PROTEOMIC AND PROBIT ANALYSES OF GLUFOSINATE-AMMONIUM-RESISTANT GOOSEGRASS *(Eleusine indica* (L.) Gaertn.) BIOTYPES IN MALAYSIA

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ABSTRACT

PROTEOMIC AND PROBIT ANALYSES OF GLUFOSINATE-AMMONIUM RESISTANT GOOSEGRASS (*Eleusine indica (*L.) Gaertn.) BIOTYPES IN MALAYSIA

Goosegrass (*Eleusine indica* [L.] Gaertn.), regarded as one of the world's worst weeds is highly pernicious to cash crop growers in Malaysia. Following reports in 2009 that glufosinate ammonium failed to adequately control goosegrass populations in Kesang, Malacca and Jerantut, Pahang, Malaysia, on-site field trials were conducted to assess the efficacy of glufosinate-ammonium and glyphosate towards goosegrass from both places. Glufosinate-ammonium at 495 g ai ha^{-1} managed to provide 82% control of the weed at the vegetable farm while the same rate failed to control goosegrass at the oil palm nursery. Glyphosate failed in controlling goosegrass population at both places where the highest rate (4320 g ae ha^{-1}) produced 13% and 3% control, respectively. The efficacy of both herbicides was also tested on the Kesang and Jerantut goosegrass grown from seeds. Glufosinate-ammonium at the recommended rate provided satisfactory control of the Kesang biotype while the same rate failed to control Jerantut biotype. Glyphosate at 540 g ae ha^{-1} again failed in damaging both biotypes. The highest rate used managed to control the Kesang biotype but still did not effectively damage the Jerantut biotype. Comparison with susceptible goosegrass showed that the 'Kesang' biotype was 1 and 6-fold more resistant to glyphosate and glufosinate-ammonium respectively while the 'Jerantut' biotype was 3- and 30-fold more resistant to glyphosate and glufosinate-ammonium respectively. The low glyphosate resistance index (R.I) value for both biotypes were believed to be caused by the significant tolerance of the susceptible biotype against glyphosate. Proteomic analysis was

conducted to see any differences in the proteins expressed by the susceptible, the Kesang and the Jerantut biotypes. There were 150 matched spots between the susceptible and the Jerantut biotypes, with 4 spots differentially expressed. Between the susceptible and the Kesang biotypes, a total of 145 spots were matched, but only 3 spots were differentially expressed. Most of the differences in abundance were due to the presence or absence of a protein in either the susceptible or the Jerantut and Kesang biotypes. MALDI-TOF analysis successfully identified the identities of ten spots from the Jerantut biotype proteome. They include peptidyl-prolyl cis-trans isomerase, ferredoxin NADP+ reductase, peroxiredoxin, granule bound starch synthase, WD-repeat protein and a small subunit of RuBisCO. The remaining four proteins were unknown and hypothetical proteins. The functions of these protein ranges from folding of proteins, electron transfer, storage, DNA and RNA related processes, antioxidants and even stress-related functions. The occurrence of glufosinate-ammonium resistance in goosegrass calls for more research to better understand the resistance mechanism of this particular weed and more integrated management of the weed to prevent escalating resistance and further proliferation in the country.

ABSTRAK

ANALISIS PROTEOMIK TERHADAP BIOTIP-BIOTIP RUMPUT SAMBAU(*Eleusine indica (*L.) Gaertn.) RINTANG GLUFOSINATE-AMMONIUM DI MALAYSIA

Rumput sambau (*Eleusine indica* [L.] Gaertn), salah satu rumpai paling teruk di dunia, merupakan satu ancaman kepada para petani tanaman kontan di Malaysia. Berdasarkan laporan pada tahun 2009 berkenaan racun rumpai glufosinat-ammonium gagal memberi kawalan memuaskan terhadap populasi rumput sambau di Kesang, Melaka, dan di Jerantut, Pahang, beberapa siri ujian lapangan telah dilakukan. Ujian-ujian ini adalah untuk menilai keupayaan glufosinat-ammonium serta glaifosat terhadap rumput sambau di kawasan-kawasan tersebut. Glufosinat-ammonium pada 495 g ai ha^{-1} berjaya memberikan kawalan ke atas rumput sambau sebanyak 82% di ladang sayur tersebut manakala kadar yang sama gagal mengawal populasi rumput sambau di nurseri kelapa sawit. Glaifosat gagal sama sekali dalam mengawal populasi rumput sambau di kedua-dua lokasi, dengan kadar tertinggi (4320 g ae ha^{-1}) sekadar mencatatkan peratusan kawalan masing-masing sebanyak 13% dan 3%. Keupayaan kedua-dua racun rumpai juga telah dinilai ke atas rumput sambau daripada Kesang dan Jerantut yang ditanam daripada biji bejih. Glufosinat-ammonium pada kadar yang disyorkan berjaya memberikan kawalan memuaskan terhadap biotip Kesang manakala kadar yang sama gagal membunuh biotip Jerantut. Sekali lagi glaifosat pada kadar 540 ae ha^{-1} gagal dalam merosakkan kedua-dua biotip. Perbandingan dengan biotip kawalan mendapati biotip Kesang adalah 1- dan 6-kali ganda lebih tahan, masing-masing terhadap glaifosat dan glufosinat-ammonium manakala biotip Jerantut pula 3- dan 30-kali lebih tahan,

masing-masing terhadap glaifosat dan glufosinat-ammonium. Nilai indeks rintangan (R.I) yang rendah yang dicatatkan kedua-dua biotip terhadap glaifosat dipercayai adalah disebabkan oleh toleransi biotip kawalan terhadap glaifosat.

Analisis proteomik telah dilakukan untuk melihat sebarang perbezaan antara protein-protein yg dihasilkan oleh biotip rentan, biotip Kesang dan biotip Jerantut. Terdapat sebanyak 150 titik padanan diantara proteom biotip rentan dan biotip Jerantut, dengan hanya 4 titik yang mempunyai perbezaan ekspresi. Diantara biotip rentan dan biotip Kesang pula, sebanyak 145 titik padanan diperolehi, dengan hanya tiga titik yang mempunyai perbezaan ekspresi. Kebanyakan perbezaan adalah disebabkan kewujudan dan ketidakhadiran protein-protein samaada dalam biotip kawalan, biotip Jerantut dan biotip Kesang. Analisis MALDI-TOF berjaya mengenal pasti sepuluh protein daripada proteome biotip Jerantut. Antaranya ialah peptidyl-prolyl cis-trans isomerase, ferredoxin NADP+ reductase, peroxiredoxin, granule bound starch synthase, WD-repeat protein dan subunit kecil RuBisCO. Baki empat protein adalah protein-protein yang tidak diketahui dan protein-protein hipotetikal. Fungsi protein-protein ini merangkumi penglipatan protein-protein, perpindahan electron, simpanan, proses-proses berkenaan DNA dan RNA, antioksida serta fungsi melibatkan stress. Kejadian rumput sambau rintang glufosinat-ammonium menampakkan keperluan untuk lebih penyelidikan dalam memahami mekanisma ketahanan racun rumpai serta pengurusan rumpai yang bersepadu untuk mengelakkan peningkatan kes-kes seumpamanya di negara ini.

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Adam Jalaludin

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LIST OF COMMON ABBREVIATION

2-DE	Two dimesional electrophoresis
ACN	Acetonitrile
ae	Acid equivalent
ai	Active ingredient
APS	Ammonium persulphate
BPB	Bromophenol blue
BSA	Bovine serum albumin
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
dH_2O	Distilled water
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
g	Gram
h	hour
ha	Hectare
L	Liter
IAA	Iodoacetamide
LC	Liquid chromatography
LC_{50}	Lethal concentration that can kill 50% of the population

LIST OF COMMON ABBREVIATION (*cont.*)

ml	Mililiter
mm	Milimeter
NL	Non-linear
Nm	Nanometer
kDa	Kilo Dalton
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation-Time of Flight
min	minute
PMF	Peptide mass fingerprinting
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TFA	Trifluoroacetic acid
V	Volts
α-CHCA	α-cyano-4-hydroxycinnamic acid

CHAPTER 1

GENERAL INTRODUCTION

1.1 THE ADVENT OF RESISTANCE

"*Survival of the fittest*" (Spencer 1864). It is the one rule that all living organism that is subjected to on this planet. Living organisms have evolved to be biologically flexible and ecologically adaptable to adverse conditions in order to survive. Not all make the cut. It is a constant battle of balance in nature with survival of the species at its stake.

The use of chemical control has a long association with agriculture industry. The inception of pesticides increases crop yields while remaining economically viable. Due to this, farmers embraced the use of chemical controls with open arms. As technologies improved, more pesticides are created and usage of chemical controls includes fungi and in 1945, weeds, with the introduction of 2,4-D. Before long, chemical control became an integral part of the agricultural environment.

As nature would have it, the heavy usage of chemicals as solvers for agriculture problems, pests, fungi and weeds allow these very own problems to biochemically adapt. Insects were the first to develop resistance towards pesticidal chemicals. The first reported case was the San Jose scale resistance towards lime sulfur in 1908 (Melander 1914). In 1940, plant pathogens resistant to fungicides were cited.

Observing these trends, Harper, in 1956, was the first to predict that weed would one day develop resistance to herbicides. His assumptions, although did not have firm foundations in plant-herbicide studies, were based on current theories and preliminary data available from other biological systems. A year later, a case of 2,4-D resistance was reported (Hilton 1957). However, the first confirmed herbicide-resistance case was for *Senecio vulgaris* against triazine herbicide in 1968 (Ryan 1970).

Since then, the number of weed biotypes resistant to herbicides has been on the rise. According to the International Weed Survey of Herbicide Resistant Weeds, there are 335 biotypes from 190 species (113 monocots and 77 dicots) have been reported resistant to various herbicides (Heap 2009) worldwide (Table 1.11). In Malaysia alone, 18 biotypes belonging to 13 species were reported to be resistant against several herbicides (Heap 2009). However, it is believed more biotypes are still to be listed into the survey's database. It is estimated that there are at least 48 biotypes that are resistant to herbicides (Seng, C. T., *unpublished data*).

Table 1.1. Mechanism of herbicide resistance, and HRAC grouping with examples (Heap 2009).

Herbicide Group	Mode of Action	HRAC Group	Example Herbicide	Total
ALS inhibitors	Inhibition of acetolactate synthase ALS (acetohydroxyacid synthase AHAS)	B	Chlorsulfuron	103
Photosystem II inhibitors	Inhibition of Photosynthesis at photosystem II	C1	Atrazine	68
ACCase inhibitors	Inhibition of acetyl CoA carboxylase (ACCase)	A	Diclofop-methyl	38
Synthetic Auxins	Syntheic auxins (action like indolacetic acid)	O	2,4-D	28
Bipyridiliums	Photosystem I electron diversion	D	Paraquat	24
Ureas and amides	Inhibition of photosynthesis at photosystem II	C2	Chlorotoluron	21
Glycine	Inhibition of EPSP synthase	G	Glyphosate	16
Dinitroanilines and others	Microtubule assembly inhibition	K1	Trifluralin	10
Thiocarbamates and others	Inhibition of lipid synthesis – not ACCase inhibition	N	Triallate	8
Triazoles, ureas, isoxazolidiones	Bleaching: Inhibition of carotenoid biosynthesis (unknown target)	F3	Amitrole	4
PPo inhibitors	Inhibition of protoporphyrinigen oxidase	E	Oxyfluorfen	3
Chloroacetamides and others	Inhibition of cell division (inhibition of very long chain fatty acids)	K3	Butachlor	3
Carotenoid biosynthesis	Bleaching: Inhibition of carotenoid biosynthesis at the phytoene desaturase step (PDS)	F1	Flurtamone	2
Arylaminopropionic acids	Unknown	Z	Flamprop-methyl	2
Nitriles and others	Inhibition of photosynthesis at photosystem II	C3	Bromoxynil	1
Mitosis inhibitors	Inhibition of mitosis/ microtubule polymerization inhibitor	K2	Propham	1
Cellulose inhibitor	Inhibition of cell wall (cellulose) synthesis	L	Dichlobenil	1
Unknown	Unknown	Z	(chloro)-flurenol	1
Organoarsenicals	Unknown	Z	MSMA	1
Total Number of Unique Herbicide Resistant Biotypes				**335**

1.2.1 HERBICIDE RESISTANCE

Herbicide resistance, as defined by the Weed Science Society of America (WSSA), is the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to its wild type. In a plant, resistance may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue-culture or mutagenesis.

It is clear that herbicide-resistant weeds fall under this definition. At the same time, it must be noted that not all herbicide resistant plants are herbicide resistant weeds. There are plants that have been genetically modified to be resistant to herbicides, such as the case of glyphosate-resistant and glufosinate-resistant crops. These herbicide resistant crops (HRCs) also falls under the same definiton mentioned earlier.

Realizing the ambiguity posed by this definition, Heap and LeBaron (2001) defined herbicide-resistant weeds as "the evolved capacity of a previously herbicide-susceptible weeds population to withstand a herbicide and complete its life cycle when the herbicide is used at its normal rate in an agricultural situation".

Generally resistance towards herbicides is grouped into two, i.e. cross-resistance and multiple resistances. Cross-resistance is defined as the expression of a genetically endowed mechanism conferring the ability to withstand herbicides from different chemical classes. Cross-resistance is further categorized into two; target site cross resistance and non-target site cross-resistance.

