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HPLC and colourimetric quantification of shikimic acid levels in crops after glyphosate treatment

By

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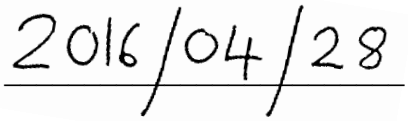
DECLARATION

I, the undersigned, hereby declare that this dissertation, which I hereby submit in partial fulfilment of the requirements for the degree Magister Scientiae (MSc. Biotechnology) in the Department of Plant and Crop Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Johan Bestbier



Date

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ABSTRACT

The shikimic acid pathway is one of the major biosynthetic pathways in higher plants responsible for the biosynthesis of aromatic amino acids (tryptophan, tyrosine and phenylalanine) and multiple secondary metabolites, such as lignin, phytoalexins and indoleacetic acid (IAA). The herbicide glyphosate [N-(phosohonomethyl)glycine] is a non-selective, broad spectrum, post emergence, foliar applied, systemic herbicide that is used globally to control over 180 weed species. Glyphosate is a potent inhibitor of a key enzyme in the shikimic acid pathway namely; 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is the only cellular target for this herbicide. Upon inhibition of EPSPS by glyphosate, shikimate, the dephosphorylated substrate of EPSPS which is upstream of this enzyme in the pathway, cannot be converted to 5-enolpyruvylshikimate-3-phosphate (EPSP). Blockage of the shikimic acid pathway consequently results in the accumulation of high levels of shikimate. Since shikimate accumulation is a direct result of herbicide inhibition of EPSPS, shikimate can be used as a convenient biomarker to measure glyphosate exposure, glyphosate damage as well as the degree of glyphosate resistance. Glyphosate resistance is conferred in glyphosate resistant (GR) crops also known as Roundup Ready® (RR) crops by incorporating a glyphosate tolerant CP4-EPSPS gene from the CP4 strain of *Agrobacterium* which encodes for a bacterial version of the EPSPS that is highly insensitive to glyphosate. When this enzyme (CP4-EPSPS) is expressed and present in RR crops it enables the plant to bypass the glyphosate inhibited native EPSPS in the shikimic acid pathway, thereby allowing the plant to complete the shikimic acid pathway (aromatic amino acid biosynthesis) by making use of the alternative enzyme, thus preventing aromatic amino acid and protein starvation and deregulation of this metabolic route, both of which follow glyphosate treatment in susceptible plants. Thus, RR crops are unaffected by herbicide treatment. Since glyphosate inhibits the EPSPS in susceptible (non-RR) crops, but not in a RR crop line, differences in the shikimate levels occur between these crop lines after glyphosate exposure. The main aims of this study were to quantify shikimic acid levels in Roundup Ready® and non-RR crops after being treated with glyphosate (Roundup Turbo®) by making use of high performance liquid chromatographic (HPLC) analysis as well as a colourimetric assay, and to use these two assays to differentiate between glyphosate resistant and susceptible plants after being exposed to glyphosate. These assays were also used to indicate whether glyphosate was responsible for herbicide damage in maize plants due to drift.

Plant tissues sensitive to glyphosate accumulate shikimic acid to high levels after glyphosate treatment. The detection of shikimic acid has been shown to be a useful marker as a measure of glyphosate injury or to score for glyphosate sensitive and resistant weed biotypes. Up to now, the most common methods for shikimic acid assay include: spectrophotometry, capillary zone electrophoresis, HPLC with UV detection, and the periodate oxidation, or Cromartie and Polge, method. Here we introduce a new method for shikimic acid detection which has a broad application, is colourimetric, sensitive, simple and very quick to use. The method can be used for quantification in plant extracts using a microtiter plate, and can be further adapted for detection of shikimic acid in intact leaf discs or other plant tissues.

Keywords: Aromatic amino acids, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), glyphosate, non-RR crops, RR crops, shikimic acid accumulation

DISSERTATION COMPOSITION

Chapter 1 of this dissertation describes the shikimic acid pathway and the enzymes involved in this pathway—the shikimic acid pathway is the only cellular target site for the herbicide, glyphosate. Chapter 1 also covers the discovery, history, uses and practises of the broad spectrum herbicide, glyphosate. This chapter also discusses how glyphosate resistance is conferred in crops i.e. how genetically modified (GM) glyphosate resistant, Roundup Ready® crops were produced by making use of genetic engineering (biotechnology). **Chapter 2** describes the materials and methods used in this study. This chapter encompasses maize and soybean greenhouse trials, glyphosate (Roundup Turbo®) application rates, maize and soybean plant tissue collection, shikimic acid extraction and shikimic acid quantification by making use of High Performance Liquid Chromatographic (HPLC) and photo-spectroscopic analysis. **Chapter 3** describes and illustrates the results obtained from maize and soybean plants treated with various concentrations of glyphosate over time, results from HPLC and photo-spectroscopic quantification analysis of shikimic acid concentrations and also illustrates the results from the colourimetric assay used to detect the presence of shikimate. This chapter also discusses two case studies; one performed on *Conyza bonariensis* weeds and the other on maize plants from Pioneer seeds which exhibited symptoms of herbicide damage, however from an unknown herbicide. **Chapter 4** discusses the results obtained from this study and proposes future perspectives for refining and further development of the colourimetric assay and states/focusses on the relevance and application of the colourimetric assay in the modern agricultural industry to quickly and accurately differentiate between resistant and susceptible plants. **Chapter 5** finally concludes the findings of this literature study and research. The literature cited in this study is listed in **Chapter 6** entitled **References.** The **Annexure/Appendix** contains photos of plants before and after treatments, photos of the shikimic acid extraction procedure, additional HPLC chromatograms, and colourimetric assay results.

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Lastly, The Almighty God for giving me strength and guiding me through this research study.

Dedication: *I would like to dedicate this dissertation to my late brother Dawie Bestbier* (MBChB) – *what a great inspiration he was to me.*

May his soul rest in peace.

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MONSANTO

NRF National Research Foundation

FABI

ABBREVIATIONS AND SYMBOLS

%	Percent/percentage
˚C	Degree Celsius
µl	Microliter(s)
µm	Micrometer/micron
µmol	Micromole
µmol/l	µM
2PGA	2-phosphoglycerate
3PGA	3-phosphoglycerate
Å	Ångström/angstrom/10^{-10} m
AAAs	Aromatic amino acids
ACCase	Acetyl-CoA carboxylase
ADCS	Aminodeoxychorismate synthase
ADH	Arogenate dehydrogenase
ADT	Arogenate dehydratase
ADP	Adenosine diphosphate
AE	Acid equivalent
Ala	Alanine
AMP	Adenosine monophosphate
AMPA	Aminophosphonic acid
Arg	Arginine
AroAT/TyrB	Aromatic amin acid aminotransferase
AS	Anthranilate synthase
Asp	Aspartic acid

AspC	Aspartate aminotransferase
ASα	Anthranilate synthase α-subunit
ASβ	Anthranilate synthase β-subunit
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosomes
bar gene	Bialaphos resistance gene
Bt	*Bacillus thuringiensis*
BX1	BENZOXAZINELESS 1
C termini	Carboxyl/COOH terminus
C_2H_3N	Acetonitrile/ACN
C3	Carbon 3
C6	Carbon 6
ca $50	Preceding the amount of $50
CARG	Compound annual growth rate
CdRP	1-(*o*-carboxyphenylamino)-1-deoxy-ribulose 5-phosphate
CDS	Coding sequence
CM	Chorismate mutase
cm	Centimeter(s)
CM-DAHP synthase	Chorismate mutase-3-deoxy-D-arabino-heptulosonate-7-phosphate synthase
CM-PDH	Chorismate mutase-prephenate dehydrogenase
CM-PDT	Chorismate mutase-prephenate dehydratase
Co.	Company
Co^{2+}	Cobalt
CoA	*p*-coumaroyl-coenzyme A
CoASH	Coenzyme A
COI1	CORONATINE INSENSITIVE 1

CS	Chorismate synthase
C-terminal ACT	Aspartokinase, chorismate mutase, and TyrA
CTP	Chloroplast transit peptide
DAHP	3-deoxy-D-arabino-heptulosonate-7-phosphate
DAHPS	3-deoxy-D-arabino-heptulosonate-7-phosphate synthase
DAT	Days after treatment
DHD	3-dehydroquinate dehydratase
DHQS	3-dehydroquinate synthase
E. coli	*Escherichia coli*
E4P	Erythrose-4-phosphate/D-erythrose 4-phosphate
ENO1	Enolase
EOBII	EMISSION OF BENZENOIDS II
EPSP	5-enolpyruvylshikimate-3-phosphate
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
F6P	Fructose-6-phosphate
FMN	Flavin mononucleotide
g	Gram
g/kg or g kg^{-1}	Gram per kilogram
G101A	Alanine at position 101
G3P	Glyceraldehyde-3-phosphate
G6P	Glucose-6-phosphate;
GaMV	Cauliflower mosaic virus
GC	Gas chromatography
GFP	Green fluorescent protein
Gln	Glutamine
Glu	Glutamate

GM	Genetically modified
GOX	Glyphosate oxidoreductase
GPT	G6P/phosphate translocator
GR	Glyphosate-resistant
GRCs	Glyphosate-resistant crops
h	Hour
H_2O	Water
H_3PO_4	Orthophosphoric/phosphoric acid
Ha	Hectare
HAT	Hours after treatment
HCl	Hydrochloric acid
HCT	Hydroxycinnamoyl-CoA shikimic acid/quinic acid hydroxycinnamoyltransferase
His	Histidine
HPLC	High performance liquid chromatography
HPP-AT	4-hydroxyphenylpyruvate aminotransferase
HR	Herbicide-resistant
IAA	Indoleacetic acid
ICS	Isochorismate synthase
IGL	Indole-3-glycerol phosphate lyase
IGPS	Indole-3-glycerol phosphate synthase
INT	Iodonitrotetrazolium chloride
IWM	Integrated weed management
JA	Jasmonic acid
K_a	Acid dissociation constant
Kg	Kilogram

*K*i	Inhibitor constant
*K*m	Michaelis-Menten constant
L/ha	Liter per hectare
L-arogenate	Pretyrosine
LC	Liquid chromatography
LCMS	Liquid chromatography mass spectrometry
LD_{50}	Lethal dose, 50%
M	Molarity/mol/L
MEP	Mevalonate/mevalonic acid pathway or 2-C-methyl-D-erythritol 4-phosphate pathway
mg	Milligram
Mg^{2+}	Magnesium
$MgCl_2$	Magnesium chloride
Million ha	Million hectare
min	Minute
MjCM1 gene	*Meloidogyne javanica* chorismate mutase
ml	Milliliter(s)
mM	Millimolar/ 10^{-3} mol/dm^3/ 100 mol/m^3
Mn^{2+}	Manganese
MO	Missouri
MOA	Mode of action
MQ.H_2O	Milli-Q water
mRNA	messenger RNA
MurA	UDP-*N*-acetylglucosamine enolpyruvyl transferase
N termini	Amino/NH_2 terminus
N	Normality
N-(phosohonomethyl)glycine	Glyphosate/Roundup®

NAD^+	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate – reduced form of $NADP^+$
NahG	Salicylate hydroxylase gene
NaOH	Sodium hydroxide
NH_2	Amine group
NH_3	Ammonia
nm	Nanometer/ $1\times10-9$ m
NMR	Nuclear magnetic resonance
Non-RR	Non-Roundup Ready®
OA	Oxaloacetate
OPPP	Oxidative pentose phosphate pathway
P106S	Proline with serine at position 128
PAI	Phosphoribosylanthranilate isomerase
PAL	Phenylalanine ammonia lyase
PAT	Phosphoribosylanthranilate transferase
pat gene	Phosphinothricin acetyltransferase gene
P-CaMV E35S	Cauliflower mosaic virus
PDH	Prephenate dehydrogenase
PDT	Prephenate dehydratase
PEP	Phosphoenolpyruvate
PGyM	Phosphoglyceromutase
Phe hydroxylase	Phenylalanine hydroxylase
Phe	Phenylalanine
Pi	Inorganic phosphate

PK	Pyruvate kinase
PLP	Pyridoxal-5-phosphate
PPA-AT	Prephenate aminotransferase
PPCK	PEP carboxykinase
PPDK	Plastidic pyruvate orthophosphate dikinase
PPi	Inorganic diphosphate
PPT	PEP/phosphate translocator
PPY-AT	Phenylpyruvate aminotransferase
Pro	Proline
Rib5P	Ribose-5-phosphate
RNAi	RNA interference
rpm	Rotations per minute
RR	Roundup Ready®
S3P	Shikimate-3-phosphate
S7P	Sedoheptulose-7-phosphate
SA	Salicylic acid
SAR	Systemic acquired resistance
SDH	Shikimate dehydrogenase
Ser	Serine
Shikimate	Shikimic acid
SHKDH	Shikimate dehydrogenase
SK	Shikimate kinase
STS	Sulfonylurea soybean
TA	Transaldolase
TAT	Time after treatment
TCA	Tricarboxylic acid

TFs	Transcription factors
TK	Transketolase
TPT	Triosephosphate translocator
t_R	Retention time
Trp	Tryptophan
TS	Tryptophan Synthase
TSα	Tryptophan synthase α-subunit
TSβ	Tryptophan synthase β-subunit
Tyr	Tyrosine
USD	US dollar ($)
UV	Ultraviolet light (1-400nm)
X5P	Xylose-5-phosphate
XPT	X5P/phosphate translocator
α-KG	α-ketoglutarate
µM	Micromolar/ 10^{-6} mol/dm^3/ 10^{-3} mol/m^3

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MOTIVATION AND OVERVIEW OF STUDY

With the increasing development of genetically modified (GM) plants, new regulations for the manipulation, growth and use of these organisms are being implemented in several countries. These regulations demand reliable methods for detecting small differences between conventional and genetically modified plant species. In general, such methods include nucleic acid amplification methods, protein based methods and detection of enzymatic activities, among others (Deisingh and Badrie, 2005; Querci *et al*., 2010).

The shikimate pathway, is one of the major biosynthetic pathways in higher plants primarily responsible for the biosynthesis of aromatic amino acids; tyrosine, tryptophan and phenylalanine. The shikimate pathway is localised in plastids of all plant tissue types, including non-green tissues such as most roots (Bonini *et al*., 2009). The shikimate pathway has been an attractive target for the design of herbicidal agents. Its penultimate step results in the reversible formation of 5-enolpyruvylshikimate-3-phosphate (EPSP) and inorganic phosphate from shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP). The reaction is catalyzed by 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is the only cellular target for *N*-(phosphonomethyl)glycine, the active ingredient of the most extensively used foliar-applied, broad-spectrum, non-selective herbicide, glyphosate, which is particularly active against annual and perennial plants (Franz *et al*., 1997).

The inhibition of EPSPS by glyphosate reduces the biosynthesis of aromatic amino acids, which leads to several metabolic disturbances, including the arrest interruption of protein production due to amino acid starvation, prevention of secondary product formation and general metabolic disruption, followed by death (Singh and Shaner, 1998). Upon inhibition of EPSPS by the herbicide, shikimate, the metabolite upstream of this enzyme in the pathway, cannot be converted to EPSP. Blockage of the shikimate pathway consequently results in the accumulation of high levels of shikimate (Marchiosi *et al*., 2009).

Since shikimate accumulation is a direct result of herbicide inhibition of EPSPS, increased interest has arisen in using shikimate as a biomarker for glyphosate exposure (Singh and Shaner, 1998). Glyphosate resistance is conferred in crops by incorporating a gene that encodes a glyphosate-insensitive EPSP synthase (CP4-EPSP synthase) enzyme. The gene encoding this enzyme was obtained from *Agrobacterium* species (strain CP4) of soil bacteria. This enzyme,

when expressed in GM crops, allows the crop to bypass the glyphosate-inhibited native EPSPS in the shikimate pathway, thereby preventing aromatic amino acids starvation and deregulation of this metabolic route, both of which follow glyphosate treatment in susceptible plants. Thus, the glyphosate-resistant (expressing CP4-EPSP synthase) crop plant is unaffected by herbicide treatment (Marchiosi *et al*., 2009). If this herbicide inhibits EPSPS in susceptible (non-glyphosate-resistant) crops, but not in a glyphosate-resistant crop line, differences in shikimate levels should occur after glyphosate exposure.

In plant tissues, shikimate quantification has been assayed by spectrophotometry, capillary zone electrophoresis, micellar electrokinetic capillary chromatography, gas chromatography and high performance liquid chromatography (HPLC) (Bonini *et al*., 2009; Buehring *et al*., 2007). HPLC has been considered a favorable and reliable technique for separation and determination of traces from a wide range of compounds. Efficiency, simplicity, sensitivity and reproducibility are all advantages of HPLC for the separation of extremely small quantities of complex mixtures.

This research project was aimed to develop a simple, rapid and sensitive colourimetric field test to measure shikimate levels in glyphosate-exposed crop tissues, and to apply this assay to quickly and accurately distinguish between glyphosate-resistant and susceptible crop cultivars and weed biotypes. Furthermore, to also evaluate this much faster and simpler colourimetric assay against HPLC analysis. Such a quick field test would be very useful to detect glyphosate injury and would also be very handy to indicate whether a crop variety is resistant to glyphosate. Additionally, since many glyphosate resistant weeds (*Conyza candensis*, *C. bonariensis*, *Lolium* spp, *Plantago lanceolata* and *Chloris virgata*) have been reported, such a test may be important to have a means of quickly identifying and characterising glyphosate resistant weed biotypes to avoid their spread and to facilitate their effective management.

AIMS AND OBJECTIVES

The main aim of this research study was to develop and refine a simple and sensitive colourimetric test which could be used to accurately and rapidly measure shikimate levels in glyphosate exposed plants. This assay must be much faster and simpler than the conventional methods, and must be handy in establishing the degree of sensitivity to glyphosate based on a colour change.

Main objectives of this research study included:

- Evaluate and compare the results from the colourimetric assay to that of HPLC analysis.
- Is there any correlation between glyphosate application rate and the degree of shikimate build-up.
- Is shikimate accumulation affected by time after glyphosate treatment.
- Is there a variance in the degree of shikimate accumulation between different plant tissue.
- Is it possible to use a simplified colourimetric assay to accurately measure shikimate levels in plants.

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 The shikimic acid pathway and aromatic amino acid synthesis

The shikimic acid pathway is named after the central intermediate that is unique to aromatic amino acid synthesis, shikimic acid (shikimate). Shikimate was first isolated from fruits of aniseed (*Illicium anisatum*) in 1885 and was named after the Japanese name of the plant shikimi-no-ki (Bentley, 1990). The shikimate pathway is one of the major biosynthetic pathways in higher plants responsible for linking metabolism of carbohydrates to the production of the aromatic amino acids (AAAs), L-phenylalanine (Phe), L-tyrosine (Tyr), and L-tryptophan (Trp). All three AAAs are derived from intermediates of the same series of reactions in the shikimic acid pathway (Tohge *et al*., 2013). The reactions in the shikimic acid pathway constitutes a part of metabolism that is found only in plants and microorganisms (bacteria and fungi) and never in animals i.e. these three AAAs cannot be produced by animals and thus are essential amino acids which needs to be obtained from their diet. This novel pathway is therefore an important target for herbicides, antibiotics, and live vaccines. The penultimate step in this pathway is inhibited by N-[phosphonomethyl]glycine, the active ingredient of the broad spectrum, non-selective herbicide, glyphosate (Herrmann and Weaver, 1999).

The component enzymes of the pathway are homologous in the microorganism and plant kingdoms. In plants, the enzymes are found in plastids and are presumably all soluble in the stroma. The genes for these enzymes are encoded in the nucleus (Pittard and Yang, 2008). An overview of the pathway is shown in Figure 1.1. In plants the three AAAs (end products) and the intermediates of the shikimic acid pathway are also used as precursors in the synthesis of a variety of other specialised secondary metabolites that play crucial roles in plant growth, development, reproduction, defense, and environmental responses (Figure 1.2; Maeda and Dudareva, 2012).

Tryptophan is a precursor of alkaloids, phytoalexins, and indole glucosinolates as well as the plant hormone auxin, whereas tyrosine is a precursor of isoquinoline alkaloids, pigment betalains, and quinones (tocochromanols and plastoquinone; Figure 1.2 and Figure 1.3).

Phenylalanine is a common precursor of numerous phenolic compounds, which include flavonoids, condensed tannins, lignans, lignin, and phenylpropanoid/benzenoid volatiles. Of the three AAAs, the highest carbon flux is often directed to Phe, as Phe derived compounds can constitute up to 23% of organic matter in some plant species (Herrmann, 1995). All three AAAs are required for protein biosynthesis in living cells and are produced from the final product of the shikimate pathway, chorismate, which is also a precursor for vitamins K_1 and B_9 and the plant defense hormone salicylic acid (Figure 1.2 and Figure 1.3; Maeda and Dudareva, 2012; Romero *et al*., 1995).

The importance of the shikimate pathway is demonstrated by the estimation that 32% of the carbon fixed by plants flows through this pathway (Mir *et al*., 2015). It is estimated that lignins, the most abundant plant biopolymers incorporated in the cell wall which are derived from the shikimate pathway, account for approximately 30% of the organic carbon in the biosphere (Saes Zobiole *et al*., 2010).

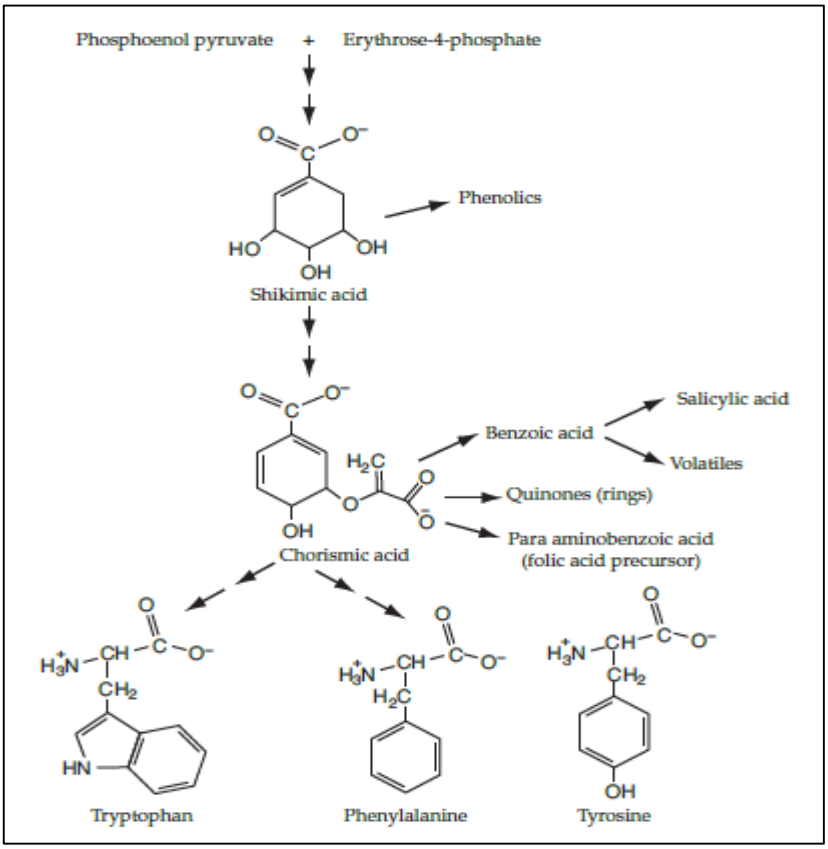


FIGURE 1.1 Simplified overview of the shikimic acid pathway. This diagram highlights the main intermediates and products of the shikimic acid pathway. The precursors are phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P). Shikimic acid and chorismic acid are key intermediates. These intermediates can be removed from the pathway and used to synthesize a variety of additional phenolic compounds (Herrmann and Weaver, 1999).

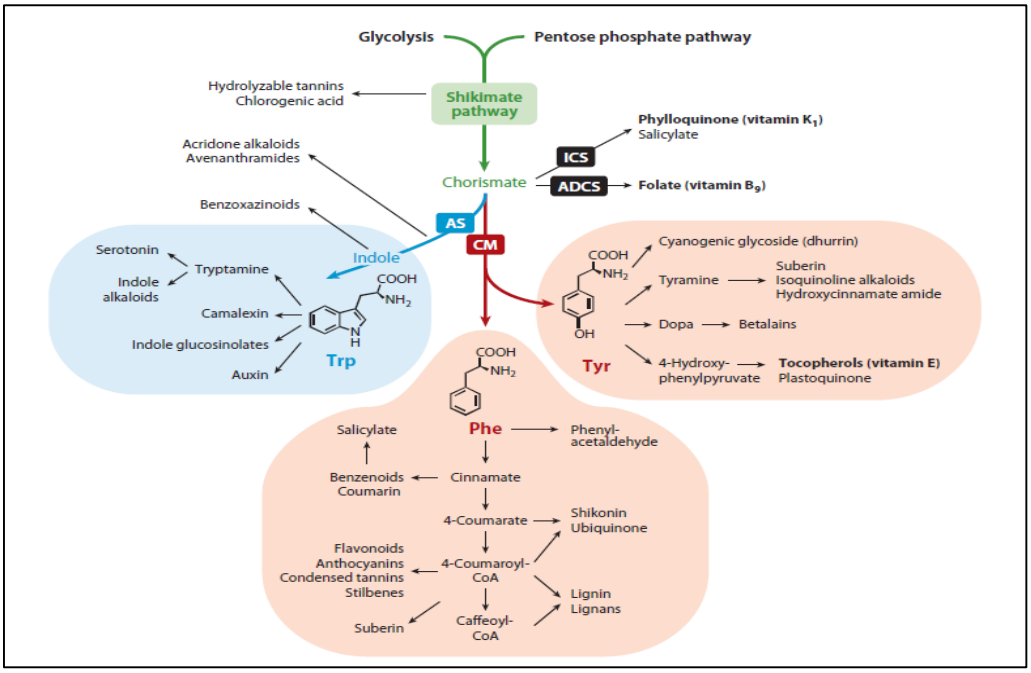


FIGURE 1.2 The aromatic amino acid pathways support the formation of numerous natural products in plants. The shikimate pathway (shown in green) produces chorismate, a common precursor for the tryptophan (Trp) pathway (blue), the phenylalanine/tyrosine (Phe/Tyr) pathways (pink), and the pathways leading to folate, phylloquinone, and salicylate. Trp, Phe, and Tyr are further converted to a diverse array of plant natural products that play crucial roles in plant physiology, some of which are essential nutrients in human diets (bold). Other abbreviations: ADCS, aminodeoxychorismate synthase; AS, anthranilate synthase; CM, chorismate mutase; CoASH, coenzyme A; ICS, isochorismate synthase (Maeda and Dudareva, 2012).

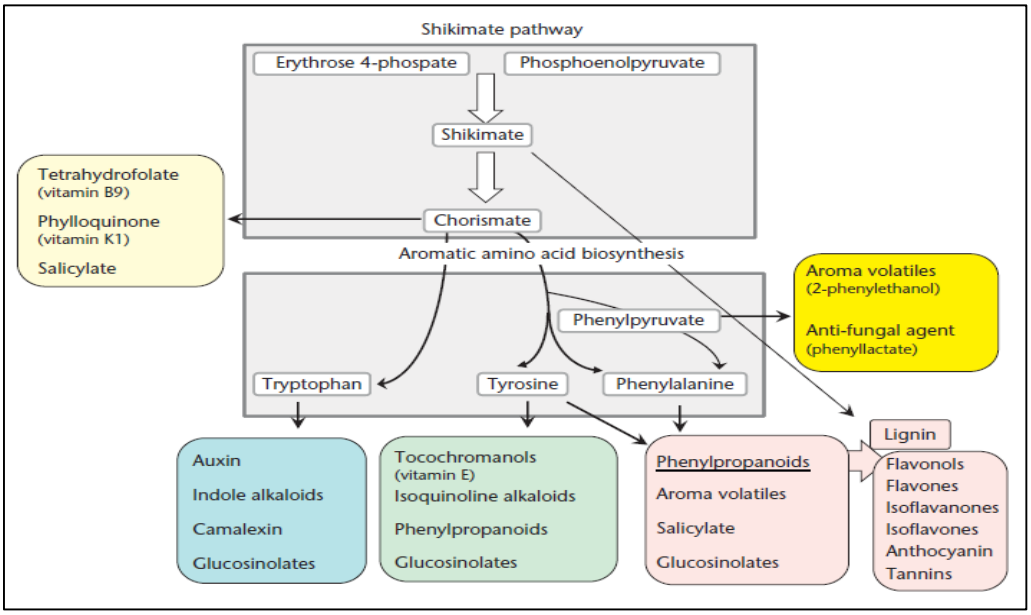


FIGURE 1.3 Major classes of specialised metabolites derived from shikimate, chorismate, phenylalanine, tyrosine and tryptophan (Maeda *et al*., 2010).

The shikimate pathway consists of seven enzymatic reactions beginning with the condensation of two metabolites, erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) to produce chorismate (Figure 1.1). The E4P precursor is an intermediate in the C-3 cycle and the non-oxidative branch of the pentose phosphate pathway in the chloroplast stroma and PEP is an intermediate in glycolysis and may be imported into chloroplasts (Herrmann and Weaver, 1999). Alternatively, PEP can be a product of chloroplast pyruvate metabolism via pyruvate kinase and pyruvate, P_i dikinase (Tohge *et al*., 2013). The first reaction in the pathway is an aldol condensation that results in the synthesis of a 7-carbon ketose, 3-deoxy-arabino-heptulosonic acid- 7-phosphate (DAHP).

DAHP synthases in plants and bacteria show only about 20% amino acid identity. The plant enzyme, however, complements bacterial mutants lacking this synthase. The bacterial enzyme exhibits feedback inhibition by the aromatic amino acid end products, phenylalanine, and tyrosine (Pinto *et al*., 1988). This does not appear to be true of the plant synthases. Expression of different isozymes of DAHP synthase in plants, however, is influenced by environmental factors such as high light intensity or wounding and the presence of hormones, gibberellic acid, and jasmonic acid (Dyer *et al*., 1986).

The second step in the pathway is the formation of a cyclic intermediate, 3-dehydroquinic acid, from DAHP. The enzyme, dehydroquinate synthase, is an oxidoreductase that requires NAD as a cofactor. The enzyme in this case catalyses both an oxidation followed by a reduction, thereby regenerating the oxidized cofactor (Bochkov *et al*., 2012).

Dehydroquinic acid is the substrate for a dehydratase that catalyses removal of water and introduces a double bond into the ring. This is followed by reduction of the ketone to an alcohol forming shikimic acid, the first unique intermediate of the pathway (Figure 1.4). In some plants, shikimic acid is the starting material for the synthesis of a variety of phenolic natural products. The most common are the water-soluble gallotannins, which are complexes of phenolics with sugars, usually glucose. Plants make these compounds to protect their tissues from UV damage and to deter herbivores. Tannins can bind irreversibly to proteins and inhibit enzyme activity. Humans have traditionally used tannic acid–rich plant extracts to tan and preserve animal hides. Some humans also like the bitter flavour of gallotannins in beverages such as tea (Bonini *et al*., 2009; Tohge *et al*., 2013).

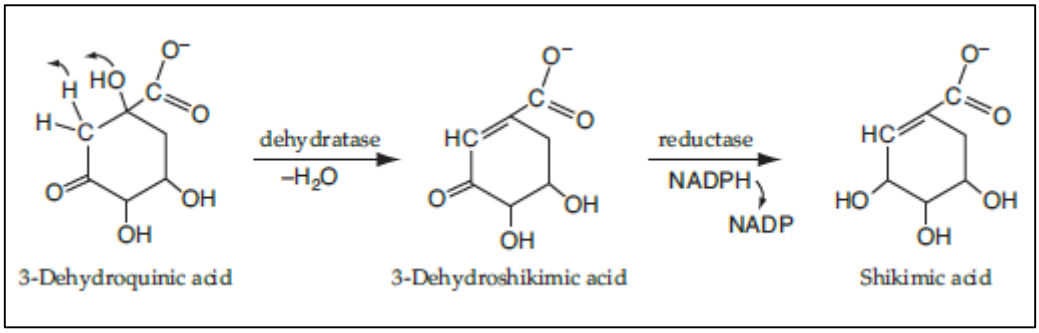


FIGURE 1.4 Synthesis of shikimic acid. A dehydratase-reductase binds dehydroquinic acid. It catalyses removal of the hydroxyl next to the carboxyl group on C-1 and a proton from the adjacent C-6 carbon, generating a double bond in the ring. The NADPH-dependent reductase activity then reduces the keto group to a hydroxyl group to generate shikimic acid (Bonini *et al*., 2009).

Shikimic acid is further metabolized to chorismic acid, another key intermediate in this pathway. A kinase adds a phosphate group to one of the meta hydroxyls to produce shikimic acid-3-phosphate. This intermediate is then condensed with another molecule of PEP to produce 5-enolpyruvyl-shikimate-3-phosphate (EPSP) (Figure 1.5). The enzyme, EPSP synthase, is inhibited by an amino acid analog, *N*-[phosphonomethyl] glycine, also known as glyphosate (Reddy *et al*., 2010). Although the structures do not appear to be similar, glyphosate competes with PEP for the same binding site on the synthase (Berg *et al*., 2008).

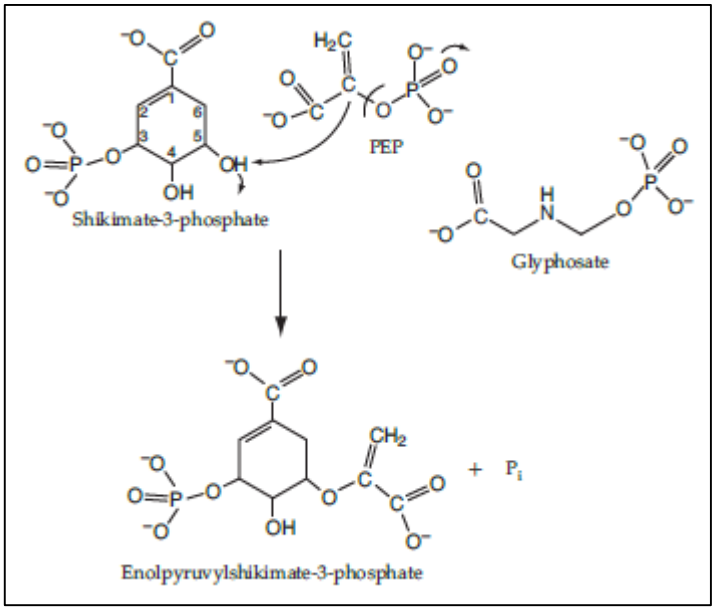


FIGURE 1.5 Synthesis of 5-enolpyruvylshikimate-3-phosphate (EPSP). The EPSP synthase catalyses removal of the phosphate from PEP. The resulting three-carbon fragment is added to the hydroxyl group on C-3 of shikimate-3-phosphate. The amino acid analog, glyphosate, is a competitive inhibitor of PEP (Tzin *et al*., 2009).

The EPSP synthase enzyme consists of two globular domains (Figure 1.6). Binding of shikimate-3-phosphate triggers a global conformational change to the more closed structure seen in Figure 1.6. PEP then binds to the active site formed at the interface of the two domains (Sost *et al*., 1984). This is also the target of glyphosate, a broad spectrum herbicide that is the active component of Roundup®. Both kinetic and crystal structure analyses showed that glyphosate competes with PEP for this same site (Boocock and Coggins, 1983). The unique nature of this structural change is further illustrated by the fact that glyphosate does not compete with PEP in any of the many other reactions that use PEP as a substrate (Alibhai *et al*., 2010).

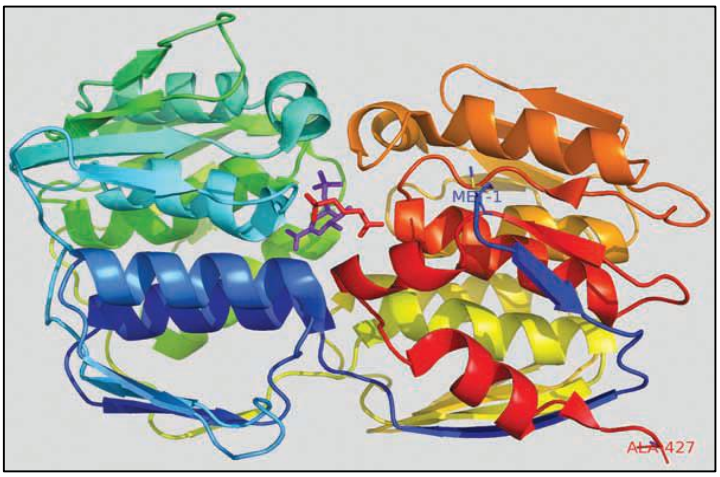


FIGURE 1.6 Structure of EPSP synthase. The enzyme consists of two domains shown in blue-green and red-yellow. In the absence of substrate, a wide gap separates the two domains. After binding shikimate-3-phosphate (shown as purple sticks), the enzyme undergoes a large conformational shift to narrow the gap between the domains as shown. The constricted active site can now bind pyruvate or glyphosate, shown as red sticks (Alibhai *et al*., 2010).

EPSP is the substrate for a synthase that removes the phosphate from C-3 and introduces a second double bond into the ring. This results in the production of chorismic acid. Chorismic acid can be a precursor for benzoic acid and its derivatives and also salicylic acid in some plants. Methylated benzoic and salicylic acids are volatile and are responsible for many floral scents that attract pollinators (Khan *et al*., 2003). Salicylic acid is also a signalling molecule that initiates responses to abiotic stress and pathogen invasion, a process referred to as systemic acquired resistance (SAR). Additionally, chorismic acid in some plants provides the ring portion of quinones and *para*-amino benzoic acid, the precursor for folic acid synthesis. In many plants some of these compounds can also be derived from phenylalanine (Tzin *et al*., 2009).

1.1.1 Focusing on 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase

EPSP synthase (also referred to as 3-phosphoshikimate 1-carboxyvinyltranferase) catalyses the penultimate step of the shikimate pathway, the formation of EPSP, by transferring the enolpyruvyl moiety of PEP to the 5-hydroxyl position of shikimate-3-phosphate (S3P). This C3 enolpyruvyl unit eventually becomes the side chain of Phe and Tyr and is removed during the biosynthesis of Trp (Yamada *et al*., 2008). Crystal structure studies have shown that the binding of the first substrate, shikimate-3-phosphate, triggers a global conformational change to form the active site in the interdomain cleft of EPSP synthase (Okunuki *et al*., 2003).

EPSP synthase is the primary target of the nonselective, broad-spectrum herbicide glyphosate. Glyphosate does not bind to the naked enzyme but rather to the enzyme-S3P complex (Lourenco and Neves, 1984). Glyphosate competitively inhibits EPSP synthase with respect to the second substrate, PEP, by occupying the PEP binding site of the enzyme-S3P complex (Malone *et al*., 2016). For a long time, the ternary complex enzyme-S3P-glyphosate has been considered a transition state analog in which glyphosate takes the place of PEP. However, other enzymes that have PEP as substrates are not inhibited by glyphosate. Enzyme ligand distances within the glyphosate-containing ternary complex were measured by NMR, and indicated that the complex may in fact not be a transition state analog, because PEP and glyphosate binding are not identical (Anderson *et al*., 1990).

EPSP synthases from different organisms have been divided into two classes based on glyphosate sensitivity: All plants and most bacteria, including *E. coli*, have glyphosate-sensitive class I EPSP synthases, whereas some bacteria, such as *Agrobacterium* sp. strain CP4, have class II EPSP synthases that are relatively resistant to glyphosate and therefore have been used to generate glyphosate-resistant crops (Ramalho *et al*., 2009).

1.1.2 Phenylalanine and tyrosine synthesis

Chorismic acid is the branch point between synthesis of phenylalanine and tyrosine or tryptophan (Figure 1.1). Chorismate mutase catalyses the transfer of the pyruvyl side chain to C-1 to produce prephenic acid, the precursor of phenylalanine and tyrosine. This mutase is allosterically regulated by the three aromatic amino acids (Yamada *et al*., 2008). In the next step, an amino group is added to prephenic acid to produce arogenic acid. The transaminase that catalyses this reaction uses glutamic acid as the amino donor. Oxidative-decarboxylation of arogenic acid by an NADP-dependent dehydrogenase yields tyrosine. To synthesize phenylalanine, decarboxylation of arogenic acid is followed by a dehydration step to produce the aromatic ring of phenylalanine (Barends *et al*., 2008; Byng *et al*., 1981).

1.1.3 Tryptophan synthesis

Chorismic acid is also the precursor for the synthesis of tryptophan (Figure 1.1). A synthase catalyses removal of the pyruvyl side chain and a transamination to produce anthranilic acid. The amino donor in this reaction is glutamine. The next step involves a glycosylation, that is, addition of 5-phospho-ribosyl-1-diphosphate to the amino group of anthranilic acid. The sugar diphosphate is a good leaving group, and the sugar is bonded to anthranilate by an *N*-glycosidic bond as seen in nucleosides. An isomerase then catalyses the formation of the ketose derivative. Decarboxylation of the aromatic ring and dehydration and cyclization produce the five-membered ring of indole glycerol-phosphate (Zhao and Last, 1996).

The final step in this branch of the pathway is catalysed by tryptophan synthase. This enzyme cleaves the glycerol-P moiety from the indole ring and replaces it with serine (Chavez-Bejar *et al*., 2012).

1.1.4 Regulation of the shikimate and aromatic amino acid biosynthetic pathways

Both microorganisms and plants regulate carbon flux toward AAA biosynthesis at the transcriptional and posttranscriptional levels. Besides basal levels of AAA production for protein biosynthesis, plants have to maintain their production for the biosynthesis of downstream natural products, including the major cell wall component lignin and defense compounds, the levels of which often drastically change under specific developmental and environmental conditions. Thus, the regulation of AAA biosynthesis in plants should be coordinated with the activities of downstream metabolic pathways and different from those of microorganisms (Bonini *et al*., 2009; Tohge *et al*., 2013).

1.1.4.1 Transcriptional regulation

In microbes, the expression of the first gene in the shikimate pathway (*DAHP synthase*) is regulated in response to the cellular levels of AAAs, playing a key role in controlling the carbon flux into the pathway (Brown and Somerville, 1971). In plants, genes from the shikimate pathway, AAA biosynthesis and phenylpropanoids are regulated by transcription factors (TFs). However, there is limited information about the effect of AAA levels on the expression of the shikimate pathway genes (Natarajan *et al*., 2001). Reduction of AAA biosynthesis through the glyphosate-mediated inhibition of EPSP synthase induces DAHP synthase protein level and activity in plant cells either transcriptionally or translationally (Benfey and Chua, 1989).

