000000
Dosage rate (L ha ⁻¹)	52883.7	3	17627.9	205.410	0.000000
TOA* Dosage rate (L ha ⁻¹)	4980.0	6	830.0	9.672	0.000000
Error	4376.7	51	85.8		

Appendix 7.3: Analysis of variance on ryegrass mortality at Roodebloem farm (2019) after glufosinate ammonium application in the morning, mid-day and evening

Effect	SS	Degree of Freedom	MS	F	p
Intercept	249185.1	1	249185.1	4706.125	0.000000
TOA	3948.8	2	1974.4	37.288	0.000000
Dosage rate (L ha ⁻¹)	54837.4	3	18279.1	345.221	0.000000
ToA* Dosage rate (L ha ⁻¹)	1704.5	6	284.1	5.365	0.000111
Error	4130.0	78	52.9		