Target site cross-resistance occurs when a change at the biochemical site of action of one herbicide also confers resistance to herbicides from a different chemical class that inhibits the same site of action in the plant. Target site cross-resistance does

not necessarily result in resistance to all herbicide classes with a similar mode of action or indeed all herbicides within a given herbicide class (Powles and Preston, 2009). For example, chemically dissimilar classes sulfonylurea and imidazolinone are both inhibitors of acetolactate synthase (ALS). Resistance of a biotype of *Lolium rigidum* through selection with sulfonylurea was caused by a change in the target site enzyme ALS (Saari *et. al*., 1994). This sulfonylurea-resistant biotype exhibits target-site resistance at various levels to other classes that are chemically dissimilar but ALS-inhibiting, nevertheless.

Non-target site cross resistance is defined as cross resistance to dissimilar herbicide classes conferred by a mechanism(s) other than resistant enzyme target sites. Non-target site cross-resistance was largely unknown in herbicide-resistant weeds but is well known in the insecticide resistance literature (Brattsten *et al.* 1986; Georghiou 1986). Only recently that non-target site cross-resistance was documented in *L. rigidum* and *A. myosuroides.* Extensive studies of biotype SLR31 of *L. rigidum* showed that resistance of this biotype to diclofop-methyl was not due to resistant ACCase. In the contrary this biotype exhibits a modest increase in the rate of diclofop-methyl metabolism (Holtum and Powles 1991).

Multiple resistance is defined as the expression (within individuals or populations) of more than one resistance mechanism. Plants with multiple resistance often possess from two to many distinct resistance mechanisms and may exhibit resistance to a few or many herbicides. Multiple resistance vary from simple to complicated cases. Simple cases are when an individual plant (or population) possesses two or more different resistance mechanisms which provide resistance to a single herbicide, or class of herbicides. More complicated and difficult to control situations

are when a number of resistance mechanisms, involving both target site and non target site resistance mechanisms, are present within the same individual.

1.2.1 Glyphosate

N-(phosphonomethyl)glycine, or glyphosate (Fig. 1.1) was first synthesized and tested as herbicide in 1971 by John E. Franz of Monsanto Company. It was then patented soon after discovering its high unit activity as an herbicide. First introduced to the commercial market in 1974 as a post-emergence, non-selective herbicide, glyphosate's popularity grew steadily over the years for several reasons and it has now become the dominant and arguably, the most important herbicide worldwide.

Glyphosate works as a herbicide by inhibiting the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) of the shikimate pathway (Fig. 1.2). This is possible as glyphosate is a transition state analog of phosphoenylpyruvate. The EPSPS inhibition causes reduced feedback inhibition of the pathway, resulting in enormous amount of carbon flow to shikimate-3-phosphate, which is then transformed into shikimate. How exactly inhibition of the shikimate pathway by glyphosate kills the plant remains vague. To date, many researchers believe that it is due to the insufficient aromatic acid production and/or attributed to the shortage of carbon flow to other essential pathways.

Being a non-selective herbicide, glyphosate works on a wide range of plant species when applied to foliage. Higher plants EPSPS are also inhibited by glyphosate. Few plant species such as conifers and *Cynodon dactylon* exerts remarkable resistance to foliage treatment with glyphosate. However, with no other analogs or alternative chemical classes that targets the EPSPS in the market, glyphosate has found usage in the

broadest of all areas, ranging from croplands to plantations and orchards, in industrial and recreational industries and even among home users.

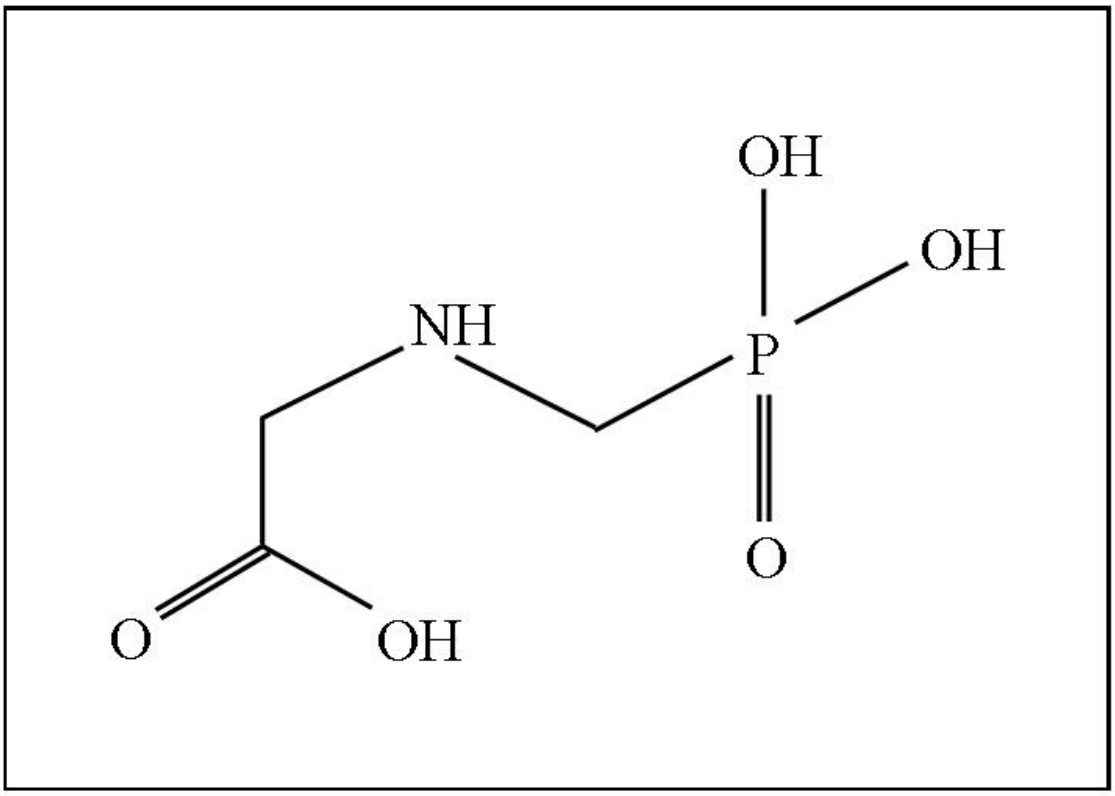

Fig. 1.1. Structure of *N*-(phosphonomethyl)glycine or glyphosate (adapted from http://www.alanwood.net/pesticides/glyphosate.html).

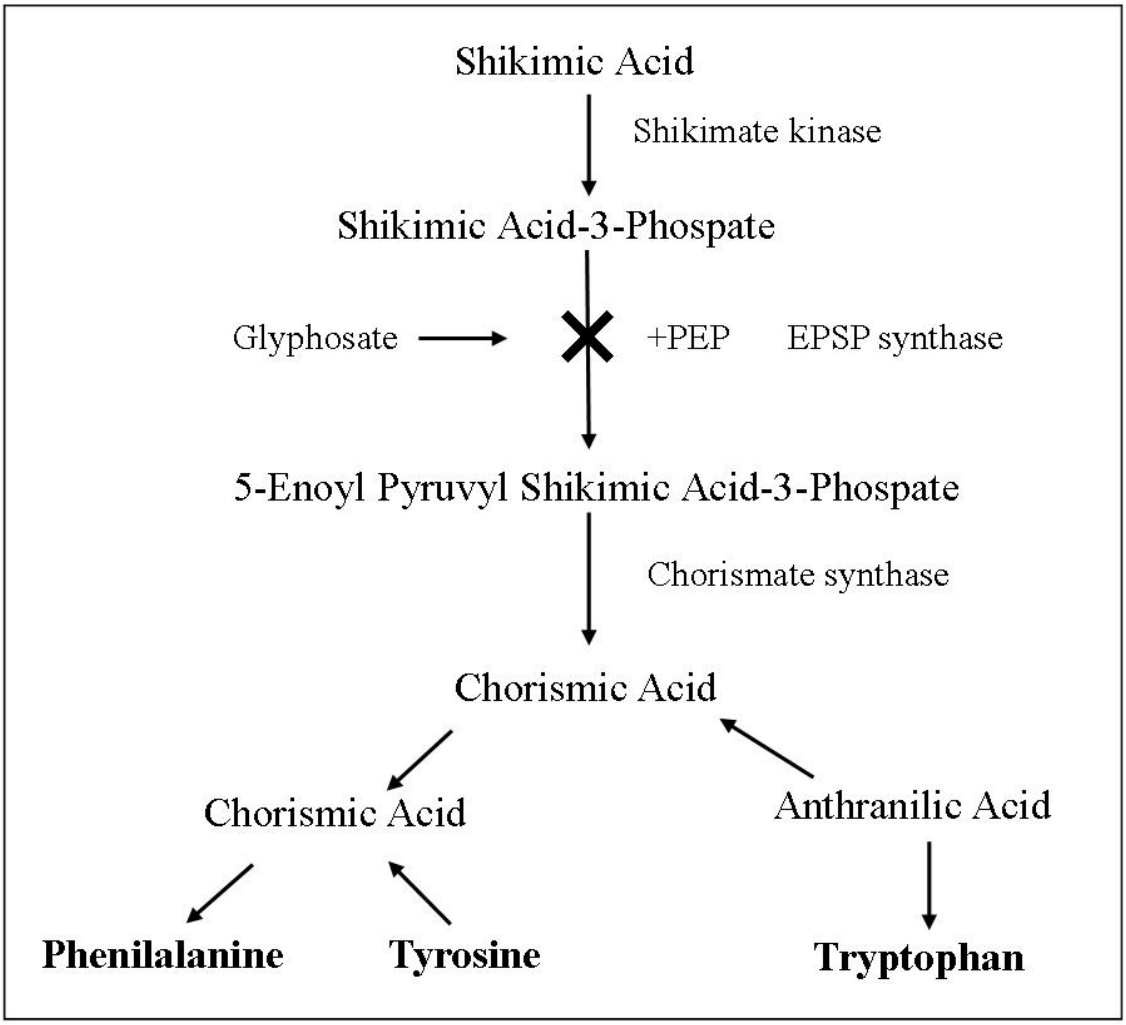

Fig.1.2. Glyphosate inhibits the 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) of the shikimate pathway (Dill 2005).

Glyphosate enters the plant through plant surfaces. It is then translocated rapidly from the foliage to the roots, rhizomes, apical meristems and other metabolic sinks for sucrose via the phloem. This property culminates in the total destruction of hard-to-kill perennial rhizome weeds such as *Sorgum halepense*, *Cyperus* spp., *Imperata cylindrica* and *C. dactylon*. In contrast with other herbicides which only destroys the above ground plant portion, glyphosate destroys both the above and the lower ground portion.

Regardless of its high unit activity as a herbicide, glyphosate shows no pre-emergence or residual soil activity (when applied post-emergence), making it an environmentally benign herbicide. This is possible since glyphosate binds tightly to soil particles. Only aminophosphonic acid (AMPA), one of glyphosate degradation product, is notably more mobile than glyphosate in soil. Glyphosate has a short environmental half-life, due to the microbial degradation in the soil into plant nutrients phosphoric acids, ammonia and carbon dioxide.

Glyphosate is also one of the least toxic herbicides to humans and animals, with an LD_{50} of 5 g/kg and above for rats. Tests carried on a range of species showed that the glyphosate has caused virtually no sub-acute, acute, chronic or neurotoxic effects when applied in the range of concentrations that is normally used or found in treated subjects (http://www.syngenta.com/country/au/SiteCollectionDocuments/Labels/INNOVA%20GLYPHOSATE%20450%20HERBICIDE%20MSDS.pdf).

Due to its non-selective nature, glyphosate could not be easily used within arable crops, since crop species are also susceptible to it. It all changed in 1996, where transgenic glyphosate-resistant crops were introduced. Transgenic glyphosate-resistant crops such as soybean, maize, canola and cotton now dominate in agriculture fields in countries such as Argentina, Brazil, Canada and the USA. This, coupled with the fact that glyphosate has become much cheaper since the introduction of its generic and the added values of glyphosate, has made glyphosate become the most important and successful herbicide in the world today.

1.2.2 Glufosinate-Ammonium

Glufosinate or glufosinate-ammonium (Fig. 1.3) was first introduced in Malaysia in 1985 under the commercial name of Basta®. It is a phosphinic acid and was listed under group H of the Herbicide Resistance Action Committee (HRAC). It is a broad spectrum, non-selective systemic herbicide.

Glufosinate-ammonium works by inhibiting the activity of glutamine synthase, the enzyme that converts glutamate plus ammonia to glutamine (Fig. 1.4). Accumulation of ammonia in the plant destroys the plant cell. This causes photosynthesis to be severely inhibited. Ammonia reduces the pH gradient across the membrane which can uncouple photophosphorylation. To date there is no known cases of weed resistant to glufosinate. However with the recent development of more than 100 varieties of glufosinate-resistant plants and increasing resistance of weeds to glyphosate and other herbicides, glufosinate ammonium usage is significantly increasing throughout the world including Malaysia.