The expression of many plant genes encoding enzymes in the AAA pathways is regulated developmentally and in response to various environmental stimuli, such as wounding, ozone and pathogen infection or elicitors (Keith *et al*., 1991).

1.1.4.2 Post-transcriptional regulation

In addition to the transcriptional regulation, AAA biosynthesis is subject to complex posttranscriptional regulations, which control carbon flux into the shikimate pathway as well as the carbon allocation toward individual AAAs (Bohlmann *et al*., 1996). Within the pathway, the partitioning of carbon flux between the Trp and Phe/Tyr pathways is controlled at the level of two enzymes, anthranilate synthase (AS) and chorismate mutase (CM), both of which compete for chorismate as a substrate AS and CM are feedback inhibited by the final product(s) of the corresponding pathways (i.e., Trp and Phe/Tyr, respectively) in both microbes and plants (Knochel *et al*., 1999; Romero *et al*., 1995). In addition, Trp activates CM to redirect flux from Trp to Phe/Tyr biosynthesis. Likewise, the enzymes localized at the branch points of Phe and Tyr biosyntheses, arogenate dehydratase (ADT) and arogenate dehydrogenase (ADH), are feedback inhibited by Phe and Tyr, respectively. In some cases, Tyr activates ADT to redirect flux from Tyr to Phe biosynthesis (Benesova and Bode, 1992).

1.1.4.3 Post-translational regulation

The synthesis of a large part of amino acids is regulated by post-translational feedback inhibition loops. Hence, enzymes from both the shikimate pathway and aromatic amino acid biosynthesis are subject to post-translational regulation (Connelly and Conn, 1986). In plants, feedback-inhibition regulation of the DAHPS enzymes by any of the aromatic amino acids is still ambiguous. A study by Tzin *et al.* (2009), revealed that ectopic expression of the *Arabidopsis DAHPS* gene with Phe feedback presents increased levels of shikimic acid, prephenate and aromatic amino acids, as well as induction of broad classes of specialised metabolites including phenylpropanoids, glucosinolates, auxin and other hormone conjugates (Jung *et al*., 1986).

Taken together with extensive biochemical and bioinformatics studies on amino acid biosynthesis, it is implied that amino acid biosyntheses are predominantly regulated by post-translational allosteric feedback loops, whereas amino acid catabolism is principally regulated at the transcriptional level (Siehl and Conn, 1988).

1.1.5 E4P and PEP precursor supply to the shikimate pathway

The supply of the DAHP synthase substrates, E4P and PEP, can also play an important role in the regulation of the carbon flux into the shikimate pathway. In photosynthetic tissues, transketolase (TK) in the Calvin cycle converts G3P and fructose-6-phosphate (F6P) to xylose-5-phosphate (X5P) and E4P (Figure 1.7). As light decreases, TK activity in transgenic tobacco leaves leads to a substantial reduction in the levels of AAAs and their downstream metabolites (e.g., phenylpropanoids, tocopherols), suggesting that the E4P supply via TK can be a limiting factor for AAA biosynthesis (Barlow *et al*., 1989). In non-photosynthetic tissues, transaldolase (TA) and TK in the oxidative pentose phosphate pathway (OPPP) likely play key roles in E4P supply to the shikimate pathway (Figure 1.7; Xiao *et al*., 2014).

In *Zea mays*, overexpression of TK rather than TA was found to be more effective in directing the carbon flux into the AAA pathways (Gan *et al*., 2007). The broad substrate specificity of TK and TA and the presence of additional intermediates potentially involved in the OPPP suggest that the OPPP and its regulation may be much more dynamic and complex than currently thought to meet the high demand of E4P for biosynthesis of AAAs, especially in plants (Basset *et al*., 2004).

Plastidic PEP can be derived from (*a*) plastidic glycolysis via phosphoglyceromutase (PGyM) and enolase (ENO1), (*b*) import from the cytosol via the PEP/ phosphate translocator (PPT), and/or (*c*) phosphorylation of pyruvate catalysed by plastidic pyruvate orthophosphate dikinase (PPDK) (Figure 1.7; Maeda and Dudareva, 2012). Although analysis of *ENO1*, *PGyM*s, *PPT*s, and *PPDK* expression revealed that the relative contributions of different pathways are tissue specific, mutant analysis showed that multiple pathways can simultaneously contribute to the plastidic PEP internal pool in plants (Rippert and Matringe, 2002). The *Arabidopsis ppt1* (*cue1*) knockout mutant displays a mesophyll-specific defect in chloroplast development that can be rescued by the constitutive overexpression of *PPDK* in the plastids, suggesting that sufficient levels of pyruvate exist in the chloroplasts to compensate for the lack of PEP transport from the cytosol (de la Torre *et al*., 2009; Tzin *et al*., 2009).

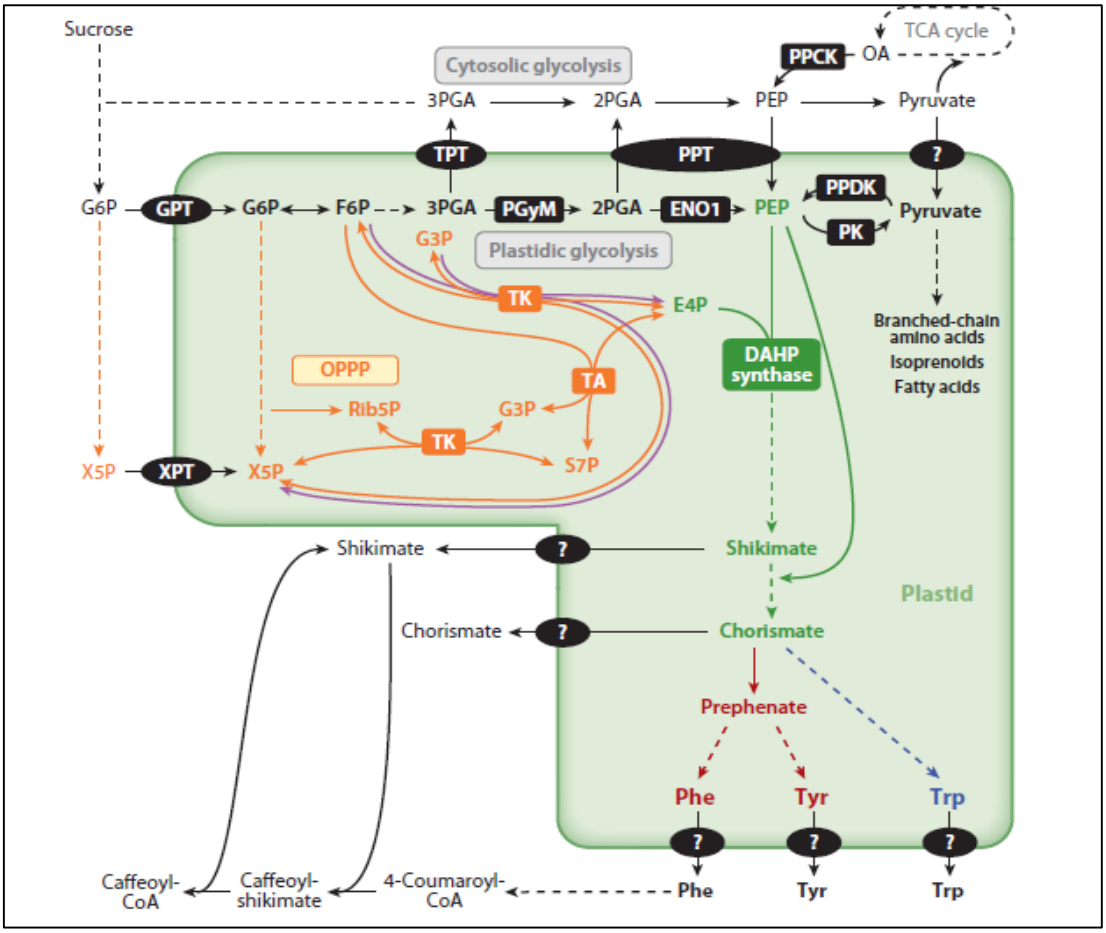


FIGURE 1.7 Supply of the shikimate pathway precursors and transport of AAAs, precursors, and pathway intermediates across the plastid membranes. E4P can be synthesized by TK as part of the Calvin cycle (purple lines) or by either TA or TK through the OPPP (orange). Plastidic PEP can be generated from three different routes: via plastidic enolase (ENO1), via PPT, and/or via PPDK. Within the plastids (light green shape), PEP is used not only for the shikimate pathway (green) but also for the synthesis of branched-chain amino acids, isoprenoids, and fatty acids after being converted to pyruvate by pyruvate kinase (PK). PEP as well as precursors for PEP and E4P biosyntheses are imported into the plastids through membrane-localized transporters (filled circles). The intermediates and products of the AAA pathways are exported from the plastids via unknown transporters (filled circles with question marks). Dotted arrows indicate that multiple reactions are involved (Maeda and Dudareva, 2012).

1.2 The herbicide glyphosate

1.2.1 Discovery and global use

Glyphosate or N-(phosphonomethyl)glycine is a phosphonomethyl derivative of the amino acid glycine (Figure 1.8). Glyphosate was discovered in 1950 (not as a herbicide) by a Swiss chemist, Dr. Henri Martin, who worked for the small Swiss pharmaceutical company, Cilag, (Franz *et al*., 1997). The molecule showed no pharmaceutical properties and consequently was not investigated any further by Cilag. In 1959, Cilag was acquired by Johnson and Johnson, which sold its research samples, including glyphosate, to Aldrich Chemical Co. In 1960 Aldrich sold small amounts of the glyphosate compound to several companies for undisclosed purposes, however no claims of biological activity were reported. This is how it came to the attention of Monsanto Company (St. Louis, MO), in its inorganic division, Monsanto was researching, and developing phosphonic acid type compounds as potential water softening agents, through testing over 100 chemical substances related to aminomethylphosphonic acid (AMPA; Ibanez *et al*., 2005). When these compounds were tested as herbicides by Dr. Phil Hamm, two of the tested compounds showed potential herbicidal activity against perennial weeds (Fraiture *et al*., 2015). However, the activity was too low to be used as a commercial herbicide. Dr. Hamm, enlisted the efforts of Monsanto chemist Dr. John Franz, to synthesis analogs and derivatives of the two compounds with stronger herbicidal activity, glyphosate was the third analog he made (Baylis, 2000). Glyphosate was officially discovered for its herbicidal activity and was first synthesised in May 1970 by Dr. Franz and was tested in the greenhouse in July 1970. The herbicidal effect of glyphosate was described by Baird and co-workers in 1971.

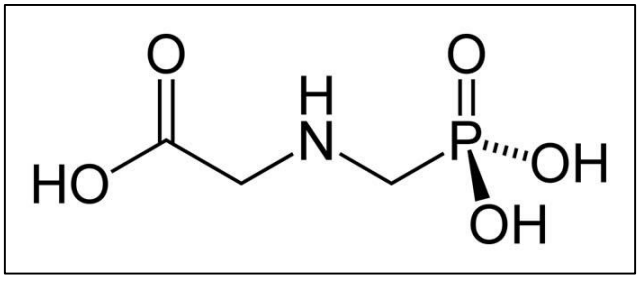


FIGURE 1.8 The chemical structure of glyphosate (Franz *et al*., 1997).

Glyphosate rapidly advanced through greenhouse screens and field testing systems and was first introduced under the tradename Roundup® by Monsanto in 1974. Glyphosate was quickly adopted by farmers, even more so when Monsanto introduced glyphosate-resistant soybean (Roundup Ready®) in 1996 which, enabled farmers to kill weeds without killing their crops (Cakmak *et al*., 2009).

Since its release as a commercial, non-selective, foliar applied, systemic herbicide, glyphosate jumped to a leading position on the pesticide market, became the most marketed herbicide active ingredient by the nineties, and has more or less held that position ever since. Glyphosate is currently labelled for use in over 130 countries and represents the largest selling agrochemical product worldwide. As of 2009, glyphosate accounted for a share of about 25% of the global herbicides market (Tong *et al*., 2009). In 2012, the global glyphosate market was valued at USD 5.46 billion and is expected to reach USD 9.09 billion by 2019, growing at a Compound Annual Growth Rate (CAGR) of 7.4% over the forecast period from 2013 to 2019. In terms of volume, the global glyphosate market demand was 794 kilotons in 2012 and is expected to reach 1350 kilotons in 2017 (Green, 2012). Figure 1.9 shows the world annual glyphosate usage (kilotons) between 2000-2014, it is evident that there is an increase in glyphosate usage. However, if glyphosate resistant crops are approved in the European countries, glyphosate usage will inevitably increase even more drastically. Based on evidence from the US, it has been predicted that the introduction of GM glyphosate resistant sugar beet, maize and soybean could lead to an 800% increase in glyphosate use by 2025, with overall herbicide use going up 72% compared to current levels (Fraiture *et al*., 2015, Green, 2012).

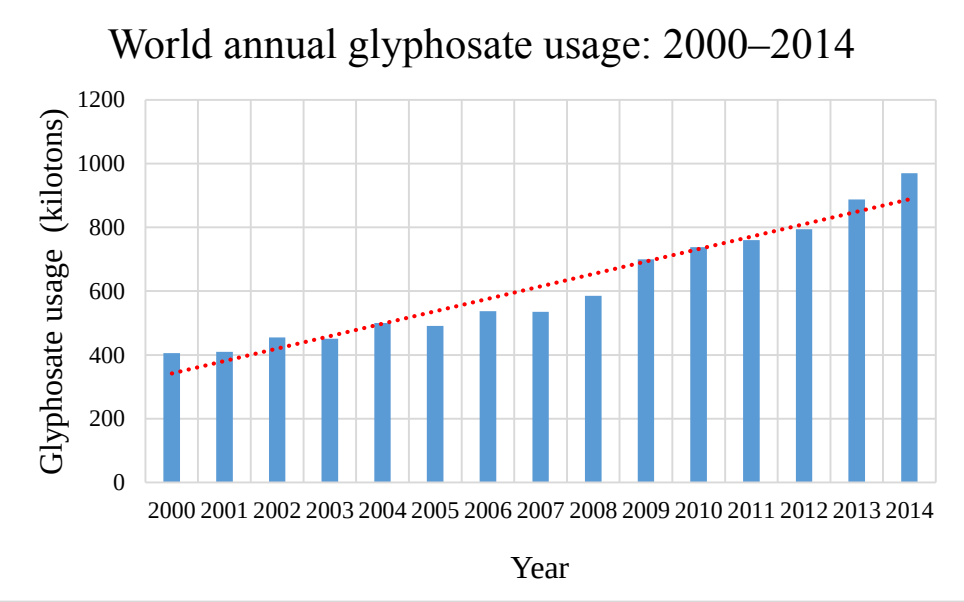


FIGURE 1.9 The global annual usage of glyphosate (Fraiture *et al*., 2015).

1.2.2 Mechanism and mode of action

In 1972, scientists at Monsanto led by Dr. E. Jaworski observed that the application of glyphosate resulted in the inhibition of aromatic amino acid biosynthesis in plants (Pline *et al*., 2002). In 1980, Professor N. Amrhein and co-workers discovered that glyphosate is unique in that it is the only molecule that is highly effective at targeting and inhibiting the enzyme 5-enolpyruvoylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19) in the shikimate pathway (Figure 1.10; Schulz *et al*., 1985). There are two forms of EPSPS in nature; EPSPS I, which is found in plants, fungi, and most bacteria, which is sensitive to glyphosate, and EPSP II, which is found in glyphosate resistant bacteria and is not inhibited by glyphosate. It is the gene from an EPSPS II bacteria that has been used to genetically engineer resistance in crops (Alibhai *et al*., 2010).

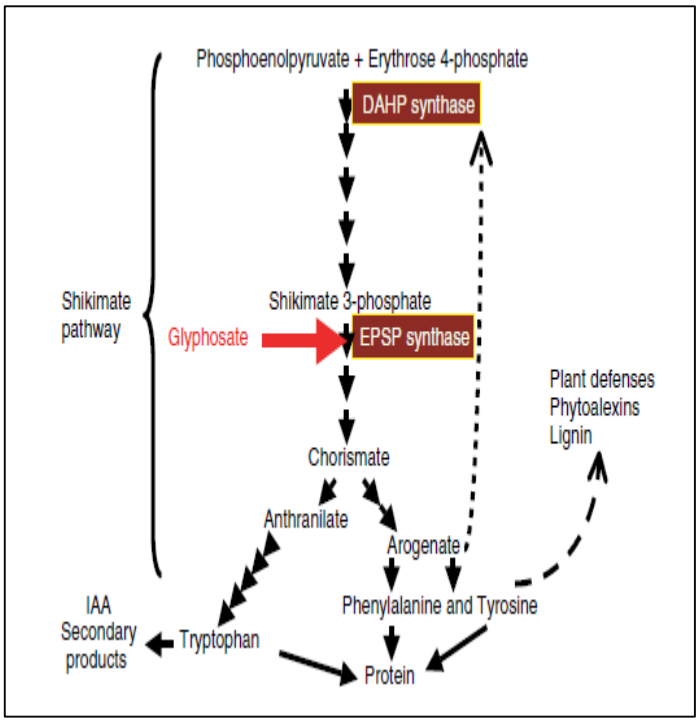
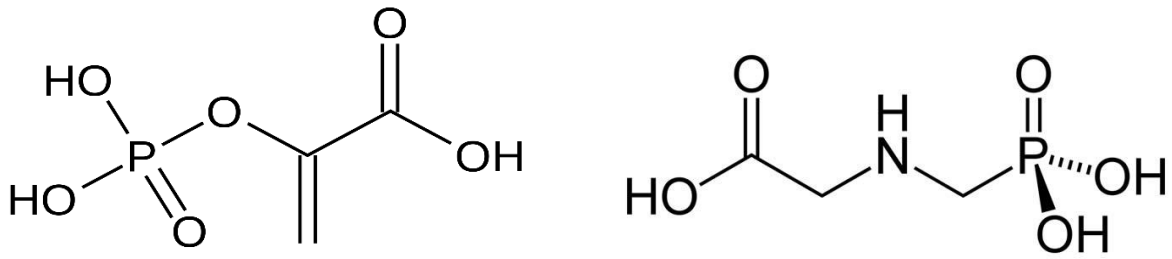


FIGURE 1.10 The shikimate pathway and the site of enzyme inhibition by glyphosate. End products of the pathway and regulatory feedback inhibition (dotted arrow) are shown (Duke and Powles, 2008).

Glyphosate kills plants by inhibiting a key enzyme in the shikimate pathway EPSPS. This enzyme is the only molecular target for glyphosate and is only present in plants (chloroplast or plastids), fungi and bacteria, but not in animals (Franz *et al*., 1997). The enzyme catalyses the reversible reaction, wherein the enolpyruvyl moiety from phosphoenolpyruvate (PEP) is transferred to the 5-hydroxyl of shikimate-3-phosphate (S3P) to form the products, 5-

enolpyruvylshikimate-3-phosphate (EPSP) and inorganic phosphate (Pi; Berg *et al*., 2008). Inhibition of EPSPS by glyphosate has been shown to proceed through the formation of an EPSPS-S3P-glyphosate ternary complex (enzyme-substrate complex) and the binding is ordered with glyphosate binding to the enzyme only after the formation of a binary EPSPS-S3P complex. The binding of glyphosate to EPSPS is competitive with PEP and uncompetitive with respect to S3P (Gao *et al*., 2014). Glyphosate is a transition state analog of PEP, which is one of the normal substrates of EPSPS (Figure 1.11). Glyphosate inhibits the function of the EPSPS enzyme by acting as competitive inhibitor of PEP through occupying the binding site on EPSPS from PEP. Glyphosate achieves this by mimicking an intermediate state of the enzyme-substrate complex and by binding more tightly to the EPSP synthase-S3P complex than does the normal substrate PEP (Dogramaci *et al*., 2014). Interestingly, glyphosate is not known to inhibit any other PEP dependent enzymatic reactions. However, like PEP, glyphosate has no affinity for the EPSPS enzyme alone. A major difference between glyphosate and PEP is that the dissociation rate for glyphosate is 2,300 times slower than that of PEP. Therefore, once glyphosate binds the enzyme-substrate complex (EPSP synthase-S3P) the enzyme is essentially inactivated (Dev *et al*., 2012).



(A) **(B)**

FIGURE 1.11 Glyphosate is a transition state anlog of PEP. **(A)** The chemical structures of phosphoenol pyruvate (PEP) and **(B)** glyphosate (Gao *et al*., 2014).

Glyphosate's mechanism of action as an herbicide is to block the penultimate step in the shikimate pathway by inhibiting the EPSPS enzyme. The reaction catalysed by EPSPS is a key step in the shikimate biosynthetic pathway which is necessary for the production of the aromatic amino acids (tyrosine, tryptophan, and phenylalanine), hormones, auxin, phytoalexins, folic acid, lignin, plastoquinones, and many other critical secondary plant metabolites. The AAAs are important in the synthesis of proteins that link primary and secondary metabolism (Bresnahan *et al*., 2003). Since the EPSPS enzyme is not present in

animals, these three AAAs and some of their derivatives (vitamins) are essential nutrients to animals, which they need to obtain from their diet. The absence of the shikimate pathway in animals is the reason this pathway is such an attractive target for antimicrobial agents and herbicides such as the glyphosate (Bochkov *et al*., 2012; Maeda *et al*., 2010).

Over 32% of the carbon fixed by plants passes through the shikimate pathway. Inhibition of EPSPS by glyphosate leads to reduced feedback inhibition and deregulation of the pathway (Mir *et al*., 2015). This in turn leads to a metabolic disruption resulting in massive carbon flow through the pathway which consequently results in the accumulation of very high levels of shikimate and shikimate-3-phosphate (Caretto *et al*., 2015). The high levels of shikimate that rapidly accumulate in glyphosate treated plant tissues was the clue that let to N. Amrhein and his co-workers to discover EPSPS as the molecular target site of glyphosate (Gorlach *et al*., 1995). In some plant species this accumulation can account for up to 45% of plant dry weight in sink tissues. Important building blocks for other metabolic pathways are reduced by uncontrolled carbon flow through the shikimate pathway and reduced levels of aromatic amino acids causes significant reductions in protein synthesis (Bongaerts *et al*., 2001).

There are other factors that contribute to glyphosate's herbicidal activity. The shikimate pathway is normally controlled by a process called feedback inhibition (Siehl and Conn, 1988). In the shikimate pathway, arogenate (a product of the pathway) is a potent inhibitor of the first enzyme in the shikimate pathway, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHP synthase; Figure 1.10). Inhibition of EPSP synthase by glyphosate results in the decreased levels of arogenate causing the deregulation of the shikimate pathway due to increased DAHP synthase activity (Jones *et al*., 1995).

1.2.3 Uptake, translocation and metabolism in plants

The shikimate pathway is most active in leaves and meristematic tissues (thus it is expected that shikimate build-up would be the greatest in these tissues). Hence, glyphosate has to translocate to these tissues to be effective (Ghosh *et al*., 2012). Glyphosate is taken up relatively rapidly through plant surfaces. Leaf uptake rates vary considerably between species, accounting for at least some of the differences in glyphosate sensitivity between species. Glyphosate uptake is dependent on several interdependent factors: droplet size and droplet spread, plant cuticle composition and thickness, surfactant and concentration, ionic strength and salt concentration, humidity and most importantly glyphosate concentration (DellaCioppa *et al*., 1986). The herbicidal efficacy of glyphosate is strictly dependent on the dose of glyphosate delivered to the symplastic or living portion of the plant (Ge *et al*., 2010).

Diffusion is the most likely mode of transport across the plant cuticle. Glyphosate loads the phloem passively and not via active transport. The physiochemical properties of glyphosate enable it to be easily loaded in the phloem where it is translocated from the leaf (source) via the phloem to the same tissues that are metabolic sinks for sucrose, it usually does not leave the symplastic assimilant flow (Figure 1.12). Up to 70% of absorbed glyphosate can translocate out of the treated leaves to the root and shoot apices (Schrubbers *et al*., 2016). Thus, phytotoxic levels of glyphosate reach meristems, young roots and leaves, storage organs and any other actively growing tissue or organ. Good uptake, excellent translocation to growing sites, no or limited degradation and a slow mode of action are the primary reasons for excellent efficacy of glyphosate. In species in which it acts faster, such as sugarbeet, glyphosate can limit its own translocation (Franz *et al.,* 1997). However, glyphosate translocation is self-limiting and only occurs for the first 48 to 72 hour after application. Glyphosate create a self-limitation of translocation by killing the phloem loading site, thus stopping phloem loading prematurely and also because of its toxicity by shutting down photosynthesis and sucrose metabolism (Dogramaci *et al*., 2015).

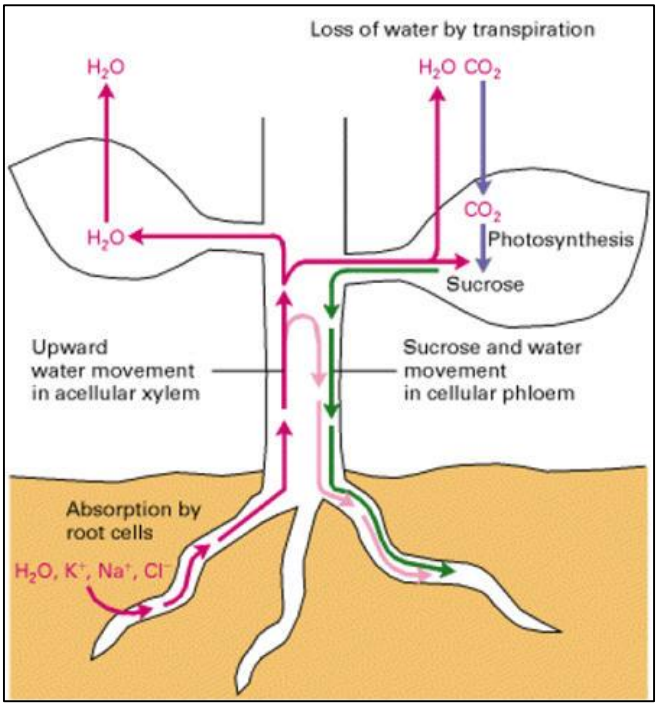


FIGURE 1.12 Movement of photoassimilate (sucrose) and water in plant vascular tissue. Glyphosate moves with photoassimilate from source (leaf) to sink meristematic regions such as roots (shown) and the shoot (not shown; Schrubbers *et al*., 2016).

1.2.4 Toxicity

Glyphosate is one of the least toxic pesticides to animals. Accordingly, it is used for weed control throughout the world in urban and recreational areas, as well as on industrial and agricultural land. Glyphosate is less acutely toxic than common chemicals such as sodium chloride or aspirin, with an LD_{50} for rats greater than 5 g kg^{-1} (Hori *et al*., 2003). Some formulation materials and cationic salt ions used with glyphosate are more toxic than the glyphosate anion. Glyphosate is not a carcinogen or a reproductive toxin, nor does it have any sub-acute chronic toxicity. In a lengthy review, Fuchs *et al*. (2002), conclude that, when used according to instructions, there should be no human health safety issues with glyphosate.

1.2.5 Degradation in plants

The extremely broad spectrum of activity of glyphosate is primarily due to the inability of most plant species to rapidly metabolise the herbicide to non-toxic forms. Until recently, the metabolic degradation of glyphosate by plants was not accepted. However, in some species of plants, glyphosate is degraded (cleaved) slowly to aminophosphonic acid (AMPA) and glyoxylate by a glyphosate oxidoreductase (GOX) and glyphosate acetyltransferase enzymes, however the rate of metabolism and degradation is not rapid enough to provide tolerance (Baylis, 2000). These glyphosate breakdown products/residues are very handy in measuring the presence of glyphosate in plants. Transgenic RR plants are able to survive the effects of glyphosate, because these plants are protected from the glyphosate toxicity by a resistant form of EPSPS (CP4-EPSP synthase), so that the healthy plant can metabolically degrade glyphosate to non-toxic breakdown products (Kang *et al*., 2010). In RR soybeans treated with commercial doses of glyphosate at late developmental stages, glyphosate metabolism is evident as substantial AMPA levels are present in harvested seeds. Similar studies have not been done with RR maize or cotton (Pline *et al*., 2002). This experiment cannot be done with RR canola because it contains a transgene that encodes a bacterial GOX and therefore very little glyphosate is found after treatment, compared to RR soybean. High levels of AMPA would be expected in canola tissues, but the levels were no higher than in RR soybeans, suggesting that canola readily degrades AMPA (Duke and Powles, 2008).

1.2.6 Techniques to measuring the presence of glyphosate in plants

Herbicides containing glyphosate undergo decomposition mainly by microorganisms to yield AMPA and glyoxylate breakdown products. A great variety of analytical methods have been applied for determination of glyphosate and its breakdown products/residues in plants (Castellino *et al*., 1989). However, the chemical properties of glyphosate that contributes to its effectiveness as a herbicide also make its determination difficult, especially at residue level. Its polar nature and high water solubility make solvent extraction difficult and limit the options to successfully use gas chromatography (GC). The similarity of glyphosate to naturally occurring amino acids (especially glycine) and amino sugars further contributes to the difficulty in determining residues in plant products. Measuring glyphosate usually require the use of lengthy clean up procedures, involving both anion and cation exchange columns (Gard *et al*., 1997).

The lack of a chromophore and fluorophore also makes it necessary to use derivatisation techniques for the determination of glyphosate by liquid chromatography (LC), which also typically involves several clean up steps (Ibanez *et al*., 2006).

Glyphosate could be measured by either direct or indirect techniques. Direct methods include nuclear magnetic resonance (NMR) spectroscopy and chromatography (GC, HPLC and liquid chromatography mass spectrometry—LCMS) techniques and involve directly measuring the presence of the glyphosate molecule or its breakdown residues, AMPA and glyoxylate (Yamamoto *et al*., 2000). Indirect techniques include HPLC, spectrophotometry, and colourimetric enzymatic assays and involve indirect measurement of glyphosate by determining whether elevated levels of shikimic acid is present in the sample (Cartigny *et al*., 2004). To measure glyphosate directly both GC and LCMS are used with various detection systems. GC analysis is performed after a derivatisation procedure that converts glyphosate to a sufficiently volatile and thermally stable derivative. In LCMS methods derivatisation procedures, producing fluorescent derivatives, are often employed to enhance the sensitivity and selectivity of detection. In many cases derivatisation procedures are quite complicated and require special equipment (Cornish-Bowden and Hofmeyr, 2002). Glyphosate can be measured by gas chromatography, by pyrolysis GC or by gas chromatography/ liquid chromatography mass spectrometry after derivatisation by perfluoroacetylation, but these methods are limited by the formation of products of degradation. HPLC methods are the most widely used to directly determine glyphosate in plants (Zelaya *et al*., 2011). NMR spectroscopy is a quantitative technique for glyphosate determination by using ^{31}P NMR spectroscopy, in an effort to eliminate the need for pre-or post-column derivatisation procedures to improve on both the chromatographic behaviour and the detection ability by GC, LCMS, and HPLC (Ge *et al*., 2011; Granby *et al*., 2003). A great advantage of NMR spectroscopy over chromatography is that it is non-destructive, meaning that the sample can be analysed without consuming it during the process, unlike GC, HPLC, or LCMS techniques, and the sample can be stored after the analysis for later studies (Christensen and Schaefer, 1993).

All of the analytical methods used for direct determination of glyphosate and glyphosate residues are expensive, complicated, and time consuming and also requires advanced instruments/equipment. The best overall technique for glyphosate determination is via the indirect method of HPLC analysis. This method is very accurate in indirectly measuring whether glyphosate is present in sensitive plants by quantifying the degree of shikimate build-up. Elevated levels of shikimate would indicate glyphosate inhibition of EPSPS.

1.2.7 Glyphosate resistance

1.2.7.1 Adoption of Roundup Ready® (RR) crops

The ability to use biotechnology to engineer glyphosate-resistant crops (GRCs) also known as Roundup Ready® (RR) crops, was a scientific breakthrough that helped to revolutionise weed management, and provided much of the impetus to restructure the seed business (Owen, 2008). For the past 18 years, the most effective choice to manage weeds for many farmers has been to use glyphosate in RR crops. The broad spectrum of weeds controlled by glyphosate and the positive environmental and safety profile of the product have made the use of glyphosate in crops for weed control an attractive consideration (Franz *et al*., 1997; Green and Owen, 2011). RR crops became available when weeds were becoming widely resistant to commonly used selective herbicides and farm size was increasing while the number of farm workers was decreasing (Rubin, 1991). Weed management was becoming too complicated, time consuming and costly for the new agricultural systems. The ability to use glyphosate in GRCs made weed management easy, efficient, economical, and environmentally compatible – exactly what growers wanted (Green, 2012).

Control of weeds with herbicides is critical in food production because weeds compete with crops for water, nutrients, and soil. In addition, weeds can harbour insects and disease pests, noxious weeds and weed seeds can greatly undermine crop quality (Main *et al*., 2004). The benefits of RR technology are very evident. The use of crops containing the RR genes enabled farmers to utilise glyphosate for effective control of weed pests and take advantage of this herbicide's beneficial features (Dill *et al*., 2008). Crops with the RR gene positively impacted current agronomic practices by: (1) offering the farmer a new wide spectrum of weed control options, (2) allowing the use of an environmentally sound herbicide (3) providing a new herbicidal mode of action for in crop weed control (4) increasing the ability to treat weeds on an "as needed" basis - flexibility of use (5) offering less dependence on herbicide used before planting (6) providing an excellent fit with no-till system, which results in increased soil moisture, while reducing soil erosion and fuel use (7) providing cost-effective weed control, not only because Roundup® herbicides may be less expensive than most alternative options, but because the total number of herbicides used may be reduced, compared to the farmer's current weed management program (Deisingh and Badrie, 2005). Thus, the utility of glyphosate in high-efficiency, and high-production agricultural systems in addition to the economic benefits, production efficiency and flexibility, enhanced weed control and the facilitation of

conservation tillage are the main drivers for the rapid adoption of GRCs (Duke and Powles, 2009).

Glyphosate provided great weed control for more than a decade, but it became a victim of its own success. Too many growers used glyphosate alone too often on too many hectares. In retrospect, glyphosate and GRCs were overused, and an important lesson was learnt about weed resistance (Ge *et al*., 2012). All experts now fully understand and agree that weeds will adapt to any single highly effective weed management practice and thus farmers need to use diverse integrated weed management (IWM) systems by combining chemical and non-chemical management systems (Gianessi, 2005; Green, 2012).

Transgenic RR crops undergo extensive phenotypic, agronomic, morphological, and compositional analyses, and they must be found to be substantially equivalent to their conventional (non RR) counterparts before they can be approved for commercialisation (Dill, 2005). The herbicide resistance traits of RR crops do not give any agronomic advantage or disadvantage to the crop until the herbicide is applied. However, opponents often frame the technology as a threat to sustainable agriculture by objecting to potential unknown long-term effects on human health and the environment, the potential to create 'super weeds', and raise ethical questions about global agribusiness, the control of seed supplies and scientists interfering with the natural order (Owen, 2008). In contrast, supporters claim that RR crops allow growers to be more productive, are safe to eat, are better for the environment (fewer herbicide applications), and enable better weed management options. RR crops have an impeccable history of safe commercial use, and the United States National Academy of Sciences concluded without equivocation that GM crops do not pose any health risks that are not present in conventionally produced crops. However, small but influential groups of activists maintain that long-term uncertainty justifies extreme precaution and are continuing the debate (Dill, 2005; Gonzáles-Torralva *et al.*, 2012).

Since the introduction of RR soybeans, growth of this herbicide resistance trait has been very rapid in soybean, cotton, maize, and canola. Statistics indicate that, in 2005 alone in the United States, genetically modified crop production systems accounted for reductions in pesticide use compared with non-GM crop production systems by over 31 million kg, with 27 million kg of that reduction being attributed to herbicide resistance traits (Dill *et al.*, 2008). While yield performance appeared to equal to non-RR crop production systems, net income gains from RR

maize, RR soybean, RR canola and RR cotton were reported to be USD 1491 million. Conversely, the proliferation of RR crops has also led to concerns of weed shifts and weed resistance as the number of hectares and use of glyphosate increase. To date, biotypes of more than 12 species have been reported to be resistant to glyphosate worldwide, strategies to manage these biotypes as well as delay further development in other species will depend on growers' judicious use of multiple element including crop rotation, alternative modes of action and varied cultural practices (Green, 2012; Owen and Zelaya, 2005; Yanniccari *et al*., 2016).

In 2013, GM crops were planted in 27 countries on over 175 million hectares by more than 18 million farmers. Of these 27 countries 19 were developing countries and 8 were developed countries. Currently 29 more countries (56 in total) have granted regulatory approval for import for food and feed use in 2014. Furthermore, 68% or 113.75 million hectares of the total were planted to RR traits. The overall growth of GM crop hectares in 2013 was 17%, or 12million hectares, and RR technology was the dominant of GM technology planted (Dill, 2005; Dill *et al.*, 2008).

1.2.7.2 Approaches of developing GR crops

There are three basic strategies that have been evaluated in order to introduce glyphosate resistance into crop species: (1) over-expression of the sensitive EPSPS target enzyme, (2) detoxification of the glyphosate molecule and (3) expression of an insensitive form of the target EPSPS enzyme (Bhatti *et al*., 2013). Various genetic engineering and biotechnology approaches to producing RR crops were attempted, but with limited success until the *CP4* gene of *Agrobacterium* sp. was found to encode a GR form of EPSPS (Haghani *et al*., 2008). When this *CP4* gene plus a promoter was placed into the genome of soybean, maize, cotton and canola crops, high levels of glyphosate resistance were expressed. In addition to the *CP4* gene, a gene from *Ochrobactrum anthropic* encoding GOX was employed to contribute to resistance in canola. The resistance factors for GR *CP4* soybean and *CP4* plus GOX canola are each about 50-fold (Querci and Mazzara, 2010). For maize, the EPSPS has been altered by site-directed mutagenesis of a maize gene to provide a form of GR EPSPS that is used in some GR maize varieties (Burke *et al*., 2008). Genes that encode other forms of GR EPSPS and glyphosate detoxification enzymes are being proposed for future RR crops, but at present, the *CP4* gene is responsible for glyphosate resistance in most commercial RR crops. The three different approaches of producing RR crops are discussed below (Pollegioni *et al*., 2011).

1.2.7.2.1 Over-expressing of the EPSPS protein

The strategy of over-expressing the EPSPS protein in the hope of overcoming the herbicidal effects of glyphosate has been attempted in both cell and whole plant systems. Amplification of the endogenous EPSPS gene has been accomplished in cell culture of several species including *Aerobacter aerogenes*, *Daucus carota* and *Nicotiniana tobacum* (Yuan *et al*., 2002). EPSPS activity that has been elevated up to 800-fold has been shown in *Nicotiniana* cell culture. However, glyphosate-resistant whole plants have not been generated from any of these cell lines to date (Berg *et al*., 2008). The alternative approach of genetically engineering overexpression of native EPSPS in a variety of systems has also been attempted with limited success. In soybean plants overexpression of glyphosate-sensitive EPSPS conferred glyphosate tolerance, but the tolerance was not sufficient to withstand commercial glyphosate application rates (Gao *et al*., 2014; Pollegioni *et al*., 2011). Petunia plants were also generated that could withstand a fourfold dose of glyphosate. However, these plants also exhibited significantly reduced growth rates compared to wild-type. Thus, no glyphosate-resistant crop species are marketed today using over-expression of native EPSPS as the mechanism of resistance (Dill *et al*., 2008; Duke and Powles, 2008).

1.2.7.2.2 Detoxification of glyphosate

Detoxification of the glyphosate molecule is the second strategy that has been employed to confer glyphosate resistance. Glyphosate detoxification has been demonstrated via two routes, one resulting in the formation of phosphate and sarcosine, while the other results in the formation of aminomethylphosphonic acid (AMPA) and glyoxylate, and is referred to as glyphosate oxidase (GOX). Scientists have proved that neither of these two mechanisms has been shown to occur in higher plants to a significant degree (Yuan *et al*., 2002). While GOX is employed in glyphosate-resistant canola, it is used in combination with a glyphosate-insensitive EPSPS. This approach was necessary, as using the detoxification mechanism alone provided insufficient resistance to glyphosate in commercial applications (Pollegioni *et al*., 2011).

1.2.7.2.3 Incorporation of glyphosate insensitive CP4-EPSPS

The method that resulted in commercial glyphosate resistance and is marketed in multiple crops under the Roundup Ready® brand was the introduction of an insensitive EPSPS (Haghani *et al*., 2008; Yi *et al*., 2016). The strategy employed in the development of these crops is shown in Figure 1.13 (Yi *et al*., 2015). Several approaches to attaining this result have been tried. Treating *Arabidopsis thaliana* with ethanemethosulfate was attempted by several laboratories without generating a glyphosate-resistant mutant (Querci and Mazzara, 2010). Extensive functional mutagenesis of bacterial and plant EPSPS enzymes has also failed to produce a commercially resistant EPSPS. Some studies have indicated that the level of resistance afforded by single-point mutations in the EPSPS molecule would be unlikely to produce commercially acceptable levels of glyphosate resistance. Because of the close overlap of the binding sites of PEP and glyphosate on the EPSPS enzyme, and the highly conserved sequence found in that particular binding domain, obtaining altered target sites that will bind PEP, exclude glyphosate, result in commercial levels of glyphosate resistance and result in plants that develop normally has been difficult (Alibhai *et al*., 2010).

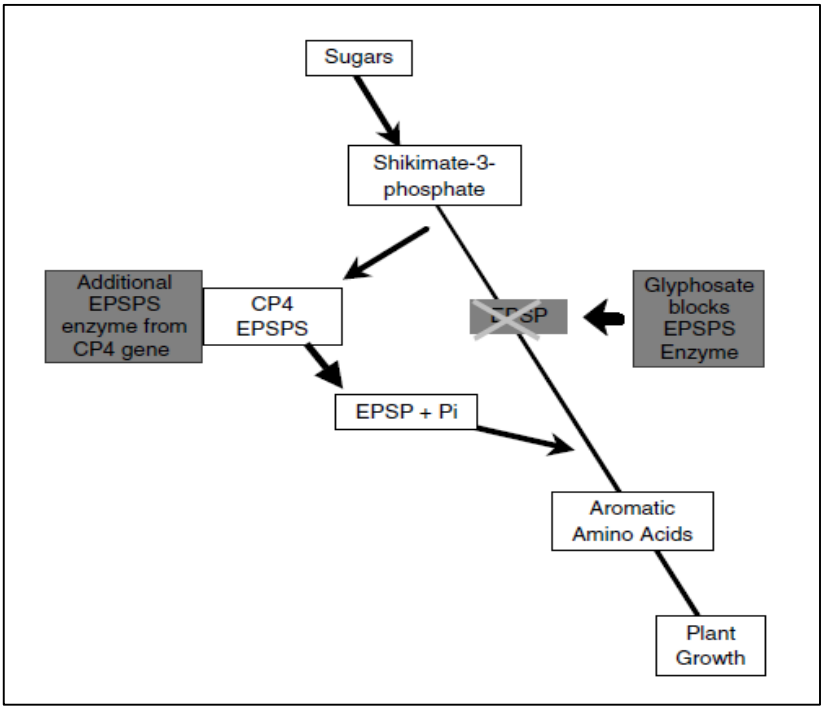


FIGURE 1.13 Strategy for the development of glyphosate-resistant crops (Yi *et al*., 2015).

Kinetic data for a select group of EPSPS enzymes are shown in Table 1.1 (Boocock and Coggins, 1983; Dill, 2005). The data show that the two single point mutations substituting glycine with alanine at position 101 (G101A) or substituting proline with serine at position 128 (P106S, Figure 1.14) have enzyme kinetics that do not meet the theoretical ideal, and result in commercially unacceptable levels of glyphosate resistance in plants. Only a single multiple missense mutation in endogenous maize EPSPS has been utilized to date to generate commercial glyphosate resistance (Kang *et al*., 2010). The mutation was generated via site directed mutagenesis of a maize cell line. This variant of maize EPSPS is a transgene with substitution of threonine at position 102 with isoleucine and substitution of proline at position 128 with serine (Figure 1.14) that is presently sold commercially in some maize hybrids and known as GA21 (de la Torre *et al*., 2009). The vast majority of the commercial glyphosate-resistant products on the market today contain the bacterial EPSPS known as CP4-EPSPS (Yi *et al*., 2016). The CP4 enzyme was isolated from *Agrobacterium sp* and is insensitive to glyphosate (Table 1.1). The substrate and glyphosate binding region of CP4-EPSPS is identical to the substrate and glyphosate binding region of sensitive EPSPS found in most plant species (Haghani *et al*., 2008). The CP4-EPSPS protein overall is 50.1% similar and only 23.3% identical to native maize EPSPS. This suggests that binding of glyphosate is excluded by conformational changes resulting from those amino acid sequence changes outside the glyphosate/PEP binding region. As shown in Table 1.1, CP4-EPSPS combines a high affinity for PEP coupled with a very high tolerance for glyphosate. The result is an ability to 'bypass' the endogenous EPSPS system with the CP4-EPSPS insertion that allows the shikimate pathway to function normally (Figure 1.13). The CP4-EPSPS enzyme is employed in nearly all glyphosate resistant crops currently sold (Dill, 2005).

Table 1.1 Kinetic properties for selected EPSPS enzymes (Dill, 2005).

Enzyme source	Km (PEP; µM)	*Ki* (glyphosate; µM)	*Ki*/Km
Petunia (wild type)	5.0	0.4	0.08
Theoretical Ideal	<15	~1500	100
G101A	210	2000	9.5
T102I/P106S	10.6	58	5.5
P106S	17	1	0.06
Agrobacterium sp CP4	12	2720	227

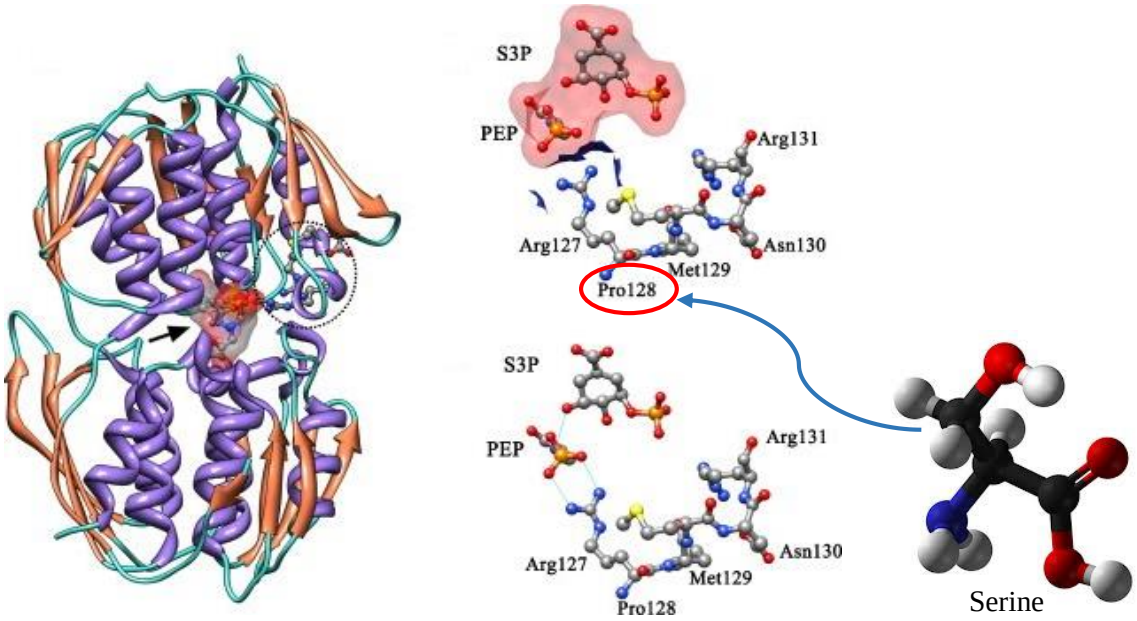


FIGURE 1.14 Single missense mutation occurring in the active site of endogenous EPSPS of plants to generate commercial glyphosate resistance. In this point mutation the amino acid Proline 128 (circled in red) is replaced by Serine, this conformational change in the protein structure of the active site result make it impossible for glyphosate to bind to EPSPS, thereby generating a glyphosate resistant EPSPS enzyme (Kang *et al*., 2010).

1.2.7.3 Development of modern day Roundup Ready® GM crops

Resistance to the herbicide glyphosate can be achieved by producing a transgenic plant via the introduction of an alternative gene encoding a protein/enzyme that is not sensitive to the actions of the glyphosate herbicide (Dill, 2005). The plant enzyme targeted by glyphosate is EPSP synthase, thus to obtain a plant that is resistant to the actions of this herbicide an alternative EPSPS enzyme which is not sensitive to glyphosate must be incorporated into the plant (Padgette *et al.*, 1995). The gene most commonly introduced into transgenic plants with herbicide resistance is the CP4 gene which was isolated from *Agrobaterium* species, this gene encodes for an additional EPSPS enzyme (CP4-EPSPS) which is resistant to the actions of glyphosate because this enzyme has a slight conformational difference when compared to the native EPSPS plant enzyme (Yi *et al*., 2016). When this alternative CP4-EPSPS transgene/enzyme is present (expressed) in a plant the shikimate pathway is able to continue functioning uninterruptedly even in the presence glyphosate. This is the mode of action employed by plants that are Roundup Ready®. *Agrobacterium* mediated transformation was

used to create the event for glyphosate resistance in plants. The gene cassette used to produce Roundup Ready® crops is shown in Figure 1.15 (Padgette *et al*., 1995).

The alternative EPSPS enzyme does not bind to the herbicide glyphosate, but is still able to combine conversion of shikimate-3-phosphate (S3P) with phosphoenolpyruvate (PEP) to produce 5-enolpyruvylshikimate-3-phosphate (EPSP) and consequently preventing aromatic amino acids starvation and deregulation of this metabolic route, both of which follow glyphosate treatment in susceptible plants, however the yield of the crop may be reduced by this treatment (Bochkov *et al*., 2012; Dill 2005; Dill *et al.*, 2008).

Thus, when the bacterial CP4 gene is expressed by a plant an alternative EPSPS enzyme is encoded. Expression of this enzyme allows the RR crop to bypass the glyphosate-inhibited native EPSP synthase in the shikimate pathway thereby allowing plants to synthesis aromatic amino acids (Phenylalanine, Tyrosine, and Tryptophan) and survive glyphosate application (Maeda and Dudareva, 2012; Querci and Mazzara, 2010).

FIGURE 1.15 Schematic representation of the Roundup Ready® soybean gene cassette (Adapted from Padgette *et al*., 1995).

1.2.7.4 Glyphosate resistant weeds

Given the mechanism of action of glyphosate and the difficulty in genetically engineering glyphosate resistant crops, it was speculated that selection of resistance in weeds would be a very rare event. However, overuse of glyphosate with the introduction of RR crops and lack of proper weed management practice lead to weed resistance (Hart and Wax, 1999; Powles, 2008). Cases of weed resistance around the world keeps increasing annually (Figure 1.16) due to continuous and in many cases misuse of the herbicide. There are currently 32 weed species worldwide in which glyphosate resistant biotypes have been selected (Figure 1.17). However, taking glyphosate's long history into account as the most widely used herbicide in the agricultural industry since 1974 it is phenomenal (ranked 8th out of top 15 in Figure 1.17) that there are not more resistant species when compared to other herbicides such as atrazine and imazethapyr, with 66 and 44 resistant species respectively (Szigeti and Lehoczki, 2003; Zhou *et al*., 2016). This again demonstrates the uniqueness and class of this herbicide. The 32 resistant weed species are shown in Figure 1.18 and the three resistant weed species found in South Africa namely *Conyza bonariensis*, *Lolium rigidum* and *Plantago lanceolata* are circled in Figure 1.18.

There are currently four known mechanisms of resistance in weeds: (1) alterations of the target site (Dinelli *et al*., 2006); (2) enhanced metabolism (Michitte *et al*., 2007); (3) compartmentalisation or sequestration i.e. decreased uptake/translocation of glyphosate to the meristematic tissues (Preston and Wakelin, 2008) and (4) over expression of the target protein. All four mechanisms of resistance appear to be overcome by increasing the rate of glyphosate (Zelaya *et al*., 2004).

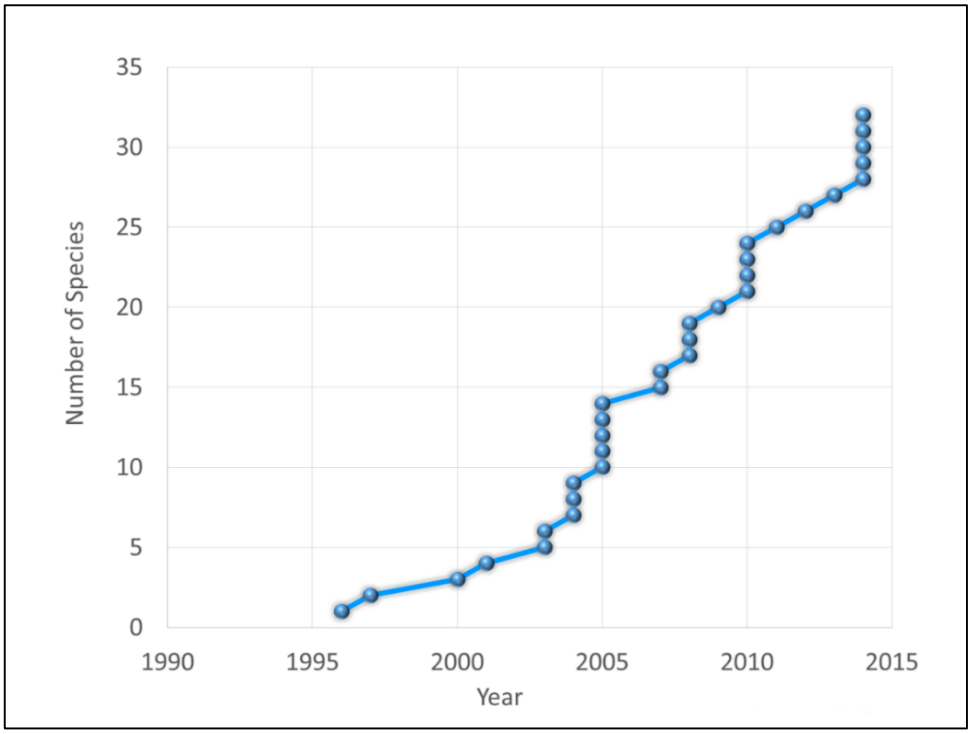


FIGURE 1.16 The increase of glyphosate resistant weeds worldwide (http://www.weedscience.org, Dr. Ian Heap, 2015/11/19).

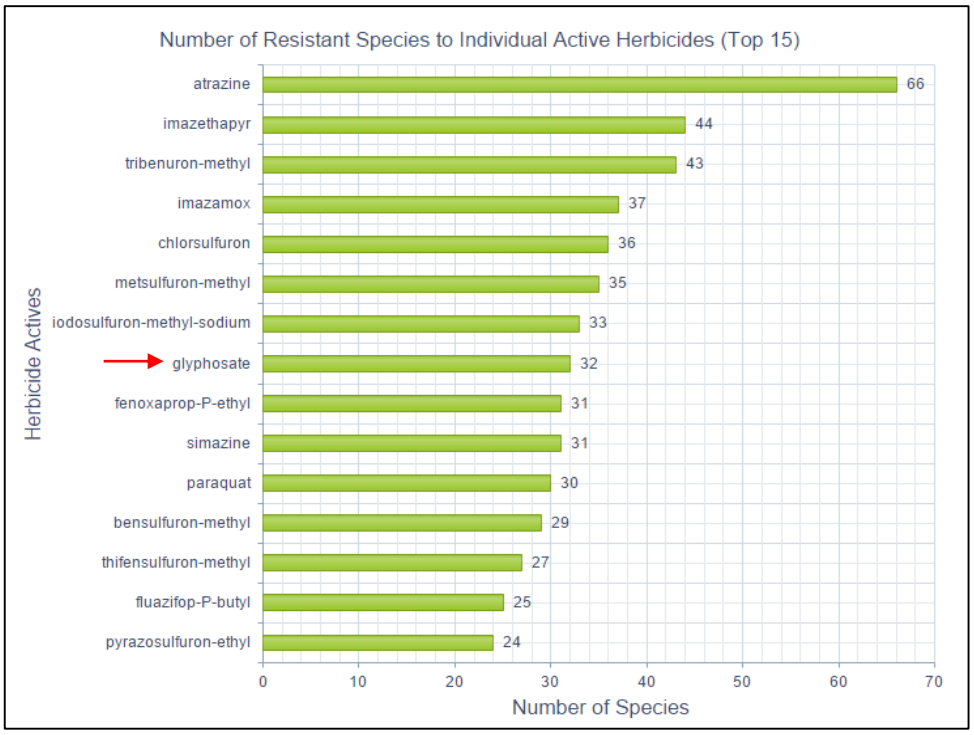


FIGURE 1.17. Number of resistant weed species to individual herbicides. Glyphosate is ranked 8th out of the top 15 herbicides in which weed resistant have been recorded, with 32 resistant species (http://www.weedscience.org/graphs/activebyspecies.aspx, Dr. Ian Heap, 2015/11/19).

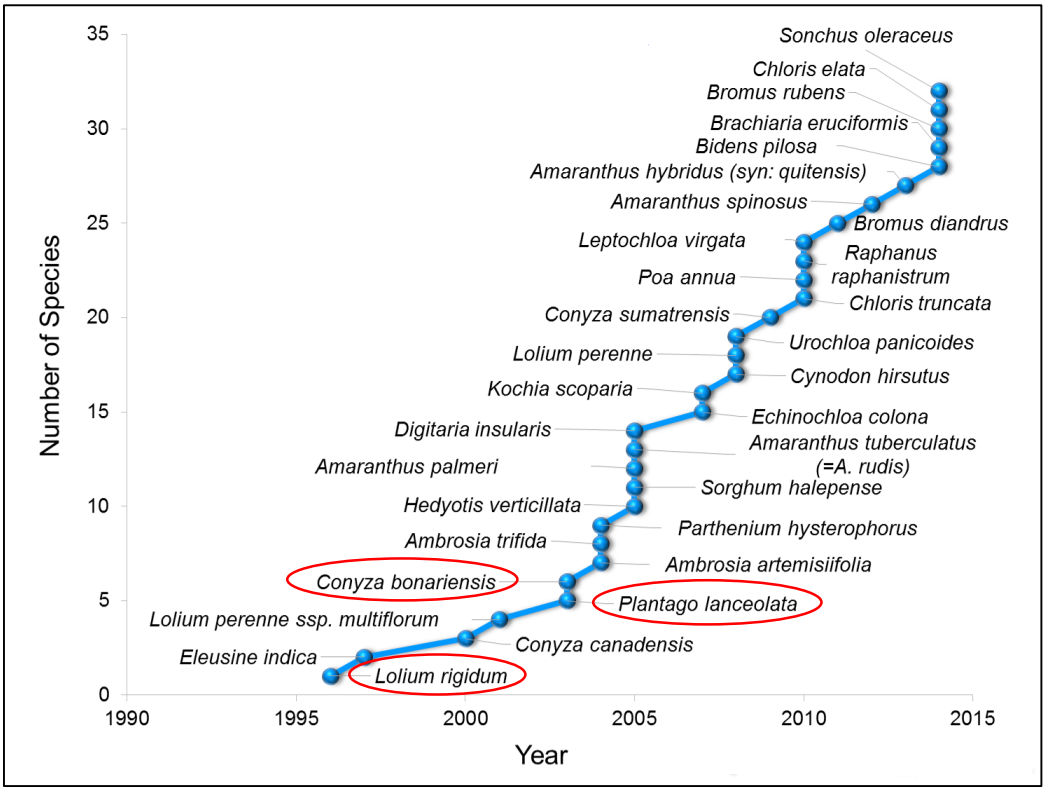


FIGURE 1.18 Resistance to glyphosate by species. Currently only three (circled in red) of the 32 resistant weed species are found in South Africa (http://www.weedscience.org/Summary/Country.aspx, Dr. Ian Heap, 2015/11/19).

An interesting question that has been asked is "Why aren't there more resistant biotypes?" There may be several reasons for this. The first is that genes encoding for glyphosate resistance appear to be a very rare. Second, the time for selecting resistance is still relatively short. Until the introduction of glyphosate-resistant crops less than 19 years ago, glyphosate was rarely used as a stand-alone product on millions of hectares. The continued intensive and widespread use of glyphosate will unfortunately inevitably select for more resistant biotypes in the future. The ultimate effect will most likely be that glyphosate will continue to be the foundation of many weed management programs and additional herbicides or other methods will be used to manage resistant biotypes (Zhou *et al.*, 2016).

1.2.7.5 Glyphosate-resistant weed management strategies (WMS)

Sustainable weed management is the foundation of sustainable agriculture. The debate over the sustainability of RR crops is primarily over the sustainability of the weed management systems in large-scale production agricultural systems and the evolution of resistant weeds (Osuna and De Prado, 2003). The increasing adoption of RR crops has exerted tremendous herbicide selection pressure on weed populations over the past decade. Farmers should not rely completely on RR crops to manage weeds to the exclusion of other weed management options (Zhou *et al*., 2016). Alternating herbicides with different modes of action or tank mixing herbicides with different modes of action are common recommendations in resistance management programs; however, these practices are often ignored by farmers for economic reasons as the cost of weed control is cheaper with RR crops using glyphosate alone. Increased awareness of weed resistance by farmers, field scouting for early detection, monitoring of farms for weed species and population shifts, and record keeping are critical to delay or prevent resistance from occurring (Hurley *et al*., 2009). Cultural (crop rotation, winter crops in rotation, cover crops), mechanical (tillage before planting, cultivation), and chemical (herbicide rates, tank mixtures, sequences, application timing, herbicide rotation with different modes of action) weed control practices must be used as dictated by grower needs to control resistant weeds (Mueller *et al*., 2003). The practice of using herbicides with alternate modes of action in a given weed management system also offers the advantage of extending the lives of current programs, such as in weed resistance management. Rotation between RR cultivar and non-RR cultivar of the same crop could aid in delaying the evolution of glyphosate-resistance in weeds. Thus, prudent use of glyphosate with other chemistries based on weeds, farm size, and economics by growers is critical in managing GR weeds (Chodova *et al*., 2009; Dewar, 2009).

A method to rapidly detect glyphosate resistance would aid farmers tremendously in planning the correct weed management strategy (WMS). The colourimetric assay is very accurate to easily and rapidly measure shikimate build-up in plants and thus to differentiate between glyphosate resistant and sensitive weed biotypes (Nol *et al*., 2012; Shaner *et al*., 2005). Therefore, this assay may be very advantageous for farmers in the future by avoiding the spread of weeds, facilitating their effective management and by saving the farmer time and money (Kretzmer *et al*., 2011).

1.2.8 Herbicide damage caused by glyphosate

Glyphosate kills plants by inhibiting the shikimic acid pathway responsible for aromatic amino acid biosynthesis and many plant secondary compounds (flavonoids, lignins and other phenolic compounds). Glyphosate applications leads to the starvation and deregulation of this metabolic route, thereby killing the plant. Treated plants stop growing rapidly, but symptom development occurs very slowly (Hensley *et al*., 2013).

Symptoms of glyphosate injury include; leaf rolling and chlorosis on leaves, which occurs gradually, often in a mottled or interveinal pattern. Stunting is the symptom when there is severe cessation of growth. Symptoms usually are most evident on new growth - meristematic areas. Necrosis and tissue destruction follow more slowly after cessation of growth and chlorosis (Figures 1.19 and 1.20). These symptoms develop gradually (1-4 weeks) after treatment in perennial plants, but in small annual plants they can occur very rapidly in a matter of days, depending on the rate (dosage) of glyphosate and environmental conditions (temperature, wind, humidity, rain etc.). Death ultimately results from dehydration and desiccation.

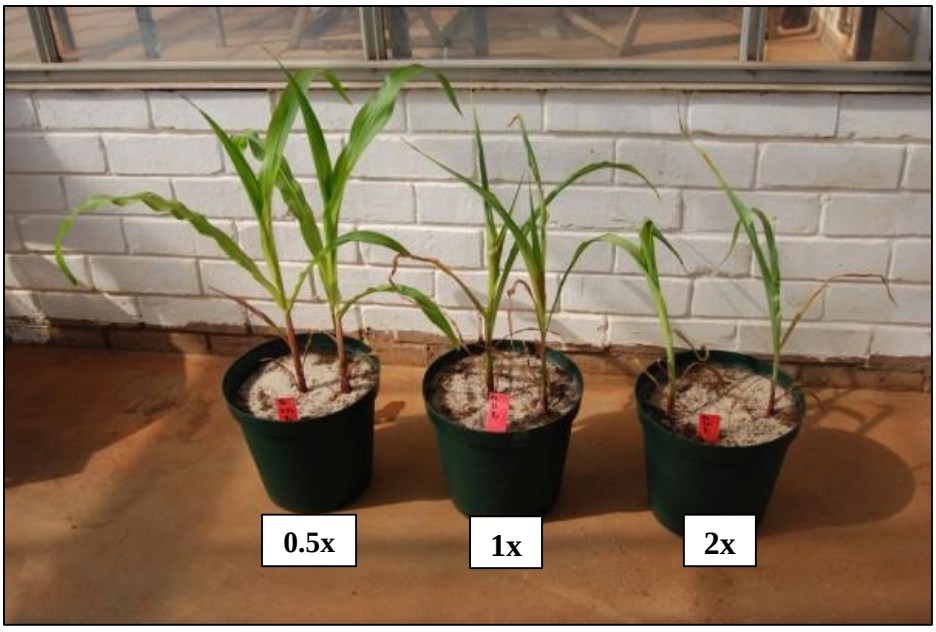


FIGURE 1.19 Conventional (non-RR) maize plants 7 days after glyphosate treatment. From left to right: Plants treated with 0.5x, 1x and 2x (double) the recommended dosage of the Roundup Turbo®. As can be seen the leaves are flaccid; leaf rolling and leaf firing (necrosis of the leaf tips) are clearly visible.— glyphosate damage to the plants is directly proportional to the dosage applied. The plants treated with 2x the recommended dosage (right) are the most severely affected (leaf rolling and necrosis are very evident) by the herbicide.

FIGURE 1. 20 Conventional soybeans 10 days after being treated with 2x the recommended dosage of Roundup Turbo®. As can be seen the plant suffers from severe glyphosate damage in the form of leaf and stem necrosis. Symptoms may resemble those of potassium deficiency or soybean cyst nematode.

CHAPTER 2. MATERIALS AND METHODS

2.1 Plant growth conditions

Seven glyphosate-resistant (Asgrow AG2901; AG2901RR), seven susceptible (Asgrow A2833; A2833) soybean seeds and seven glyphosate-resistant (DeKalb 545RR; DK545RR) and seven susceptible (Gast8550Bt, G8550Bt) maize seeds were planted in 23 cm diameter (7 kg) pots containing a sand/coir (8:2) soil mix medium (a total of 56 pots were used per treatment, which was performed in triplicate). The pots were watered thoroughly and covered with plastic bags to reduce evaporation. Following emergence, the plastic bags were removed and crops were thinned to five seedlings per pot. The seedlings were grown under the following greenhouse conditions: natural diurnal and nocturnal light, 28-35 ºC diurnal and 20-25 ºC nocturnal conditions; plants were irrigated as needed and fertilised (Miracle Gro® Excel, Scott Sierra, Marysville, OH) one week after emergence using a fertigation system (liquid fertiliser solution). Planting was done in triplicate for both dose and time responses over a 9 month period to include multiple seasons and to eliminate as many environmental conditions as possible. The same planting procedure and greenhouse conditions were used/followed for plants used in both the HPLC and colourimetric assay experiments.

2.2 Glyphosate application

Twenty-eight pots with 15-18 cm tall soybean (V3-V4) and 28 pots containing 21-25 cm tall maize (V6-V8) plants were used in this study for time and dose response respectively. Dose response: for both maize and soybean plants, 8 pots of each were treated with the isopropylamine salt of glyphosate (Roundup Turbo®, Monsanto, St. Louis, MO), using half (0.5x) the manufacture's recommended field rate (1L/ha i.e. 0.102 ml glyphosate/m^2), the recommended (1x) rate (2L/ha i.e. 0.204 ml glyphosate/m^2 or 765g/ha acid equivalent (AE) per hectare) and double (2x) the recommended field rate (4L/ha i.e. 0.408 ml glyphosate/m^2). Time response: for both maize and soybean plants, 8 pots of each were treated with the isopropylamine salt of glyphosate using half the manufacturer's recommended field rate, the recommended rate of 2 L/ha and double the recommended field rate of Roundup Turbo®. This

application of glyphosate was performed by an air powered hand sprayer, which delivered 187 L/ha at a pressure of 2.8 kg/cm through an 80015-E nozzle. Spraying was done 35-50 cm above plant canopy. Care was taken to ensure uniform spray coverage and to minimize foliage overlap. Plants were sprayed in the morning and were allowed to grow in full sunlight during the initial 12h after treatment (HAT). In addition to the pots sprayed with glyphosate, for both dose and time responses six maize and six soybean pots were not sprayed/treated, and these served as untreated control plants for the study. The experiment was repeated in time (January, May, July and October).

2.3 Plant tissue collection and assessment of phytotoxicity

For both maize and soybean, three randomly selected glyphosate treated pots and the untreated control pots were assessed at each of the following time points: 0, 5, 12, 24, 72, 144 and 216 hours after treatment (HAT). Glyphosate efficacy was determined by comparing the degree of visual crop injury in the glyphosate treated plants to the degree of crop injury in the untreated control plants (where 0% = asymptomatic and 100% = completely necrotic). Plant height was also determined for both glyphosate treated and untreated control plants by measuring the distance from the soil surface to the collar of the uppermost expanded leaf in maize and the apex of soybean. Samples for shikimate analysis were collected as follows: Time response: for both maize and soybean plants the control and treated plants were collected 3, 6, 9 and 12 days after treatment (DAT). The plant parts that were collected include: roots, actively growing portion of stem (basal 3 cm of the coleoptile for maize), the apex (for soybean), and the youngest fully expanded leaves. Tissue samples from two plants per treatment were used to create a pooled sample in order to minimise the biological variance, this was done in triplicate. Dose response: for both maize and soybean plants the control and treated plants were collected 3 DAT. The same plant parts were excised as described above for *time response*. The plant parts were transferred (stored) to labelled falcon tubes and immediately frozen in liquid nitrogen. The tubes were then transferred and stored at -80°C in a chest freezer.

2.4 Shikimic acid extraction for high performance liquid chromatography

Plant material was ground in liquid nitrogen with a mortar and pestle into a fine powder, weighed, and stored at -80°C. For HPLC analysis of shikimic acid, shikimate was extracted from 100mg of ground tissue using 900µl 0.25N HCl (Sigma Aldrich, 37%) for 5 min by means of a micropestle in a 1.5 ml microfuge tube. This was followed by vortexing the extracts for 5 min and finally the extracts were sonicated for 8 min at 25 ºC (2210 Bransonic Ultrasonicator, Markham, OH, Canada). Plant cell components were then separated by centrifugation at maximum speed using a desktop microcentrifuge for 10 min. The pellet was discarded and the supernatant was filtered through a 0.22 µm syringe filter (Whatman™ Spartan® Syringe Filter) and used directly for shikimate analysis by HPLC (Kretzmer *et al.*, 2011).

2.5 Shikimic acid extraction for colourimetric assay

Plant material was ground in liquid nitrogen into a very fine powder by mortar and pestle weighed and stored at -80°C. Shikimic acid was extracted from 100 mg frozen powder using 900µl 0.1N NaOH (Sigma Aldrich) using three cycles of freeze-thawing. The extracts were further ground for 5 min using a micropestle in a 1.5 ml microfuge tube, followed by vortexing the extracts for 5 min and finally the extracts were sonicated for 8 min at 25 ºC (2210 Bransonic Ultrasonicator, Markham, OH, Canada). The extracts were then centrifuged at maximum speed in a desktop microcentrifuge for 10 min in order to separate the plant cell components. The pellet was discarded and the supernatant was filtered through a 0.45 µm syringe filter (Whatman™ Spartan® Syringe Filter) and used directly for shikimate analysis in the colourimetric assay.

2.6 High-performance liquid chromatography analysis of shikimate

Maize and soybean extracts (250-300 µl) were filtered through a 0.22 µm nylon membrane syringe filter (Whatman™ Spartan® Syringe Filter) to remove any particulate matter prior to chromatographic analyses. An aliquot of 20µl of the filtered extracts was injected into an Agilent-Hewlett-Packard (Wilmington, DE) series 1100 liquid chromatograph equipped with Chemstation software and diode array detector. Separation utilized a LiChrosorb/luna NH_2

100A (250 x 4 mm, 5 µm internal diameter, Phenomenex, Torrance, CA) analytical column with a flow rate of 1 ml/min mobile phase (95% acetonitrile: 4% water: 1% orthophosphoric acid). The elution of shikimic acid was monitored by a photodiode array detector using a detection wavelength of 215nm, resulting in a retention time (t_R) of 8.45min The total run time per sample was 20 min. Samples were assayed in triplicate, and standard curves were prepared (20 µl) with shikimic acid standard (> 98% pure, Sigma Aldrich) at a range of 1 – 1200 µmol/l.

2.7 Coupled enzymatic colourimetric assay

The colourimetric assay was conducted according to the protocol* provided by Kretzmer *et al.* (2011). The colourimetric assay for shikimic acid is a coupled enzymatic assay (Figure 2.1) combining the enzymes; shikimate dehydrogenase [SHKDH; provided by Monsanto (St. Louis, MO)] and diaphorase along with the tetrazolium dye, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium (INT), which, in the presence of shikimic acid, forms a coloured formazan product. The colour intensity of the formazan salt is directly correlated to the concentration of shikimic acid present. Thus, the presence of shikimic acid is detected visually and the concentration can be accurately quantified with a spectrophotometer. In the first part of the coupled assay (Figure 2.1), SHKDH converts (oxidizes) shikimic acid to dehydroshikimate and reduces NADP to NADPH. In the second part of the assay, diaphorase transfers electrons from NADPH to reduce the tetrazolium dye INT which becomes coloured when reduced. Keith Kretzmer and Douglas Sammons (Monsanto, St. Louis, MO) provided the protocol and SHKDH enzyme for the colourimetric assay.

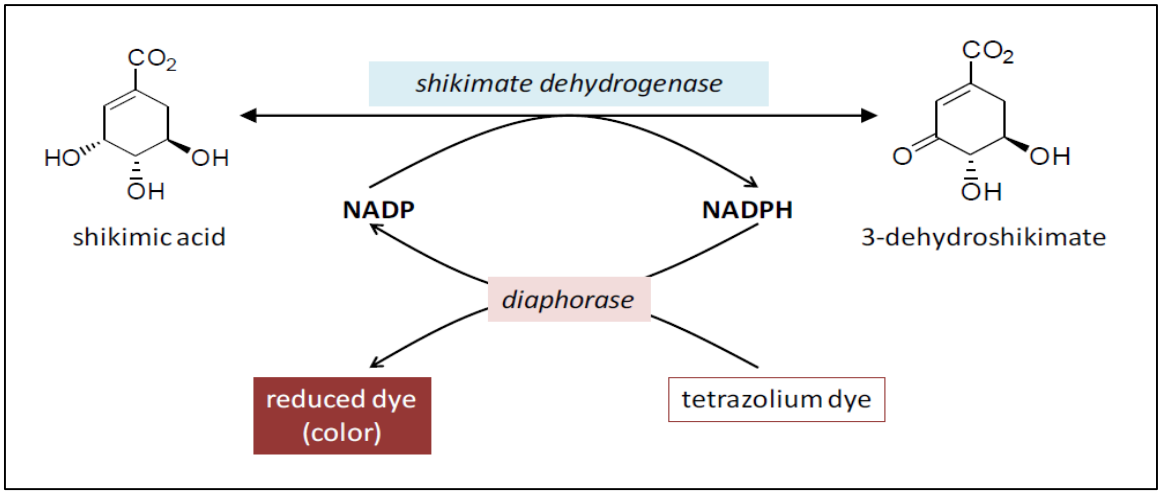


FIGURE 2.1 Schematic representation of the chemical reactions involved in colourimetric assay (Kretzmer *et al*., 2011).

Colourimetric assay for leaf extracts: the couple assay solution was freshly prepared prior to assay as per Table 2.1. After extraction, 10µl of the extract was transferred to a sterile 96 well microtiter plate (Costar, 3596). 100µl of 2x couple assay incubation solution containing components as per Table 2.1 (Tris buffer, $NADP^+$, $MgCl_2$, INT dye, diaphorase enzyme) and 80µl MQ.H_2O was added to the extract in the well. Plate absorbance was read at 500nm to establish the background absorbance. The enzymatic colourimetric assay was started by adding 10µl of the enzyme, shikimate dehydrogenase (Monsanto, St. Louis, MO), to add to a final well volume of 200µl. After shikimate dehydrogenase was added the plate absorbance (500 nm for INT) was read in intervals of 2 minutes over a total period of 60 minutes. Colour changes were observed and absorbance (OD) values were recorded.

TABLE 2.1 Components of incubation solution used for leaf extracts.

				Volume (µl)	
Assay component	**Stock (mM)**	**Final Assay (mM)**	**2x Coupled Assay Solution (mM)**	**For 1 ml (8 wells)**	**For 10 ml (1 plate)**
Tris buffer, pH 9.0	500	50	100	200	2000
$NADP^+$	18	0.9	1.8	100	1000
$MgCl_2$	200	5	10	50	500
INT	6	0.6	1.2	200	2000
Diaphorase	100 U/ml	1 U/ml	2 U/ml	20	200
MQ.H_2O				430	4300
Total µl				1000	10 000
Shikimate dehydrogenase (Dilute 1:1000 in 1M Tris, pH 9.0)				10 µl/well	10 µl/well

Colourimetric assay for whole leaf discs/quick field test kit: the necessary volumes of incubation solution as per Table 2.2 for tissue to be submerged/immersed in was prepared. Typically, solutions for whole plant incubation was diluted to 0.5x for best results. The absorbance of the assay incubation solution was read at 500nm before immerging the leaf discs in the solution to establish the background absorbance. Leaf discs (6 x Ø3mm) excised from fully expanded leaves of untreated control and glyphosate treated plants were immersed in microfuge tubes containing 0.5x coupled assay solution (Table 2.2). The tubes containing leaf disc were then incubated in the assay solution in an incubation chamber for 2, 5, 10, 15, 20, 30, 45 and 60 minutes at 25˚C. After the latter incubation times, leaf discs were removed from the solution. The solutions were transferred to a sterile 96 well microtiter plate (Costar, 3596). The plate absorbance was read at 500 nm in intervals of 2 minutes over a total period of 60 minutes. Colour changes were observed and absorbance (OD) values were recorded.

TABLE 2.2 Components of incubation solution used for leaf discs.

			Volume (ml)	
Incubation solution component	**Stock (mM)**	**Final 1x incubation solution (mM)**	**For 10 ml**	**For 20 ml**
Borate buffer, pH 9.0	500	50	1	2
$NADP^+$	18	0.9	0.500	1
$MgCl_2$	200	5	0.250	0.5
INT	6	0.6	1	2
Diaphorase	100 U/ml	1 U/ml	0.200	0.400
MQ.H_2O			6.55	13.1
Shikimate dehydrogenase (Dilute 1:1000 in 500 mM Borate Buffer, pH 9.0)			0.500	1
Total ml			10	20

Shikimic acid (> 98% pure, Sigma Aldrich) standard curves were established/determined according to the concentration range as per Table 2.3 (Annexure page 148). Unknown shikimic acid values from leaf extracts and leaf disc assay solutions can be extrapolated from a linear or polynomial curve derived from the absorbance (OD) values from the shikimic acid standard curves.

CHAPTER 3. RESULTS

3.1 High performance liquid chromatographic quantification/assay of shikimic acid levels

HPLC analysis was used to establish the retention time of shikimic acid in order to identify the location of the peak, and also to quantify shikimic acid concentrations in plant samples. The HPLC analytical method provided a clear distinctive chromatographic peak without interference, which represented shikimic acid. Shikimic acid eluted at 8.45 min (i.e. a retention time (t_R) of 8.45 min), with a total analysis time of 20 min. The shikimic acid absorbance spectrum is shown in Figure 3.1. The UV spectra of both shoulders of the shikimic acid peak maximum were essentially identical and the similarity index of the spectra in a peak equals to 0.9999 to confirm the purity of the shikimic acid peak. This purity was also demonstrated by the linear relationship between the signals obtained at 202, 207, 210, 212, 217 and 222 nm, showing the consistency of the peak and specificity of the method, although the use of DAD (Diode array detection) alone is by no means conclusive in establishing peak purity. Shikimic acid standard solutions with varying concentrations (10, 25, 50, 75 and 100µg/ml) were used to establish the shikimic acid standard curve which is illustrated in Figure 3.2.

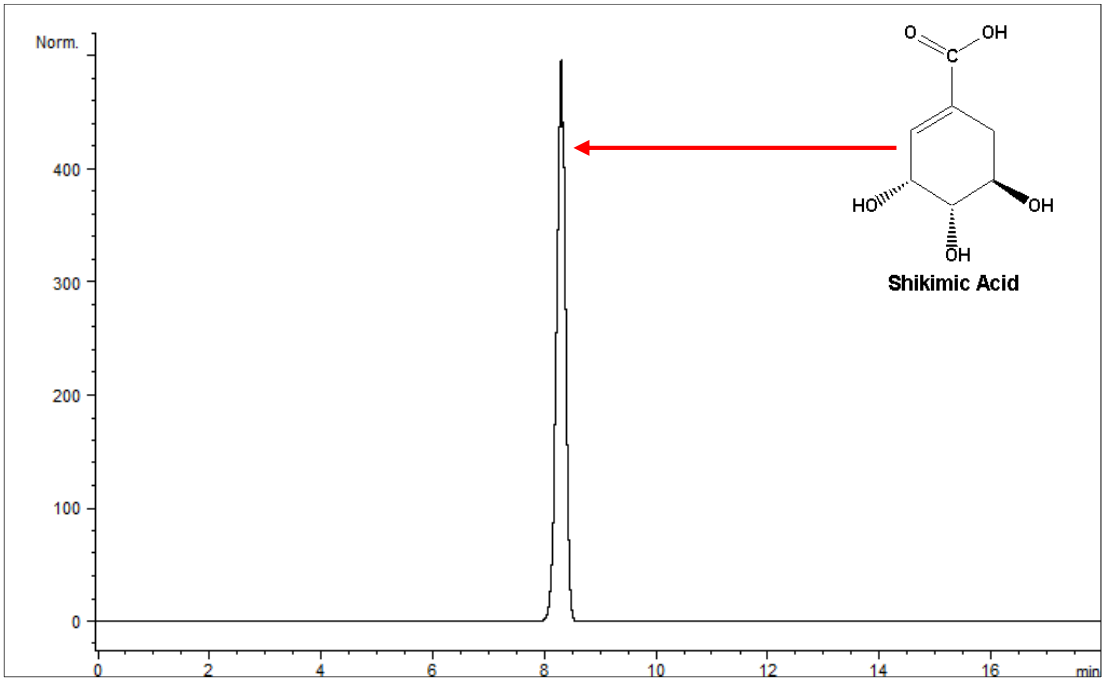


FIGURE 3.1 High performance liquid chromatogram (absorption spectra) of shikimic acid standard solution (Sigma-Aldrich; S5375 SIGMA). The shikimic acid peak is indicated on the figure.