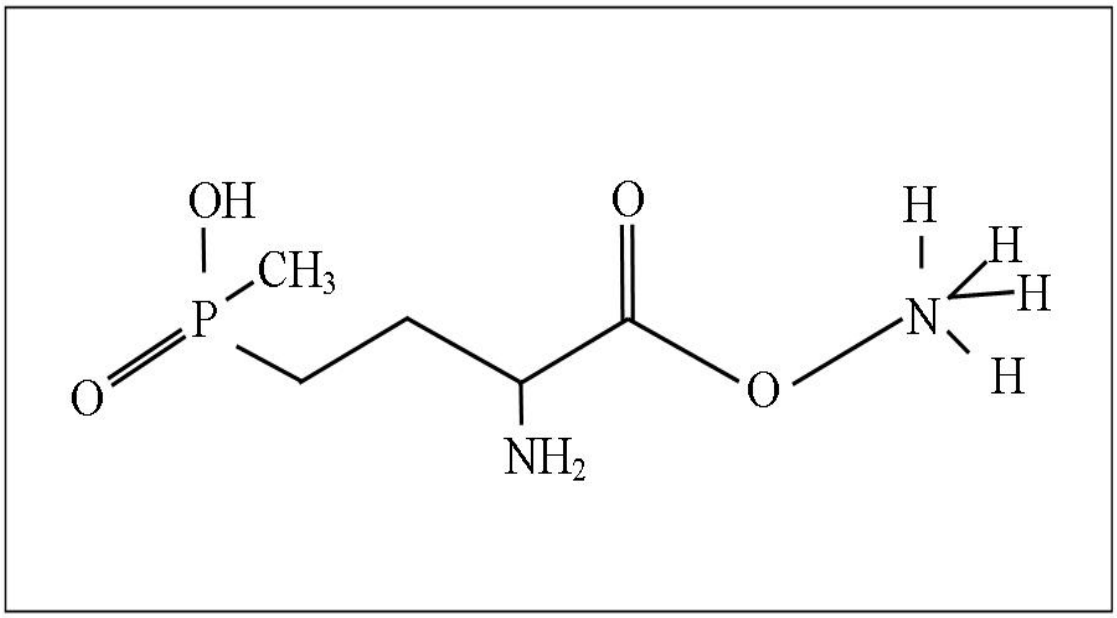

Fig. 1.3. Structure of glufosinate-ammonium (adapted from http://www.chemblink.com/products/77182-82-2.htm).

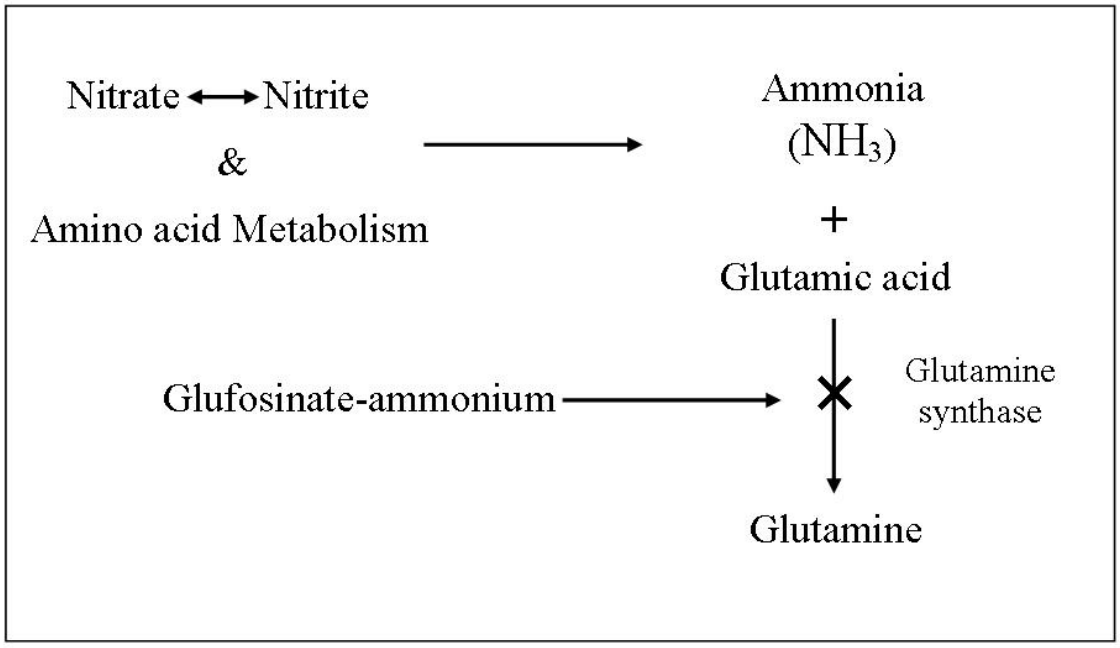

Fig. 1.4. Glutamine synthase inhibition by glufosinate-ammonium (adapted from Ahn 2008)**.**

1.3 GOOSEGRASS (*Eleusine indica*)

*Eleusine indica (*L.) Gaertn is a monocot weed that belongs to the Poaceae family. Common names for it includes goosegrass and/or wiregrass and Malaysians call it 'rumput sambau' or 'rumput kuda' and sometimes 'cakar ayam'. Its culms are erect, prostrate and branching from 5 to 50 cm long. The foliar are linear and smooth, and can reach up to 20 cm long. Inflorescence are digitate, with spikelets subdigitately arranged and contains 3 to 9 fertile flowers. Although *E. indica* have a rather short lifespan, they flower all year round. They prefer low-moistured soils and can also be found in wastelands, roadsides and croplands throughout Malaysia. It grows best in moist, fertile, cultivated soil in full sunlight, and once established is difficult to eradicate (Swarbrick 1997).

A single plant of *E. indica* may produce more than 50,000 small seeds, which move readily by wind, in mud on the feet of animals and in the tread of machinery. The seeds are eaten by wild and domestic animals. It is believed that *E. indica* was an introduced invasive and not an original weed of Malaysia, although the place/country of origin still remains a mystery.

Known as a sun-loving weed, *E. indica* is harmful to crops during the seedling stage. Being a rhizomatous weed, it matures, propagates and spreads very rapidly. As such, they are very competitive to crop seedlings in acquiring nutrients from soil. Due to this, goosegrass is very undesirable to farmers and is often weed out with herbicides, as exemplified by glyphosate or glufosinate.

1.3.1 Resistant Goosegrass in Malaysia

Intensive use of herbicides with the same mode of action and lack of integrated weed management has given rise to goosegrass that are resistant to herbicides. In 1989, the first case of goosegrass resistant to fluazifop-butyl was recorded in Malaysian farm due to repetitive usage (Leach *et al*. 1993). Acquiring resistance to fluazifop-butyl suggested that they may also be cross-resistant to other herbicides in the A/1 Group. It was then discovered a year later that there are goosegrass biotypes resistant to group D/22 herbicides. Group D/22 is the Bipyridillums (Photosystem-I-electron diversion). Research has shown that these particular biotypes are resistant to paraquat and they may be cross-resistant to other Group D/22 herbicides.

Group A/1 herbicides on the other hand are known as ACCase inhibitors (Inhibition of acetyl CoA carboxylase (ACCase). Studies have proved that these particular biotypes are resistant to fluazifop-P-butyl, and propaquizafop and they may

also be cross-resistant to other herbicides in the A/1 Group. The multiple resistance of *Eleusine indica* further evolved when in 1997 resistance of this biotype to glyphosate (herbicide group G/9) was reported.

Although it already developed multiple resistances to herbicides from group D/22 and Group A/1, the inclusion of glyphosate in the list is truly worrying. This is because unlike other herbicides, glyphosate's mode of action is non-selective.

1.4 PROTEOMICS

The word proteomics originated from the word proteome, which was introduced by Wilkins *et al.* (1995) to describe the protein complement of the genome. Simply put, proteomics refers to the study of the proteome. A more refined definition of the word would be the high-throughput identification and analysis of proteins. Normally the objectives of proteomic research are to investigate protein expressions, quantification, function under specific biological function and protein identification of resolved proteins (Zazali 2004; Thelen 2007). A normal approach in most proteomic research involves separating the proteins (two dimensional gel electrophoresis), visualising and quantification of the protein spots (staining and scanning) and identification of the proteins (mass spectrometry).

1.4.1 Two Dimensional Gel Electrophoresis

The two dimensional gel electrophoresis (2-DE) were first applied (1975), around the same time at which SDS-PAGE was introduced. It separate proteins on the basis of their isoelectric point (pI) by isoelectric focusing (IEF) and molecular weight (PAGE or SDS-PAGE), hence the two dimensional term. Extremely powerful in its

resolving capacity, it suffers major drawbacks from reproducibility issues due to the fragile tube gels used for IEF. Only after the introduction of immobilized pH gradient (IPG) strips (Görg *et al.* 1978, 2000) saw the resurgence of this technique.

In IEF, protein samples were first solubilised in rehydration buffer. A typical solution generally contains urea, non-ionic or zwitterionic detergent such as CHAPS, TRITON X100 or NP-40, DTT, carrier ampholytes and a tracking dye. Urea solubilises and denatures proteins while thiourea further improves protein solubilisation, especially for hydrophobic proteins. The non-ionic/ zwitterionic detergents help solubilise hydrophobic proteins and minimize protein aggregation. Dithithreitol (DTT) acts as a reducing agent. Carrier ampholytes were used to improve protein separation, enhance protein solubility and produce more uniform protein conductivity across the pH gradient.

IPG strips were then rehydrated prior to focusing. The sample is applied along with the rehydration solution or by cup loading onto hydrated IPG strips. Following focusing, IPG strips undergo a two-step equilibration process. The equilibration solution contains urea, glycerol and SDS. Urea together with glycerol reduces the effects of electroendosmosis by increasing the viscosity of the buffer (Görg 2000). SDS denatures proteins and forms negatively charged protein-SDS complexes. In the first step, DTT was added to the equilibration solution to ensure the proteins are fully reduced. Iodoacetamide (IAA) was introduced in the second step to alkylate thiol groups on proteins, preventing their reoxidation during electrophoresis. It also alkylates residual DTT and minimizes unwanted reactions of cysteine residues with acrylamide monomers (Bonaventura *et al*. 1994).

In the second dimension, isoelectrofocused proteins are separated by molecular weight in polyacrylamide gels containing sodium dodecyl sulphate (SDS-PAGE). The

tris-glycine buffer system described by Laemmli (1970) was used. Equilibrated IPG strip(s) is pushed down until it touched the gel surface. Bubbles between the gel surface and the strips are eliminated and the strip(s) is sealed with agarose sealing solution to prevent movement of the strip.

1.4.2 In-Gel Detection of Proteins

There are various staining procedures for visualisation of proteins. Important considerations include the ease of use, reliability, sensitivity and compatibility with mass spectrometry (MS) analysis. Two of the more preferred staining methods are silver staining and coomassie staining using coomassie brilliant blue (CBB).

Silver staining is often preferred due to its high sensitivity which is up to 1 ng (Ocbs *et al.* 1981; Shevchenko *et al.* 1996). Because silver forms complexes with nucleophilic groups, such as the $–NH_2$ of lysine (Rabilloud 1990), silver staining intensity correlates with lysine content in the protein (Mortz et *al.* 2001). Originally it was not compatible with MS analysis due to the incorporation of glutaraldehyde in its procedures. The use of aldehyde-based sensitizers, which promotes the binding of silver to proteins, prevents total digestion of peptides and reduced the efficiency of peptide extraction. This is because aldehyde(s) modify and crosslink with lysine residues (Shevchenko *et al.* 1996). Shevchenko *et al.* (1996) described a method where he overcomes this problem by replacing the aldehyde(s) with sodium thiosulfate. However, silver staining still suffers from other problems such as inferior reproducibility, poor linear dynamic range and non-quantitative negative staining of some modified proteins (Wilkins and Gooley 1998; Görg *et al.* 2000; Westermeier and Naven 2002). Silver staining has a linear dynamic range of one order of magnitude (Patton 2000).

Coomassie brilliant blue (CBB) staining is, traditionally preferred, due to its ease of use and compatible with subsequent mass spectra analysis. There are two chemical forms of CBB, the R-250 and the G-250. Both variants have a linear dynamic range up to one order of magnitude, but they differ greatly in their sensitivity, quantitative linear range and destaining properties. Since G-250 is better than R-250 in all of these aspects, it is recommended for proteomic applications. However, the limitation of CBB dye is its sensitivity, which ranges from 200 – 500 ng protein/spot with conventional methods using R-250 (Wilson 1979). However, this limit is overcome when Neuhoff *et al.* (1985, 1988) reduce the detection limit to about 10 – 30 ng protein/spot by using large amount of ammonium sulfate in acidic alcoholic media where the dye molecules are aggregated into colloidal particles. Kang *et al.* (2002) reported improved sensitivity and faster staining time of colloidal CBB staining by adding aluminium sulphate and replacing methanol with ethanol. Another modified colloidal CBB staining by Candiano *et al.* (2004), called 'Blue Silver' reported even higher sensitivity, comparable to that of silver staining.

Fluorescent protein stains, such as SyproRuby™, Deep Purple™ and ruthenium II, are also becoming more prominent as the method of choice for protein visualisation. These broad dynamic range fluorescent protein stains have higher sensitivities than CBB (some as sensitive as silver staining), and often have a linear dynamic range of more than one order of magnitude (Rabilloud *et al.* 2000, 2001; Steinberg *et al.* 2000; Chevalier *et al.* 2004). They are also compatible with MS analysis. Cyanine-based fluorescence dyes, which are used in difference gel electrophoresis (DIGE), enables detection of protein differences in two samples/populations (Tonge *et al.* 2001).