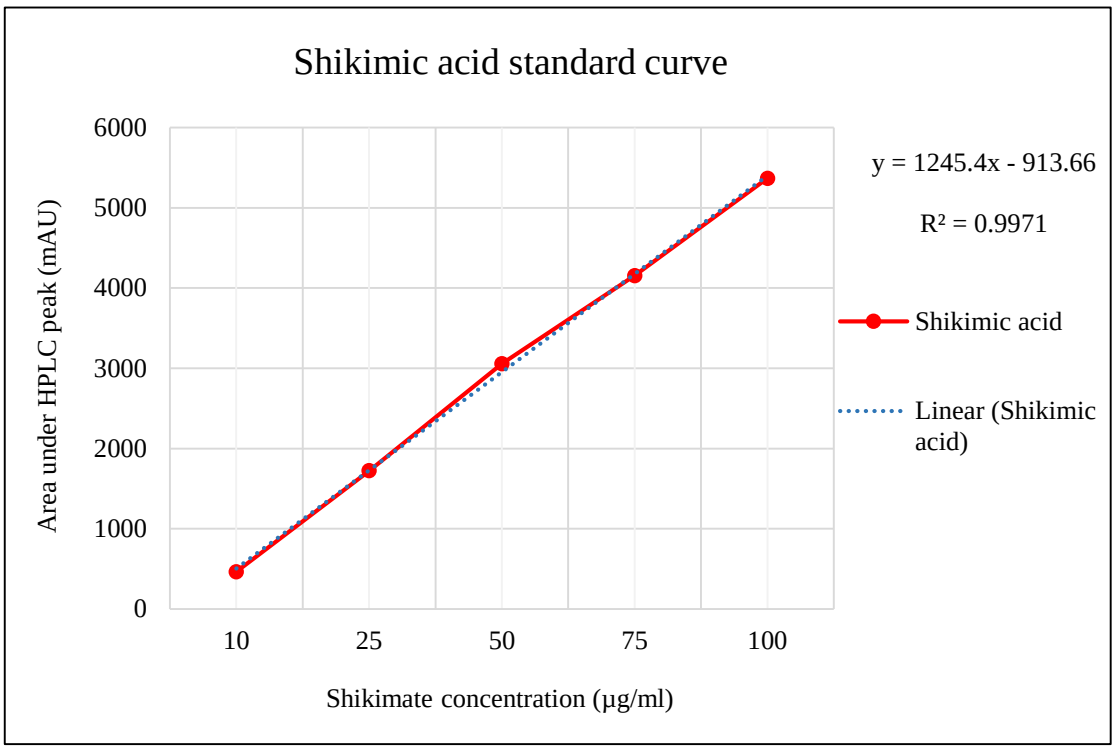


FIGURE 3. 2 Shikimic acid standard curve.

3.1.1 Accumulation of shikimic acid in glyphosate-treated plants

Shikimic acid levels (i.e. concentrations) were determined in the leaves of untreated and treated maize and soybean plants from day 0, just prior to the glyphosate application, until 12 days after treatment (DAT). The height of the peak representing shikimic acid is directly correlated to the degree of shikimate accumulation, a higher peak is indicative of higher shikimate concentrations in a sample. The average background level of shikimate on day three observed in untreated maize plants (Figure 3.3 A) was slightly higher than that of untreated soybean plants (Figure 3.4 A). Changes in shikimic acid concentrations differed substantially between RR and non-RR cultivars/varieties. The shikimic acid peak of treated RR maize (Figure 3.3 C) and soybean (Figure 3.4 C) plants were significantly lower when compared to that of treated non-RR maize (Figure 3.3 D) and soybean (Figure 3.4 D) plants. Shikimate concentrations in non-RR plants were on average more than 4.5-fold (maize plants) and 9-fold (soybean plants) higher in non-RR maize and soybean plants respectively, when compared to that of RR plants three days after glyphosate treatment. Similar results were obtained for 6, 9 and 12 DAT.

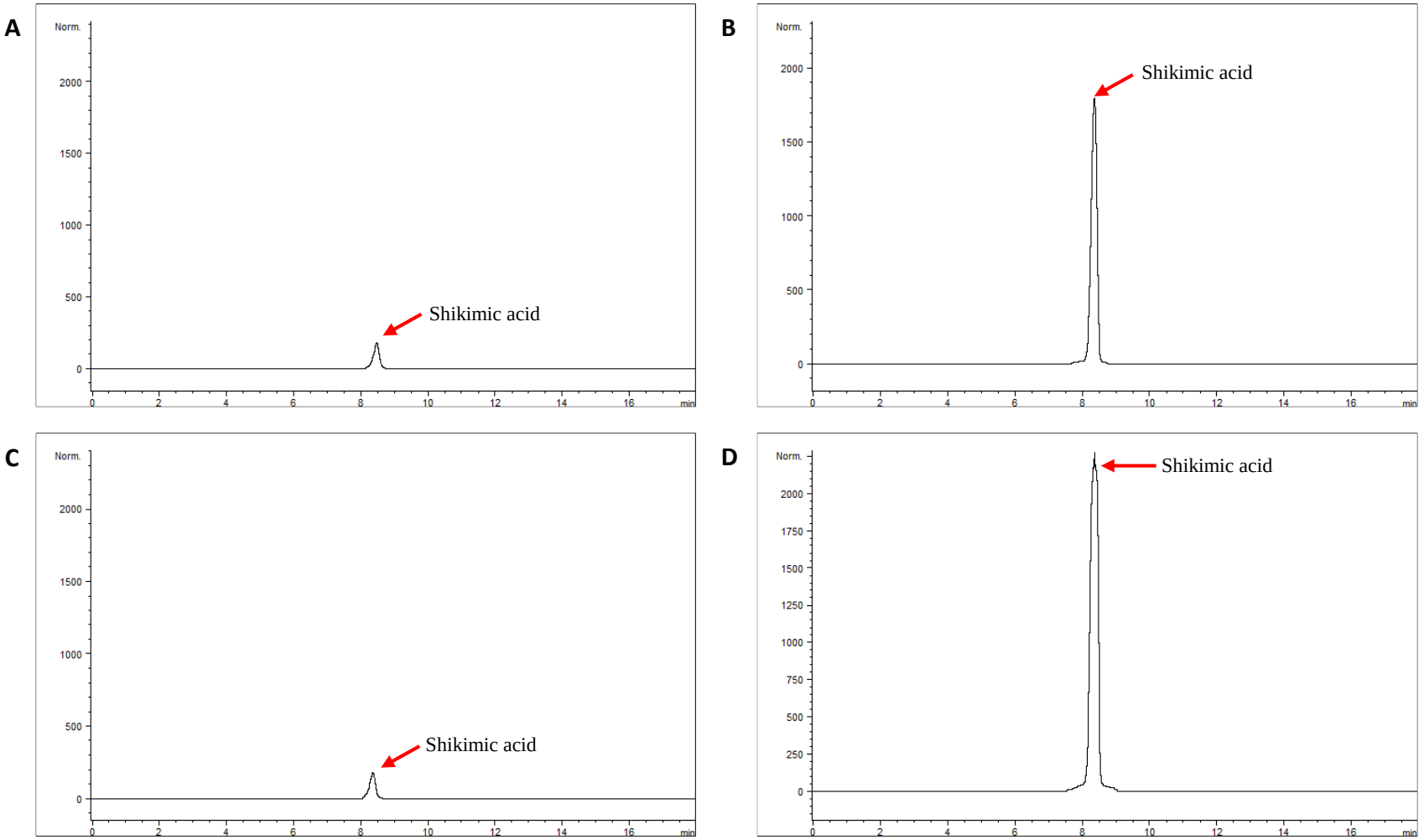


FIGURE 3.3 Representative chromatograms for shikimic acid accumulation and quantification in RR and non-RR maize leaves 3 days after glyphosate treatment. (**A**) Untreated control maize plants; (**B**) Shikimic acid standard solution with a concentration of 150µg/ml; (**C**) RR maize cultivar treated with the recommended dosage (2L/ha) of Roundup Turbo®; (**D**) Non-RR (Yieldgard®) maize cultivar treated with the recommended dosage of Roundup Turbo®.

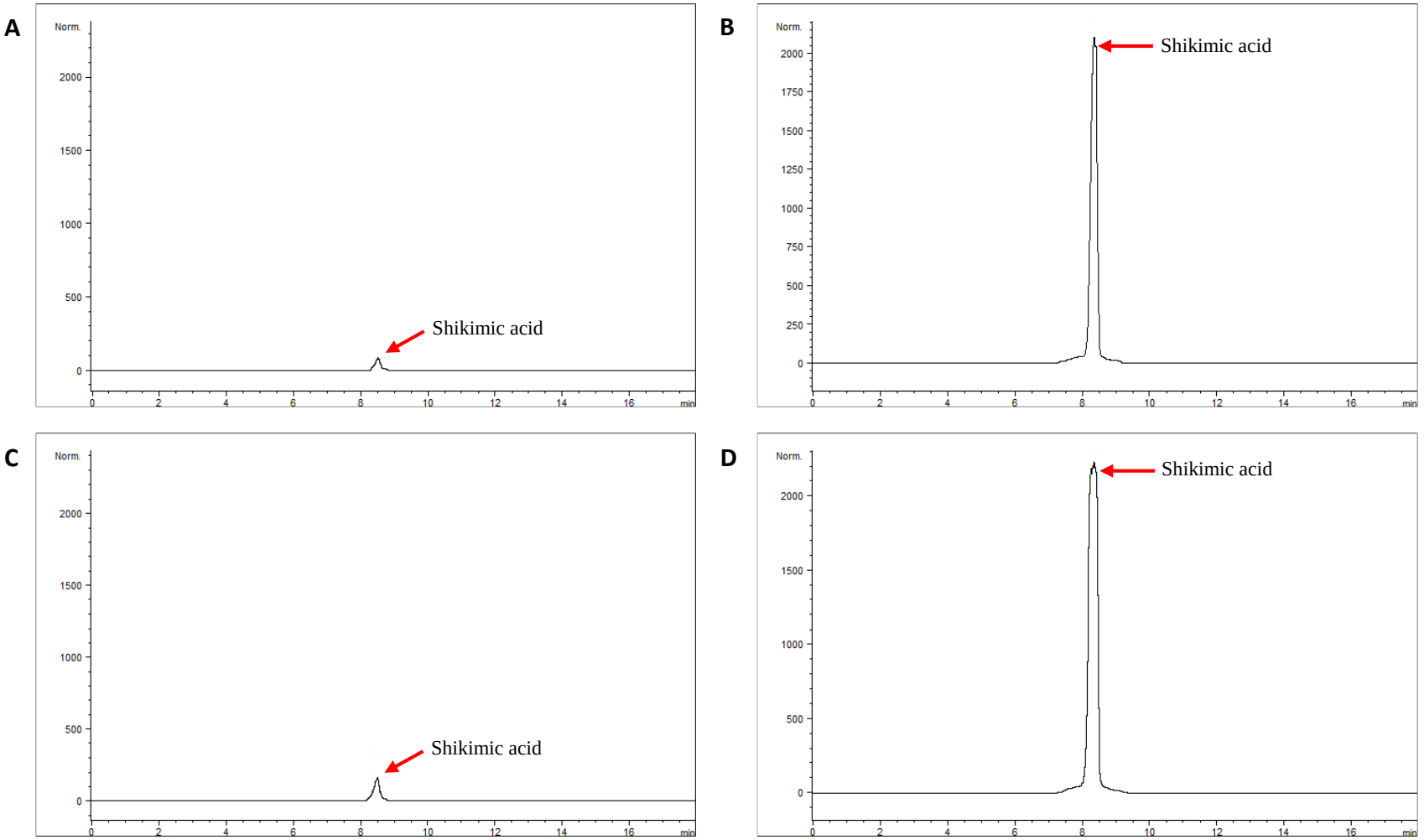


FIGURE 3.4 HPLC chromatograms for quantification of shikimic acid in RR and non-RR soybean leaves in response to glyphosate application 3 DAT. (**A**) Untreated control soybean plants; (**B**) Shikimic acid standard solution with a concentration of 150µg/ml; (**C**) RR soybean cultivar treated with the recommended dosage (2L/ha) of Roundup Turbo®; (**D**) Non-RR soybean cultivar treated with the recommended dosage of Roundup Turbo®.

3.1.2 Correlation between glyphosate dosage and shikimic acid accumulation

Shikimic acid concentrations in leaves of RR and non-RR maize and soybean plants were determined in response to treatment with various dosages of glyphosate. This dose response analysis was performed to establish the relationship between glyphosate dosage and shikimic acid accumulation within leaves. The mean background level of shikimate concentration (mg/g) observed in untreated control maize plants (green bars in Figure 3.5 A) were marginally higher, but not significant at $p \leq 0.05$, when compared to that of untreated soybean plants (green bars in Figure 3.5 B). The marginal difference, statistically not significant at $p \leq 0.05$, in shikimate concentrations between soybean (C3) and maize (C4) plants were also reported in several research papers, where the authors demonstrated that shikimate concentrations occurring naturally in C4 plants are slightly higher than in C3 plants, however also not significant at $p \leq 0.05$ (Bazzaz *et al*., 1974; Gowik and Westhoff, 2011; Mera *et al*., 2006; Singh and Shaner, 1998).

Glyphosate had no significant effect on shikimate concentrations in treated RR maize (blue bars in Figure 3.5 A) or soybean (blue bars in Figure 3.5 B) plants, regardless of dosage applied, when compared to untreated control plants. At a significance level of $p \leq 0.01$ there was no significant difference in shikimate concentrations between untreated control and treated RR maize or soybean plants regardless of the glyphosate dosage applied.

For non-RR maize and soybean plants there was a stong correlation between shikimic acid concentration levels and the glyphosate application rate. This correlation is clearly illustrated in Figure 3.5 A and 3.5 B by the regression lines with R^2 values of 0.99 (non-RR maize) and 0.97 (non-RR soybean) respectively. The shikimate concentrations in the leaves of non-RR maize and soybean (red bars in Figure 3.5 A and 3.5 B respectively) plants were directly proportional to glyphosate dosage i.e. shikimic acid concentrations (mg/g) increased as the glyphosate application rate increased. At a significance level of $p \leq 0.05$ there was a significant difference in shikimate concentrations between treated RR (blue bars) and treated non-RR (red bars) maize and soybean plants. Also, for non-RR soybean there was a significant ($p \leq 0.05$) difference in shikimate concentrations between plants treated with different dosages of glyphosate (red bars in Figure 3.5 B).

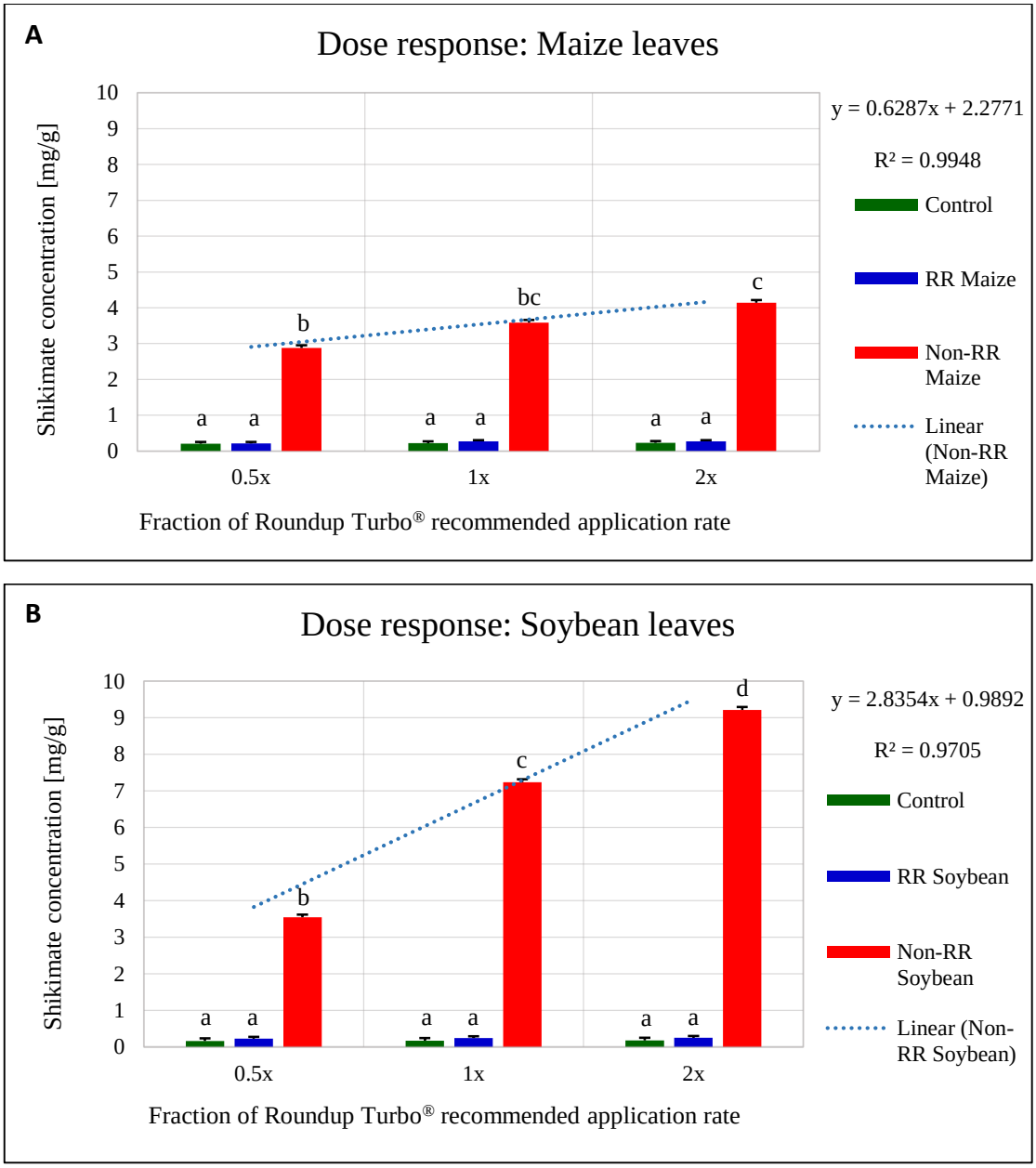


FIGURE 3.5 HPLC results for dose response of **(A)** maize and **(B)** soybean leaves, 3 days after being treated with with various dosages (0.5x, 1x and 2x the fraction of recommended application rate) of Roundup Turbo®. Bars represent means (+SE) of three (triplicate) biological replications. Means with different letters indicate significant differences at $p \leq 0.05$ Fisher's protected LSD.

3.1.3 Comparison between different plant tissue and shikimic acid accumulation

Shikimic acid concentrations (mg/g) in the roots, stems, and leaves of RR and non-RR maize and soybean plants were determined in response to glyphosate treatments. The primary objective of this analysis was to determine the degree in which shikimic acid accumulated in different plant parts i.e. in which tissue accumulation was the greatest. Overall, shikimic acid concentrations was the highest in the leaves, followed by the stems and was the lowest in roots of both untreated and treated maize and soybeans plants, regardless of the cultivar/variety.

Shikimic acid concentrations in untreated maize plants were slightly higher in comparison to soybean control plants (green bars in Figure 3.6 A and B) irrespective of tissue type. This once again demonstrates the fact that the concentration of naturally occurring shikimate is marginally higher in C4 plants (Bazzaz *et al*., 1974; Gowik and Westhoff, 2011; Mera *et al*., 2006; Singh and Shaner, 1998). At a significance level of $p \leq 0.05$ there was not a significant difference in shikimate concentrations between untreated maize and soybean control plants. At the same stringency levels of $p \leq 0.05$ there was also no significant difference in shikimic acid levels between untreated control plants and treated RR maize or soybean (blue bars in Figure 3.6 A and B) plants, regardless of the tissue type.

A significant difference ($p \leq 0.05$) in shikimic acid concentrations between treated RR maize and soybean plants (blue bars in Figure 3.6 A and B) and non-RR maize and soybean plants (red bars in Figure 3.6 A and B) was evident irrespective of the tissue type.
Also, for treated non-RR maize and soybean plants (red bars in Figure 3.6 A and 3.6 B) at $p \leq 0.05$ there was a significant difference in shikimic acid levels between root, stem, and leaf tissue.

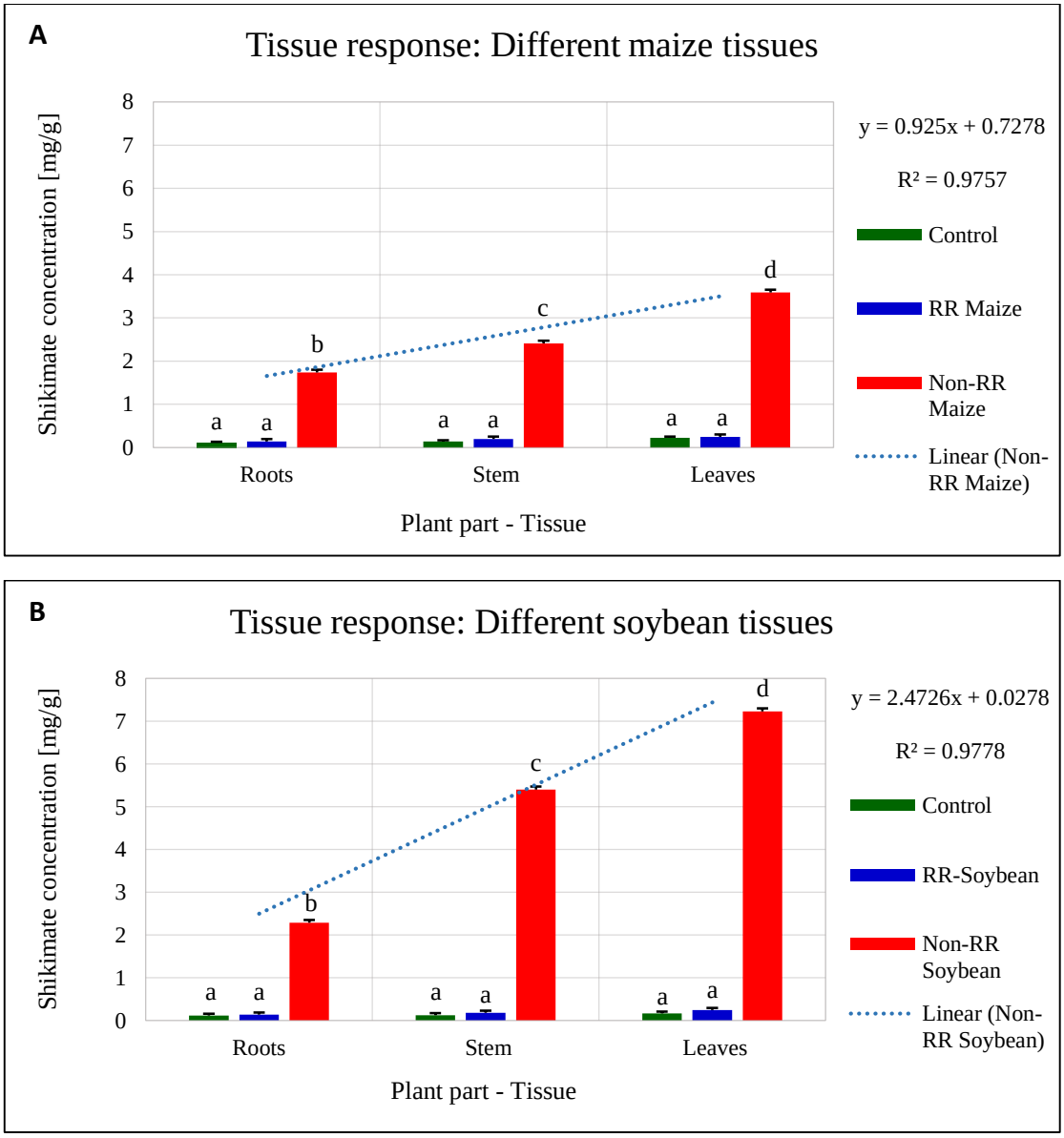


FIGURE 3.6 HPLC results for dose and tissue response of different; **(A)** maize and **(B)** soybean tissues, 3 days after being treated with the recommended rate of Roundup Turbo®. Bars represent means (+SE) of three (triplicate) biological replications. Means with different letters indicate significant differences at $p \leq 0.05$ Fisher's protected LSD.

3.1.4 The relationship between shikimic acid accumulation in different plant tissue types and time after treatment

Shikimic acid concentrations (mg/g) in various tissues (roots, stems and leaves) from RR and non-RR maize and soybean plants were measured over a period of 12 days after glyphosate treatment. The aim was to establish if there is any correlation between shikimic acid build-up in different plant tissues and time after glyphosate treatment.

As can be seen from Figure 3.7 A and B, it is evident that there was no significant difference ($p \leq 0.05$) or increase in shikimic acid concentrations in leaf tissue of untreated control (green lines) and treated RR (blue lines) maize and soybean plants over the 12 day period after treatment. In fact, the lines representing shikimic acid from control and RR plants overlap (Figure 3.7 A and B). However, there was a significant ($p \leq 0.05$) increase in shikimate concentration when comparing treated RR (blue lines) and treated non-RR (red lines) from 3 to 12 DAT in both maize and soybean leaves.

The time courses of subsequent changes in shikimic acid concentrations from leaf tissue differed substantially: in non-RR maize plants levels increased more than 3-fold from 6 to 9 DAT, but increased 4.5-fold on average in non-RR soybean plants from 3 to 6 DAT. At 9 DAT, the level of shikimate was more than 4 and 9 times higher in non-RR maize and soybean plants respectively in comparison to RR plants.

For both non-RR maize and soybean plants at $p \leq 0.05$, there was a significant increase in shikimate levels from 3 to 6 DAT as well as from 6 to 9 DAT. Thus, the greatest increase in shikimate concentrations for both maize and soybean leaves occurred between 3 and 9 DAT (red line in Figure 3.7 A and B). However, for both non-RR maize and soybean leaf tissue the increase in shikimic acid levels between days 9 to 12 was not as noteworthy and at $p \leq 0.05$ there was not a significant increase in shikimate concentrations as shikimate levels reached a relatively stable level. Thus, time after glyphosate treatment only played a significant role in the degree of shikimic acid accumulation in the leaves of non-RR plants.

For non-RR maize and soybean leaf tissue there was a direct correlation between shikimic acid concentration levels and time after glyphosate treatment. This correlation is clearly illustrated in Figure 3.7 A and B by the regression lines with R^2 values of 0.97 (non-RR maize) and 0.96 (non-RR soybean) respectively.

For non-RR maize root tissue (yellow line in Figure 3.7 C) there was a significant increase ($p \leq 0.05$) in shikimic acid levels between 3 to 6 DAT. However, there was no significant increase ($p \leq 0.05$) in shikimic acid concentrations between 6 to 9 DAT. For non-RR soybean root tissue (Figure 3.7 D) there was an increase in shikimic acid concentrations for the 3 to 9 DAT period, however the 6 to 9 DAT period was the most significant (at $p \leq 0.05$).

Increases in shikimic acid levels for non-RR maize stem tissue was significant ($p \leq 0.05$) for the period between 3 to 9 DAT (blue line in Figure 3.7 C), but the most significant period of shikimate accumulation was between 3 and 6 DAT. For non-RR soybean stem tissue there was a significant difference in the increase of shikimic acid levels between 3 to 9 DAT however, the most significant increase ($p \leq 0.05$) in shikimic acid levels for was between 6 and 9 DAT.

For both non-RR maize and soybean cultivars, shikimic acid concentrations in root, stem and leaf tissue reached a plateau in shikimic acid build-up between 9 and 12 DAT, i.e. there was no significant increase in shikimate concentration between 9 and 12 DAT at significance level of $p \leq 0.05$.

At a stringency level of $p \leq 0.05$ there was a significant difference in shikimic acid concentrations between all three tissue types from RR and non-RR maize and soybean plants. Thus, there was significant differences ($p \leq 0.05$) in shikimic acid concentrations between the root (purple), stem (turquoise) and leaf (green) tissue from both RR maize and soybean (Figure 3.7 C and D) when compared to the root (yellow), stem (blue) and leaf (red) tissue of non-RR maize and soybean over the 12 day period.

There is a relationship between the level of shikimate build-up in different tissue and the time after glyphosate treatment. This data clearly illustrated that shikimic acid accumulation was the greatest and occurred the most rapidly in leaf tissue of both maize and soybean plants.

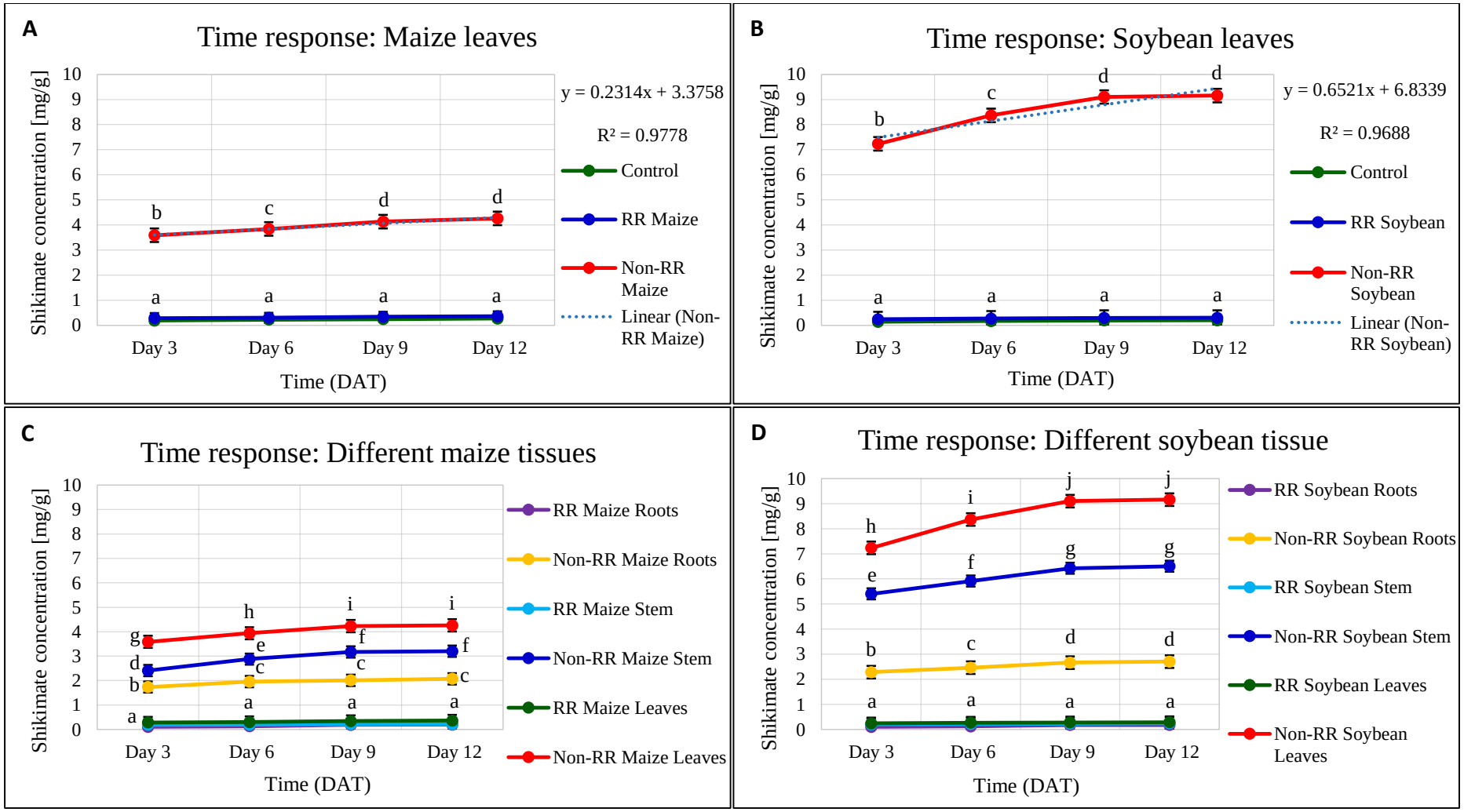


FIGURE 3.7 HPLC results for time response of **(A)** maize; **(B)** soybean leaves and time and tissue response of different; **(C)** maize (**Note**: The Y-axis of this graph is to the same scale as that of Figure D) and **(D)** soybean tissues, 3, 6, 9 and 12 days after being treated with the recommended rate of Roundup Turbo®. Markers represent means (+SE) of three biological replications. Means with different letters indicate significant differences at $p \leq 0.05$ Fisher's protected LSD.

3.2 Colourimetric assay of shikimic acid levels

3.2.1 Colourimetric assay performed in assay plates: Shikimate standard

Colourimetric analysis was performed on shikimate standard solutions to calibrate the spectrophotometer and to setup standard curves. Absorbance readings from shikimate standards were used to establish a link between shikimate concentrations and the corresponding absorbance range (Figure 3.8 and Table 3.1). Optimization was done with shikimate dehydrogenase enzyme ratios of 1:1000, 1:2000, and 1:5000, results showed no significant variation between shikimate concentration and enzyme concentration and a ratio of 1:1000 was used for further reactions.

The colourimetric test is specific to shikimic acid and fairly sensitive to changes in shikimic acid concentrations (Kretzmer *et al.*, 2011). The presence of shikimic acid within a sample was indicated by a colour change, and the intensity of the colour change indicative of the shikimate concentration. An increase in shikimic acid concentrations within a sample resulted in a more intense shading of deep red to maroon. The colourimetric test however has limits in detecting exceptionally high shikimate concentrations, but the assay threshold is higher than one would find in plant species.

Figure 3.8 shows the colourimetric assay performed on a wide concentration range (0µM - 574µM) of shikimic acid standard solutions. The red arrow indicates the direction of the shikimate concentration gradient. It is clear that there was an increase in colour intensity (deeper shading) as shikimate concentrations increased. Thus, a colour change indicates that shikimate is present in the sample solution and the degree of colour change i.e. the colour intensity is directly correlated to the amount of shikimate present.

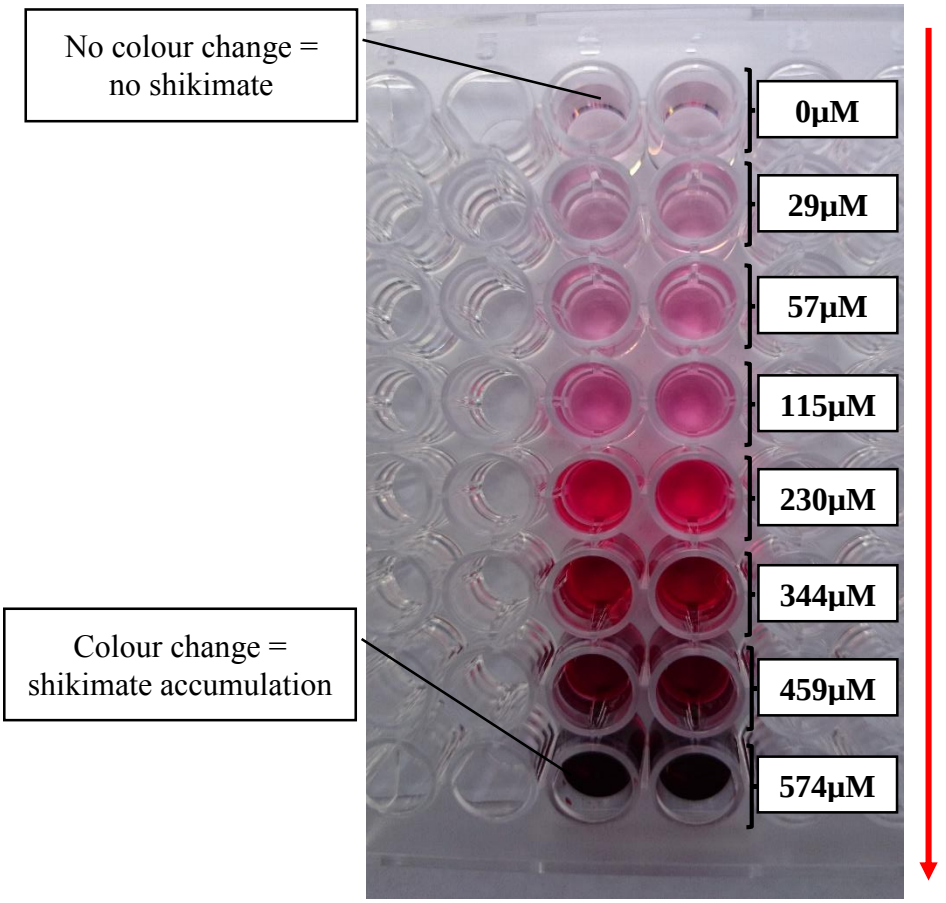


FIGURE 3.8 Results from a colourimetric assay performed with various concentrations (µM) of shikimate standard.

TABLE 3.1 Shikimate standard solutions with corresponding absorbance values.

Shikimate standard concentration (µM)	Absorbance (500nm)
0	0.1
29	0.21
57	0.53
115	1.11
230	1.65
344	2.01
459	2.47
574	2.83

The colourimetric assay is a simple assay developed to detect and quantify shikimic acid in plant tissues. The advantages of the assay are: it is specific for shikimic acid, it is rapid, and it is colourimetric (Kretzmer *et al.*, 2011). The assay is most useful for determining the sensitivity of plants and plant tissues to the herbicide, glyphosate.

The colourimetric test for shikimic acid is a coupled enzymatic assay (Please refer to Figure 2.1) combining the enzymes; shikimate dehydrogenase (SHKDH) and diaphorase along with the tetrazolium dye, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium (INT), which, in the presence of shikimic acid, forms a formazan product which is coloured. The colour intensity of the formazan salt is directly correlated to the concentration of shikimic acid present. Thus, the presence of shikimic acid is detected visually and the concentration can be accurately quantified with a spectrophotometer. In the first part of the coupled assay (Please refer to Figure 2.1), SHKDH converts (oxidizes) shikimic acid to 3-dehydroshikimate and reduces NADP to NADPH. In the second part of the assay, diaphorase transfers electrons from NADPH to reduce the tetrazolium dye, INT, which becomes coloured when reduced (Kretzmer *et al.*, 2011).

For the colourimetric assay any shikimic acid present in a sample would react with SHKDH and diaphorase enzymes in the assay solution, consequently resulting in a visible colour change produced by the INT dye. The optical density (OD) of colourimetric assay solutions containing various concentrations of shikimic acid standard solutions were established by making use of a spectrophotometer at an absorbance of 500nm (OD500), this was performed to yield a shikimic acid standard curve (Figure 3.9). The R^2 value (0.99) obtained from the data shows that there is a direct correlation between absorbance (intensity of colour produced by INT dye) and shikimate concentration. Figure 3.10 compares the shikimic acid standard curve obtained from HPLC (red line) and colourimetric (blue line) analysis. The figure illustrates the relationship between the results and it is clear that the standard curves of these two analyses were comparable. However, HPLC analysis yielded higher absorbance values, but this was expected as it is a more precise technique.

In order to establish the relationship between absorbance, concentration, and time, OD values (OD500) were measured as a function of time and increasing shikimate concentration (Figure 3.11). Absorbance of the colourimetric assay solutions were measured at 0 min, just prior to adding the enzyme SHKDH to the assay solution or extract in order to determine/obtain the

background absorbance of the sample. After adding SHKDH, absorbance was again measured at the following time intervals: 3, 5, 10, 15, 20, 30, 45, and 60 minutes (Figure 3.11).

Shikimic acid present in a sample would react with the enzymes in the assay solution, resulting in a visible colour change produced by the INT dye. The degree of the colour change (shade) intensified over time and as shikimic acid concentrations increased until an optimum reaction time was reached. From Figure 3.11 it is clear that at 0 min, the absorbance (Y-axis) remained constant as there was no change in colour, regardless of the increase in shikimic acid concentrations (X-axis). This was because the SHKDH enzyme, which activated the colourimetric assay was not available to react with shikimate to yield a colour change.

In general, samples to which the colourimetric assay solution was added, exhibited an increase in absorbance (more intense colour) over time and as shikimate concentrations (0, 1, 2, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 54 µg/ml) increased (Figure 3.11). However, after a certain time and concentration a stable state in the OD values were reached (indicated by a turquoise arrow on Figure 3.11 and Figure 3.12) i.e. there was no significant increase in OD values after this time period or concentration.

The data used for statistical purposes was recorded 15 min after adding SHKDH (Figure 3.12). This time interval was relevant since it was earliest time period that the absorbance of shikimate concentrations peaked and plateaued. Also, at this interval there was no significant difference between OD values when compared to later measured time intervals such as 20, 30, 45, and 60min (Figure 3.11).

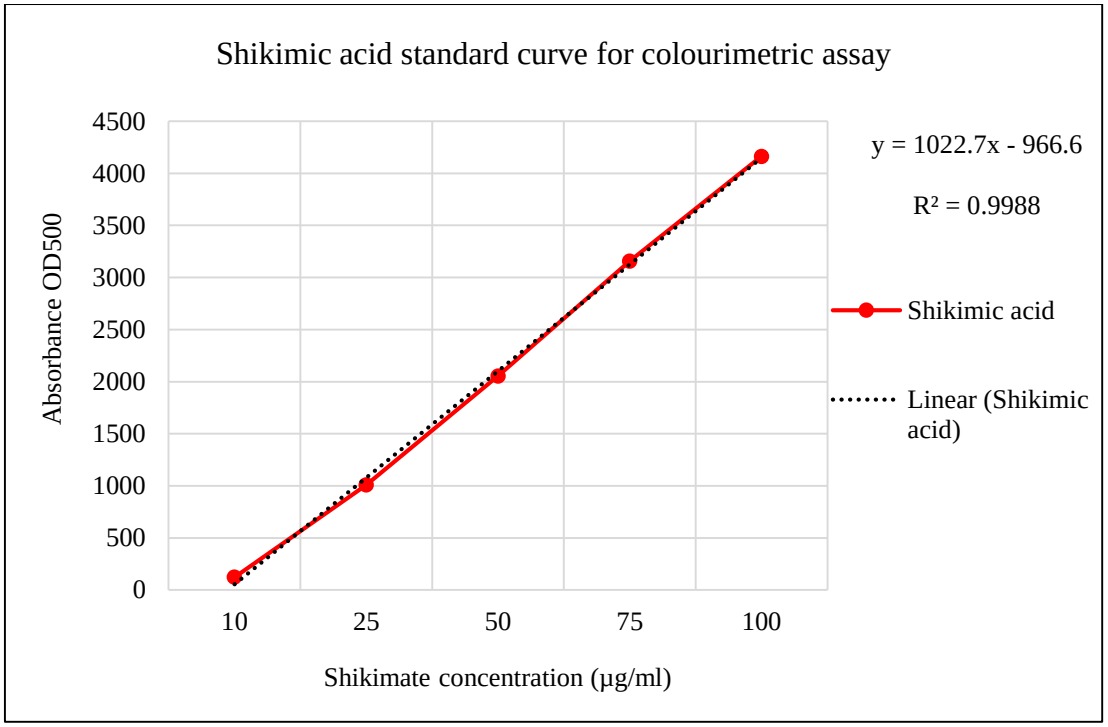


FIGURE 3.9 Shikimic acid standard curve obtained from the colourimetric assay.