1.4.3 Peptide Mass Fingerprinting (PMF)

Peptide mass fingerprinting (PMF) is a technique for protein identification. Proteins are cleaved by protease into smaller peptides, which are measured by mass spectrometry such as MALDI-TOF (Matrix Assisted Laser Desorption/ Ionization-Time of Flight) or ESI-TOF (Electrospray Ionization-Time of Flight). Identification is accomplished by matching the observed peptide masses to the theoretical masses derived from a sequence database (Pappin *et al.* 1993; Henzel *et al.* 1993; Mann *et al.* 1993; James *et al.* 1993; Yates *et al.* 1993; Clauser *et al.* 1993). Because only the mass of the peptides need to be known, PMF is less time consuming compared to the conventional de novo sequencing of peptides/ proteins.

1.4.4 MALDI-TOF Mass Spectrometry

Matrix assisted laser desorption/ ionization is a technique most commonly used to ionize proteins or peptides for MS analysis. MALDI instruments are often coupled together with time-of-flight (TOF) analyzer, which measures the mass of intact peptides. In mass spectrometry (MS), analytes need to be ionized into a gas phase. This creates a problem for large macromolecules, like proteins and peptides. Although transforming them into gas phase is possible, it was always considered an Augean task. The development of MALDI-TOF MS tremendously simplifies analysis of large macromolecules, and enables them to be analyzed in various physical states (flowing, liquid solution or dry, crystalline state) (Fenn *et al.* 1989; Tanaka *et al.* 1988; Karas and Hillenkamp 1988).

In MALDI-TOF, samples are first excised from gels and undergo in-gel digestion by proteolytic enzymes, such as trypsin, endoprtotease Glu C (V8 protease), Endoprotease Lys C and endoprotease Asp N. These enzymes are site-specific, meaning they cleave at certain amino acids in the peptide. The most commonly used proteolytic enzyme in proteomic, trypsin, cleave at only 2 of the twenty amino acids, e.g. lysine and arginine at the C-terminal side, except if they are attached to proline in the C-terrminal direction. This site-specific property allows the production of a whole list of expected fragments masses for every protein in any sample. Accurate mass determination often requires a minimum of at least four proteolytic peptides.

The digested protein are then mixed with crystalline matrix such as 2,5-hydroxybenzoic acid (DHB), 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) or α-cyano-4-hydroxycinnnamic acid (α-CHCA), and spotted onto a plate to co-crystallize. The plate is inserted into the MALDI instrument and bombarded by a laser, volatizing and ionizing the samples to singly charged ions in a gas phase. The TOF analyzer then measures the mass of intact peptides. The mass fingerprint, i.e. the list of peptide mass derived from the mass spectrum for each protein, are identified by matching the experimentally determined peptide masses with those calculated from entries in sequence databases (Hurkman and Tanaka 2007).

1.4.5 Protein Identification

In order to identify proteins from the peptide masses, several search softwares are available. These softwares include open source programs, such as Aldente (Gasteiger et al. 2005) and ProFound (Zhang and Chait 2000), and commercial ones like MASCOT (Perkins et al.) and SEQUEST (Yates 1998). Most of the open source

programs are available online while the commercial ones often come as a package with the instrument. Some of the commercial programs are also available online for free via web interface. These programs use sophisticated algorithms and probability-based statistics in order to define the best match between the experimental data and a sequence in the database. Examples of the databases used by these search softwares include NCBI NR (National Centre for Biotechnology Information; http://www.ncbi.nlm.nih.gov/protein), SWISS-PROT and TrEMBL (http://expasy.org/sprot/). The choice of program is often based by the experience of the user. A list of protein search programs is available at http://www.peptideresource.com/proteomics.html.

For example, ProFound employs a Bayesian algorithm to identify proteins, taking into account individual properties of the proteins in the database and other relevant informations, such as molecular weight, pI, chemical modification, etc., that are relevant to the experiment. Currently the database that is used by ProFound is the NCBI NR (nonredundant) database (http:// www.ncbi.nlm.nih.gov/BLAST/blast_databases.html). The three most important criteria used in order to distinguish the highest possibility of a protein from the search result being the sample protein are the Z score, the probability and the percentage of the sequence coverage.

An estimated Z score is the distance to the population mean in unit of standard deviation. It also corresponds to the percentile of the search in the random match population. The estimated Z score is generated as an indicator of the quality of the search result. It is generated when the search result is compared against an estimated random match population. For example, an estimated Z score of 1.65 above for a search means that the search is in the 95th percentile. In other words, there are only about 5%

of random matches left that could yield higher Z scores than this search. Other values of Z score are 1.282, 2.326, and 3.090, corresponding to 90.0th, 99.0th, and 99.9th percentile (http://prowl.rockefeller.edu/prowl/profound_help.html).

The probability provided in the search result is the normalized probability that a protein in a database is the protein being analysed based on data, experimental conditions and other background information, provided prior to the search. This Bayesian probability should be viewed as a measure of the confidence level of the hypothesis that protein searched is the sample protein based on the available information. The higher the probability, the higher the confidence level is. However it should be remembered that there are no absolute certainty for any given identification, only the probability (Zhang and Chait 2000). The percentage coverage on the other hand shows how much of the protein sequence covered by matched peptides to the whole length of protein sequence.

1.5 OBJECTIVES OF STUDY

The objectives of this research are:

a) To identify and ascertain new biotypes of goosegrass that is resistant to glufosinate-ammonium in Malaysia.

b) To evaluate the resistance level of goosegrass biotype(s) that is/are resistant to glufosinate-ammonium and glyphosate.

c) To obtain 2-D gel analysis of the proteins in herbicide-resistant goosegrass biotype(s).

1.6 STRUCTURE OF THESIS

The work embodied in this thesis is divided in five chapters. Chapter 1 (General Introduction) discuss briefly on herbicide resistance status in the world while focusing on herbicide resistance status in Malaysia, primarily involving goosegrass, herbicides glyphosate and glufosinate ammonium with some notes on proteomics.

The materials used throughout this research are listed in Chapter 2 (Materials and Methods). This chapter also describes the methodology employed in evaluating the resistance of goosegrass and in obtaining the proteome map of *Eleusine indica*.

Chapter 3 (Results) focuses primarily on the preliminary evaluations of resistance level of goosegrass under both field and greenhouse conditions to glufosinate-ammonium and glyphosate. Further evaluations of goosegrass grown from seeds are also included. The proteome map of proteins in *Eleusine indica* are described. Comparisons of proteome map between susceptible and resistant biotypes of goosegrass are described and discussed.

Chapter 4 collates the findings in the preceding chapter and some discussions are included in this chapter.

Finally, Chapter 5 embodies the conclusion based on the discussions in the previous chapter.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Plant Materials

Goosegrass (*Eleusine indica*) used in this study was collected from Kesang, Malacca (subsequently called the Kesang biotype) and Tun Razak Centre for Agricultural Research (PPPRT) of Jerantut, Pahang (subsequently known as the Jerantut biotype). Susceptible goosegrass biotype were collected from urban housing areas without any history of herbicide treatments.

2.1.2 Chemicals

All chemicals used were of analytical grade unless stated otherwise.

BDH Laboratory Supples, Poole, England

- Bromophenol Blue

Bio-rad Laboratories, Richmond, USA

- 0.5M Tris-HCl buffer pH 6.8
- 1.5M Tris-HCl buffer, pH 8.8
- 30% Acrylamide/Bis solution, 37.5:1 (2.6% C)
- 10X Tris/ Glycine/ SDS buffer
- Ready Strip™ (70 mm, pH 3-10 NL)

Invitrogen™, California, USA

- BENCHMARK™ Protein Ladder
- ZOOM® Carrier Ampholytes 3-10

Merck KGaA. Darmstadt, Germany

- Dithiothreitol (DTT),
- Iodoacetamide (IAA),
- 2-mercaptoethanol
- N,N,N',N'-Tetramethylethylenediamine (TEMED)
- Sodium hydroxide (NAOH)
- Tris(hydroxymethyl)aminomethane

R & M Chemicals, Malaysia

- Ammonium persulphate (AP)
- Sodium dodecyl sulphate (SDS)

Sartorius Stedim Biotech, Germany

- Vivaspin 20 (10 000 MWCO PES)

Sigma-Aldrich, St. Louis, USA

- Brilliant Blue G (Coomassie Blue G-250)
- Protease Inhibitor Cocktail
- Thiourea

Syngenta Crop Protection Sdn. Bhd., Selangor, Malaysia

- Glufosinate-ammonium (commercial grade)
- Glyphosate (commercial grade)

Systerm, Malaysia

- Acetic acid (glacial)
- Acetone
- Ammonium sulphate
- Ethyl alcohol 95%
- Formaldehyde
- Glycerol
- Methanol
- Hydrochloric acid
- Ortho-phosphoric acid
- Sodium phosphate monobasic
- Sodium phosphate dibasic
- Sodium thiosulphate
- Urea

2.1.3 Instrumentation

- Centrifuge – Heraeus Biofuge® Stratos
- Electrophoresis cell – Mini PROTEAN® Tetra Cell, Bio-Rad
- Liquid chromatography - ÄKTA Prime Plus, Amersham Biosciences
- Column - HiPrep™ 26/10, Desalting (50 ml), GE Healthcare, USA
- Isoelectric Focusing – Ettan IPGphor 3, GE Healthcare
- Mass spectrometry – Sciex TOF/TOF 5800 Mass Spectrometer, Applied Biosystems
- Power Supply – PowerPac™ Basic, Bio-Rad
- Scanner – Image Scanner III, GE Healthcare
- Spectrophotometer – JASCO V-630 UV-Vis Spectrophotometer

- Sprayer - PB-20 Knapsack Sprayer, Cross Mark® and Hudson Planter Mist 6911.
- Weighing balance – Mettler B204-S

2.2 METHODS

2.2.1. On-site Field Trial and Greenhouse Evaluation

A field trial was set up in the farmer's vegetable farm in Kesang, Malacca (GPS coordinate 2N 19' 58.1262", 102E 21' 58.575") and in the oil palm nursery in Jerantut, Pahang (GPS coordinate 3N 51' 25.2, 102E 33' 43.92"). Plots of 2 m × 1 m were laid out with 3 replicates for each plot, and were arranged accordingly in a randomized complete block design. Glufosinate-ammonium was sprayed onto *Eleusine indica* plants using a flat fan nozzle sprayer calibrated to deliver 450 L/ha (PB-20 Knapsack Sprayer, Cross Mark®) at four different rates ranging from 247.5 g a.i. ha^{-1} to 1980 g a.i. ha^{-1} (Kesang farm), and from 495 g a.i. ha^{-1} to 3960 g a.i. ha^{-1} (Jerantut palm oil nursery) including untreated control plots. Glyphosate was also tested at both Kesang and Jerantut fields, with rates ranging from 540 g a.e ha^{-1} to 4320 g ae ha^{-1}. All herbicide spraying were conducted in early morning on a clear weather. Most of the goosegrass were matured and at seed producing stage. The goosegrass were in excess of 90% coverage and interaction with other weed species, if any, would be minimum. Interactions with other weed species were not taken into consideration in this study. Visual estimates of percentage damage due to herbicide treatment based on leaf and stem necrosis at weekly intervals for 4 consecutive weeks, based on a scale of 0 to 100% (0 = no damage, 100 = total control).

In order to rule out environmental factors (e.g. rain, humidity and light) and agronomic factors (e.g. soil type, water stress and soil pH) which may affect the efficacy of herbicides on the goosegrass, cuttings from the field that survived the herbicide treatment were collected and transplanted into pots in a greenhouse (30°C/ 25°C day/ night temperature, 75% relatuve humidity and an average light intensity of 400 $\mu Em^2 s^{-1}$) in the Institute of Biological Sciences, University of Malaya, Kuala Lumpur, Malaysia (GPS coordinate 3N 7' 52.64", 101E 39' 25.25"). In order to evaluate the resistance level of both the 'Kesang' and 'Jerantut' biotypes, susceptible samples of goosegrass towards glufosinate-ammonium were collected from urban housing areas with no history of herbicide treatments.

Cuttings of goosegrass were transplanted into unsterilized potting soil in 10 cm^2 pots with 0.3 cm of the shoot buried (a maximum period of 7 days was allowed until the cuttings are transplanted). The pots were kept inside the greenhouse and watered twice daily from above using a fine hose. After the leaves have regenerated to about 3 cm long, the pots are moved outside the greenhouse to allow maximum sun exposure. Once the leaves were about 7 to 20 cm long, the goosegrass plants were treated with glufosinate-ammonium at 495, 990, 1980, and 3960 g a.i. ha^{-1} with three replicate pots per treatment using similar spray application equipment described earlier at a spray volume of 450 L ha^{-1}. The goosegrass were also treated with glyphosate with rates ranging from 540 g ae ha^{-1} to 4320 g ae ha^{-1}. Sampling and assessment on the herbicide efficacy were based on the Syngenta's Quick Test method (Boutsalis 2001) with slight modifications. Visual estimates of percentage damage of goosegrass following glufosinate-ammonium and glyphosate treatments were carried out in the same manner as those employed in the on-site field trial.

2.2.2. Statistical Analysis

The percentage of control of goosegrass as a result of glufosinate ammonium treatment was subjected to Probit Analysis (Finney 1971) using the statistical software SPSS (SPSS Statistics 17.0) to determine the LC_{50} values. The resistance indices for each biotype were also calculated.