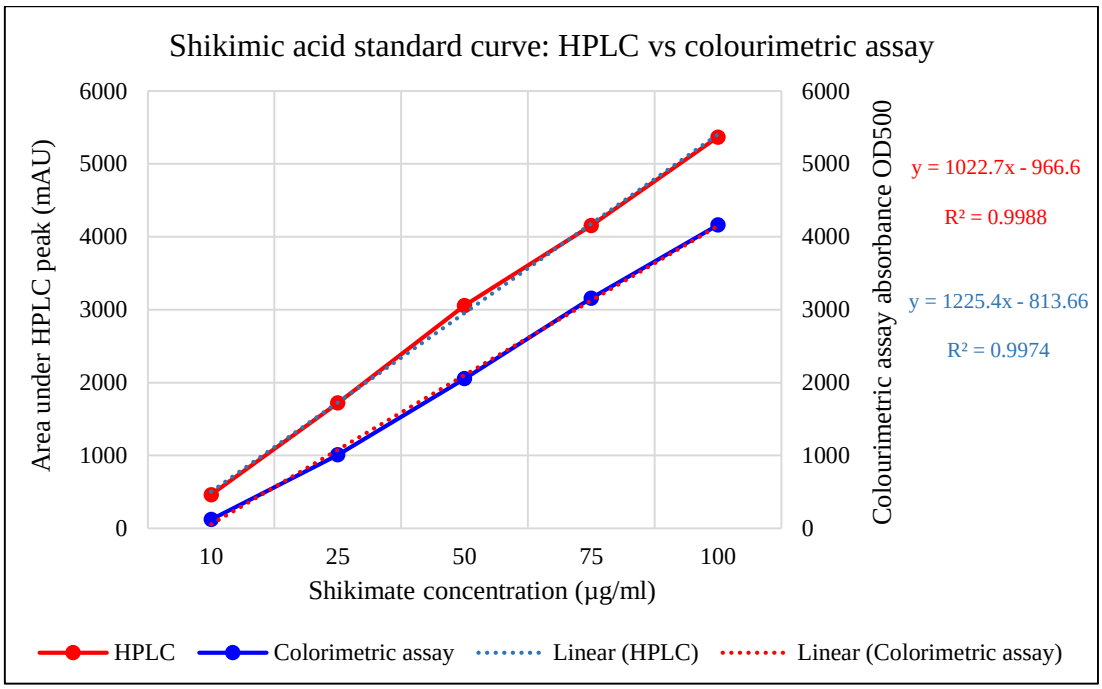


FIGURE 3.10 Comparison of shikimic acid curves obtained from HPLC (red) and colourimetric (blue) assays.

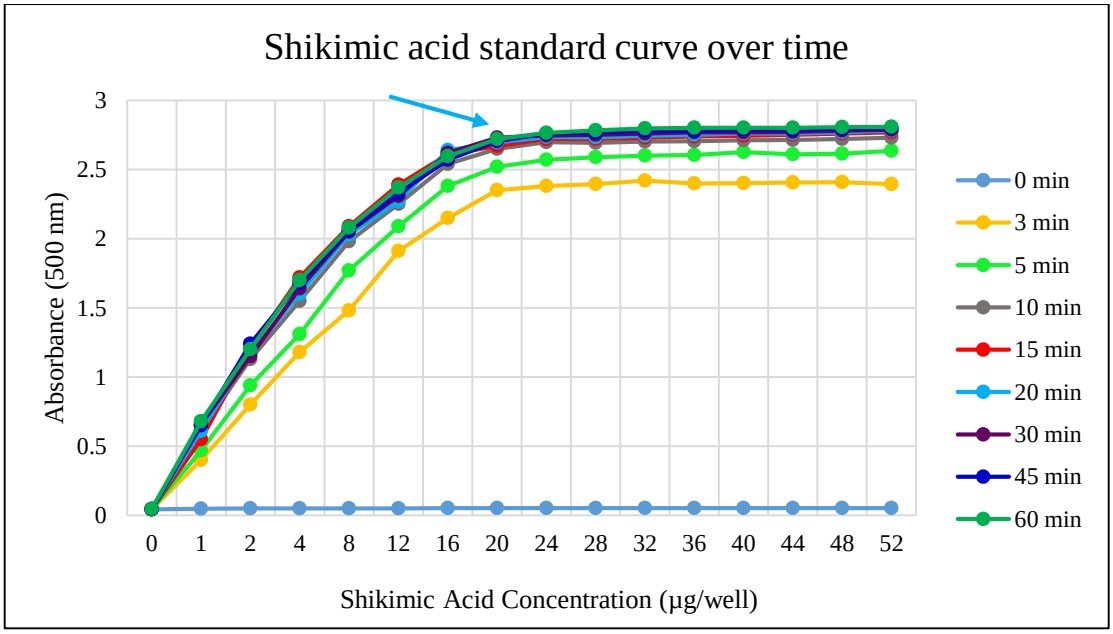


FIGURE 3.11 Shikimic acid assay in microtiter plate using shikimate standards and coupled assay solution containing INT dye. OD values for shikimic acid concentration increased with time, from 3 min up to a maximum at 60 min.

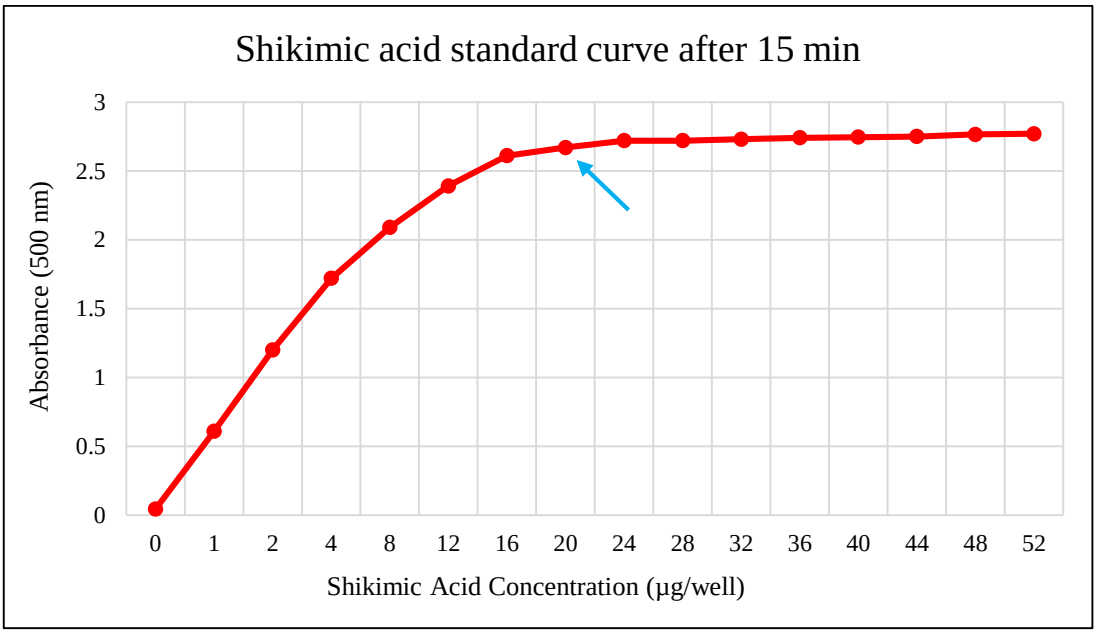


FIGURE 3.12 Shikimic acid standard curve obtained from the colourimetric assay performed with a wide range shikimic acid standard concentrations 15 min after adding the SHKDH enzyme.

3.2.1 Colourimetric assay performed on leaf discs and leaf extracts: The effect of dose on shikimate concentrations

Spectrophotometry was used to measure the absorbance of colourimetric assay solutions in order to determine the degree of shikimate build-up in leaf discs (Ø 3mm) and leaf extracts from RR and non-RR maize and soybean plants three days after being treated with various rates of glyphosate. The aim of this experiment was to determine the correlation between application rate and shikimate build-up, but in addition also to match the absorbance readings obtained from leaf discs to that of leaf extracts and compare the effectiveness of the two methods.

There was no significant difference ($p \leq 0.05$) in the absorbance readings (OD500) from assay solutions of leaf discs or leaf extracts between untreated control (green) and treated RR (blue) maize and soybean plants (Figure 3.13 A, B, C and D). However, it is evident that there was a significant difference ($p \leq 0.05$) in the absorbance values (i.e. shikimate concentration) of assay solutions from treated RR (blue) and non-RR (red) maize and soybean plants (Figure 3.13 A, B, C and D).

It was expected that leaf extracts would measure higher concentration of shikimate and yield more efficient results in the colourimetric assay when compared to leaf discs. Reasons for this include; leaf discs were only immersed in the solution, there was no maceration of tissue, centrifugation, or filtration techniques implemented for this method, hence since there was no extraction procedure applied a lower shikimate concentration was expected.

When using leaf extracts instead of leaf discs slightly higher levels of shikimic acid could be detected, however these differences was not significant at $p \leq 0.05$ and values obtained from leaf discs were sufficient to effectively indicate and differentiate between susceptible and resistant plants. Table 3.2 and Table 3.3 illustrate the differences between these two methods and also compare them to HPLC analysis.

As can be seen from Table 3.2 and Table 3.3, the shikimate concentrations (mg/g) obtained from HPLC and colourimetric (leaf discs) analysis differed by an average of 18% and 17.5% for maize and soybean plants respectively. Depending on the glyphosate dose, HPLC measured between 17-19% (maize) and 17-18% (soybean) higher shikimate concentrations than the colourimetric assay performed with leaf discs. Table 3.4 illustrate the R-square values attained from data of the colourimetric assay (leaf discs and leaf extracts) and HPLC analysis. From the R^2 values it is clear that results obtained from the colourimetric assay is supported and validated

by that obtained from HPLC analysis, as there was a direct correlation between shikimate concentration (absorbance values) and glyphosate application rate. Since there was a direct correlation between the colour intensity produced by INT dye, absorbance, and shikimic acid content, the colourimetric assay could be very efficient in signifying differences between sensitive and resistant plants.

From the outcomes illustrated in Tables 3.2, 3.3 and 3.4, it is clear that the results obtained from the colourimetric assay are accurate in differentiating between glyphosate sensitive and resistant cultivars. The results corresponded to those obtained from the HPLC assay. From both these analyses it is evident that glyphosate had no significant effect on shikimate build-up in RR crops regardless of the rate applied and that there was a strong correlation between shikimate build-up in non-RR crops and glyphosate application rate.

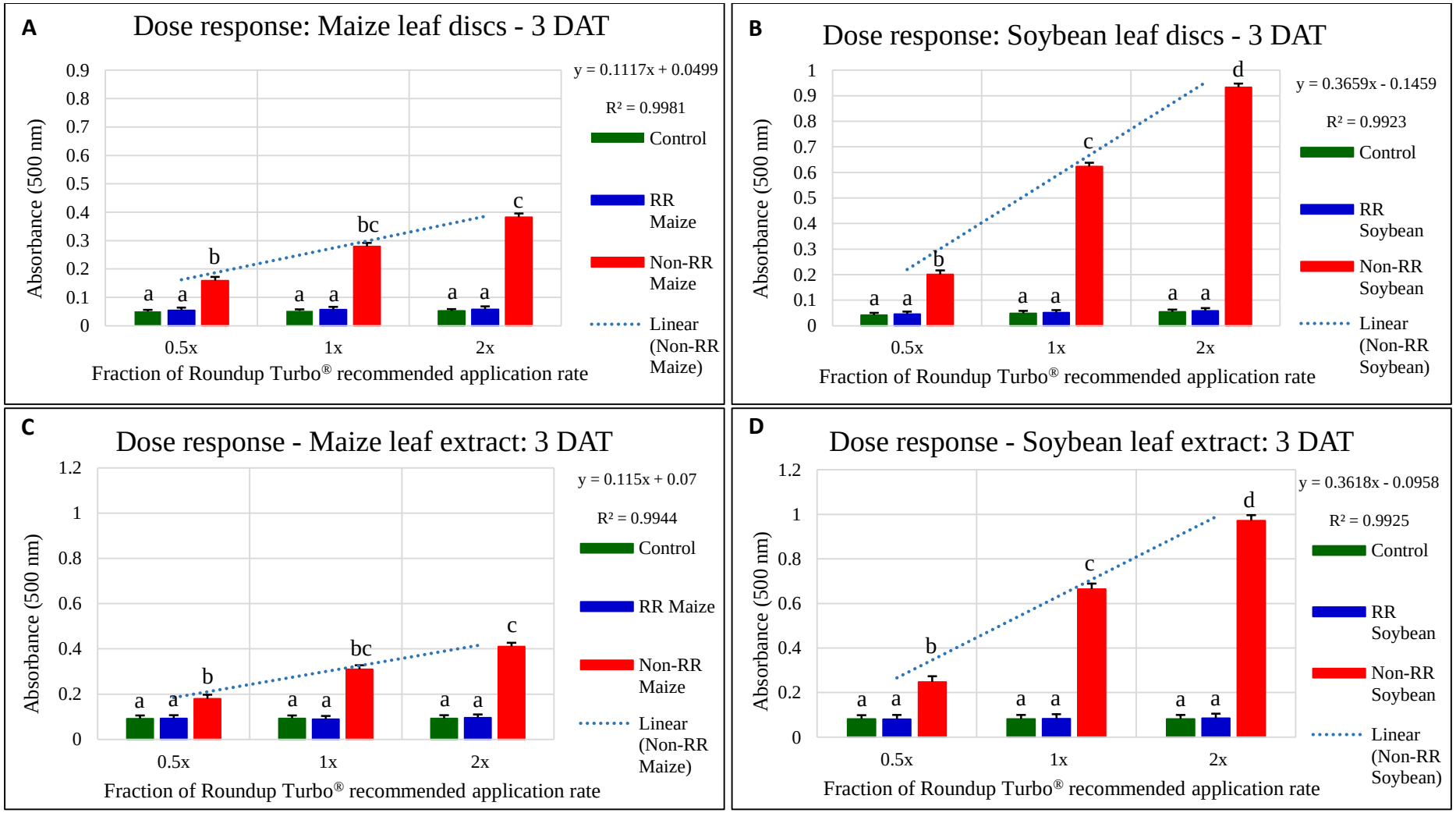


FIGURE 3.13 Photospectroscopy results for dose response of **(A)** maize leaf discs; **(B)** soybean leaf discs; **(C)** maize leaf extracts and **(D)** soybean leaf extracts 3 days after being treated with various dosages (0.5x, 1x and 2x the fraction of recommended application rate) of Roundup Turbo®. Means are from three biological replications. Means with different letters indicate significant differences at $p \leq 0.05$ Fisher's protected LSD.

TABLE 3.2 Comparison of shikimate concentrations from treated non-RR maize plants obtained from HPLC and colourimetric assays, 3 days after being treated with various concentrations of glyphosate.

	Shikimate concentration (mg/g)			Significant difference ($p \leq 0.05$) between LD and LE	Difference (%) between HPLC and LD
Glyphosate treatment	HPLC	Colourimetric assay			
		Leaf discs (LD)	Leaf extracts (LE)		
0.5x	2.88	2.36	2.41	No	18%
1x	3.59	2.98	3.03	No	17%
2x	4.14	3.35	3.40	No	19%

TABLE 3.3 Comparison of shikimate concentrations from treated non-RR soybean plants obtained from HPLC and colourimetric assays, 3 days after being treated with various concentrations of glyphosate.

	Shikimate concentration (mg/g)			Significant difference ($p \leq 0.05$) between LD and LE	Difference (%) between HPLC and LD
Glyphosate treatment	HPLC	Colourimetric assay			
		Leaf discs (LD)	Leaf extracts (LE)		
0.5x	3.54	2.94	2.99	No	17%
1x	7.23	5.93	6.0	No	18%
2x	9.21	7.64	7.72	No	17%

TABLE 3.4 R-square values obtained from data of HPLC and colourimetric assays.

	R^2 value		
Plant cultivar	HPLC	Leaf Discs	Leaf Extracts
Non-RR maize	0.99	0.99	0.99
Non-RR soybean	0.97	0.99	0.99

3.2.2 Colourimetric assay performed on leaf discs and leaf extracts: The effect of time after treatment (TAT) on shikimate concentrations

The colourimetric assay was used to determine to which degree TAT influenced shikimate concentrations in leaf discs and leaf extracts from maize and soybean plants over a period of 12 days. Shikimate concentrations occurring naturally in plants are minute, since it is only an intermediate metabolite for aromatic amino acid biosynthesis in the shikimic acid pathway. Control plants in Figure 3.14 A, B, C and D (illustrated by green lines) represents these naturally occurring levels of shikimate in maize and soybean plants. The blue line shows shikimic acid concentrations in treated RR maize and soybean plants (Figure 3.14 A, B, C and D) and it is clear that glyphosate treatments regardless of TAT, had no significant effect on shikimate concentrations in glyphosate resistant plants. There was no significant ($p \leq 0.05$) difference in absorbance between control and treated RR maize and soybean plants irrespective of TAT for both leaf discs and leaf extracts.

Comparing the absorbance of assay solutions from RR (blue) and non-RR (red) maize and soybean leaf discs as well as leaf extracts (Figure 3.14 A, B, C and D), there was a significant difference ($p \leq 0.05$) in the shikimic acid levels over the 12 day period.

The time response graphs (Figure 3.14) illustrate the correlation between absorbance of assay solutions (which directly indicate to shikimate concentration present in the solution) and DAT. In general, for both non-RR maize and soybean plants there was a correlation between absorbance and TAT. For both leaf discs and leaf extracts the most significant ($p \leq 0.05$) period of increase in absorbance (shikimate concentrations) for non-RR maize and soybean plants were between 3 and 6 DAT. The increase in absorbance between 6 and 9 DAT was also significant at $p \leq 0.05$, but not significant for 9 to 12 DAT. These results accurately support those obtained from HPLC analysis.

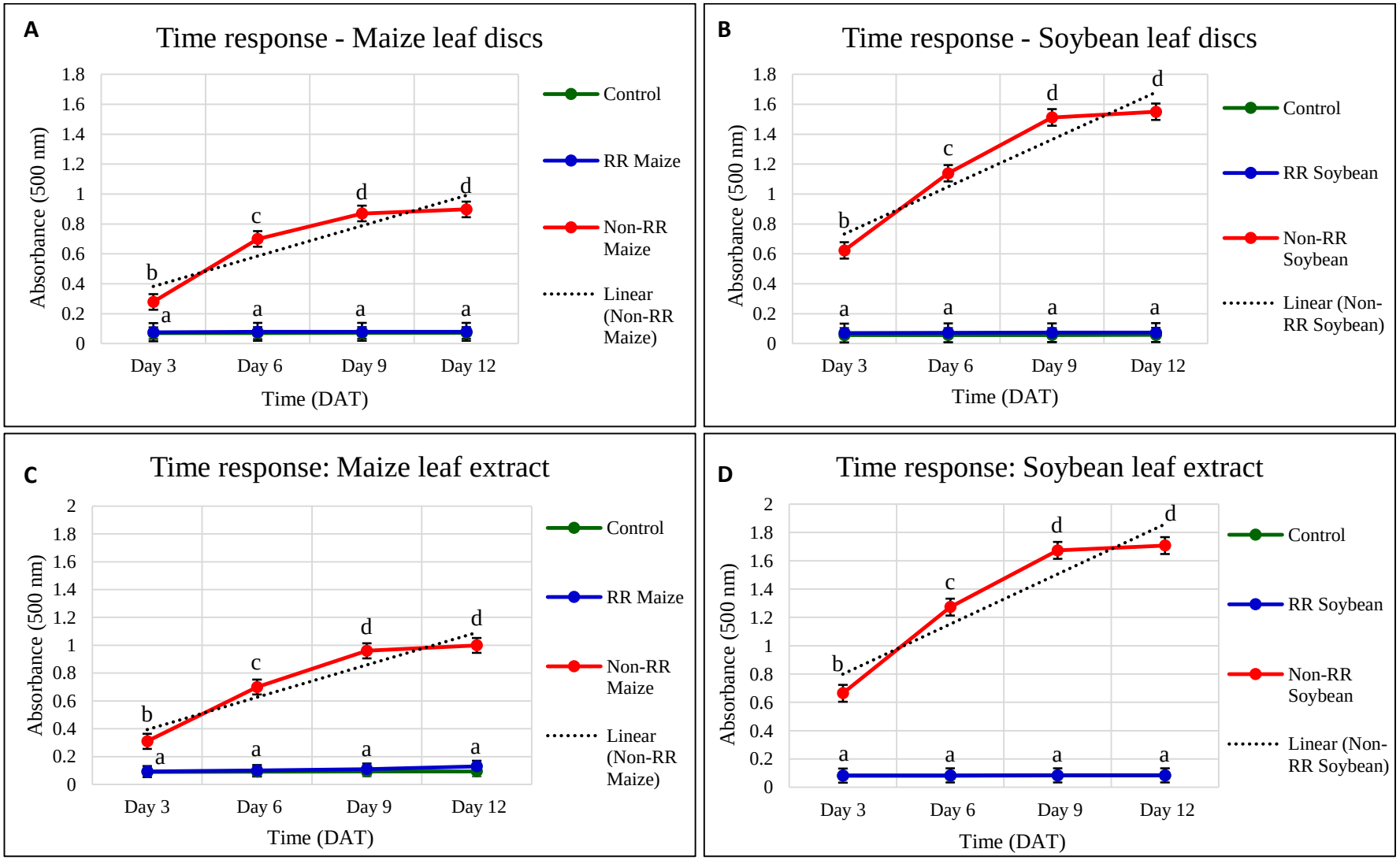


FIGURE 3.14 Photospectroscopy results for time response of **(A)** maize leaf discs; **(B)** soybean leaf disc; **(C)** maize leaf extracts and **(D)** soybean leaf extracts 3, 6, 9 and 12 days after being treated with the recommended dosage (1x) of Roundup Turbo®. Absorbance was mearured 15min after adding the enzyme, shikimate dehydrogenase. Means are from three biological replications. Means with different letters indicate significant differences at $p \leq 0.05$ Fisher's protected LSD.

3.2.3 Colourimetric assay performed on leaf discs and leaf extracts: The combined effect of dose and time after treatment (TAT) on shikimate concentrations.

This experiment was conducted to establish the relationship between absorbance, glyphosate dosage applied and TAT and to which degree each parameter influenced the absorbance. For this experiment the colourimetric assay was used to study which effect increasing rates of glyphosate had on shikimate concentrations in treated non-RR maize and soybean leaves (discs and extracts) over a period of 12 days - to investigate the effect of dose in conjunction with time on shikimate concentrations.

For control (green lines) maize and soybean leaf discs (Figure 3.15 A and B), no significant ($p \leq 0.05$) increase in absorbance values were noticeable over time. For leaf extracts (Figure 3.15 C and D) from control plants, exactly the same trend was noticed, there was no significant ($p \leq 0.05$) increase in absorbance as the TAT progressed. For treated non-RR maize and soybean plants, it is evident that there were a strong correlation between absorbance (shikimate concentrations), glyphosate application rate, and TAT (Figure 3.15 A, B, C, and D). In general, the relationship was directly proportional; shikimate build-up was directly linked to the application rate and TAT. Thus, absorbance increased as one or both of these two parameters increased.

From Figure 3.15 it is clear that absorbance increased over time and as the applied dosage increased, for instance 2x glyphosate treatments (red lines) yielded more than double or triple the absorbance obtained from 0.5x (yellow lines) treatments depending on the plant species. For maize and soybean leaf discs and leaf extracts the most significant ($p \leq 0.05$) period of increase in absorbance was between 3 and 6 DAT regardless of dose. Maize and soybean leaf discs and extracts also displayed a significant ($p \leq 0.05$) increase in absorbance between 6 and 9 DAT. However, between 9-12 DAT the increase was not significant at $p \leq 0.05$.

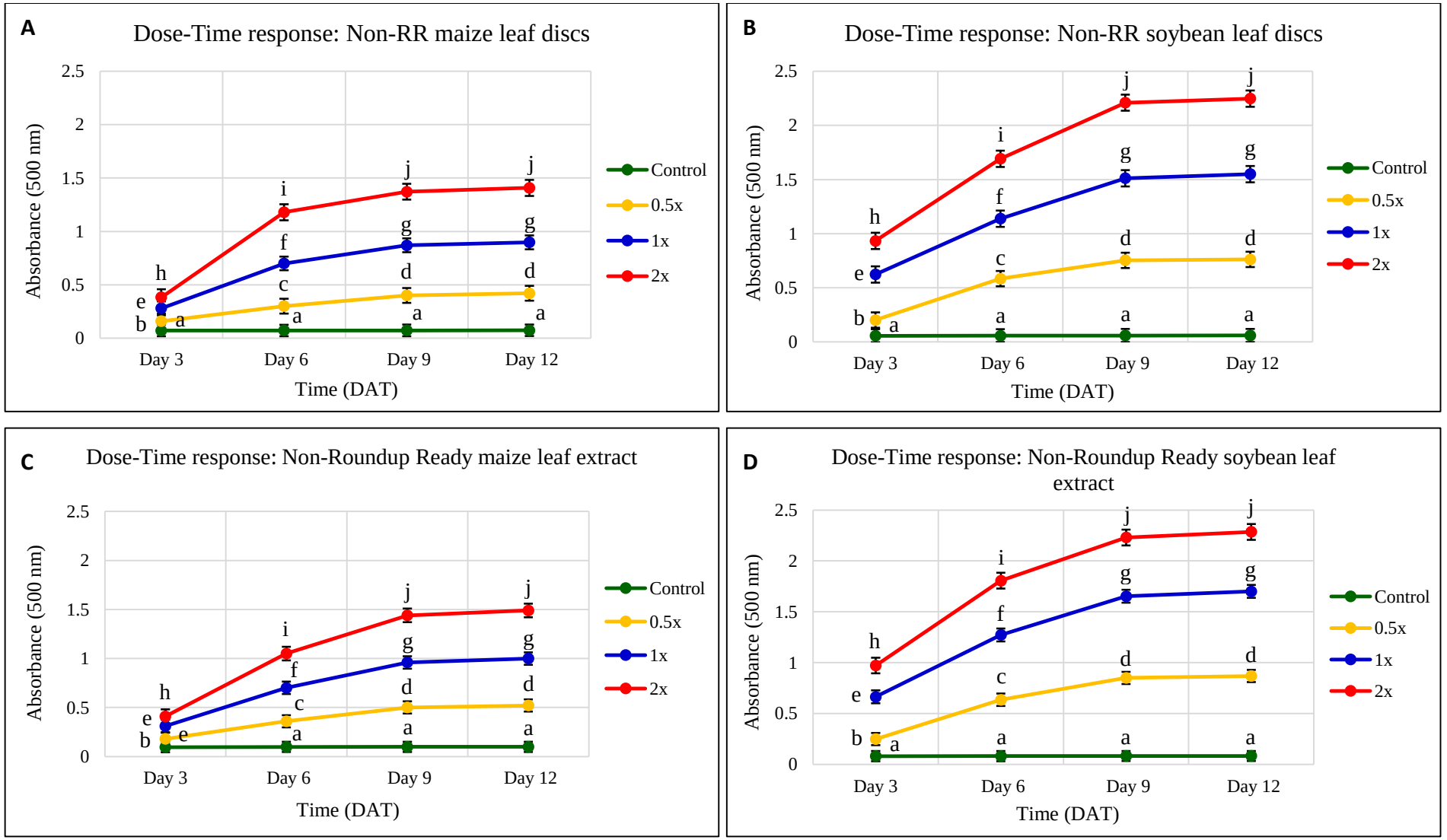


FIGURE 3.15 Photospectroscopy results for dose-time response of **(A)** maize leaf discs; **(B)** soybean leaf disc; **(C)** maize leaf extract and **(D)** soybean leaf extracts 3, 6, 9 and 12 days after being treated with various dosages (0.5x, 1x and 2x the fraction of recommended application rate) of Roundup Turbo®. Absorbance of the assay solutions in which leaf discs were submerged was measured 15min after adding the enzyme, shikimate dehydrogenase.

3.2.4 Effect of dose and time after treatment (TAT) on absorbance of assay solutions from leaf discs of different plant species: Non-RR Maize vs Non-RR Soybean

These results shows the absorbance of colourimetric assay solutions of leaf discs from maize and soybean plants as well as the effect dose and TAT have on shikimate build-up. The correlation between absorbance, glyphosate dosage applied and TAT in different plant species (maize and soybean) is indicated on the graph (Figure 3.16). There was a significant ($p \leq 0.05$) difference in absorbance between maize (green lines) and soybean (red lines) plants regardless of TAT or dosage applied. In gereral the absorbance (i.e. degree of shikimate build-up) of assay solutions from non-RR soybean leaf discs were greater than that of maize leaf discs. However, both glyphosate application rate and TAT played a significant role in shikimate build-up, the degree of shikimate accumulation was also influenced by the plant species. From Figure 3.16 it is clear that shikimate build-up between species differed at the same glyphosate rate and TAT. For maize and soybean plants increase in absorbance was the highest between 3 and 9 DAT and a stable state was reached after 9 DAT. This supports the results obtained form HPLC analysis in which shikimate build-up were much higer in soybean leaf tissue as opposed to maize leaves. Thus, shikimate build-up is directly attributed to; (1) the plant species, (2) the rate of application and (3) time after treatment (TAT).

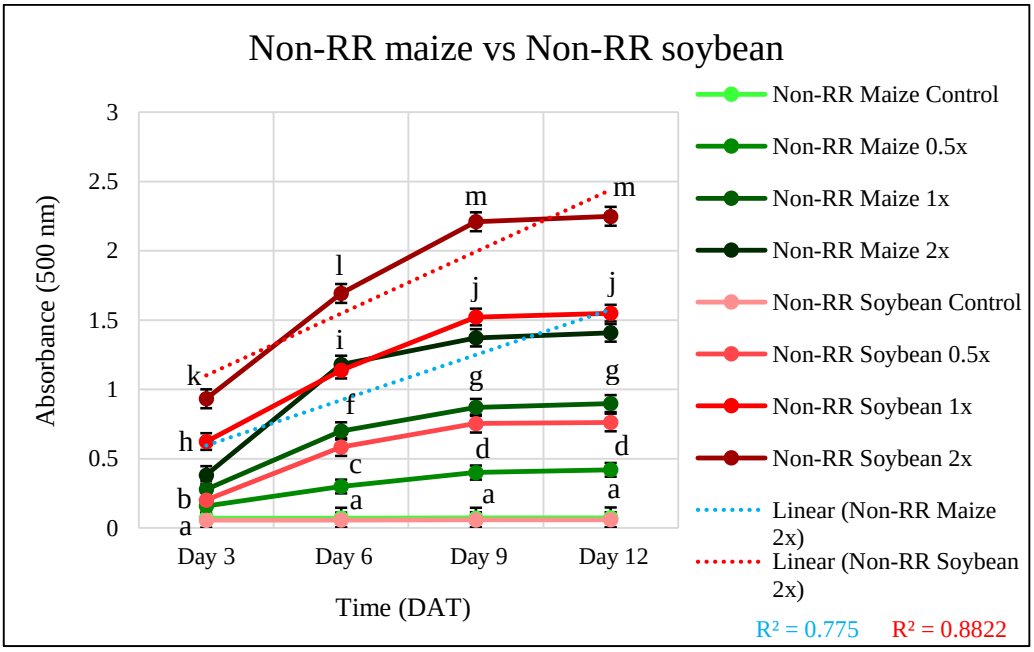


FIGURE 3.16 Photospectroscopy results for dose-time response of non-RR maize and soybean leaf discs 3, 6, 9 and 12 days after being treated with various dosages of Roundup Turbo®.

3.2.5 Colourimetric assay performed on leaf discs and leaf extracts: Non-RR maize vs Non-RR soybean

Colourimetric analysis of assay solutions from non-RR maize and soybean leaf discs and leaf extracts were performed. The aim was to illustrate differences in concentrations of the coloured substance (reduced INT dye) present in assay solutions between maize and soybean plants over time and also to compare concentrations between leaf discs and leaf extracts. By comparing Figure 3.17 A and B, it is apparent that non-RR soybean leaf discs and extracts contained significantly (at $p \leq 0.05$) higher concentrations of the coloured dye, than non-RR maize leaf discs and extracts. From Figure 3.17 A and B it is evident that shikimate build-up is strongly linked to the plant species and also that leaf extracts yielded a slightly greater absorbance than that of leaf discs.

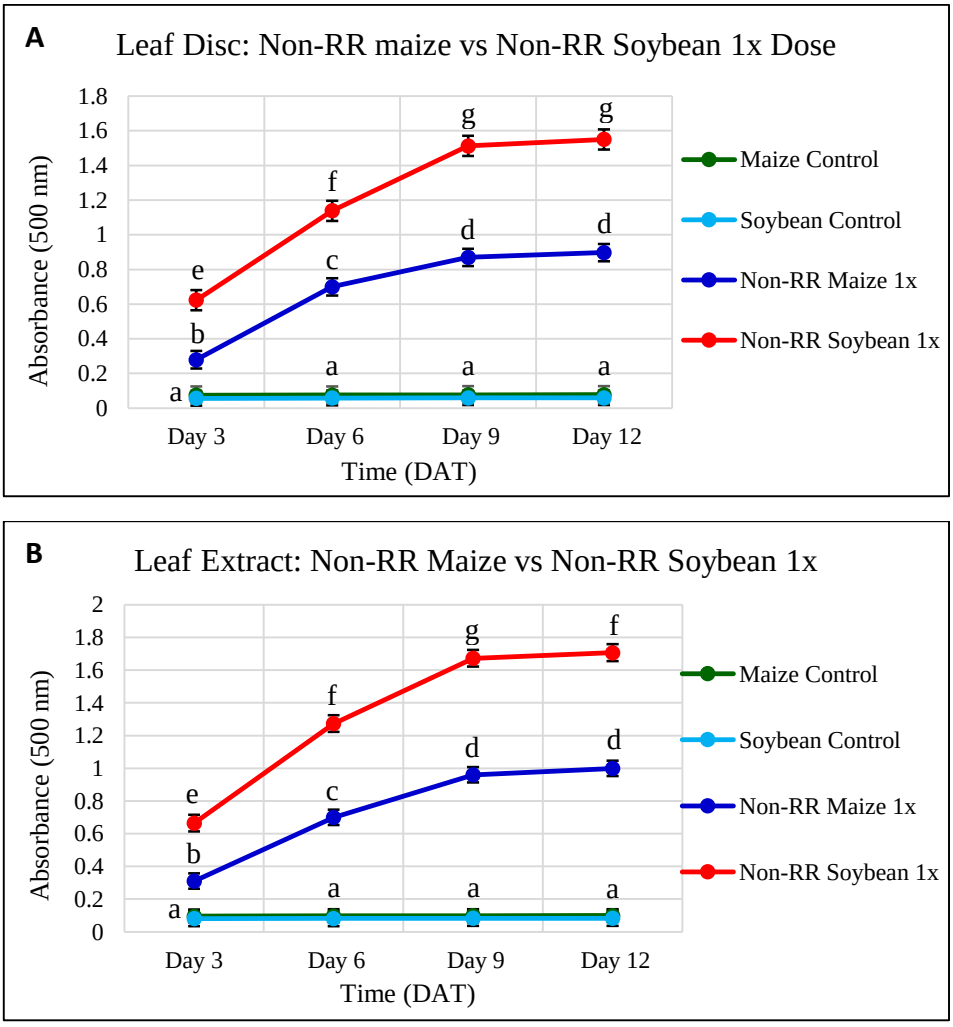


FIGURE 3.17 Photospectroscopy results for dose-time response of non-RR maize versus Non-RR soybean **(A)** leaf discs and **(B)** leaf extracts 3, 6, 9 and 12 days after being treated with the recommmended rate (1x) of Roundup Turbo®.

3.2.6 Colourimetric assay comparing leaf discs to leaf extracts at various dose treatments

The main of aim of this experiment was to illustrate differences and compare the effect of glyphosate dose on the absorbance of assay solutions obtained from immersed leaf discs and leaf extracts of treated non-RR maize and soybean plants. This experiment was relevant since it compared results obtained from the two methods in order to establish which was more precise in obtaining an accurate absorbance reading which ultimately represent shikimate concentration within a sample. A significant difference in absorbance readings between the two methods would lead to the rejection of the one method which yielded the significantly lower reading.

As can be seen from Figure 3.18 A and B, although there were slight differences (lower values from leaf discs) in the absorbance obtained from leaf discs (blue) and leaf extracts (red), the difference between the two methods were not significant at $p \leq 0.05$ regardless of dose. Small and non-significant differences would mean that the faster method using leaf discs would be accurate enough to use under field conditions.

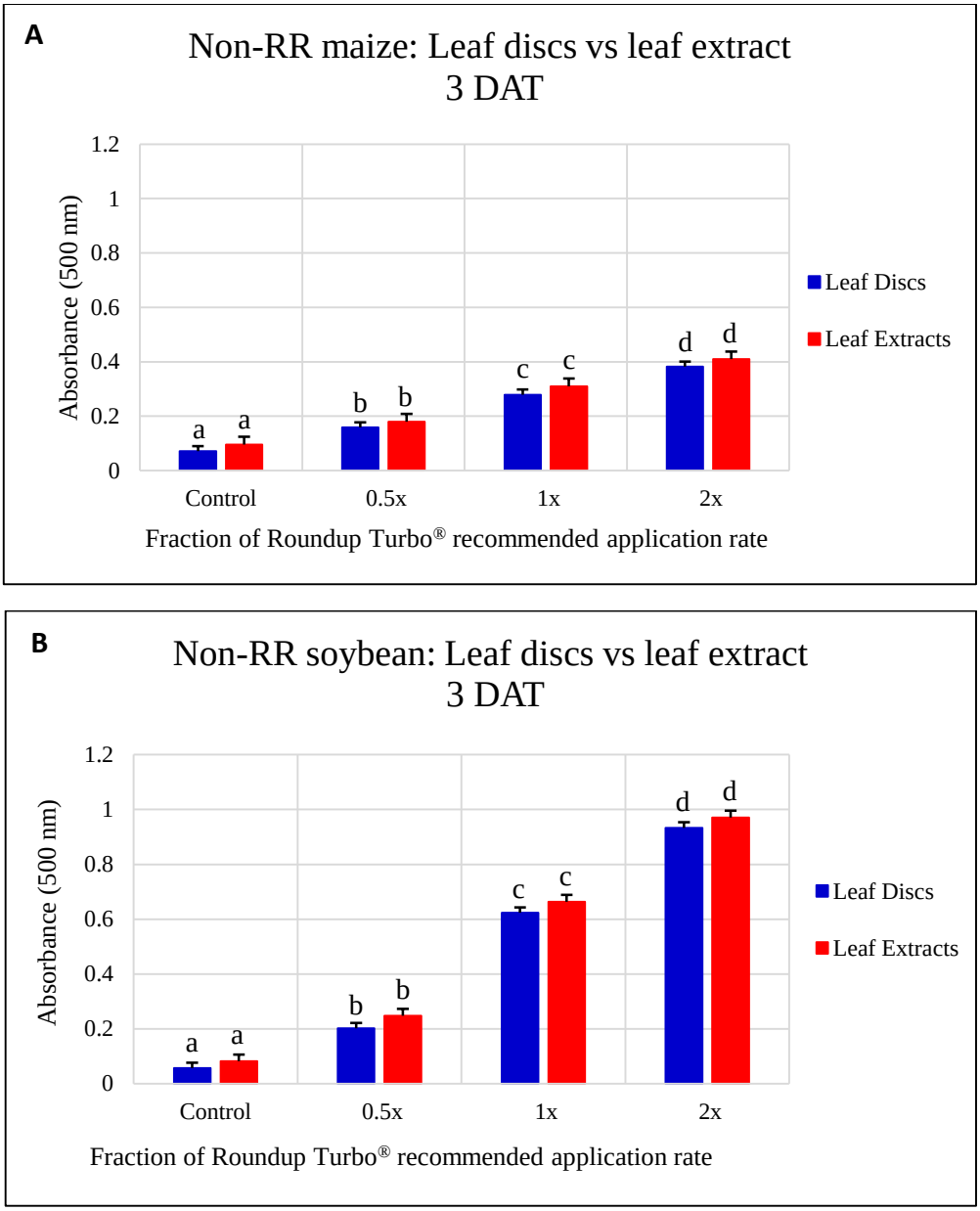


FIGURE 3.18 Photospectroscopy results comparing the absorbance of dose response from **(A)** non-RR maize leaf discs to extracts and **(B)** non-RR soybean leaf discs to extract 3 days after being treated with various dosages (0.5x, 1x and 2x the fraction of recommended application rate) of Roundup Turbo®. Means with the same letter indicate non-significant differences at $p \leq 0.05$ Fisher's protected LSD. All data were calculated in triplicate.

3.2.7 Colourimetric assay comparing leaf discs to leaf extracts at various glyphosate dose treatments and TAT

The aim of this experiment was to compare the absorbance of colourimetric assay solutions from leaf disc to that of leaf extracts in order to establish which of these two methods yielded superior results at different application rates and TAT. As can be seen from Figure 3.19 A and B, in general leaf extracts yielded greater absorbance readings regardless of the application rate or TAT. Thus, at any of the tested application rates and TAT there was a higher concentration of shikimate in assay solutions from leaf extract as opposed to solutions from immersed leaf discs. However, these differences were not statically significant at $p \leq 0.05$. As the differences between the two methods were not significant it means that the method using leaf discs would be accurate enough to use under field conditions. Advantages of using leaf discs over extracts for the assay include: they are easy and rapid to use since there is little or no sample preparation involved and requires no advanced laboratory equipment to yield accurate results.

Thus, to use leaf discs for the colourimetric assay is very effective in establishing the shikimate concentration of a sample and hence could be used to accurately differentiate between sensitive and resistant plants.