The data from field and greenhouse experiments were collated and subsequently subjected to ANOVA. Prior to ANOVA, the percentage of control data were transformed to log + 5. Treatment means were then subjected to Tukey's tests to determine significant differences between them, if any.

2.2.3 Seed Test

Prior to the on-site field trial, mature goosegrass seeds were collected from respective places. The seeds were air dried and stored in paper envelope to prevent rapid heating (Moss 2009). The seeds were germinated in unsterilized potting soil in 10 cm^2 pots and labelled accordingly. Germinated seedlings were grown outdoors and watered accordingly.

Once the leaves have grown to 7 to 20 cm long, glufosinate-ammonium and glyphosate were sprayed at four different rates for each herbicide as described in 2.2.1, using the same spray application equipment with similar spray volume (450 L ha^{-1}) as described earlier. Visual estimate of percentage damage, Probit analysis and statistical analysis were carried out similarly as in Section 2.2.2.

2.2.4 Protein Extraction

Goosegrass seeds of Kesang, Jerantut and susceptible biotypes were germinated separately in 30 cm x 65 cm x 5 cm seedling tray. Once the seedlings have reached 3 to

5 tiller stage, they were uprooted. Shooting were removed from the root, frozen (shoots) in liquid nitrogen and pulverized into fine powder with a mortar and pestle. From here on all steps were carried out at 4 °C unless stated otherwise. The procedure was adapted from Cummins *et al.* (1997), with slight modifications. The powder was suspended in extraction buffer (5 ml of extraction buffer for each gram of powder; Appendix C-1) mixed with protease inhibitor cocktail and filtered through 2 layers of muslin cloth. The homogenate was then centrifuged at 12000 rpm for 40 min at 4 °C. Ammonium sulphate precipitation was carried out, up to 80% saturation. The homogenate was centrifuged again at 12000 rpm for 10 minutes at. Protein pellets was dissolved in buffer A (Appendix C-1) and filtered using syringe filter (0.45µm) before being applied onto prepacked Sephadex G-25 column (HiPrep™ 26/10, Desalting, 50 ml). The column was connected to ÄKTA Prime Plus and was equilibrated with buffer A up to 3 times column volume. Sample was then loaded into 5 ml sample loop and injected into the column. During sample application the flow rate was set at 2.5 ml/min and the sample was eluted with buffer A. Flow rate at 5.0 ml/min were also tested to see whether there were any differences in the elution profile. The protein profile was monitored at 280 nm. Fractions of 5 ml were collected and fractions containing peaks were pooled. Pooled fractions were then concentrated with 20 ml concentrator (Vivaspin 20, MWCO 10kD) and saved for further analysis. Several flow rates were tested to determine whether there were any differences in the elution profile.

2.2.5 Protein Estimation (Bradford assay)

The protein content determination was conducted as described by Bradford (1976) and the Bradford reagent was prepared as in Appendix C-2. Each time protein estimation was carried out, a standard curve was constructed. Protein standards were

prepared in duplicates. Increasing volumes (10 to 50 µl) of stock BSA solution (2 mg/ml) were added into different test tubes and volume in each test tube was made to 100 µl with buffer A. The blank was prepared by pipetting 100 µl of buffer A into a test tube. Unknown samples were prepared in dilution of 2.5 or 5 fold. To each standard and sample, 5 ml of Bradford reagent was added and shaken well. After 5 minutes and before 1 h of incubation, absorbance reading was taken at 595 nm on JASCO V-630 UV-Vis Spectrophotometer. Data obtained were plotted as average absorbance at 595 nm against amount of BSA. The protein content of the sample(s) was estimated from the standard curve as shown in Appendix C-2. For diluted sample(s), the amount generated was multiplied with the dilution factor.

2.2.6 Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed using Mini PROTEAN® Tetra Cell electrophoresis units with a Bio-Rad PowerPac™ Basic power supply. Commercial 0.5 Tris-HCl, pH 6.8, 1.5 M Tris-HCL, pH 8.8, 30% Acrylamide/Bis solution, 37.5:1 (2.6% C) and 10X Tris/ Glycine/ SDS buffer were used throughout the experiment. The assembly and preparation of the apparatus, other buffers and reagents were as described in the instruction manual provided and listed in Appendix C-3, based on Laemmli (1970).

2.2.6.1 Gel Preparation

The 4% stacking gel and 12% resolving gel were prepared as shown in Table 2.1.

Table 2.1. Stacking and resolving gel formulations.

Solution	12% Stacking Gel	4% Resolving Gel
dH_2O (ml)	3.4	6.1
30% Acrylamide/Bis (ml)	4.0	1.3
Gel buffer* (ml)	2.5	2.5
10% (w/v) SDS (ml)	0.1	0.1

*Stacking gel buffer is 0.5 M Tris-Hcl, pH 6.8 while resolving gel buffer is 1.5 M Tris-Hcl, pH 8.8.

The monomers were prepared by mixing all the reagents except TEMED and APS. The solutions were then degassed for 15 minutes. Prior to pouring gel into gel cassettes, 5 µl of TEMED and 50 µl of 10% (w/v) APS were added (resolving gel) and 10 µl of TEMED and 50 µl APS (stacking gel) and swirl gently to initiate polymerization. Once the stacking gel has been poured, 200 µl of overlay solution was laid onto the top of the gel solution and left to polymerized. Only after the stacking gel has polymerized was the resolving gel poured. A comb was inserted to create wells and the gel was left to polymerize.

2.2.6.2 Electrophoresis

Before loading samples into the wells, the wells were rinsed 3 times with running buffer. Equal volumes of samples were loaded into the wells. The electrophoresis was run at 120 V for 2.5 h. For molecular weight estimation, a protein standard (BENCHMARK™ Protein Ladder) was loaded into a free well along with the samples.

2.2.7 Two-Dimensional (2-D) Gel Electrophoresis

2.2.7.1 Sample Application by In-Gel Rehydration

The 70 mm IPG strips used (Ready Strip™) can absorb a total volume of 125 µl of solution. As such, a minimal volume of concentrated protein sample (e.g 25 µl) was added with a volume of rehydration buffer (Appendix C-4), with a final volume of 125 µl. The immobiline strip was inserted (gel side down) into a graduated plastic pipette used as replacement to the rehydration tray. One end of the pipette was sealed with parafilm and the sample solution was pipetted underneath the strip into the gel. Care was given to avoid and minimise bubble formations and the strip was left to rehydrate overnight at room temperature.

2.2.7.2 Isoelectric Focusing (IEF)

The strip(s) in the graduated plastic pipette (rehydration 'tray') was pulled out with tweezers and placed gel side up into the lane on the IPGphor tray, with acidic end of the strip positioned at the anode end, and the basic end at the cathode end. A paper wick was soaked with approximately 100 µl of deionized water and cut into two. The paperwicks were placed on each end of the strips with half of the paperwicks covering the gel edge. The electrodes were then placed onto the paperwicks that covered the gel, and locked into position. About 3.5 ml of IPG Dry Strip Fluid were then pipetted into the lane, covering the strip and the paperwicks. The IPGphor was programmed to run in 3 stages with the first stage in gradient mode at 250 V for 10 min, second stage, also in gradient mode at 3500 V for 1:30 h and the final stage, in steep mode at 3500 V also at 1:30 h. All three stages were set to run at 2 mA and 5W. Once the IEF run was completed the strip were removed and proceed to the second dimension.

2.2.7.3 Second Dimension (SDS-PAGE)

Following the first dimension process, the IPG strips then underwent a two step equilibration process, 15 min each. Each strip required 2.5 ml of equilibration buffer (Appendix C-4). In the first equilibration step, 0.25% (w/v) of dithiothreitol (DTT) was dissolved in 2.5 ml of equilibration buffer. This solution was then poured in a 15 ml centrifuge tube and the strip is immersed in the solution, gel side down. The centrifuge tube was capped and shaken gently on a shaker for 15 min. During this time, the second equilibration solution was prepared. 4.5% (w/v) of iodoacetamide (IAA) and traces of bromophenol blue (BPB) was dissolved in 2.5 ml of equilibration buffer.

After the first equilibration step ended, the solution in the centrifuge tube was discarded and the second solution was poured in and left gentle shaking for another 15 minutes. Then the strip was rinsed with SDS running buffer and excess buffer was blotted out by letting it stand on a filter paper. The second dimension was carried out on mini-PROTEAN^{TM} Tetra Cell electrophoresis units. The strip was then lubricated in SDS running buffer and positioned in between plates. The gel edge was made sure to touch the surface of the SDS-PAGE gels with extra care to prevent bubbles between the gel strip and the SDS-PAGE gel. The molecular weight marker was placed at the acidic end of the strip. The strip was then sealed with agarose sealing solution (0.5% (w/v) agarose in SDS running buffer) to prevent it from moving. The electrophoresis was run at a constant voltage of 120 V using PowerPac™ Basic power supply unit.

2.2.8 Gel Staining

Colloidal Coomassie Blue Staining G-250 was used as due to its sensitivity (up to 10 ng of protein can be detected) and compatibility with subsequent mass spectrometry analysis. The procedure was adopted from Neuhoff *et al.* (1988). The

stock solution was prepared by firstly dissolving 100 g of ammonium sulphate in about 500 ml of water. Then, 2% (w/v) ortho-phosphoric acid was added into the ammonium sulphate solution. 5% (w/v) CBB G-250 (1 g in 20 ml, prepared separately) was then added gradually. The volume was then made up to 1 L. The solution was shaken vigorously before use for even distribution of the colloidal particles. The actual staining solution was prepared by mixing methanol and colloidal stock solution at a 1:4 ratio (methanol: colloidal CBB). 20 ml of methanol was mixed with 80 ml of colloidal stock stain solution. During staining, air tight container was used to prevent methanol evaporation and placed on a shaker to prevent evaporation. The staining solution was changed once after 12 h to enhance dye deposition on low abundance proteins. Destaining was carried out by washing the gel slab in 20% (v/v) methanol, to wash out the colloidal particle.

2.2.9 Gel Visualisation and Spot Analysis

Destained gels were scanned using Image Scanner^{TM} III with the LabScan software. The scanner was first calibrated with Kodak Step Tablets no. 2 and 3. The scanner was then set to transparent mode before scanning. Both *.mel* and *.tif* files were saved for visualization and analysis purposes.

2.2.9.1 Analysis of 2-D Gels

Coomassie blue stained gels were scanned (as described earlier) and its .mel images generated were analysed using Melanie Version 7.0 and ImageMaster^{TM} 2D Platinum software Version 7.0. Qualitative and quantitative differences between susceptible and resistant samples were sought. Qualitative differences were defined as spots that were present in the susceptible sample gels but absent in the resistant sample

gels, or *vice versa*. Quantitative differences were sought in spots that were present in both gel (susceptible and resistant) sets and the mean 'volumes' (volume = area X intensity) of scanned spots were compared.

The gels from susceptible and resistant biotypes were analysed by an automated procedure to identify spots. The smoothness, saliency and min. area was adjusted to give the best spots detection. The best image from gel of the resistant biotype was used as reference and all gels are matched to it. The background value each gel was subtracted. Spot volumes were then normalized against the total volume for all spots. Matched spot volumes were compared and analysed statistically for any significant change. A particular spot that is present in all samples were chosen as a marker to evaluate similarities in all samples tested.

2.2.10 MALDI-TOF

Protein spots (1 mm^3) were excised from the Jerantut biotype gel using a clean scalpel and transferred into 1.5 ml Eppendorf tube. The gel plugs were dried and sent to Proteomics International (Perth, Australia) for analysis. Protein samples were trypsin digested and peptides extracted according to standard techniques (Bringans *et al.* 2008). Peptides were analysed by MALDI TOF-TOF mass spectrometer using a 5800 Proteomics Analyzer (AB Sciex). Bovine serum albumin was used as standard.

Generated mass spectra of the peptides were analysed using ProFound, a tool for searching a protein sequence collections with peptide mass maps (http://prowl.rockefeller.edu/prowl-cgi/profound.exe). ProFound utilizes Bayesian algorithm to rank the protein sequences in the NCBI non-redundant (NR) database according to their probability of being the analysed protein. The Z score indicates the quality of the search, corresponding to the percentile of the candidate in the random

match population. Thus, a Z score of 1.65 (a 95th percentile) for a search means there are about another 5% of random matches that could probably be the candidate.

Several information were included in all the searches, such as a maximum of one missed cleavage allowed, digestion by trypsin, the appropriate taxa, pI and molecular weight of the samples. Partial carbamidomethylation of cysteine and partial modification of methionine (methionine oxidation) were assumed. A mass tolerance of 0.05 Dalton was set initially, with gradual increase to a maximum of 0.50 Dalton, depending on the situation.

CHAPTER 3

RESULTS

3.1 Field Evaluation of Herbicide Resistant Goosegrass

Glufosinate-ammonium provided very good control of the goosegrass populations at the Kesang farm. Even at a sub-lethal dose of 247.5 g a.i. ha^{-1} (half of the recommended rate of application), 77% of control was achieved 14 days after treatment (DAT). There were rate-mediated increases in the percentage control of goosegrass with glufosinate-ammonium, as evident in Fig. 3.3 and 3.4. Glufosinate-ammonium at the recommended rate of 495 g ai ha^{-1} registered 82% control of the goosegrass while the same herbicide at 990 g ai ha^{-1} caused 94% kill of the weed. At four times than the recommended rate (1980 g ai. ha^{-1}), 97% control was achieved (Fig. 3.1 and Table 3.1).