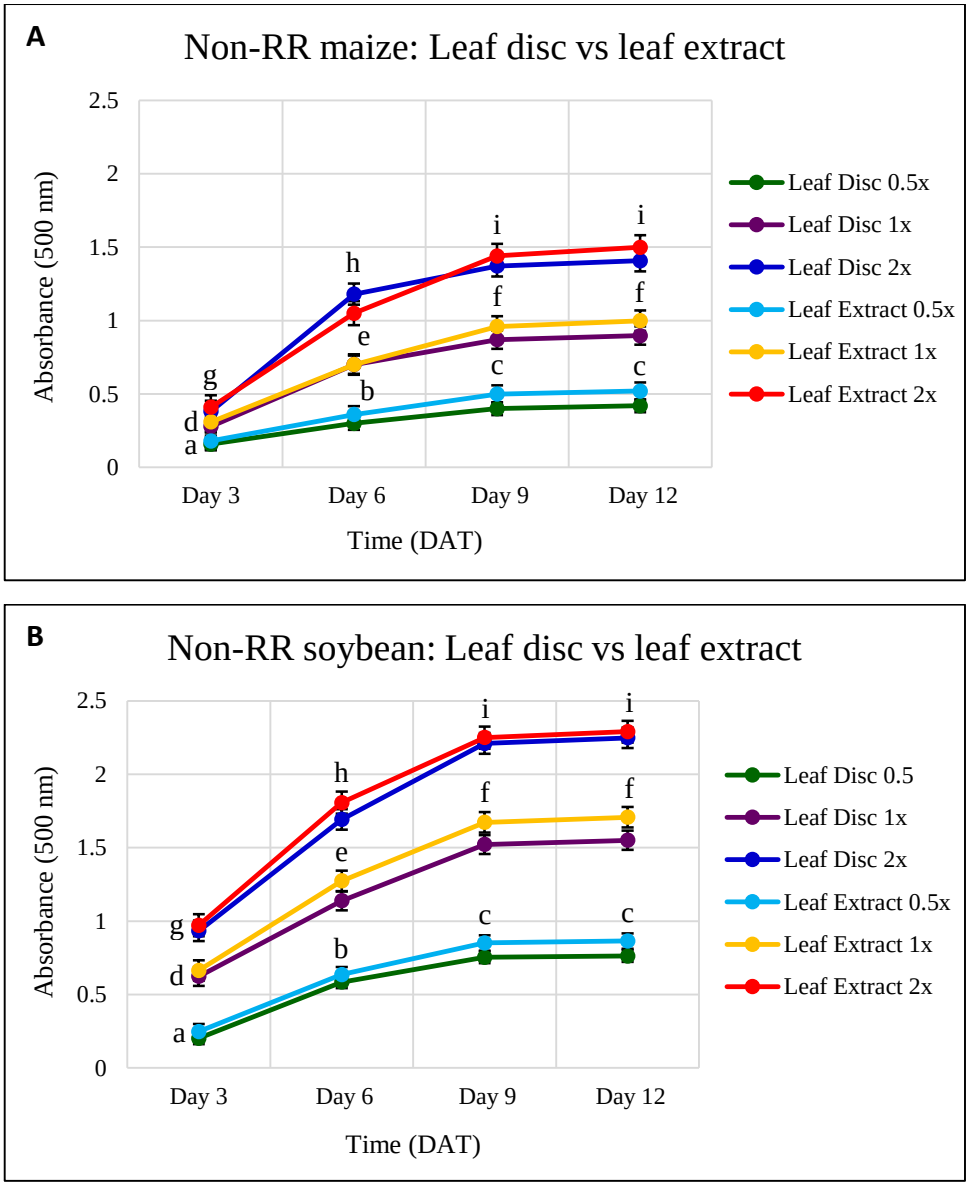


FIGURE 3.19 Photospectroscopy results comparing the absorbance from dose-time responses of **(A)** non-RR maize leaf discs to leaf extracts and; **(B)** non-RR soybean leaf discs to leaf extracts 3, 6, 9 and 12 days after being treated with various rates (0.5x, 1x and 2x the fraction of recommended application rate) of Roundup Turbo®.

3.3.1 Colourimetric assay performed on maize and soybean leaf discs – Dose response

This assay was performed to illustrate the relationship between colour change (shikimate build-up) of assay solutions containing leaf discs and glyphosate application rate. Leaves from maize and soybean plants were treated with varying concentrations (0.5x, 1x and 2x the fraction of recommended application rate) of Roundup Turbo® prior to analysis.

Six Leaf discs (Ø 3mm) from untreated control, treated RR, and treated non-RR maize and soybean plants were immersed in the colourimetric assay solution and incubated. After incubation no colour change was visible in untreated control or glyphosate treated RR maize and soybean plants regardless of dosage applied (Figure 3.20 B and Figure 3.21 B). For treated non-RR maize and soybean leaf discs a very distinct change in colour of assay solutions was visible – the colour intensity enhanced as glyphosate dosage applied increase. These results illustrated the ability of the colourimetric assay to visually indicate the presence of shikimate and differentiate between susceptible and resistant plants.

Enzymes in the colourimetric assay responsible for the colour change required time to react with shikimate to yield a colour change. Figure 3.20 A and Figure 3.21 A shows the assay solutions 30 seconds after immersing non-RR maize and soybean leaf discs, whereas Figure 3.20 B and Figure 3.21 B shows the same solutions 5 minutes after immersion. This shows that colour developed over time.

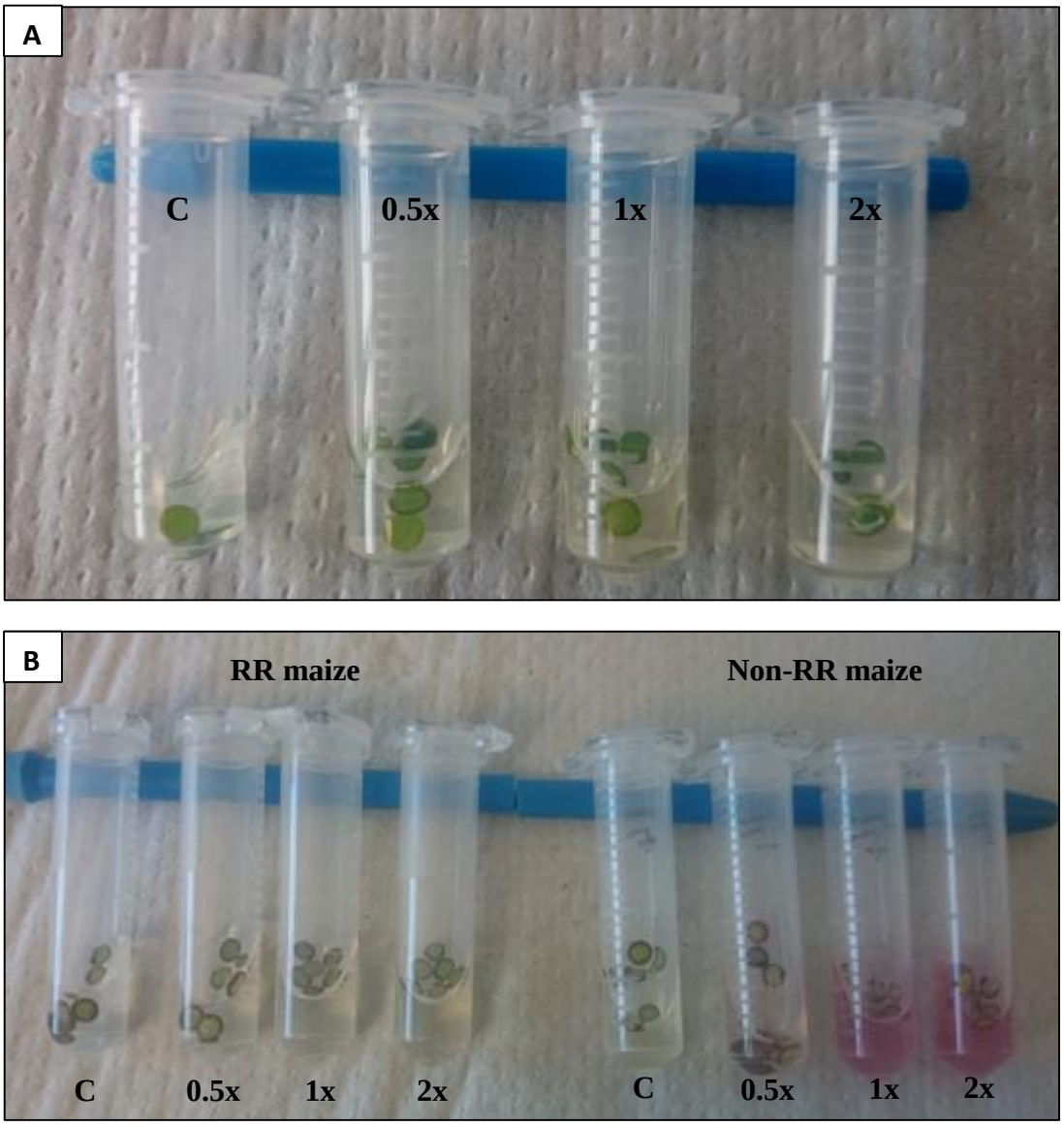


FIGURE 3.20 Colourimetric assay performed on RR and non-RR maize leaf discs 3 days after being treated with varying concentrations (0.5x, 1x and 2x the fraction of recommended application rate) of Roundup Turbo®. **(A)** Non-RR maize leaf discs 30 seconds after being submerged in the colourimetric assay solution; **(B)** RR and non-RR maize leaf discs 5 minutes after being submerged in the colourimetric assay solution.

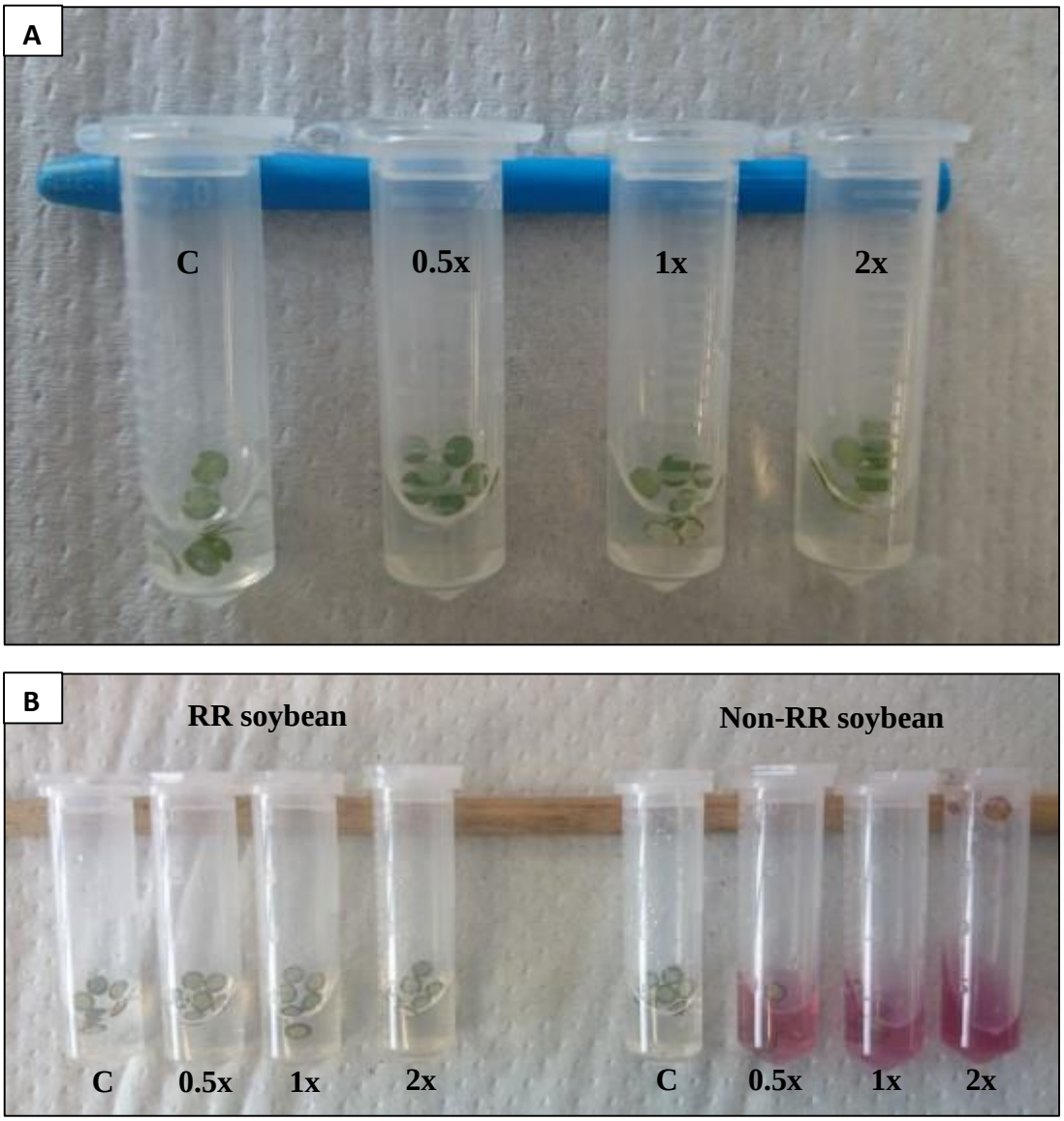


FIGURE 3.21 Colourimetric assay performed on RR and non-RR soybean leaf discs 3 days after being treated with various rates (0.5x, 1x and 2x the fraction of recommended application rate) of Roundup Turbo®. **(A)** Non-RR soybean leaf discs 30 seconds after being submerged in the colourimetric assay solution; **(B)** RR and non-RR soybean leaf discs 5 minutes after being submerged in the colourimetric assay solution.

3.3.2 Colourimetric assay performed in assay plates on maize and soybean leaf extracts – Dose response

The assay was done on leaf extracts from maize and soybean plants treated with various rates of glyphosate (0.5x, 1x and 2x) in order to demonstrate the relationship between application rate and colour intensity. There was no visible colour change in assay solutions from untreated control leaf extracts or treated RR leaf extracts regardless of the dose applied (Figure 3.22 A and 3.22 B and Figure 3.23). However, an increase in colour intensity of assay solutions from leaf extracts of non-RR maize (Figure 3.22 A and 3.22 B) and soybean plants (Figure 3.23) were observed as the application rate of glyphosate increased.

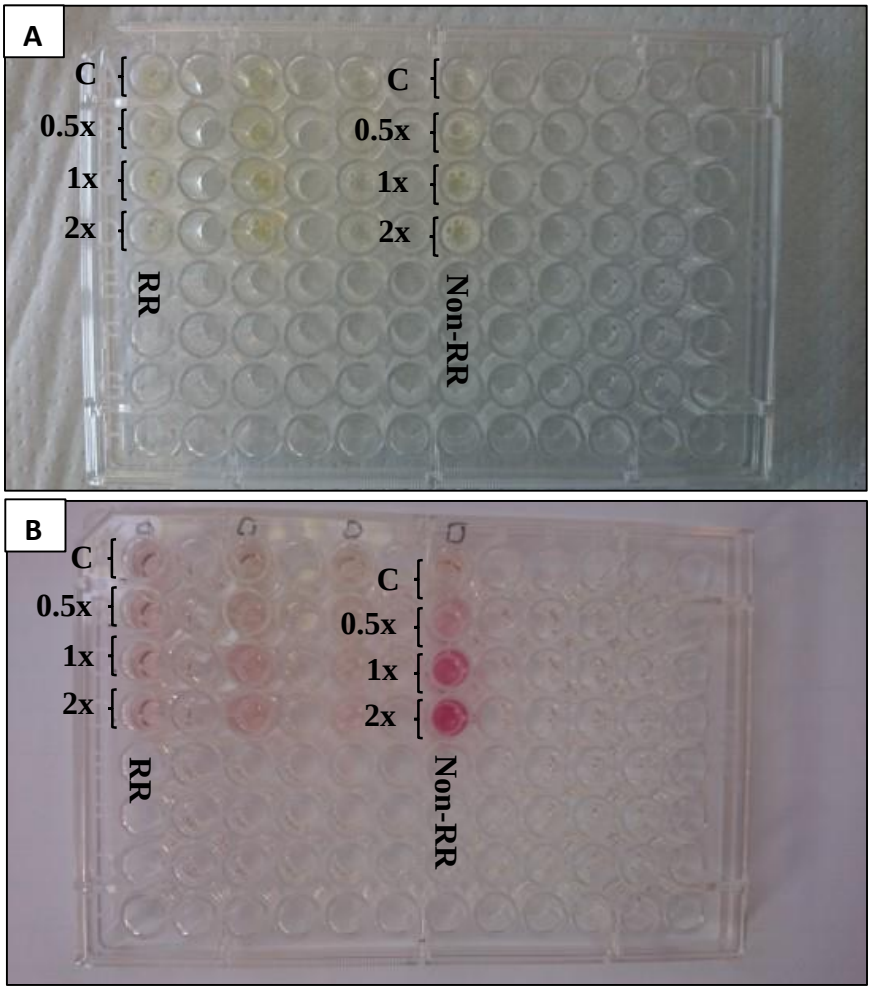


FIGURE 3.22 Colourimetric assay performed on RR and non-RR maize leaf extracts **(A)** before adding the enzyme shikimate dehydrogenase (SHKDH) to the extracts; **(B)** 5 minutes after adding SHKDH.

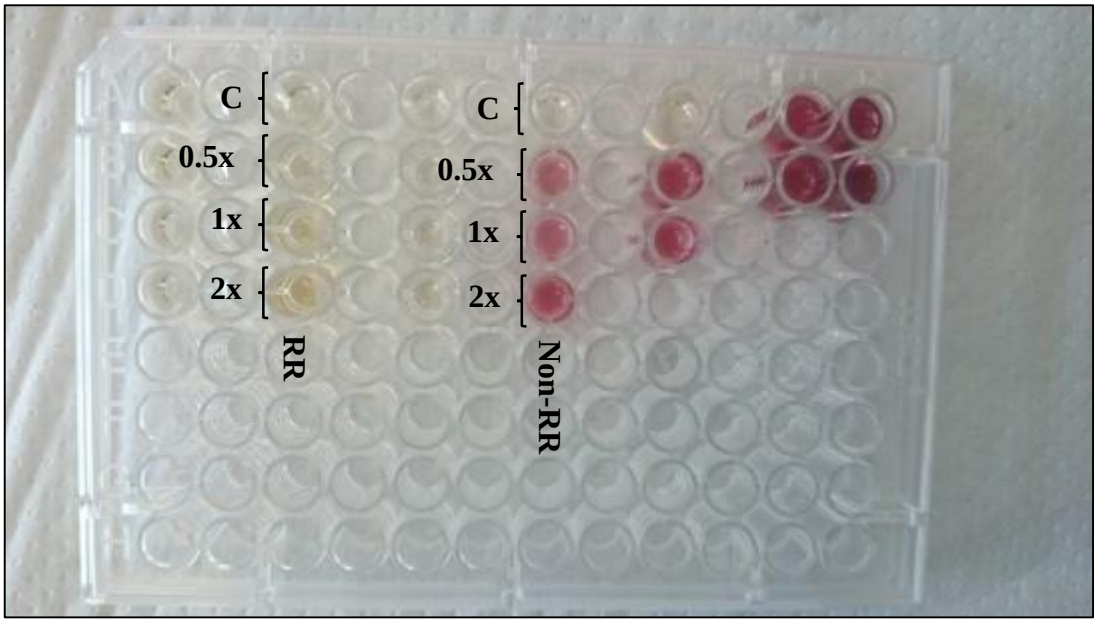


FIGURE 3.23 Colourimetric assay performed on RR and non-RR soybean leaf extracts from leaves treated with various rates (0.5x, 1x and 2x the fraction of recommended application rate) of Roundup Turbo®, 5 minutes after adding SHKDH.

3.3.3 Colourimetric dipstick

To test whether the colourimetric assay could be converted to a dipstick protocol and whether the SHKDH enzyme could be dried and reactivated, the assay solution containing SHKDH was applied to TLC plates and dried in an incubation chamber at 25˚C for 24h. The purpose of the colourimetric dipsticks were to rapidly indicate the presence of shikimic acid in a sample. This in turn could be useful to detect glyphosate damage and also to easily differentiate between susceptible and resistant plants.

The dipsticks contained a layer of dried enzymatic assay solution which would react with any shikimate present in a sample. The colour of an unused dipstick was white and the strip would remain white if dipped into a solution (containing extract or leaf discs) containing little or no shikimate, such as the minute concentrations occurring naturally in plants (untreated control plant on the left in Figure 3.24), but the dipstick will develop a red colour in the presence of shikimate.

If dried dipsticks were dipped into a solution containing shikimate, such as leaf extracts from glyphosate treated non-RR crops, the portion of the stick immersed in the solution would change colour. The intensity of colour change produced can be directly linked to the amount

of shikimate present in the sample which in turn corresponds to the degree of susceptibility. As can be seen from Figure 3.24, the strip dipped into leaf extracts from control plants exhibited no visible colour change. However, the strips immersed in leaf extracts from plants treated with 1x and 2x recommended dose of glyphosate demonstrated a colour change. It is clear that the sticks dipped into extracts made from leaves treated with higher dosages (1x vs 2x recommended application rate) of glyphosate exhibited greater colour changes (Figure 3.24). These results demonstrates that these colourimetric dipsticks are sensitive and accurate in differentiating between glyphosate sensitive and resistant plants.

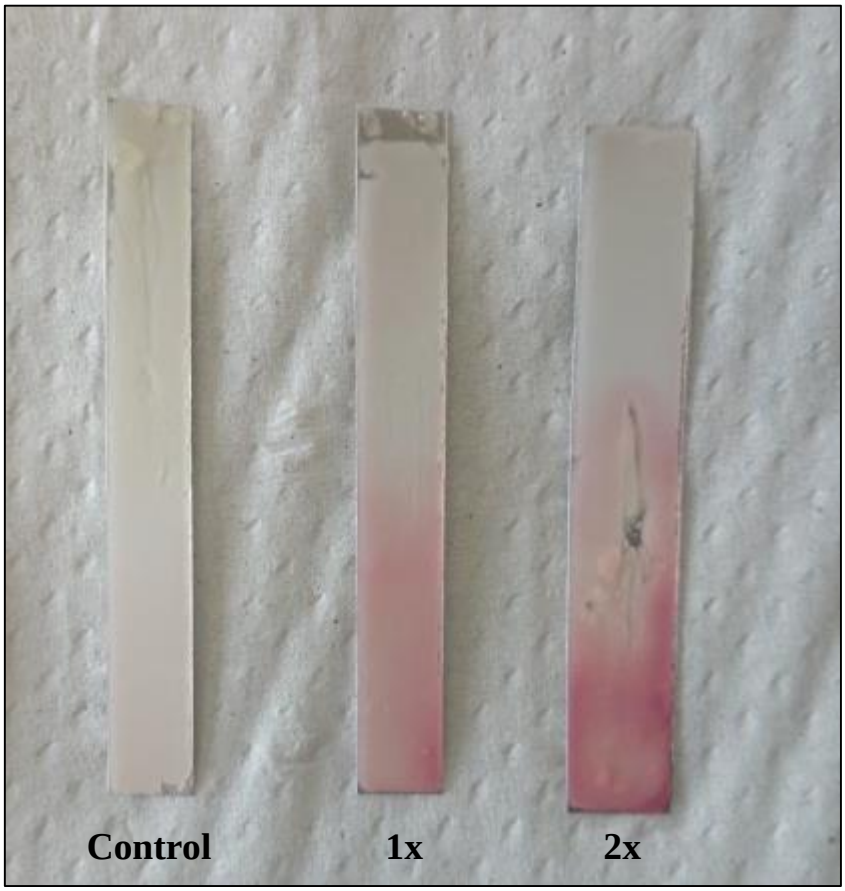


FIGURE 3.24 Colourimetric dipsticks developed in the laboratory. These dried sticks were dipped into solutions containing six leaf discs from; untreated control non-RR soybean plants and plants treated with the recommended (1x) and double (2x) the recommended application rate of Roundup Turbo®.

3.4.1 Case Study 1: Determining the source which caused herbicide damage in maize plants

The Department of Plant Production and Soil Science at UP was approached by a seed company which claimed that one of their clients maize plants were damaged in the field by glyphosate due to drift from a neighbours farm. Three randomly selected maize plants exhibiting symptoms of herbicide damage were received. HPLC and colourimetric analysis were used to establish whether the source which caused (was responsible) herbicide damage to the plants was glyphosate. It was determined that the herbicide responsible for the damage was most probably glyphosate as the shikimate levels in all three plants were elevated in comparison to untreated maize plants.

The approximate dose to which these three plants were exposed to could be determined by examining and comparing the shikimic acid content of the damage plants to the shikimate concentrations obtained from untreated control maize plants and maize plants treated with various concentrations (0.5x, 1x and 2x of the recommended application rate) of Roundup Turbo® under greenhouse conditions, where the recommended (1x) application rate of Roundup Turbo® is 2L/ha.

From this is was established that maize plant #1 (Figure 3.25 C and Figure 3.26) was exposed to glyphosate drift which was equivalent to a 350ml/ha Roundup Turbo® treatment. Maize plant #2 (Figure 3.25 D and Figure 3.26) was exposed to glyphosate drift with a formulation equal to approximately 750ml/ha. Glyphosate damage was the most significant in maize plant #3 (Figure 3.25 E and Figure 3.26) i.e. this plant exhibited the most severe damage due to drift. It was calculated that maize plant #3 was exposed to glyphosate drift which was equivalent to a 1L/ha Roundup Turbo® treatment. It is important to note that these calculations were made based on Roundup Turbo® formulations and that other manufacturers may have different AI (active ingredient) formulations and also that the G8550Bt maize cultivar was used for control plants.

Thus, it was successfully determined by HPLC and colourimetric analysis that glyphosate was the herbicide responsible for damage in all three of these maize plants.

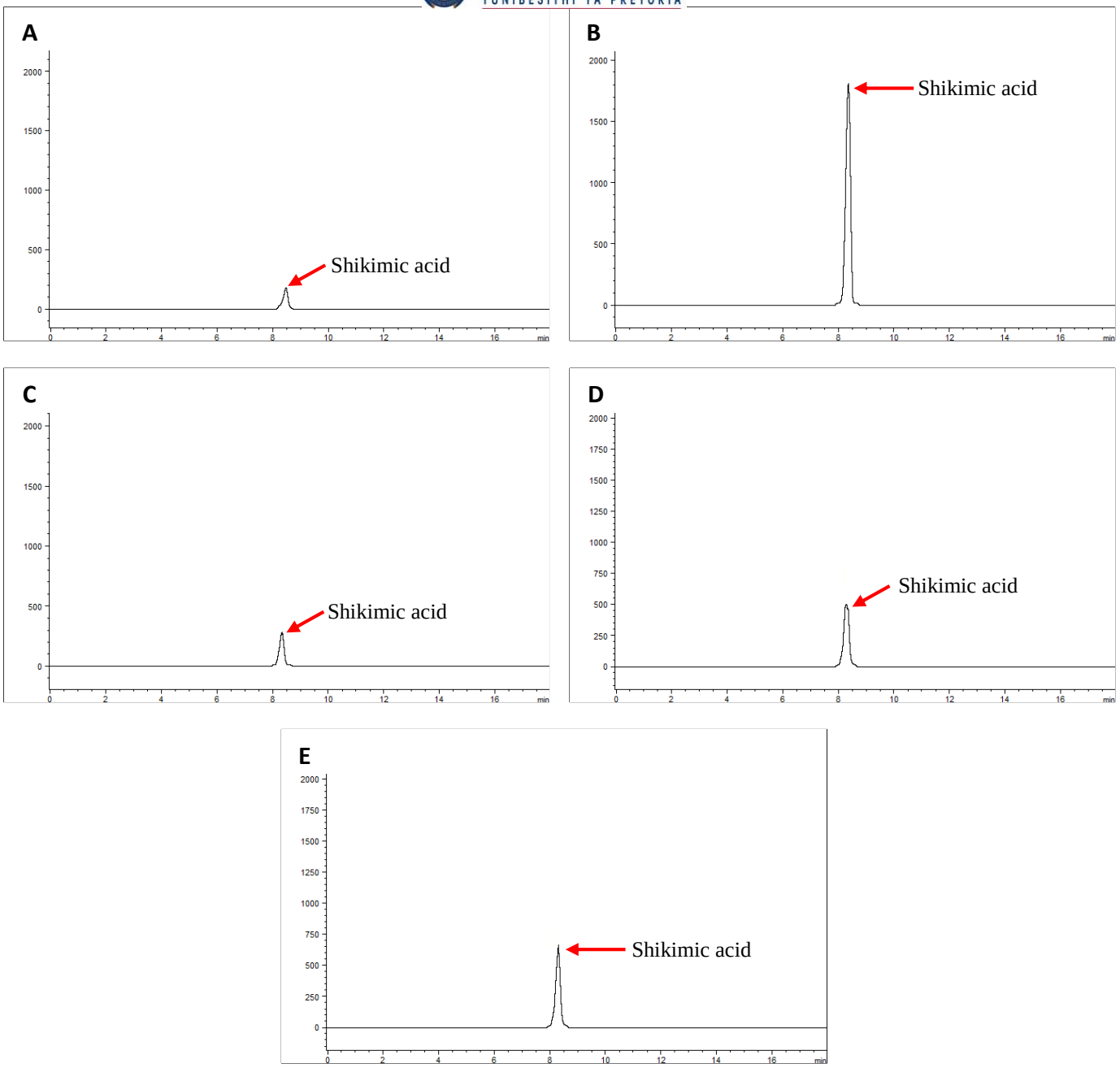


FIGURE 3.25 HPLC chromatograms presenting shikimic acid levels in leaves of non-RR maize plants. The shikimic acid peak is indicated on the chromatograms with a red arrow **(A)** Control non-RR maize plant; **(B)** Control sample spiked with 150µg/ml shikimic acid standard; **(C)** Maize plant number 1; **(D)** Maize plant number 2; **(E)** Maize plant number 3.

FIGURE 3.26 Colourimetric assay performed on the three non-RR maize plants to determine if elevated shikimic acid levels as a result of glyphosate exposure could be detected.

3.4.2 Case Study 2**:** Testing the colourimetric assay on treated *Conyza bonariensis*

Leaf discs from *Conyza bonariensis* weeds treated with various doses of glyphosate were also assayed using the quick, colourimetric test. This was performed in order to determine whether this particular weed was susceptible or resistant to glyphosate. As the colourimetric test is not plant species specific it could be used to establish the degree of shikimate build-up in this weed specimen. A colour change indicated that the plant is susceptible to glyphosate.

A resistant plant would demonstrate no colour change after being treated with the recommended (1x) rate of glyphosate. As can be seen from Figure 3.27, a colour change was visible in all three glyphosate dose treatments regardless of application rate, however the colour intensity was the most evident/significant in assay solutions from leaf discs treated with double (2x) the recommended rate. Since a colour change was visible in assay solutions from leaf discs treated with half the recommended rate (0.5x treatment), it provided clear evidence that this particular weed specimen was in fact susceptible and not resistant to glyphosate.

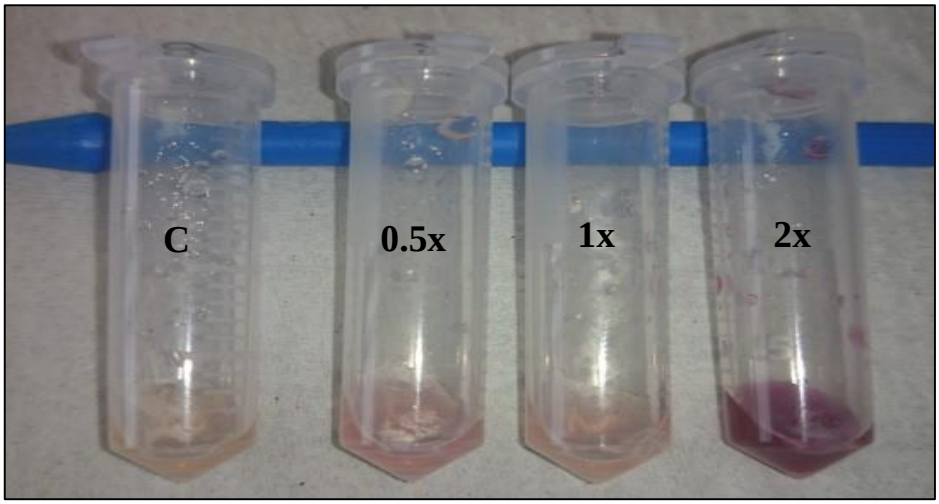


FIGURE 3.27 Colourimetric assay performed on an unknown *Conyza bonariensis* weed plant treated various rates (0.5x, 1x and 2x the fraction of recommended application rate) of Roundup Turbo®, the untreated control (C) plants is on the left. This was performed to establish whether the particular specimen was susceptible or resistant.

CHAPTER 4. DISCUSSION

In this study we used HPLC and a colourimetric assay to quantify and measure shikimic acid levels in maize and soybean plants before and after exposure to glyphosate. The main questions asked in this study were: 1) Is there a correlation between glyphosate dose and the amount of shikimate build-up? 2) How is shikimate accumulation affected by time after treatment (TAT)? 3) Are there differences in shikimate accumulation between root, stem and leaf tissues? 4) Can we use a simplified colourimetric assay to accurately measure shikimate? The results obtained will be discussed under these four points.

4.1. *Shikimate accumulation and dose*

Results attained when measuring shikimate concentrations in plants sprayed with different levels of glyphosate (0.5x, 1x and 2x the fraction of recommended dose) showed a clear correlation (R^2 value of 0.99 and R^2 value of 0.97 for maize and soybean plants respectively; Figure 3.5) between shikimate levels and dose. Untreated control and treated RR plants had very low amounts of shikimate regardless of the dosage applied. For treated non-RR plants, even the lowest rate of glyphosate (0.5x) caused a significant increase in the concentration of shikimate (Figure 3.5 and Figure 3.13). The concentration of shikimate increased markedly in the glyphosate-treated non-RR plants with increasing rate of glyphosate. Interestingly the level of shikimate build-up differed between maize and soybean plants. Shikimate build-up was significantly higher ($p \leq 0.05$) in glyphosate treated non-RR soybean plants, when compared to maize plants (Figure 3.5 and Figure 3.13). Thus, shikimate accumulation is dose related and plant species specific, similar results were also found in studies by Bazzaz *et al*. (1974), Gowik and Westhoff (2011) and Singh and Shaner (1998).

4.2. *Shikimate accumulation over time (TAT)*

Time response trials were performed to compare the relationship between shikimate build-up and time after glyphosate application. From the results, it is clear there was a strong correlation (R^2 value of 0.98 and R^2 value of 0.97 for maize and soybean plants respectively; Figure 3.7) between the degree of shikimic acid accumulation and time after glyphosate treatment. For non-RR maize and soybean plants, increases in shikimate concentrations was significant (p

≤0.05) for the nine day period following glyphosate exposure (Figure 3.7 and Figure 3.14). However, there was a distinct plateau in the degree of shikimate build-up between nine and twelve days after treatment for both Non-RR maize and soybean plants. The reason for this flattened phase might be attributed to the disruption (metabolic fatigue – slowdown and shutdown of metabolism) of the shikimic acid pathway by glyphosate, during this period little or no shikimic acid biosynthesis occurred. A study by Dill *et al*. (2008) have also reported that the concentration of shikimate decreased in velvetleaf (*Abutilon theophrasti* Medik.) during recovery from sub-lethal and lethal doses of glyphosate exposure eight days after treatment. According to the study this observation is most likely due to the metabolism of shikimate as the plant recovers from the initial injury. The most plausible explanation to why shikimate concentrations increased rapidly between 3 and 9 DAT might be because the plant was trying to synthesise and build-up aromatic amino acid reserves in order to survive and counter the effect of glyphosate injury – however, this only resulted in the overproduction of shikimate as the EPSPS enzyme in the pathway was inhibited by glyphosate (Duke and Powles, 2009).

4.3. *Shikimate accumulation in different tissues*

The results obtained by quantifying shikimate levels in various treated maize and soybean tissue types (root, stem and leaf tissues) clearly showed that shikimate accumulation was the greatest and occurred the most rapidly in leaf tissue. Accumulation was the second highest in stem tissue and was the lowest in root tissue (Figure 3.6 and Figure 3.7 C and D). The degree/relationship of increase in shikimate concentrations between these three tissue types were linear. In general shikimate levels in all three soybean tissue types were significantly ($p \leq 0.05$) higher when compared to maize plants. Shikimate concentrations in soybean stem and leaf tissue were 2-fold and 2.7-fold higher respectively than in maize stem and leaf tissue.

As shikimate accumulation occurred the most rapidly and was significantly higher in leaf tissue when compared to stem and root tissues, it is evident that leaf tissue is the superior and most effective tissue type (plant material) to use for shikimic acid analysis. In addition to the latter, leaves are generally also the easiest plant tissue to harvest and work with. As a result, leaves were used for the colourimetric field test kit. The amount of shikimate build-up in different tissues might also be used to evaluate the efficiency of glyphosate translocation to different tissues.

4.4. *Colourimetric assay*

The first step in validating and evaluating the efficacy of the colourimetric assay was to compare the standard curves obtained from HPLC analysis and the colourimetric assay. Similar results in shikimate levels were observed from HPLC and colourimetric (spectrophotometric) assays. Both analyses revealed that the degree of shikimate accumulation in treated non-RR maize and soybean plants were related to glyphosate dose and time after treatment. Results from both assays showed that there was a markedly increase in shikimate concentrations in glyphosate-treated non-RR plants with increasing rate of glyphosate and as time after treatment progressed.

From the results it is clear that both HPLC and the colourimetric assay could be used to accurately quantify minute concentrations of shikimate in untreated plants as well as measure elevated levels of shikimate in glyphosate exposed plants. Evaluation of HPLC chromatograms and the degree of colour change produced by the colourimetric assay could be used to detect glyphosate damage in plants before symptoms such as leaf necrosis and leaf rolling start to show. Thus, both assays could also be used to differentiate between glyphosate susceptible and resistant plants (Figure 3.20 and 3.21). In addition, HPLC analysis is also useful in determining what dosage of glyphosate a plant was exposed to, by comparing the shikimate concentration (mg/g) present in the unknown sample exhibiting symptoms of glyphosate damage to that of samples treated with a known dose of glyphosate under greenhouse conditions.

HPLC is the most sensitive and accurate method to consistently measure shikimic acid levels in plant samples. HPLC has many advantages to precisely quantify the concentration of shikimic acid present in a sample, however, unfortunately HPLC also have several disadvantages which include: it is a complicated, delicate, sensitive and expensive machine which could only be operated by a trained technician/scientist in an advanced laboratory. There is no quick or easy way to quantify shikimate concentration with HPLC analysis. Quantifying shikimic acid levels in samples using HPLC is expensive and very time consuming (sample preparation, runtime etc.) and in addition samples may need to be sent to a specialised laboratory for analysis.

By contrast, the colourimetric quick test is an easy and simple assay that has been developed to visually detect the presence of shikimate and accurately quantify shikimic acid levels in plants samples merely on the degree of a colour change. The test is very useful to quickly and

accurately differentiate between glyphosate susceptible and resistant plants based on a colour change (Figure 3.20 and 3.21). This test would enable farmers to rapidly establish whether weeds in their fields are sensitive or resistant to glyphosate and thus avoid their spread and facilitate more effective weed management strategies. The colourimetric assay has several advantages which include: it is specific for shikimic acid, it is colourimetric, quick, and easy to use (Kretzmer *et al.*, 2011). Results from the assay could be easily interpreted by farmers and non-scientific users, since it is merely based on a colour change.

Lastly, samples of non-RR maize provided to us by a farmer who suspected herbicide damage by glyphosate were evaluated. If the plants were exposed to glyphosate we would expect to see elevated levels of shikimate. We analysed the samples using both methods (HPLC and colourimetric) and found that all the maize plants provided were injured by low concentrations of glyphosate. As the farmer did not spray glyphosate to control weeds in his fields, the mostly likely cause of damage was due to wind drift from a neighbouring farm.

Thus, both HPLC and colourimetric assays, could be used to detect glyphosate drift damage even if a non-target plant was exposed to glyphosate. Relatively high concentrations of shikimate were detected in the brown, dead corn plant material that had been killed by glyphosate. Therefore, these two tests could be done at any time following the herbicide treatment, and shikimate accumulation due to glyphosate damage can be observed even if only brown, dead plant material is available for analysis. Furthermore, these tests can be done on plants of different ages, which makes the assay very useful because drift injury from glyphosate may occur at any time during the growing season.

CHAPTER 5. CONCLUSIONS

Shikimic acid quantification results obtained from the colourimetric assay was similar and corresponded to that obtained from HPLC analysis. The assays mutually indicated that shikimic acid accumulation was related to glyphosate dose and time after treatment. Both assays showed that shikimic acid levels were significantly higher in glyphosate treated non-RR maize and soybean cultivars when compared to control and treated RR maize and soybean cultivars. In general, for non-RR maize and soybean cultivars there was a direct correlation between shikimic acid accumulation, glyphosate application rate, and time after glyphosate application. Shikimic acid accumulation was also plant species and plant tissue specific. Shikimate build-up was significantly higher ($p \leq 0.05$) in treated non-RR soybean plants than in maize plants, also accumulation was the highest in leaf tissue, followed by stem tissue and was the lowest in root tissue.

Thus, the results obtained from HPLC and colourimetric analysis supported each other, in that there is a strong correlation between the degree of shikimate accumulation, plant species, different tissue types, glyphosate application rate, and time after treatment. Comparison of HPLC and colourimetric assay results also showed that the colourimetric assay is very accurate in quantifying shikimic acid concentrations. Since results obtained from the colourimetric assay corresponded to that of HPLC, the colourimetric assay could be used to accurately differentiate between glyphosate susceptible and resistant plants and also to indicate glyphosate damage in plants. From this it is evident that the colourimetric assay would be accurate to use in the field. Consequently, the four main questions asked in this study were successfully answered.

Although HPLC analysis is very accurate and sensitive it is an expensive, complicated and a very time consuming method of quantification. The colourimetric assay had several advantages over HPLC analysis which included; it produce rapid and colourimetric results, it is easy and simple to use. Colourimetric results are easily interpreted as it is based on a colour change. The only down side to the colourimetric assay is that production of the recombinant SHKDH enzyme is expensive, time consuming and requires an advanced laboratory. Thus, the colourimetric assay is a very efficient method to accurately quantify shikimic acid levels in plants. This assay is very handy to rapidly differentiate between glyphosate susceptible and resistant plants and also to indicate whether a plant was exposed or damaged by glyphosate.

Future prospects for the colourimetric assay would include redefining and optimising the colourimetric dipsticks. Dipsticks should enable users to simply dip the stick in a solution containing leaf discs and wait for a colour change to be produced. The colour change could then be compared to a standardised colour chart, which would indicate the degree of susceptibility of the plant species to glyphosate based on the colour produced on the stick. This can then be used for fast in-field evaluation of glyphosate applications and uptake by weeds as well as detection of glyphosate resistance in weeds, allowing farmers to react more quickly to cases of resistance.