Figure 3.2 illustrates the level of control of goosegrass biotype from Jerantut, Pahang populations subjected to the recommended rate of 495 g ai ha^{-1} *vis-à-vis* 3960 g ai ha^{-1} or eight times the recommended rate of glufosinate-ammonium. Interestingly, at 495 g ai ha^{-1} very poor control of the scourge was achieved (Fig. 3.5), apparently with no sign of breakdown of resistance with age. With 3960 g ai ha^{-1} the herbicide impacted measurable control against the weed, ranging from 65 to 85% kill. Intriguingly, there was time-mediated reduction in the ability of the herbicide to kill the weed. These phenomena were exemplified by the initial 92% kill of the weed at 7 DAT *vis-à-vis* 85, 72, and 67% kill at 14, 21, and 28 DAT, respectively.

Glufosinate-ammonium at the recommended application rate of 495 g a.i. ha^{-1} failed to inflict any damage on the goosegrass populations in Jerantut, Pahang (Table 3.1 and Fig. 3.2). Nevertheless, the rate-mediated increase in the level of control of the goosegrass populations by the herbicide prevailed. For example, with 990 g a.i. ha^{-1}, a 45% control was achieved, and the percentage control increased by 20% with a two-fold increase in rate of glufosinate-ammonium used.

Table 3.1. Percentage control of goosegrass in the field by different rates of glufosinate-ammonium 14 days after treatment.

Biotype	Rate (g ai ha^{-1})	Percentage control
Kesang	247.5	77
	495	82
	990	94
	1980	97
	3960	NA
Jerantut	495	0
	990	45
	1980	65
	3960	85

* NA – not applicable or not tested.

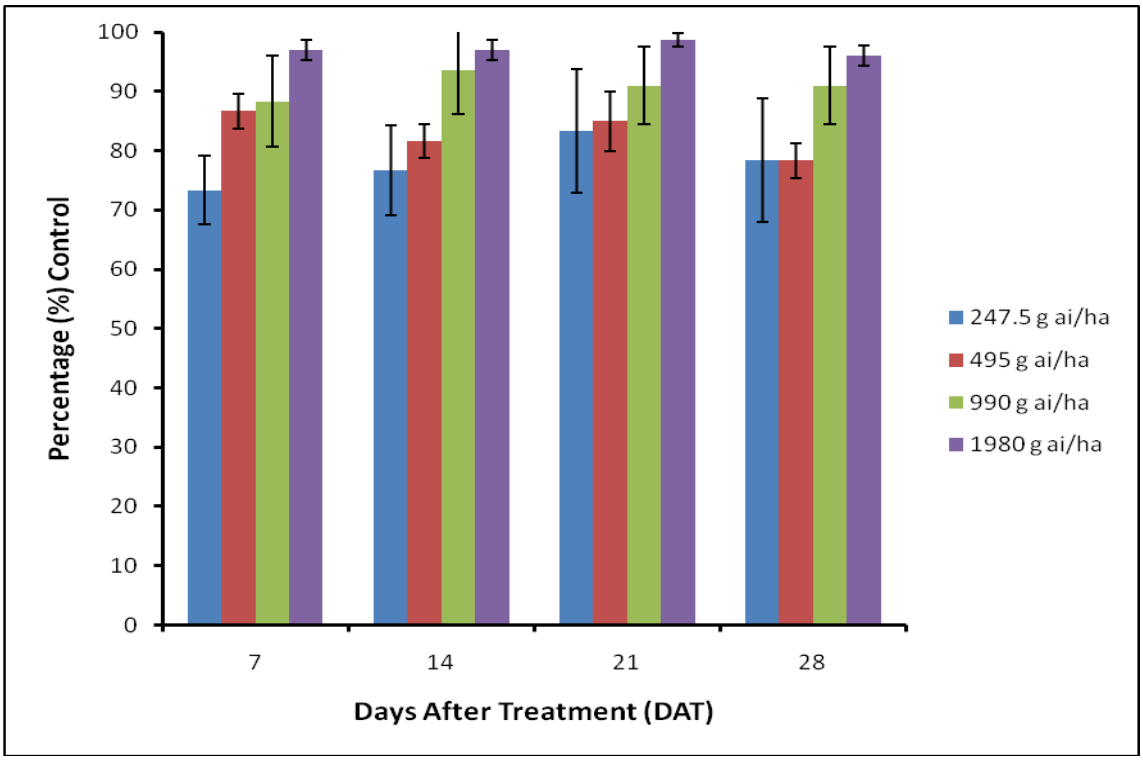

Fig. 3.1. Field evaluation on differential responsess of the goosegrass biotype from Kesang, Malacca to glufosinate-ammonium at 247.5 – 1980 g ai ha^{-1}. Bars represent 1±SD values.

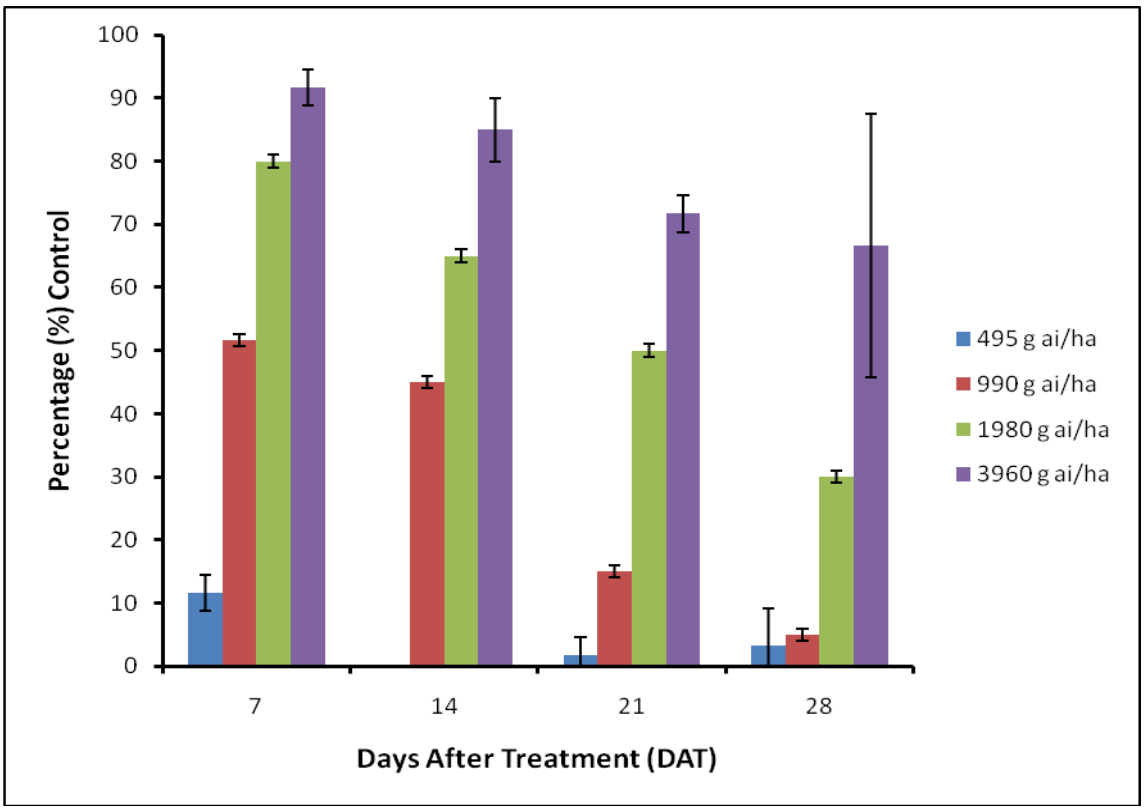

Fig. 3.2. Field evaluation on differential responses of the goosegrass biotype from Jerantut, Pahang to glufosinate-ammonium at 495 - 3960 g ai ha^{-1}. Bars represent 1±SD values.

Fig. 3.3. Control of goosegrass in Kesang, Malacca by glufosinate-ammonium at 247.5 g ai ha^{-1}.

Fig. 3.4. Control of goosegrass in Kesang, Malacca by glufosinate-ammonium at 1980 g ai ha^{-1}.

Fig. 3.5. Control of goosegrass plot in Jerantut, Pahang by glufosinate-ammonium at 495 g ai ha^{-1}.

Fig. 3.6. Control of goosegrass in Jerantut, Pahang by glufosinate-ammonium at 3960 g ai ha^{-1}.

On the other hand, it was a different story for glyphosate-treated goosegrass for Kesang and Jerantut populations. Glyphosate sprayed in the Kesang farm at twice than the recommended rate (1080 g ae ha^{-1}), produced only 10% of control, 14 days after glyphosate application. Quadrupling that rate at 4320 g ae ha^{-1} resulted in a mere increase of another 3% in control (Table 3.2 and Fig. 3.7).

As illustrated in Figure 3.8, glyphosate application had close to no effect on the goosegrass at the oil palm nursery in Jerantut, Pahang. Throughout the 4 weeks after treatment with glyphosate, the highest level of control achieved was approximately 5% and that was during the first week for the higher rates (2160 g ae ha^{-1} and 4320 g ae ha^{-1}). Figure 3.9 and 3.10 illustrate how little the effect of glyphosate, at the highest rate used, had to the goosegrass in Kesang, Malacca and Jerantut, Pahang.

Table 3.2. Percentage control of goosegrass in the field by different rates of glyphosate 14 days after treatment.

Biotype	Rate (g ae ha^{-1})	Percentage control
Kesang	540	NA
	1080	10
	2160	18
	4320	13
Jerantut	540	0
	1080	0
	2160	3
	4320	3

* NA – not applicable or not tested.

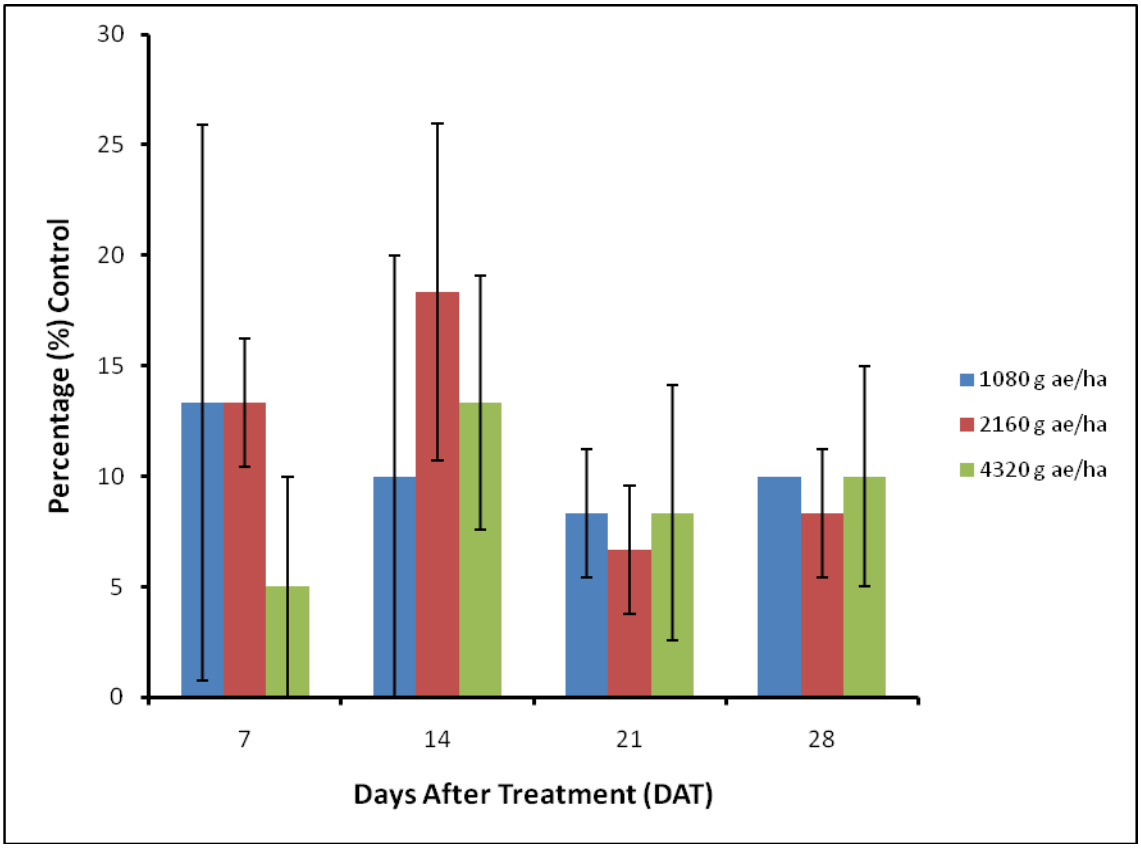

Fig. 3.7. Field evaluation on differential responses of the goosegrass biotype from Kesang, Malacca to glyphosate at 1080 - 4320 g ae ha^{-1}. Bars represent 1±SD values.