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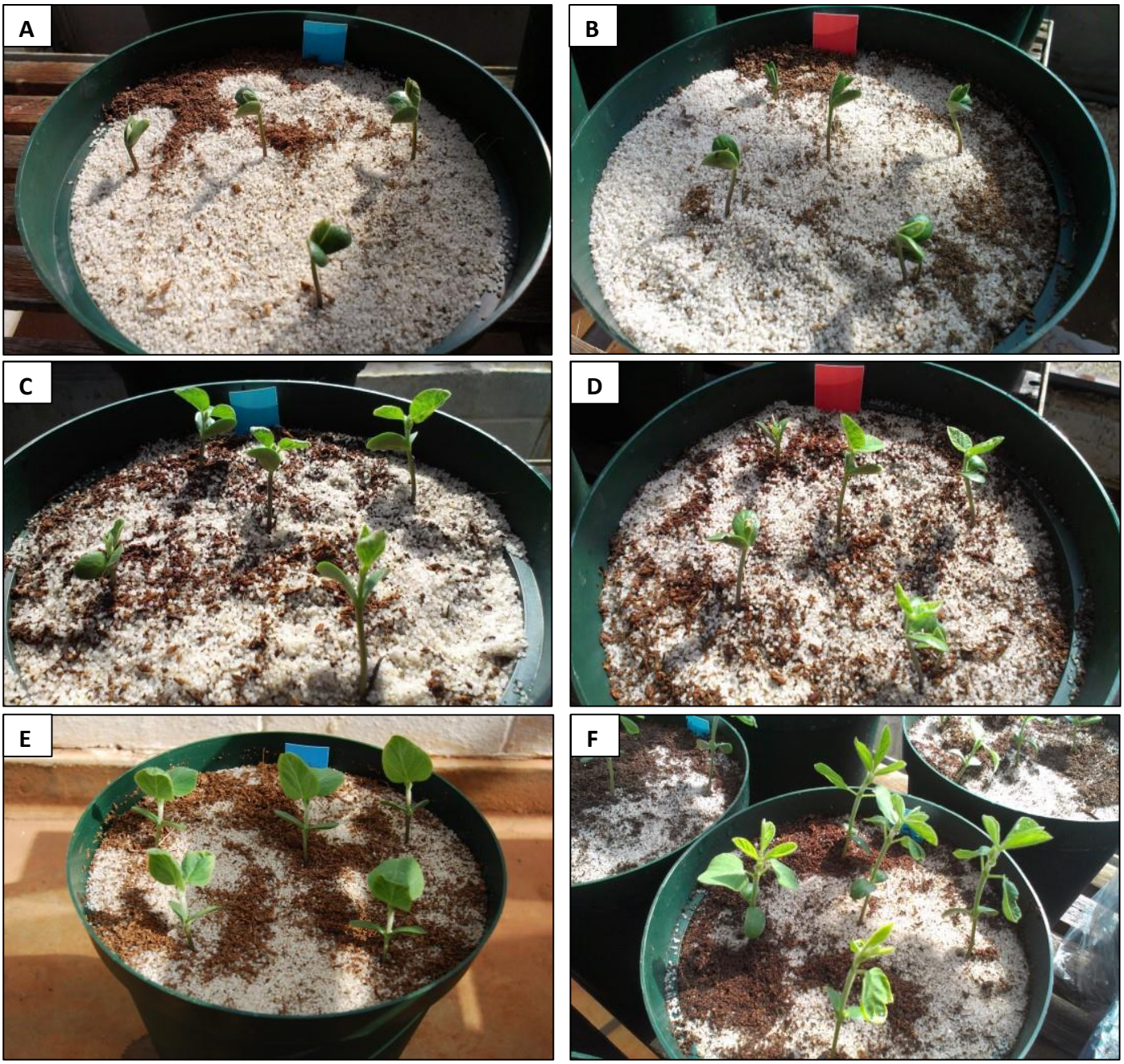
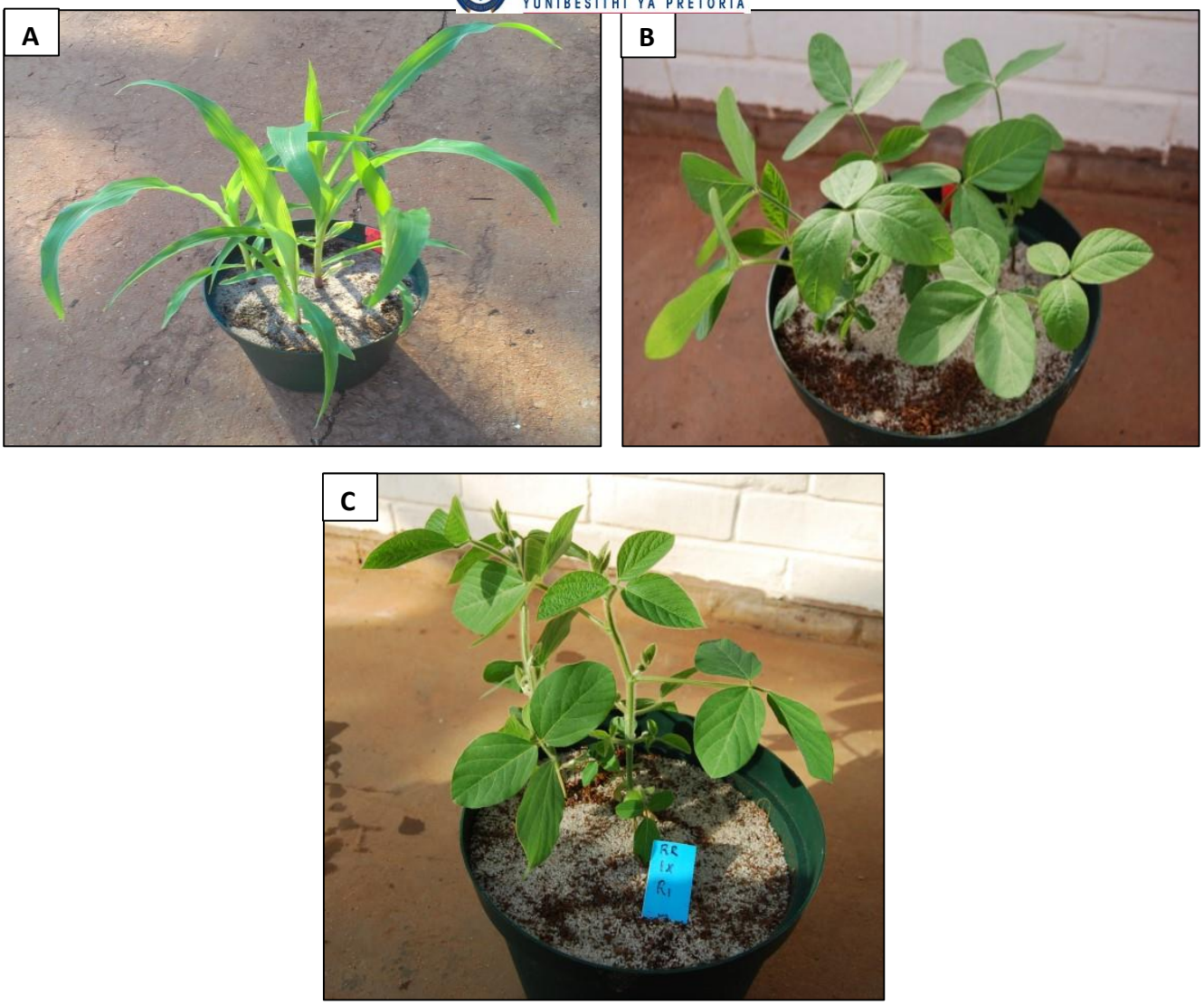
APPENDIX

1. Planting, spraying and harvesting

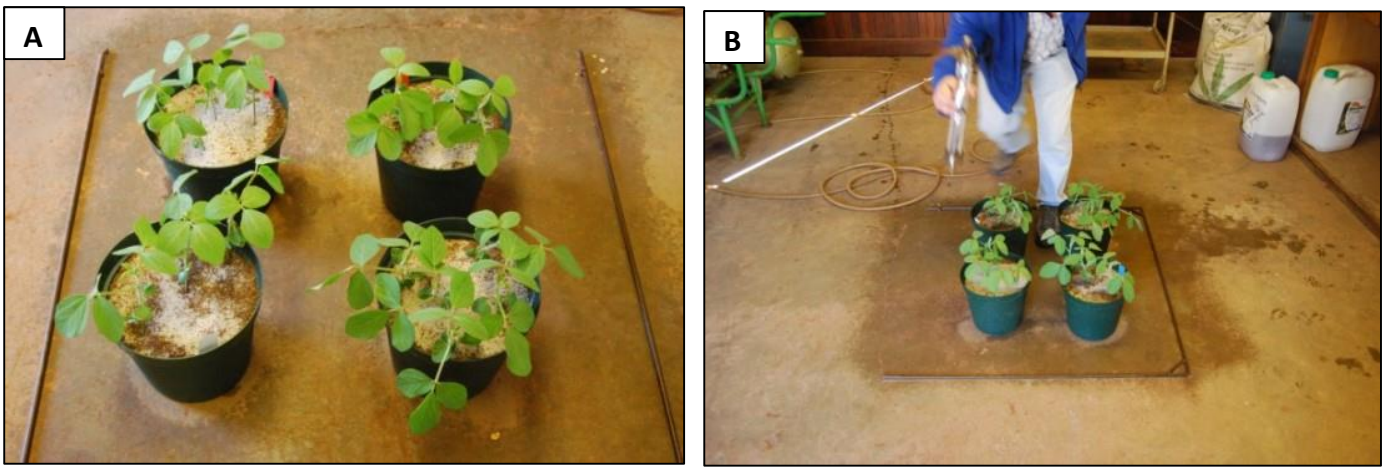
APPENDIX 1. Maize and soybean plants were grown in a sand coir mixture under greenhouse conditions. **(A)** Sand/coir medium in 7kg plastic pots; **(B)** Maize and soybean seeds in labelled bags prior to planting; **(C)** Plastics bags were used to cover pots to reduce evaporation – bags were removed once seedlings emerged; **(D)** Non-RR maize seedlings seven days after emergence; **(E)** RR maize seedlings seven days after emergence; **(F-G)** Non-RR (red tag) and RR (blue label) maize plants 15 days after emergence.



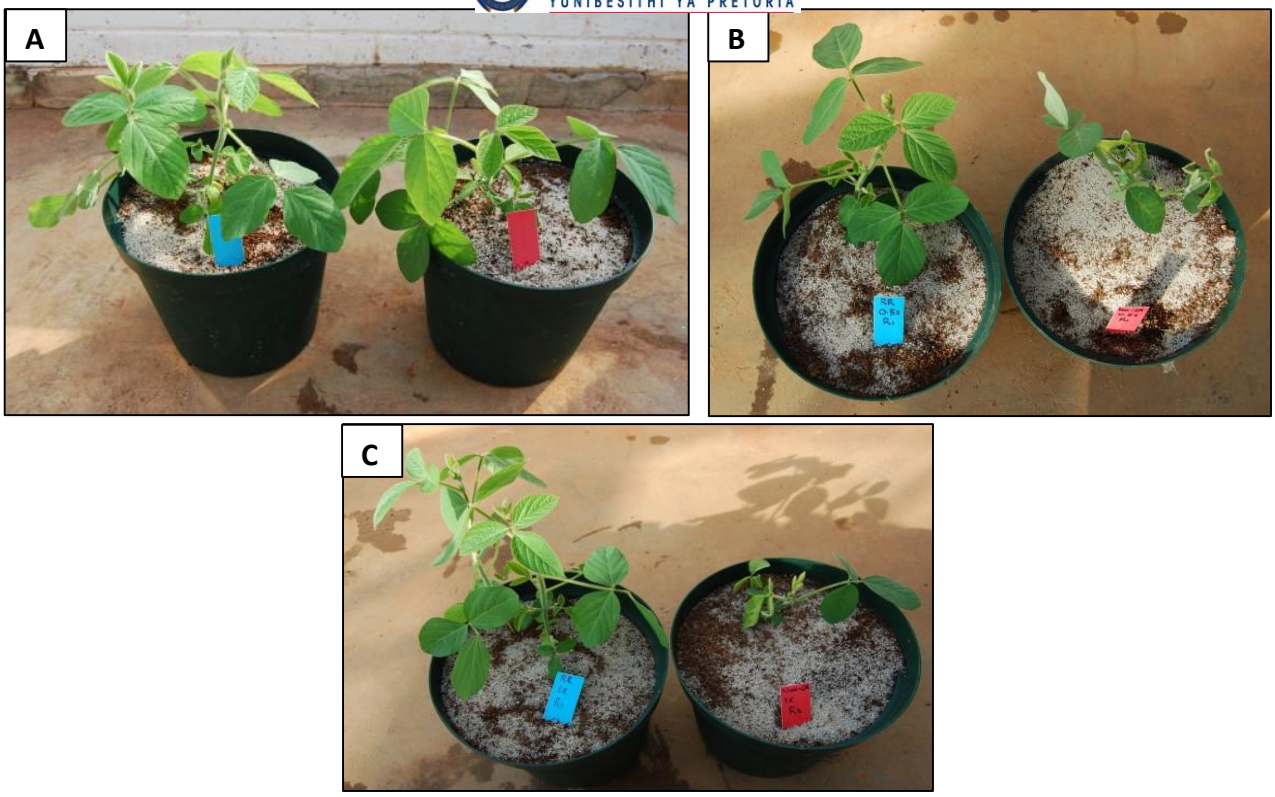
APPENDIX 2. (A-B) RR (blue tag) and non-RR (red label) soybean plants were grown in a sand/coir medium under greenhouse conditions – these soybean seedlings were seven days old (post emergence); **(C-D)** RR and non-RR soybean plants 10 days after emergence; **(E)** RR soybean plants 12 days after emergence; **(F)** RR soybean plants 15 days after emergence.



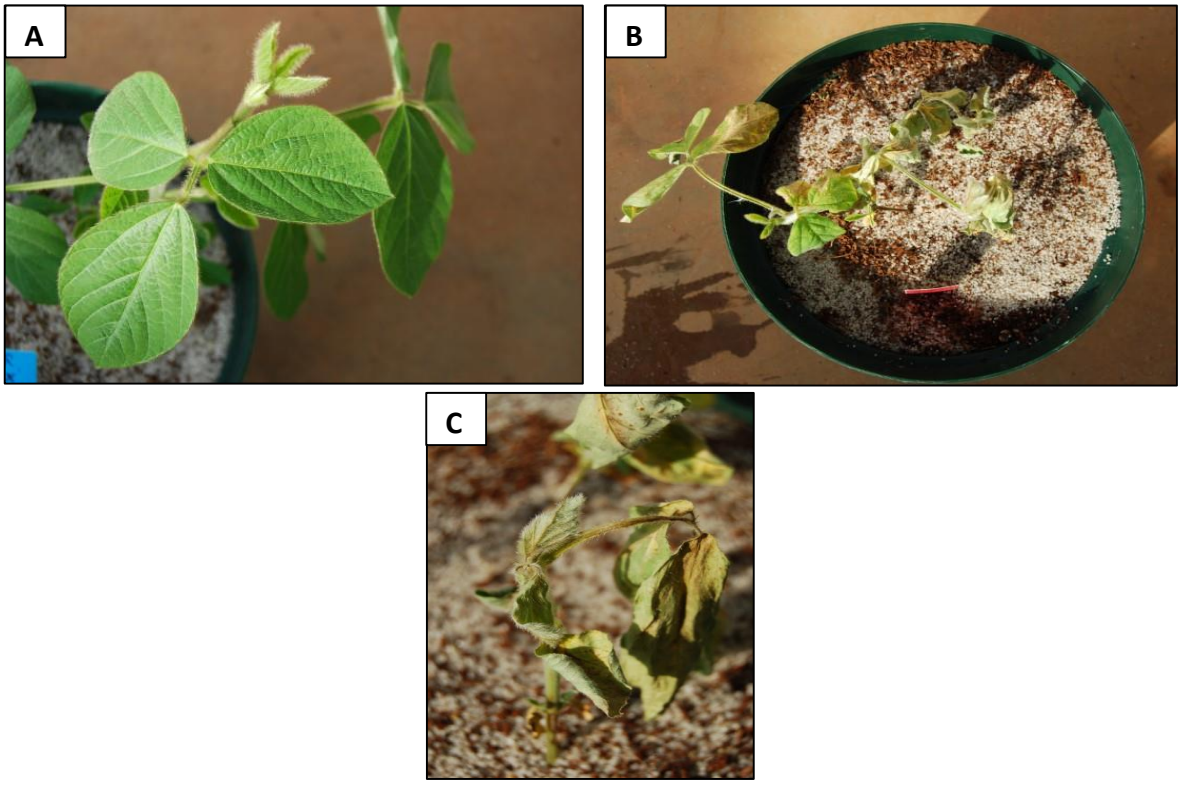
APPENDIX 3. (A) 30 day old non RR maize plants, prior to glyphosate application; **(B)** Non-RR soybean plants 30 days after emergence, this photo was taken before Roundup Turbo® treatment; **(C)** RR soybean plants 30 days after emergence prior to glyphosate spray treatment.



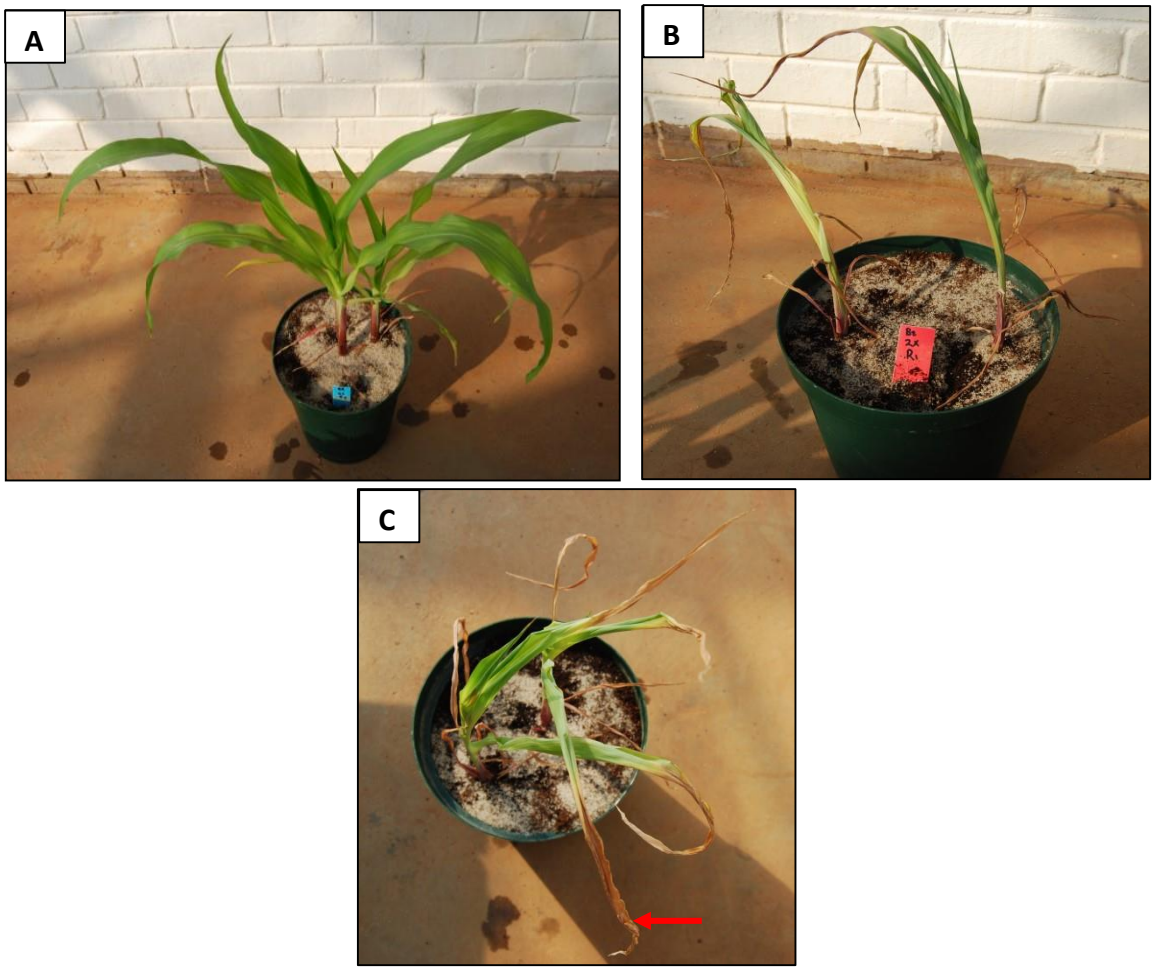
APPENDIX 4. (A-B) Soybean plants being sprayed with various concentrations of Roundup Turbo®. The same procedure was followed for maize plants.



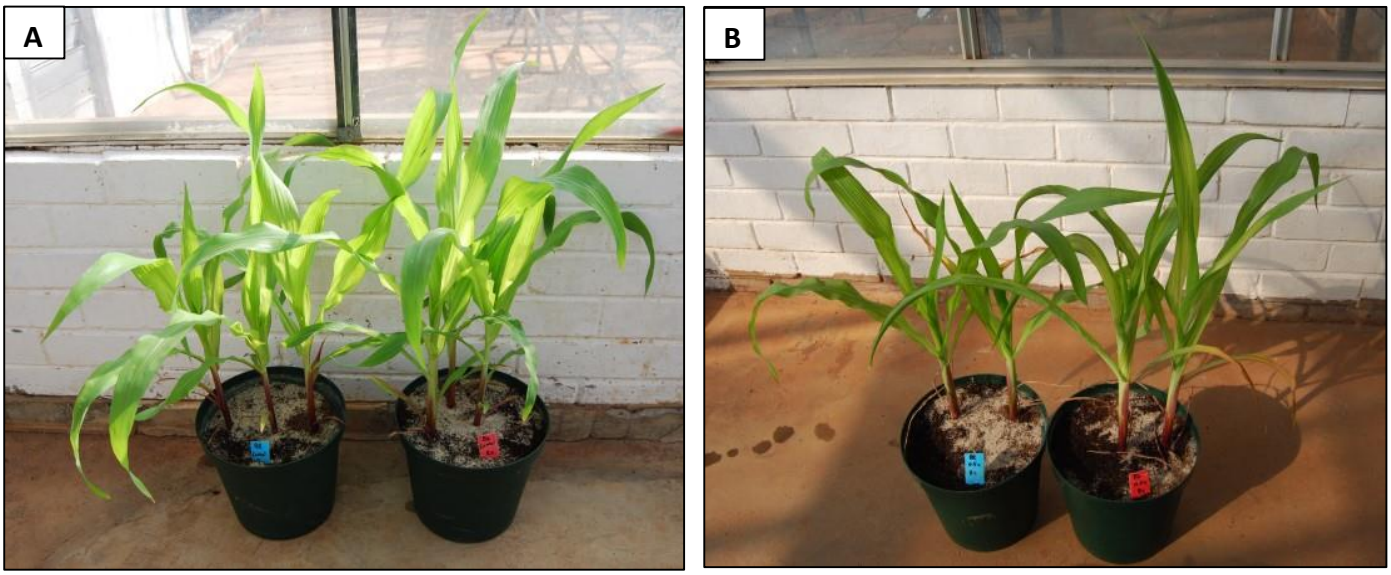
APPENDIX 5. (A) RR and Non-RR soybean control plants; **(B)** RR and Non-RR soybean plants three days after being treated with half (0.5x) the recommended dosage of Roundup Turbo®; **(C)** RR and Non-RR soybean plants three days after being treated with the recommended (1x) dosage of Roundup Turbo®.



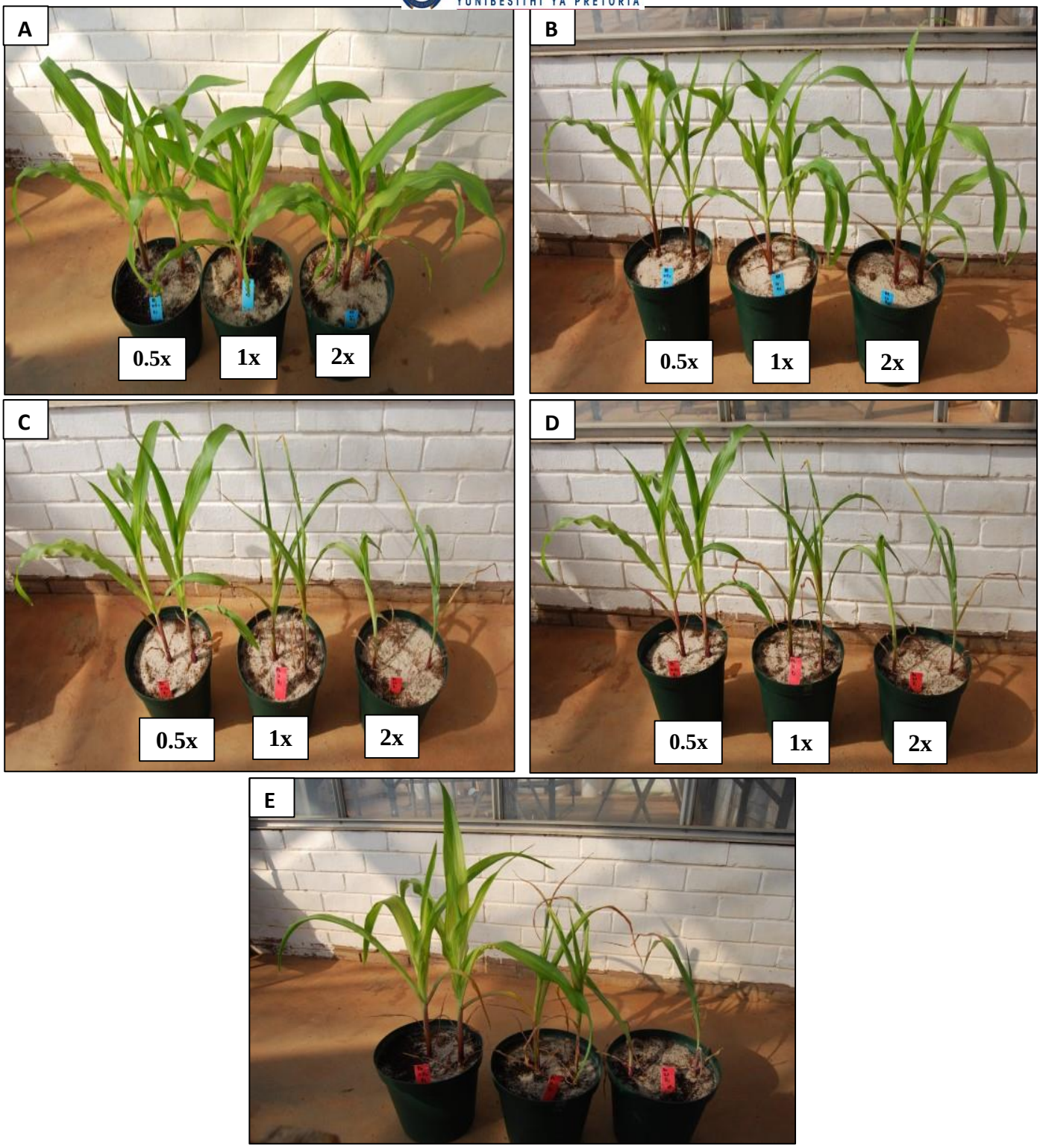
APPENDIX 6. (A) RR soybean plants nine days after being treated with the the recommended dosage of Roundup Turbo®; **(B-C)** Non-RR soybean plants nine days after being treated with the the recommended dosage of Roundup Turbo®.



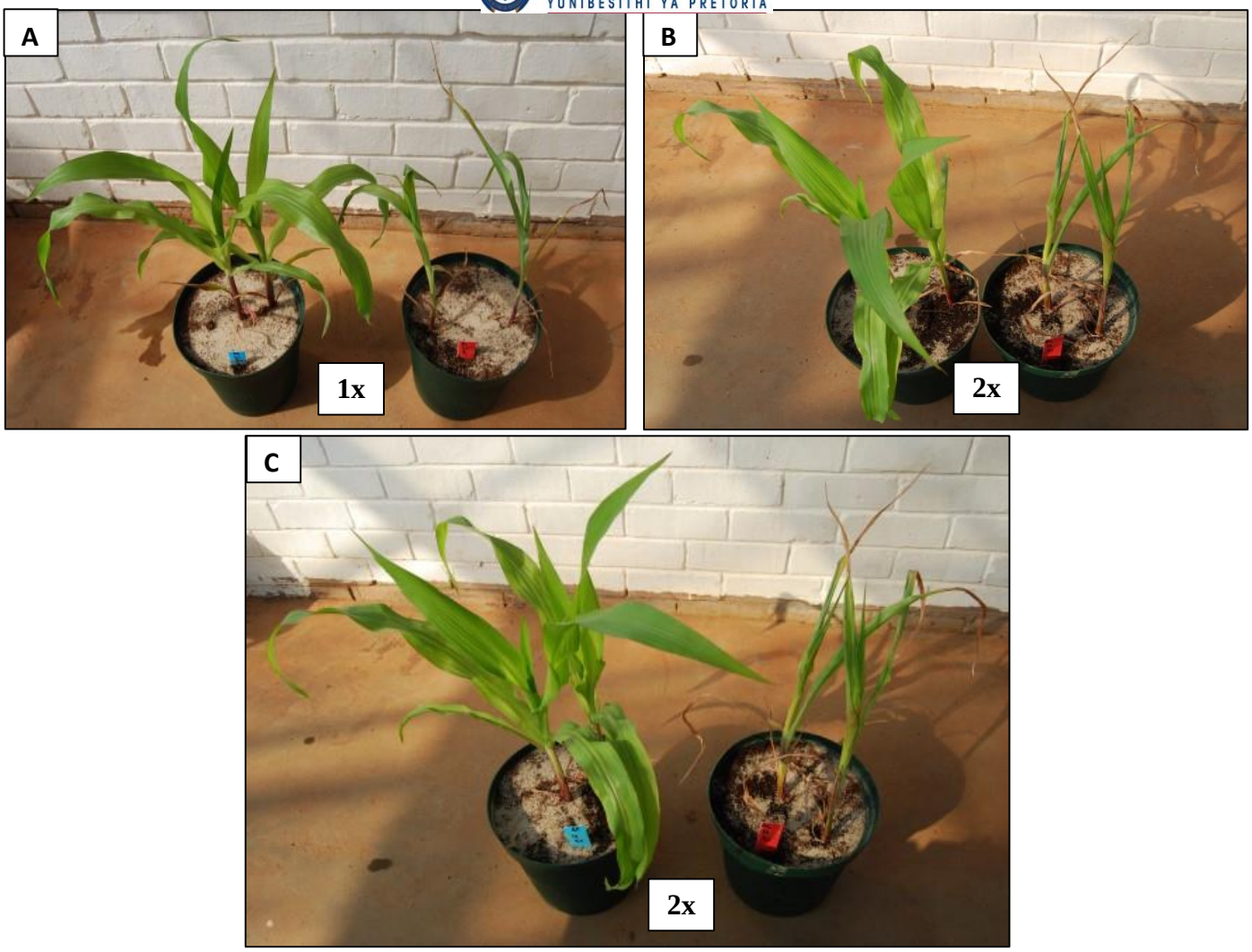
APPENDIX 7. (A) RR maize plants nine days after being treated with the recommended dosage of Roundup Turbo®; **(B-C)** Non-RR maize plants nine days after being treated with the the recommended dosage of Roundup Turbo® - leaf necrosis (indicated by red arrow) is very evident in non-RR plants.



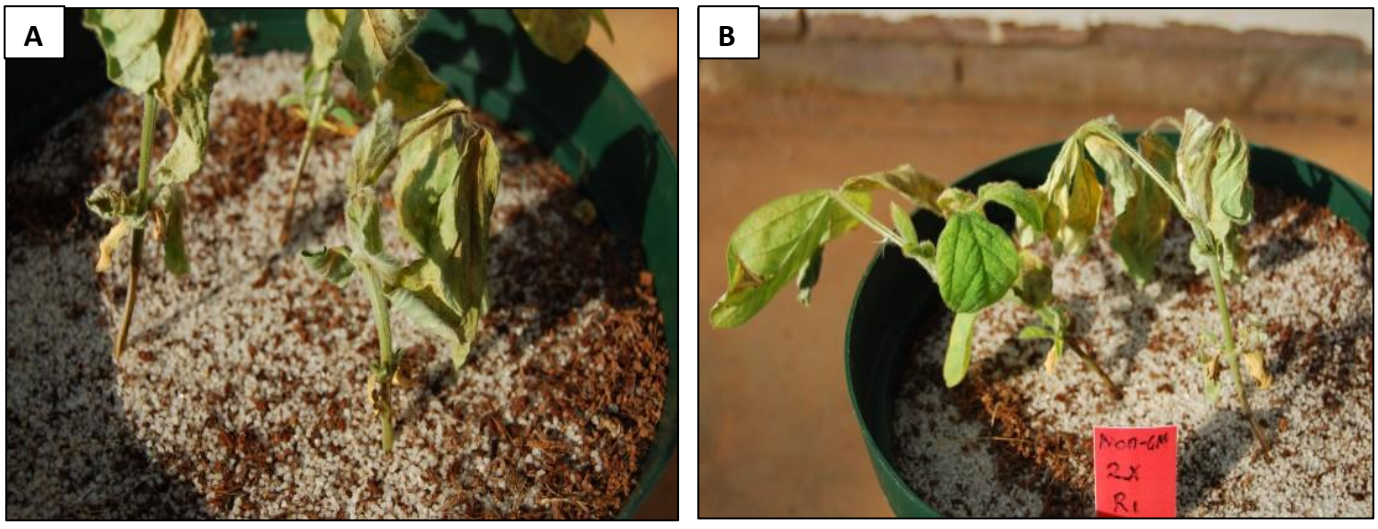
APPENDIX 8. (A) RR and non-RR maize control plants; **(B)** RR and non-RR mazie plants 3 days after being treated with half (0.5x) the the recommended dosage of Roundup Turbo®.



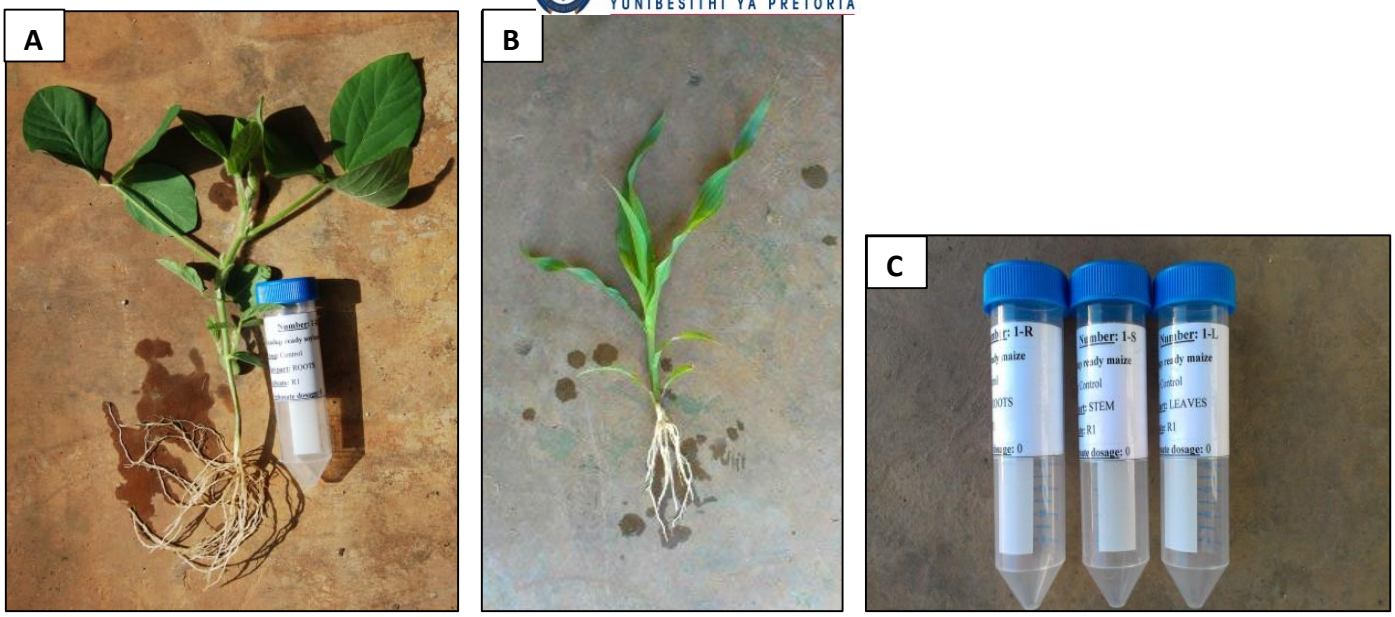
APPENDIX 9. (A-B) RR maize plants six days after being treated with half (0.5x – left) the recommended dosage, the recommended dosage (1x – middle) and double (2x – right) of Roundup Turbo®; **(C-E)** non-RR maize plants six days after being treated with half (0.5x – left) the recommended dosage, the recommended dosage (1x – middle) and double (2x – right) of Roundup Turbo®.



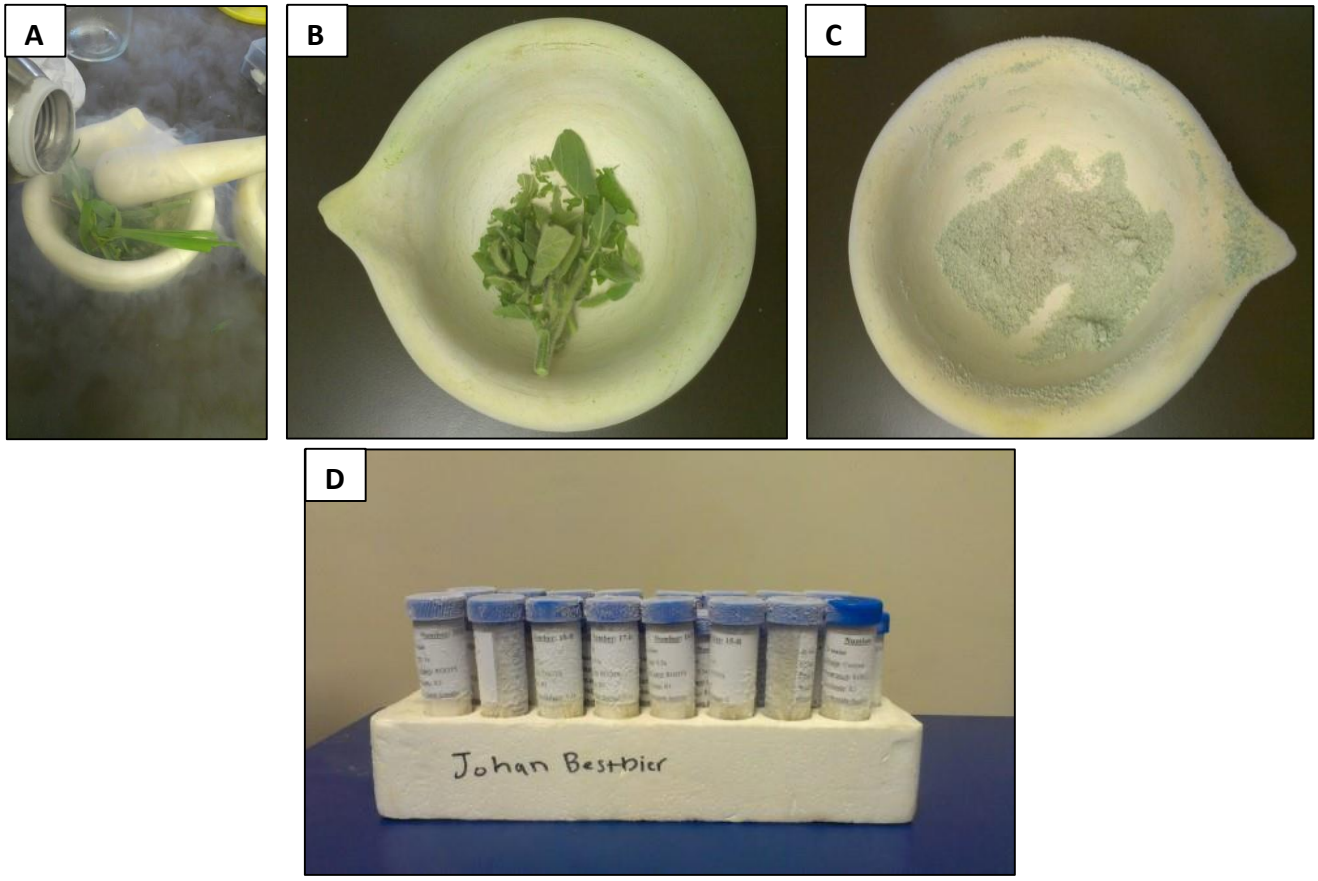
APPENDIX 10. (A) RR (blue tag) and Non-RR (red tag) maize plants six days after being sprayed with the recommended dosage (2L/ha) of Roundup Turbo®; **(B-C)** RR and Non-RR maize plants six days after being sprayed with double (4L/ha) the recommended dosage of Roundup Turbo®.



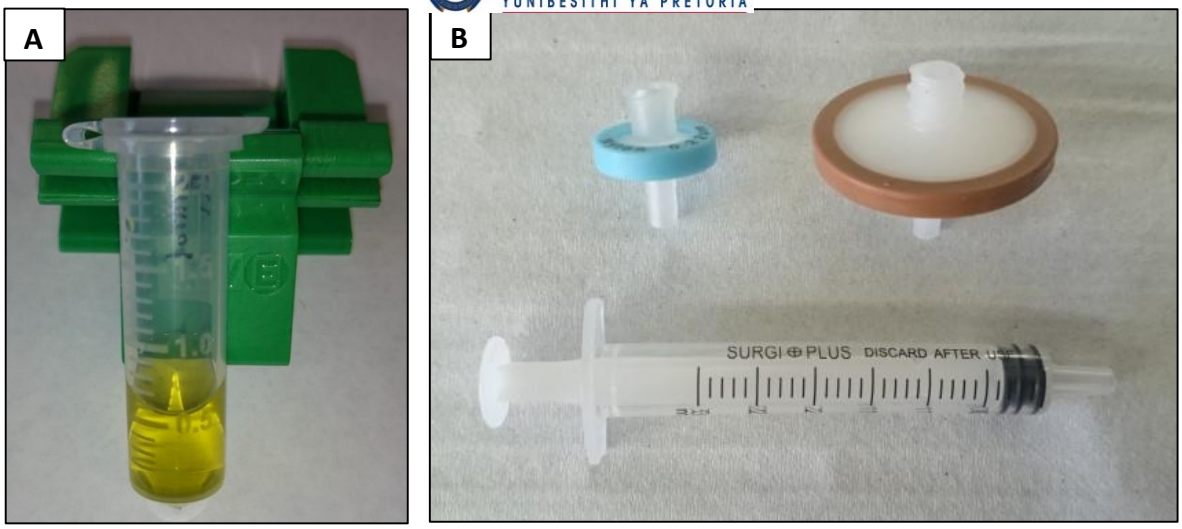
APPENDIX 11. (A-B) Non-RR soybean plants nine days after being sprayed with double (2x) the recommended dosage of Roundup Turbo®.



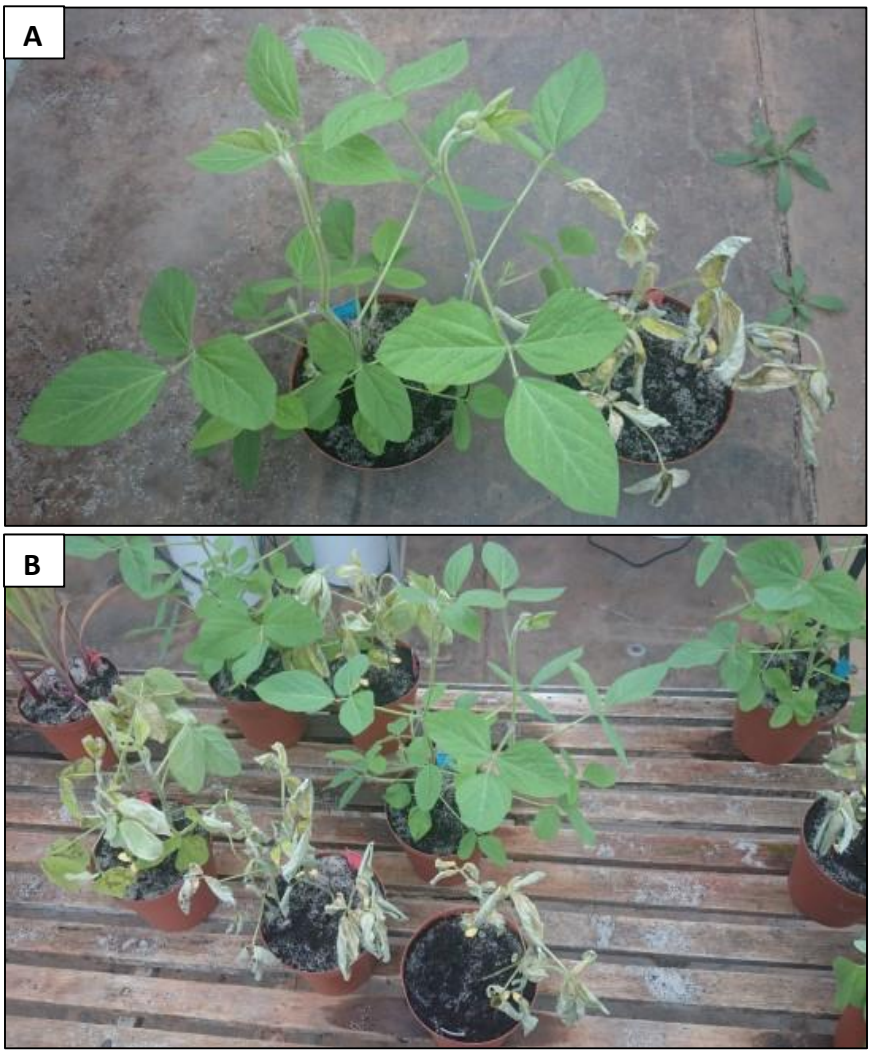
APPENDIX 12. (A-B) Harvesting of soybean and maize plant parts/material (leaves, stems/shoots and roots); **(C)** Plant material was stored in either 15ml or 50ml falcon tubes.



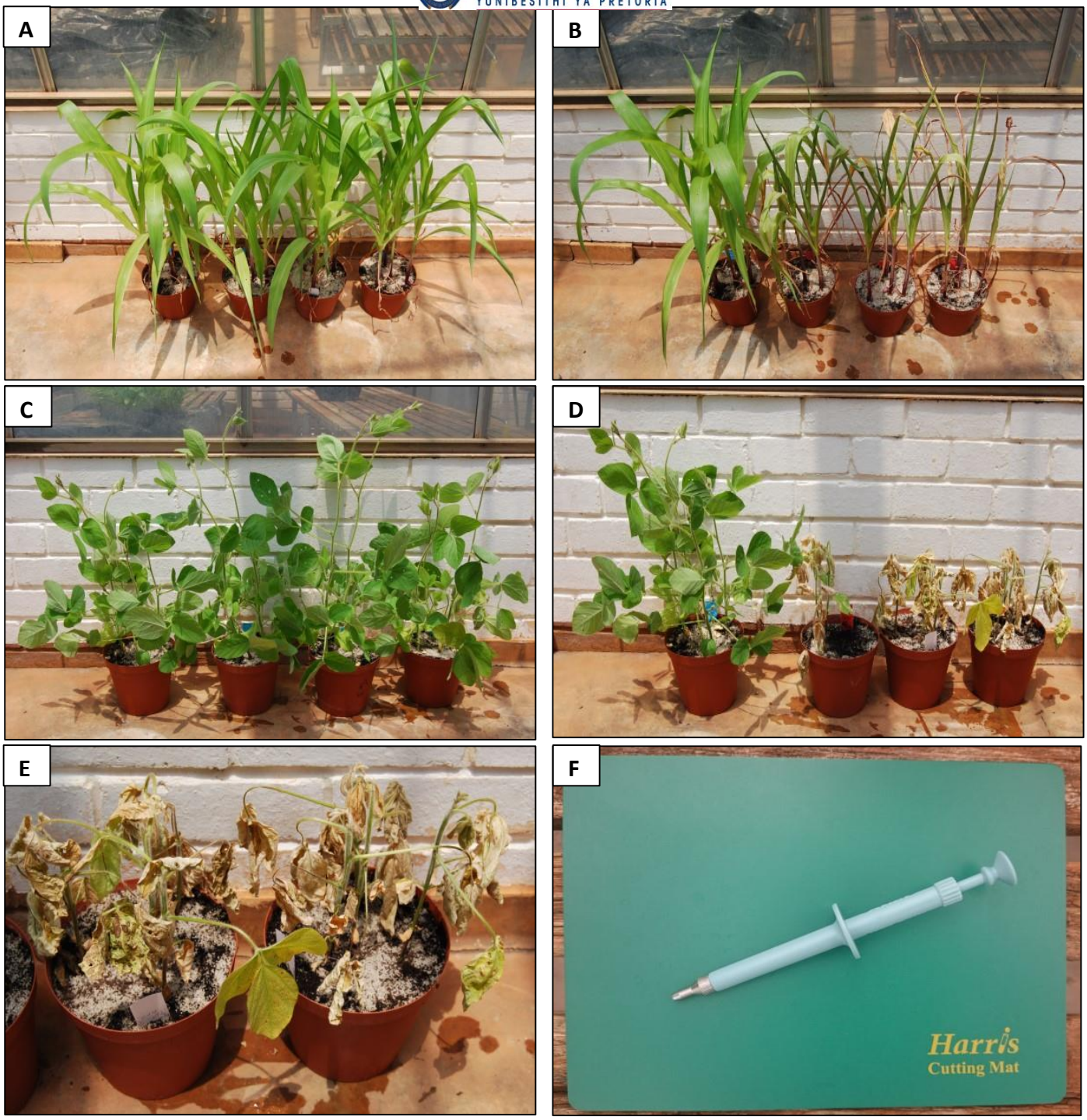
APPENDIX 13. (A-C) Plant material was ground in liquid nitrogen by mortar and pestle into a very fine powder. **(D)** Powder was weighed and transferred to labelled falcon tubes and stored at -80˚C.



APPENDIX 14. (A) Shikimic acid extracts made from the fine powder; **(B)** 0.22µm and 0.45µm Whatman syringe filters were used to filter any particles or debris from the extract solution.

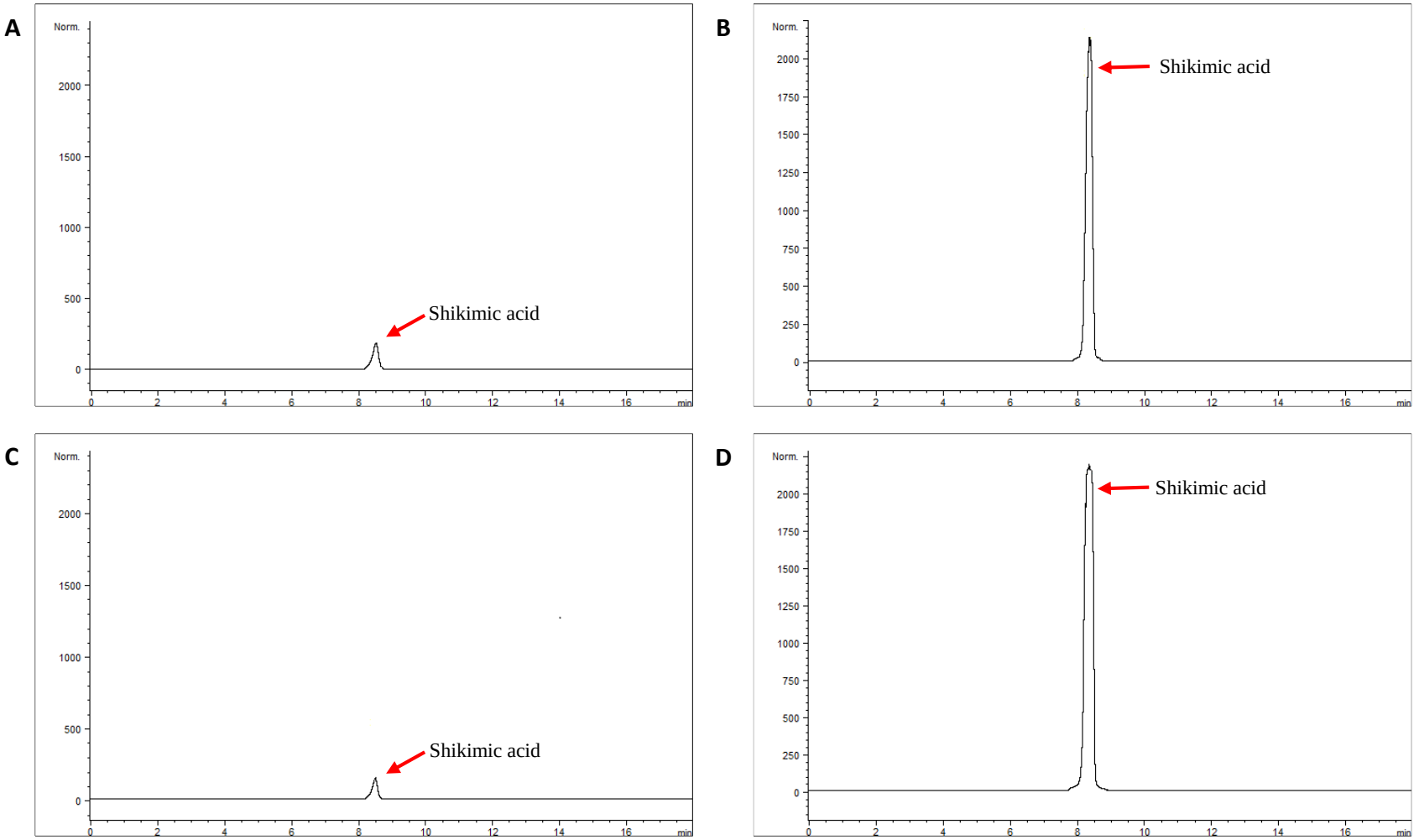


APPENDIX 15. (A-B) RR and non-RR soybean plants that were used for the colourimetric assay, seven days after being treated with various concentration of glyphosate.



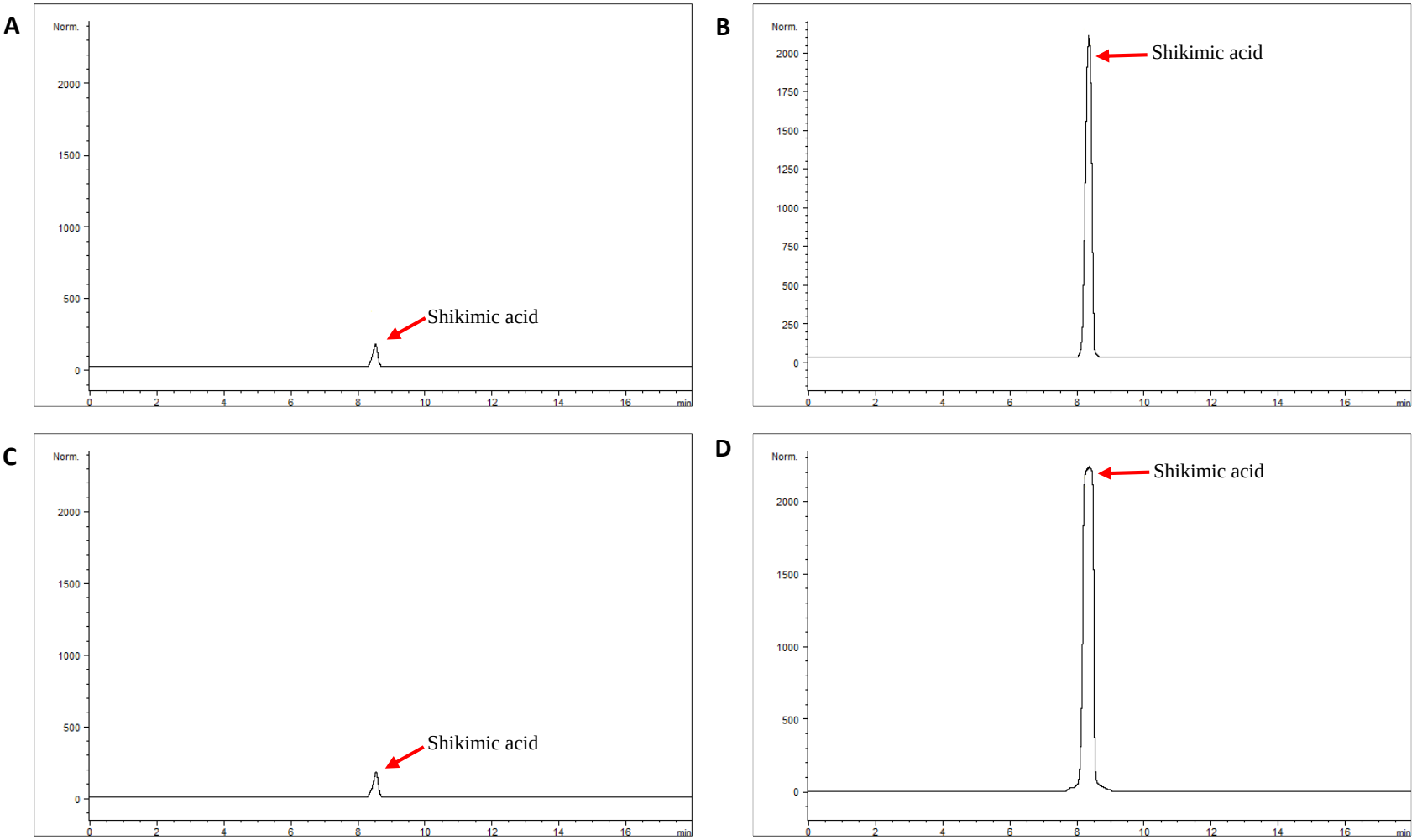
APPENDIX 16. (A) From left to right: maize control plant, RR maize plants treated with half (0.5x) the recommended, the recommended (1x) and double (2x) the recommended rate of Roundup Turbo®. **(B)** From left to right: maize control plant, non-RR maize plants treated with half (0.5x) the recommended, the recommended (1x) and double (2x) the recommended rate of Roundup Turbo®. **(C)** From left to right: soybean control plant, RR soybean plants treated with half (0.5x) the recommended, the recommended (1x) and double (2x) the recommended rate of Roundup Turbo®. **(D)** From left to right: soybean control plant, non-RR soybean plants treated with half (0.5x) the recommended, the recommended (1x) and double (2x) the recommended rate of Roundup Turbo®. **(E)** Close up of non-RR soybean plants nine days after being sprayed with the recommended rate (left) and double the recommended rate (right) of Roundup Turbo®; **(F)** Harris 3 mm diameter punch and cutting mat used to cut/excise leaf discs from maize and soybean plants.

2. HPLC chromatograms—Time response of maize leaves



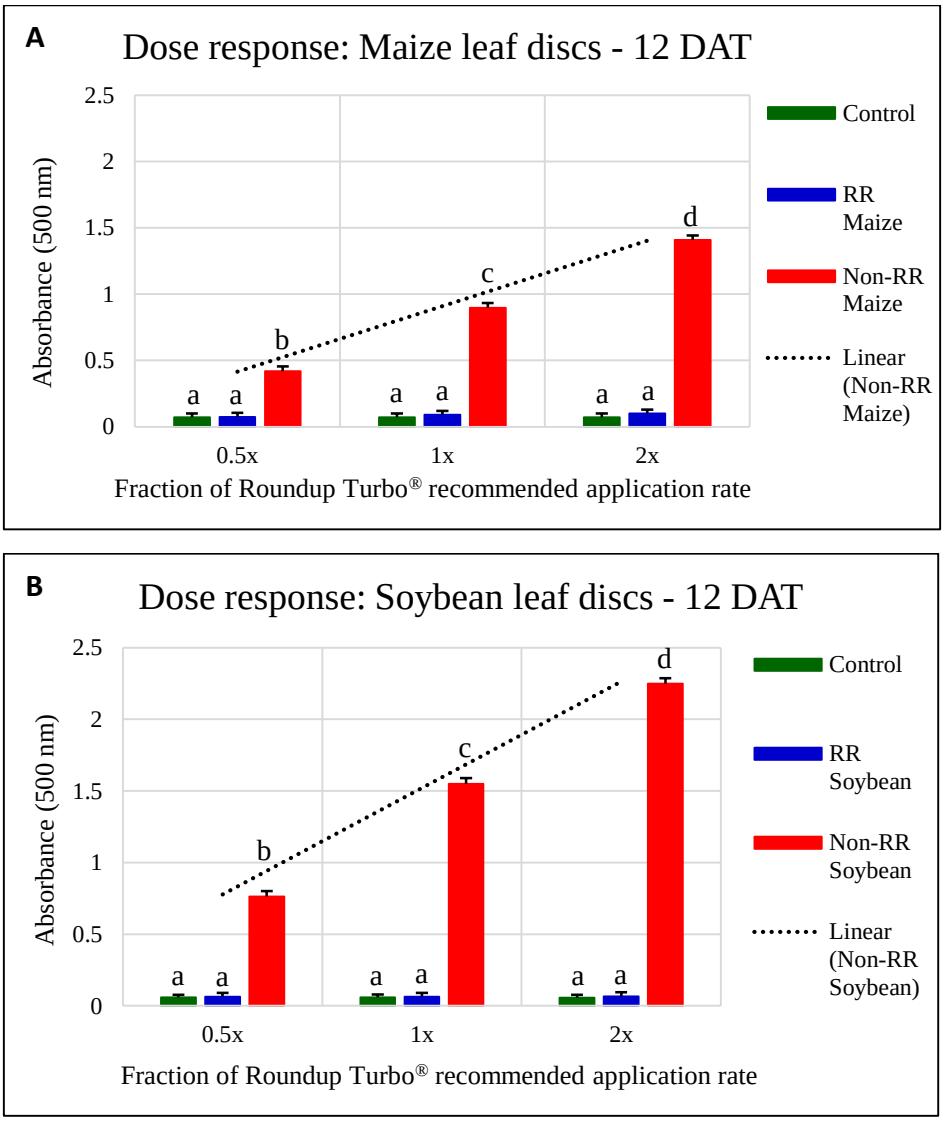
APPENDIX 17. HPLC chromatograms for time response of maize leaves. The shikimic acid peak is indicated on the chromatograms with a caption and a red arrow **(A)** Control maize plants; **(B)** Control sample spiked with 150µg/ml shikimic acid standard; **(C)** Roundup Ready® maize nine days after being treated with the recommended dosage (2L/ha) of Roundup Turbo®; **(D)** Non-roundup ready maize nine days after being treated with the recommended dosage of Roundup Turbo®.

3. HPLC chromatograms—Time response of soybean leaves



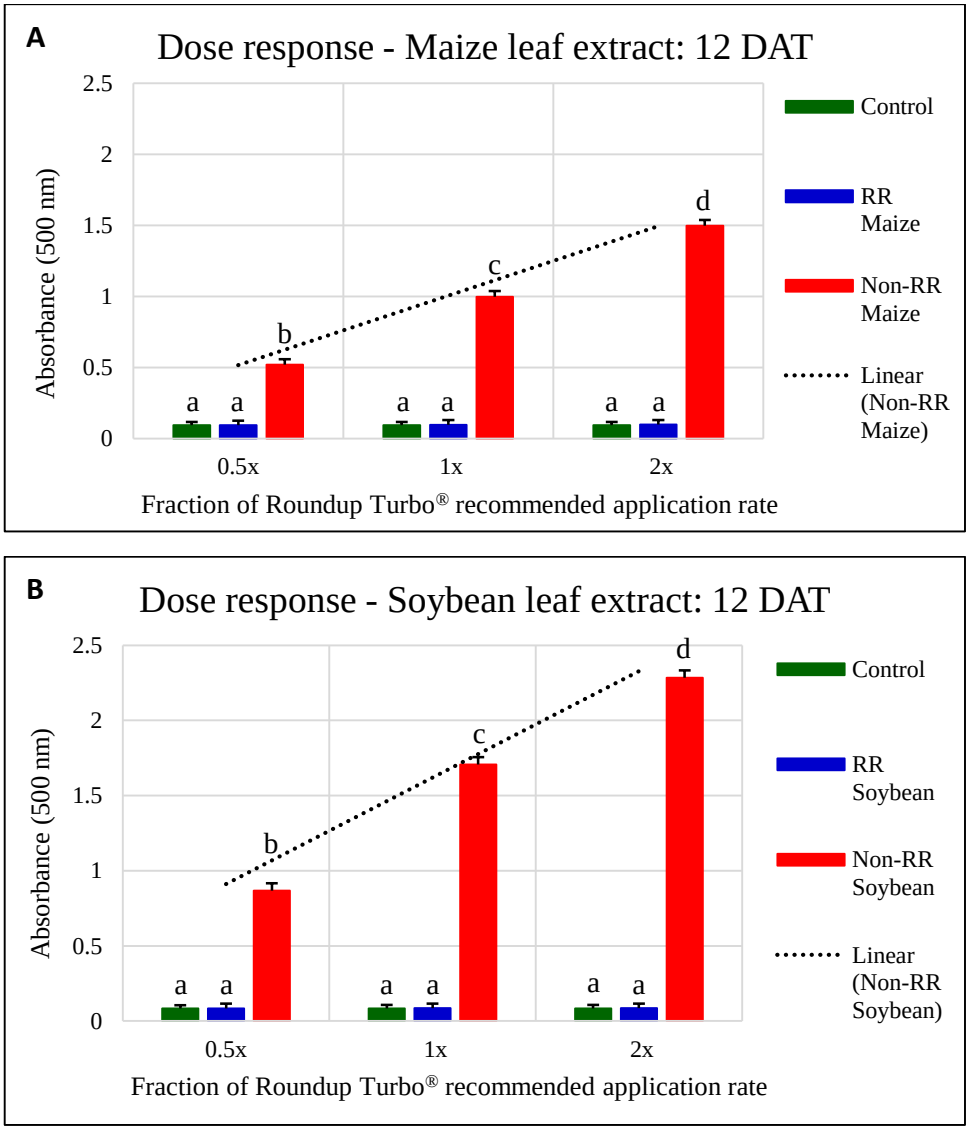
APPENDIX 18. HPLC chromatograms for time response of soybean leaves. The shikimic acid peak is indicated on the chromatograms with a caption and a red arrow **(A)** Control soybean plants; **(B)** Control sample spiked with 150µg/ml shikimic acid standard; **(C)** Roundup Ready® soybean nine days after being treated with the recommended dosage (2L/ha) of Roundup Turbo®; **(D)** Non-roundup ready® soybean nine days after being treated with the recommended dosage of Roundup Turbo®.

4. Colourimetric assay—Leaf discs: Dose response



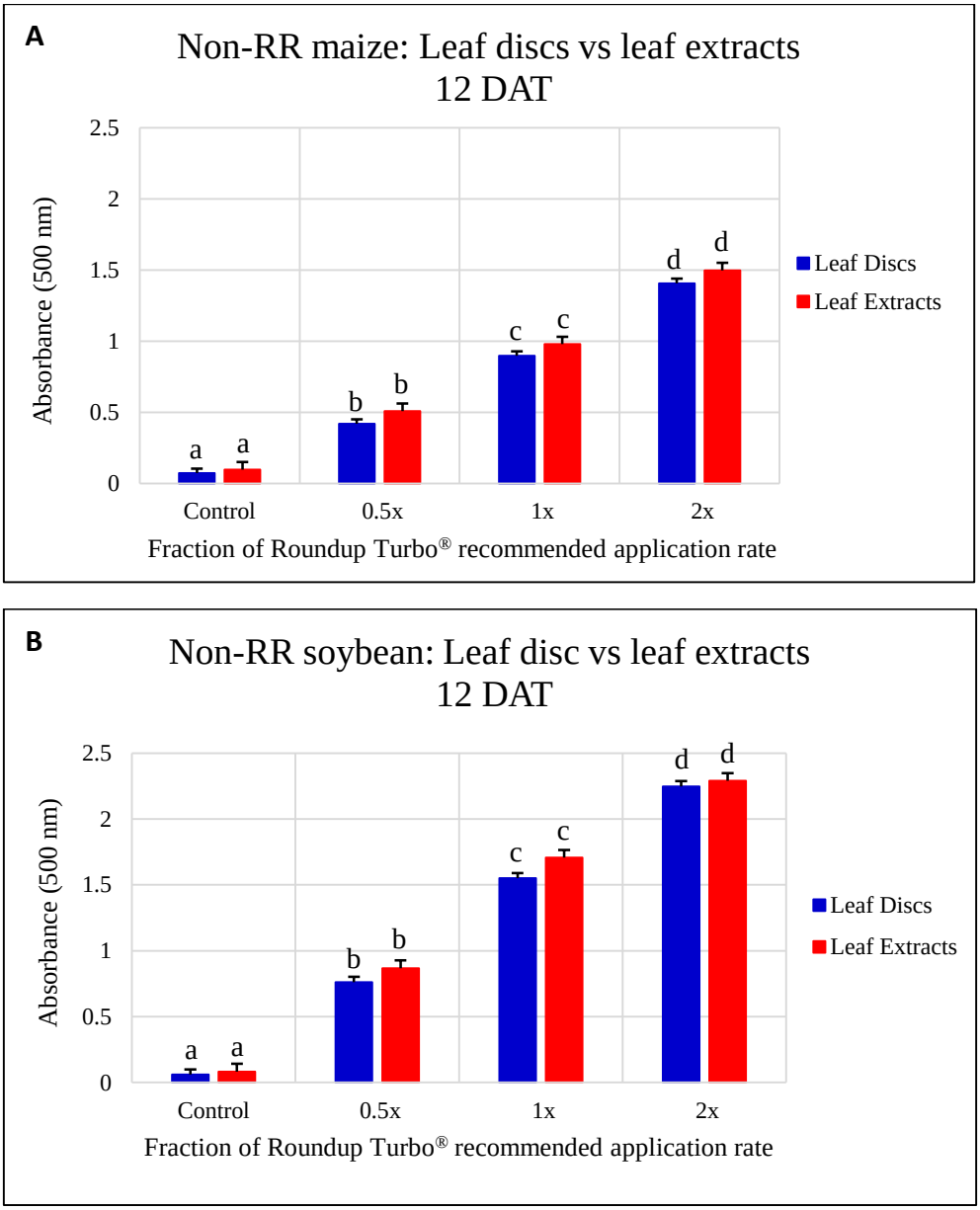
APPENDIX 19. Photospectroscopy results for dose response of **(A)** maize leaf discs and **(B)** soybean leaf discs 12 days after being treated with various dosages (0.5x, 1x and 2x the fraction of recommended application rate) of Roundup Turbo®. Absorbance of the assay solutions in which leaf discs were submerged were measured 15min after adding the enzyme, shikimate dehydrogenase.

5. Colourimetric assay—Leaf extracts: Dose response



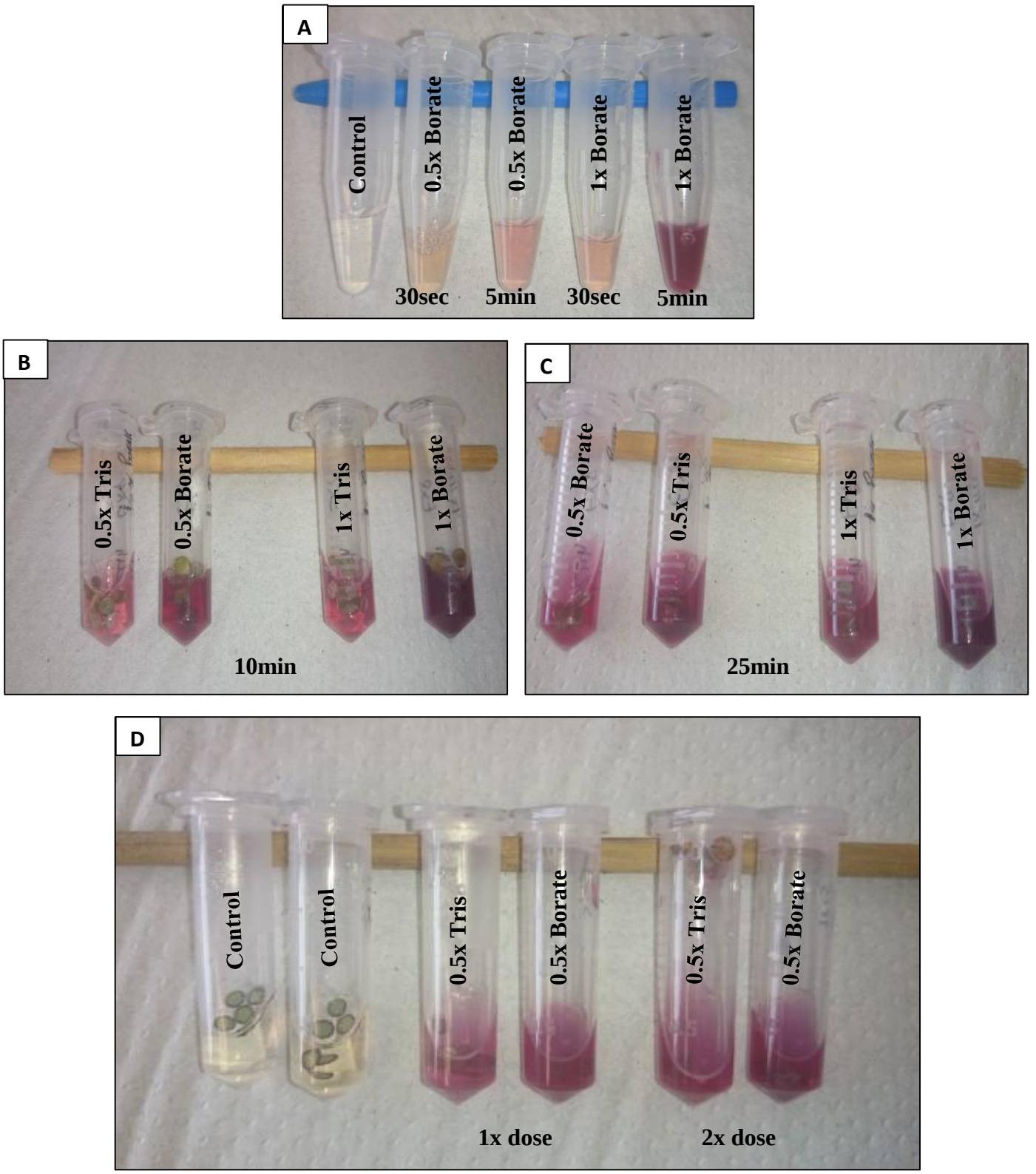
APPENDIX 20. Photospectroscopy results for dose response of **(A)** maize leaf extracts and **(B)** soybean leaf extracts 12 days after the plants were treated with various dosages (0.5x, 1x and 2x the fraction of recommended application rate) of Roundup Turbo®. Absorbance of the leaf extracts were measured 15min after adding the enzyme, shikimate dehydrogenase.

6. Dose response: Leaf discs versus leaf extracts

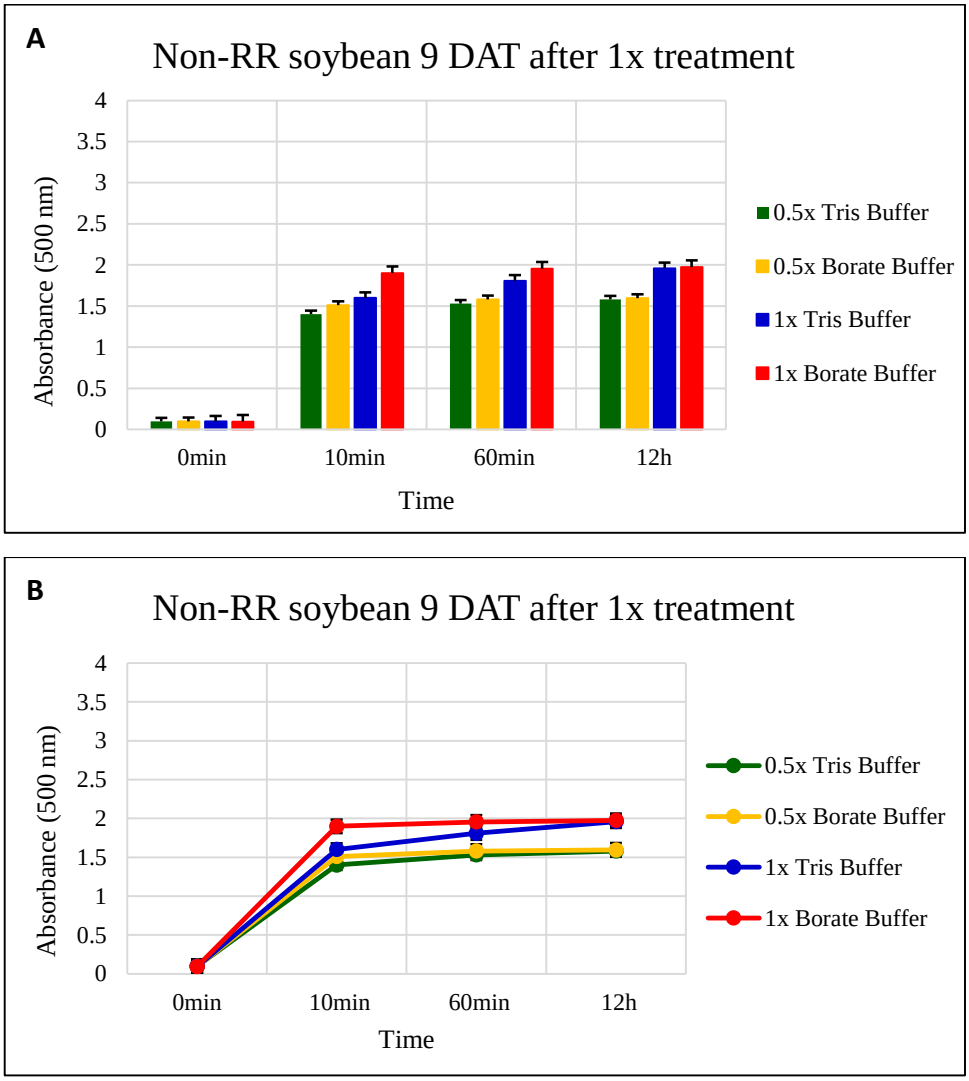


APPENDIX 21. Photospectroscopy results comparing the absorbance from dose response of **(A)** non-RR maize leaf discs to extracts and **(B)** non-RR soybean leaf discs to extract 12 days after being treated with the recommended dosage of Roundup Turbo®.

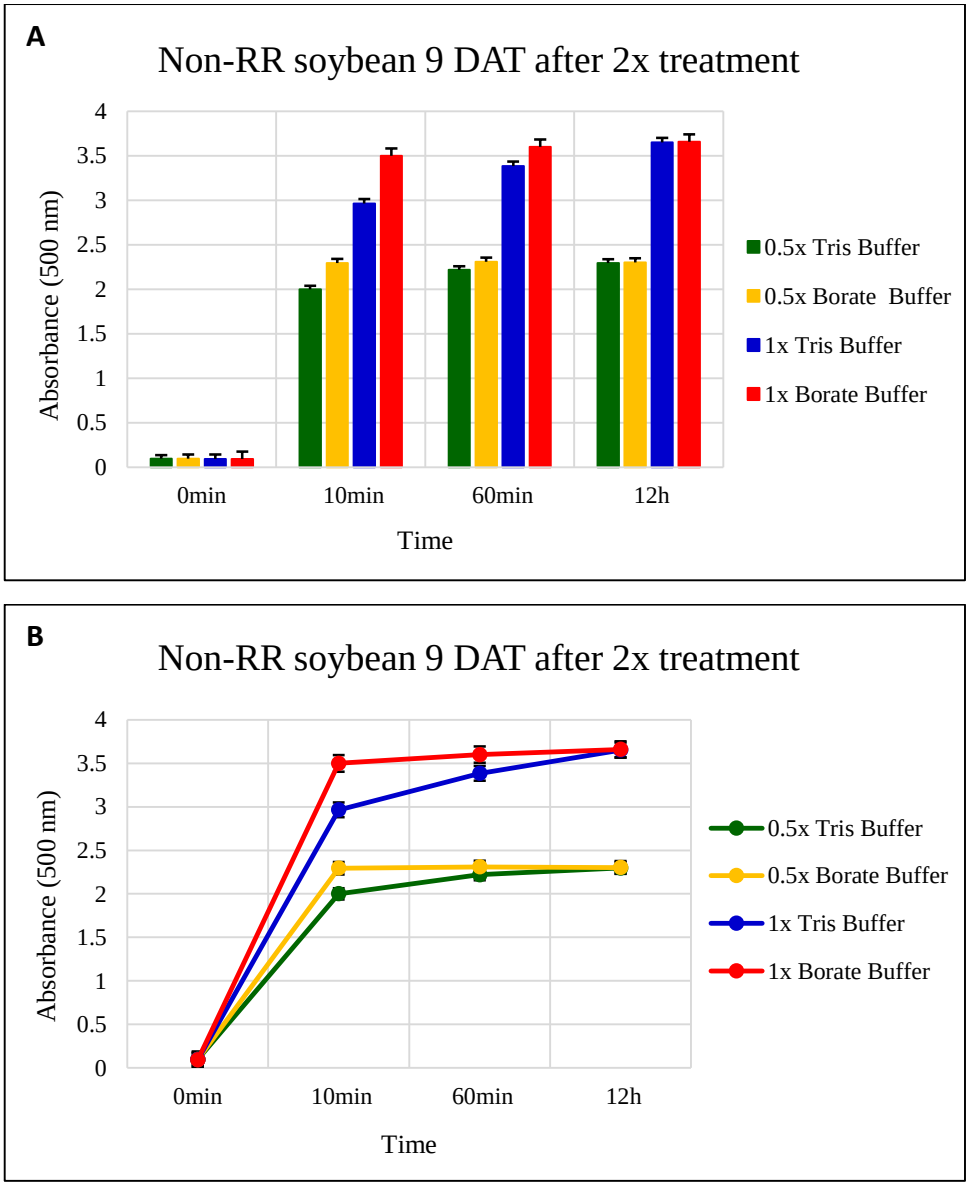
7. Leaf discs: Borate buffer versus tris buffer



APPENDIX 22. (A) Colourimetric results performed on shikimate standard using 0.5x vs 1x borate buffer; 30sec and 5min after adding SHDHD; **(B-C)** Results of borate buffer versus tris buffer performed on non-RR soybean leaf discs 10 and 25min after adding SHKDH respectively; **(D)** Colourimetric assay performed on maize leave discs treated with different glyphosate rates (1x and 2x) using 0.5x tris and 0.5x borate buffers.



APPENDIX 23. Photospectroscopy results of assay solutions from non-RR soybean leaf discs 9DAT after being treated with the recommended dosage (1x) of Roundup Turbo®. The assay was performed by using different buffers (Borate and Tris) at different concentions (0.5x and 1x). These graphs illustrates the difference in assay absorbance against time between 0.5x and 1x borate and 0.5x and 1x tris buffers; 0.5x represent half the buffer concentration and 1x the recommended buffer concentration.



APPENDIX 24. Photospectroscopy results of assay solutions from non-RR soybean leaf discs 9DAT after being treated with double the recommended dosage (2x) of Roundup Turbo®. The assay was performed by using different buffers at different concentions. These graphs illustrates the difference in assay absorbance against time between 0.5x and 1x borate and 0.5x and 1x tris buffers; 0.5x represent half the buffer concentration and 1x the recommended buffer concentration.

TABLE 2.3 Shikimic acid concentrations used to establish shikimic acid standard curves.

In assay plates					Standard curve tubes		
Number	Shikimic acid [µg/10 µL]	Final well volume (µL)	Shikimic acid [mM]	Shikimic acid [µM]	Shikimate stock [10mg/ml] (µL)	MQ.H_2O (µL)	Final volume (µL)
1	0	200	0	0	0	1000	1000
2	1	200	0.03	28.7	10	990	1000
3	2	200	0.06	57.4	20	980	1000
4	4	200	0.11	114.8	40	960	1000
5	8	200	0.23	229.7	80	920	1000
6	12	200	0.34	344.5	120	880	1000
7	16	200	0.46	459.3	160	840	1000
8	20	200	0.57	574.2	200	800	1000
9	24	200	0.69	689	240	760	1000
10	28	200	0.80	803.9	280	720	1000
11	32	200	0.92	918.7	320	680	1000
12	36	200	1.03	1033.6	360	640	1000
13	40	200	1.15	1148.4	400	600	1000
14	44	200	1.26	1263.2	440	560	1000
15	48	200	1.38	1378.1	480	520	1000
16	52	200	1.49	1492.9	520	480	1000