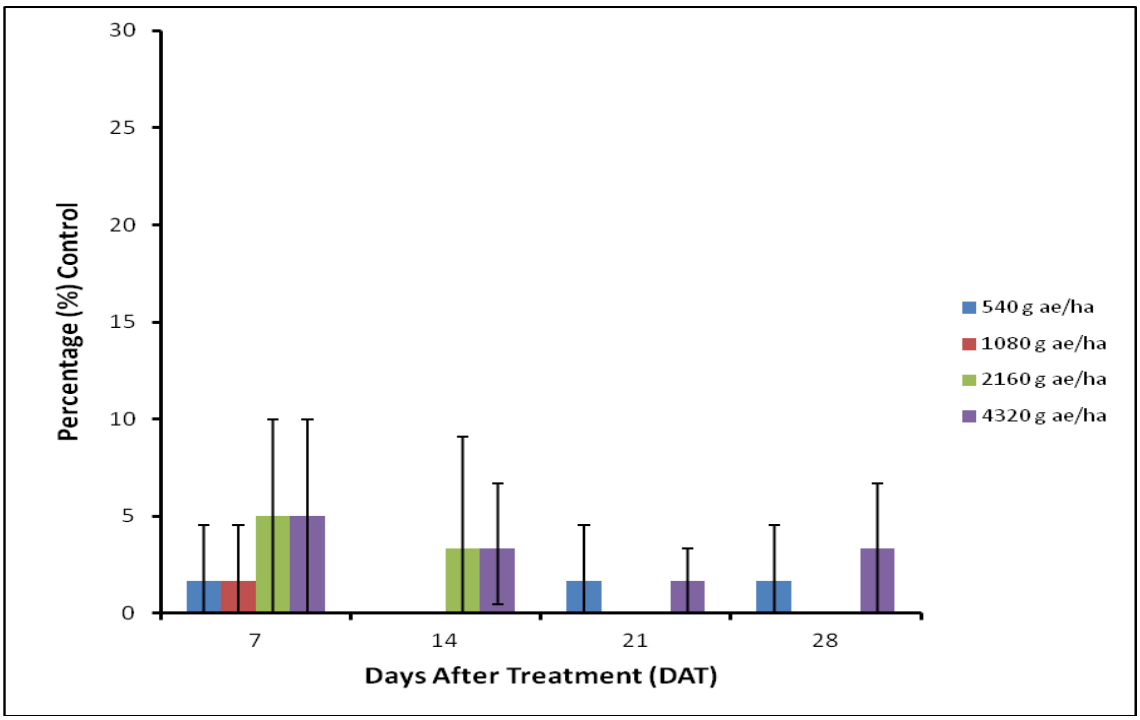

Fig. 3.8. Field evaluation on differential responses of the goosegrass biotype from Jerantut, Pahang to glyphosate at 540 - 4320 g ae ha^{-1}. Bars represent 1±SD values.

Fig. 3.9. Control of goosegrass in Kesang, Malacca by glyphosate at 4320 g ae ha^{-1}.

Fig. 3.10. Control of goosegrass in Jerantut, Pahang by glyphosate at 4320 g ae ha^{-1}.

3.2 Greenhouse Evaluation of Herbicide Resistant Goosegrass

The same level efficacy of the herbicide on the goosegrass in Kesang field was not manifested on the goosegrass populations in the greenhouse trial. At the recommended label rate of 495 g a.i. ha^{-1}, only 43% of control was achieved. As the rate(s) increased, so did the level of control (Fig. 3.11 and Fig. 3.12). A total annihilation (100% control) of the goosegrass populations for the Kesang biotype was achieved (100%) at 4- and 8-times more than the recommended rate of application of the herbicide (Table 3.3).

Glufosinate-ammonium under greenhouse studies produced a similar pattern of control against the Jerantut biotype, similar to those in the field trials. At 495 g a.i. ha^{-1}, only 3% of control was achieved. The herbicide at 990, 1980 and 3960 g a.i. ha^{-1} produced 37%, 28% and 64% control, respectively against the Jerantut biotype of goosegrass at 14 DAT (Fig. 3.13, Fig. 3.14 and Table 3.3).

While treatment with 495 g a.i. ha^{-1} failed to impact any significant kill on the Jerantut biotype of goosegrass, similar treatment afforded 35-46% kill on the Kesang biotype of goosegrass (Fig.3.15). Albeit measurable differences in the percentage kill of the scourge with time for both biotypes, such damages were not very significant.

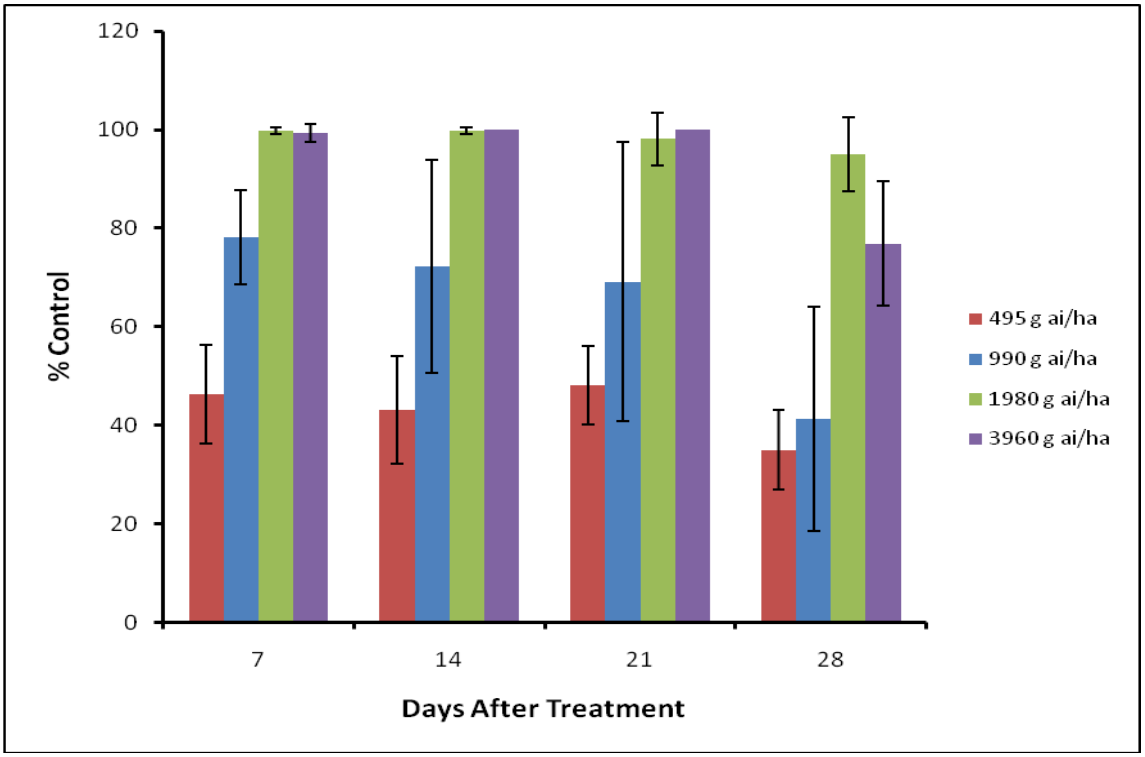

Fig. 3.11. Greenhouse evaluation on differential responses of the goosegrass biotype from Kesang, Malacca to glufosinate-ammonium at 495 – 3960 g ai ha^{-1}. Bars represent 1±SD values.

Fig. 3.12. Greenhouse evaluation of transplanted goosegrass from Kesang, Malacca with different rates of glyphosate. Plant in the black poly bag represents the untreated control.

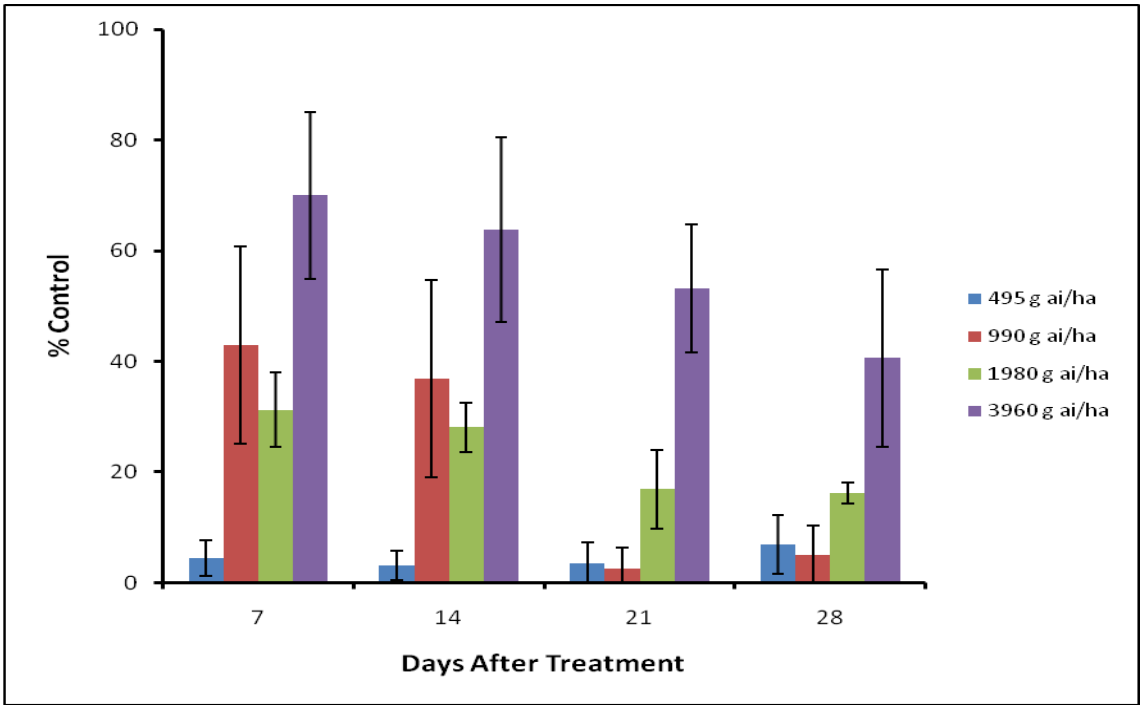

Fig. 3.13. Greenhouse evaluation on differential responses of the goosegrass biotype from Jerantut, Pahang to glufosinate-ammonium at 495 – 1980 g ai ha^{-1}. Bars represent 1±SD values.

Fig. 3.14. Greenhouse evaluation of transplanted goosegrass from Jerantut, Pahang with different rates of glufosinate-ammonium.

Table 3.3. Percentage control of goosegrass in greenhouse evaluation by different rates of glufosinate-ammonium 14 days after treatment.

Biotype	Rate (g ai ha^{-1})	Percentage (%) control
Kesang	247.5	NA
	495	43
	990	72
	1980	100
	3960	100
Jerantut	495	3
	990	37
	1980	28
	3960	64

* NA – not applicable or not tested.

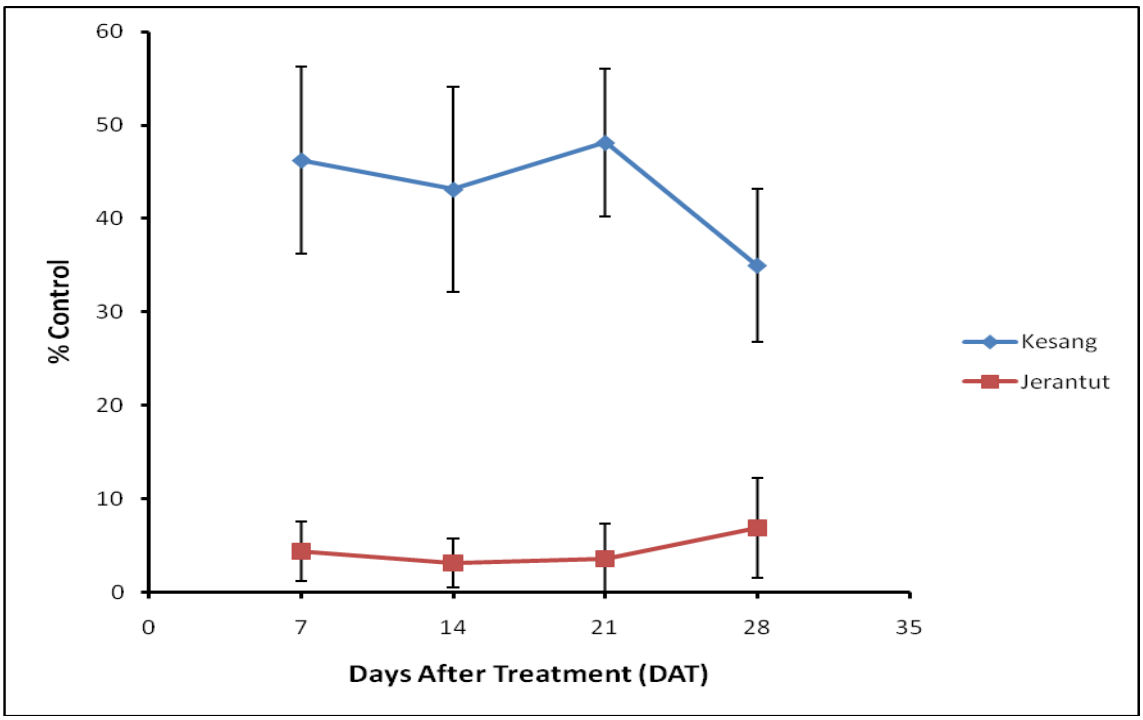

Fig. 3.15. Greenhouse evaluation on the differential responses of the Kesang and Jerantut biotypes to glufosinate-ammonium treatments at the recommended rate of 495 g ai ha^{-1}. Bars represent 1±SD values.

Greenhouse evaluation of transplanted goosegrass from the Kesang farm showed increased susceptibility towards glyphosate (Fig 3.16). At the higher rates of glyphosate (2160 and 4320 g ae ha^{-1}) applied, the control capacity on the scourge was 71% (greenhouse trial) compared to 18% in the field trial and 94% (greenhouse trial) compared to 13% (field trial) respectively at 14 DAT (Table 3.4). Time-mediated increase in terms of weed control was observed as illustrated in Figure 3.16. With the exception of the recommended rate of 540 g ae ha^{-1}, all other rates showed time-mediated increase in goosegrass control.

Subsequent greenhouse trial on the transplanted Jerantut biotype suggested higher susceptibility towards glyphosate (Fig. 3.18 and 3.19). At fourteen days after application, only glyphosate at the recommended rate of 540 g ae ha^{-1} had no effect on

the Jerantut biotype. However at twice, four and eight times more than the recommended rate, there were about 19% to 25% kill of the weed (Table 3.4).

Figure 3.20 illustrates a comparison between the Kesang and Jerantut biotypes treated with 4320 g ae ha^{-1} of glyphsosate. While treatment with 4320 g ae ha^{-1} failed to control the Jerantut biotype 14 DAT, the Kesang biotype was adequately controlled at 14 DAT with the same treatment. Despite the differences in percentage control of goosegrass, similar time-mediated response was observed.

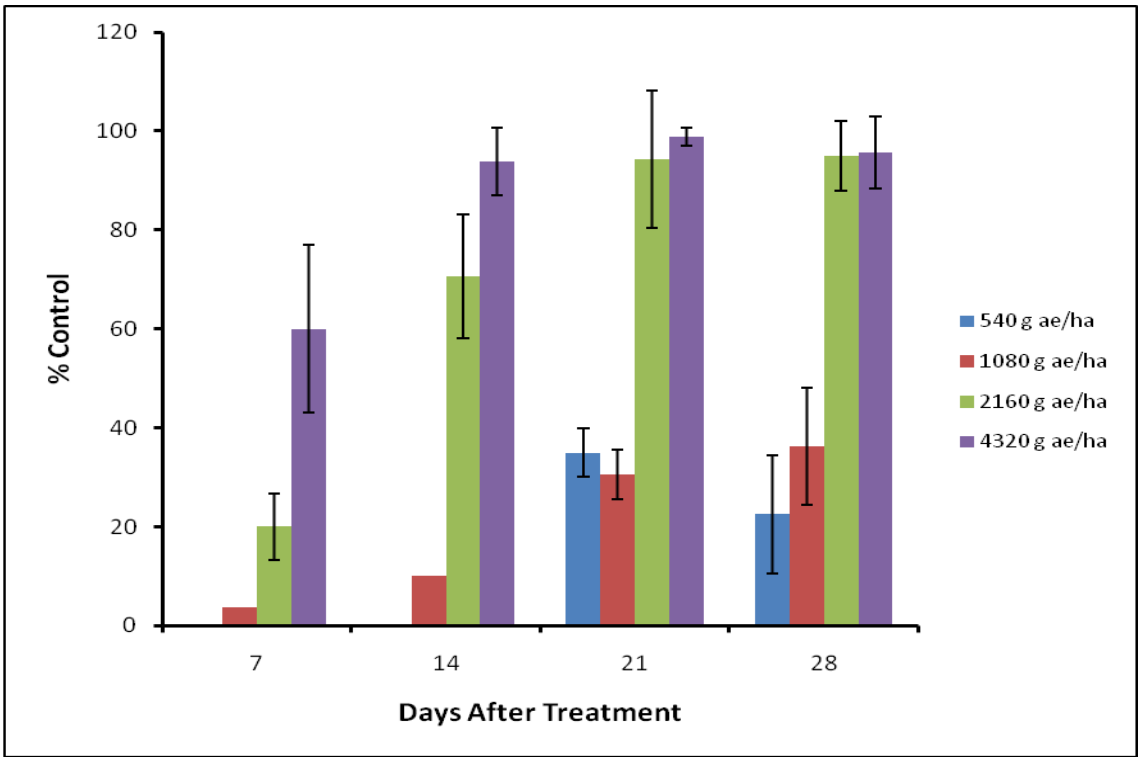

Fig. 3.16. Greenhouse evaluation on differential responses of the goosegrass biotype from Kesang, Malacca to glyphosate at 540 – 4320 g ae ha^{-1}. Bars represent 1±SD values.

Table 3.4. Percentage control of goosegrass by different rates of glyphosate at 14 days after treatment.

Biotype	Rate (g ae ha^{-1})	Percentage (%) Control	
		Field trial	Greenhouse
Kesang	540	NA*	**0**
	1080	10	**10**
	2160	18	**71**
	4320	13	**94**
Jerantut	540	0	**1**
	1080	0	**22**
	2160	3	**25**
	4320	3	**19**

* NA – not applicable or not tested.

Fig. 3.17. Greenhouse evaluation of transplanted goosegrass from Kesang, Malacca with different rates of glyphosate. Plant in the black poly bag represents the untreated control.

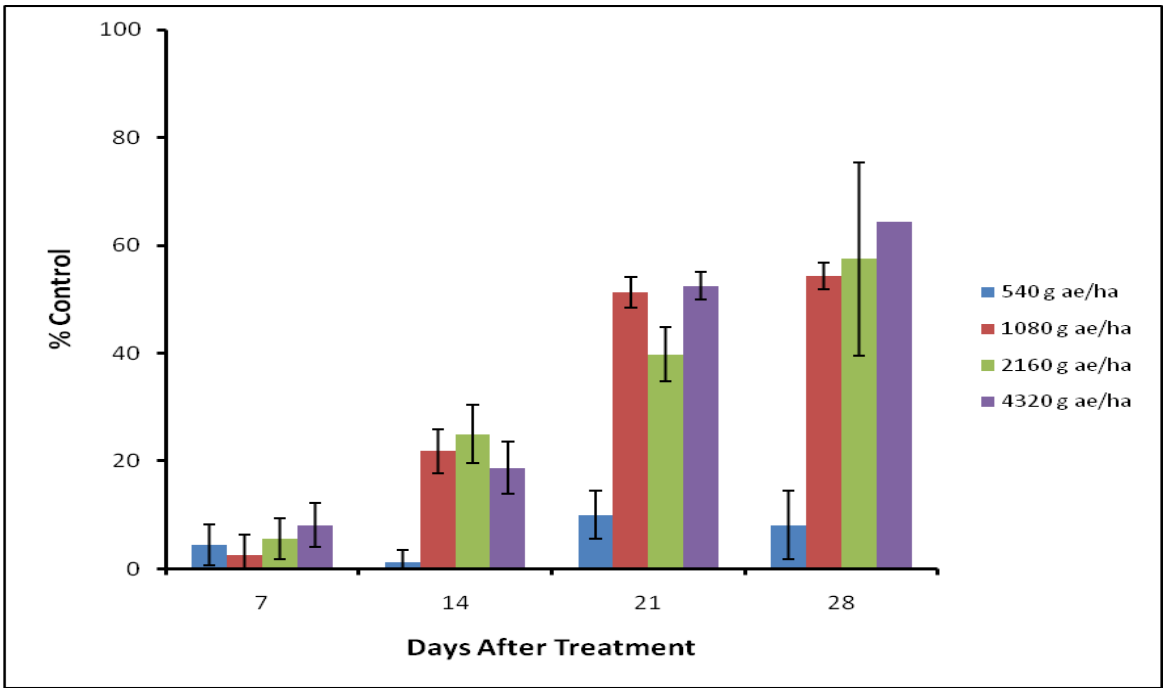

Fig. 3.18. Greenhouse evaluation on differential responses of the goosegrass biotype from Jerantut, Pahang to glyphosate at 540 – 4320 g ae ha^{-1}. Bars represent 1±SD values.

Fig. 3.19. Greenhouse evaluation of transplanted goosegrass from Jerantut, Pahang with different rates of glyphosate. Plant in the black poly bag at the left side represents the untreated control.

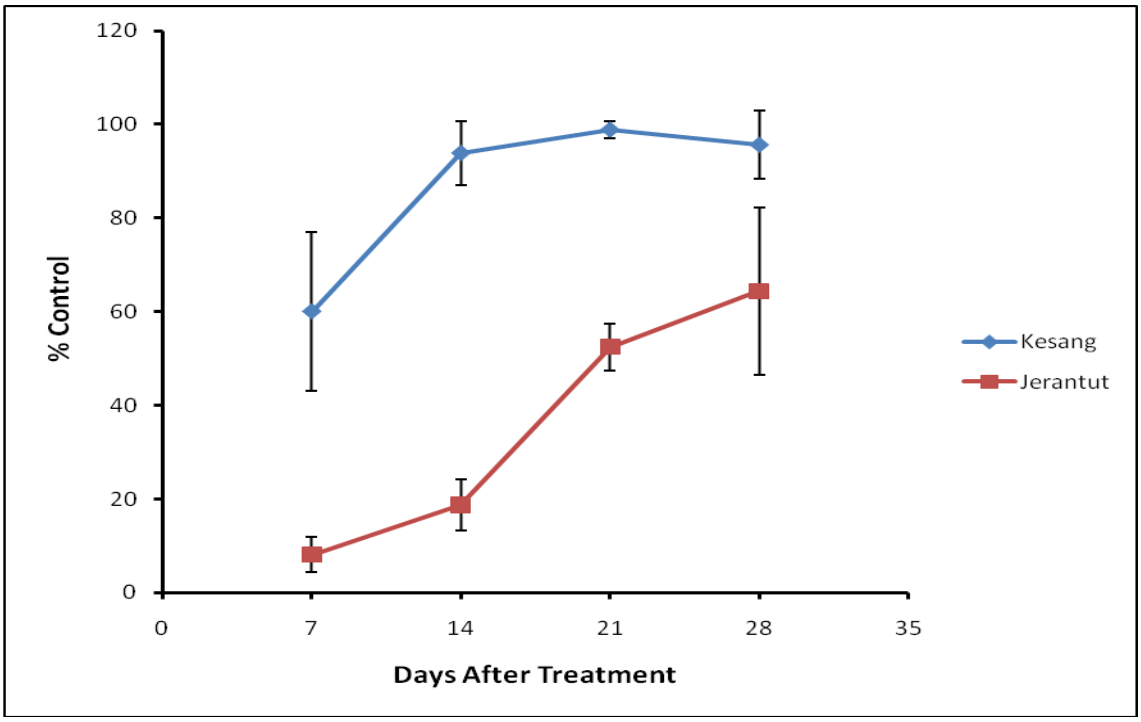

Fig. 3.20. Greenhouse evaluation on the differential responses of the Kesang and Jerantut biotypes to glyphosate treatments at 4320 g ae ha^{-1}. Bars represent 1±SD values.

The LC_{50} values together with the resistance index for all three biotypes are shown in Table 3.5. The transplanted Kesang biotype has a resistance index of 1.97 for glufosinate-ammonium. The parallel figure for the transplanted Jerantut biotype was 7.63. The same transplanted Kesang biotype recorded a resistance index of 8.41 for glyhosate and 24.37 for the transplanted Jerantut biotype.

Tukey's analysis showed that when treated with glufosinate-ammonium at various rates (495 to 3960 g ai ha^{-1}), the Kesang biotype produced significantly different level of control of the weed at the recommended label rate of 495 g ai ha^{-1}, compared to the other rates. This was also true for 990 g ai ha^{-1}. However at the higher

rates of 1980 g ai ha^{-1} and 3960 g ai ha^{-1}, the control capacity of glufosinate-ammonium on the goosegrass, are much or less the same, albeit significantly different from 495 and 990 g ai ha^{-1}. Both Jerantut and the susceptible biotypes generated similar results for treatment with glufosinate-ammonium (Table 3.6).

No significant differences were achieved, regardless of the rate used, for the susceptible biotype of goosegrass treated with glyphosate. There were significant difference in control of the weed between the two lower rates (540 and 1080 g ae ha^{-1}) of glyphosate and the two higher rates (2160 and 4320 g ae ha^{-1}) on the Kesang biotype. Interestingly, the Jerantut biotype showed significantly different level of control of the scourge, based on the rates used.

As illustrated in Table 3.6, glufosinate-ammonium at 495 g ai ha^{-1} did not display any significant difference in the control of the susceptible and the Kesang biotype. However the Jerantut biotype produced significantly different level of control compared to the other 2 biotypes when treated with 495 g ai ha^{-1}. These trends prevailed in the other two rates of 1980 g ai ha^{-1} and 3960 g ai ha^{-1}. Only the treatment with 990 g ai ha^{-1} of glufosinate seems to give significantly different control of the weed between the three biotypes (susceptible, Kesang and Jerantut).

Treatment with glyphosate at 540 g ae ha^{-1} did not show any significant affect on the susceptible and the Jerantut biotype. Only the Kesang biotype seemed to be affected significantly by glyphosate treatment at 540 g ae ha^{-1}. Glyphosate treatment at twice than the recommended rate also produced the same control capacity for all three biotypes compared to those treated with 540 g ae ha^{-1}. For the two higher rates at 2160 and 4320 g ae ha^{-1}, there were no difference in control of goosegrass between susceptible and Kesang biotype. However treatment at these rates (2160 g ae ha^{-1} and

4320 g ae ha^{-1}) produced different control on the Jerantut biotype, as compared to the susceptible and the Kesang biotypes.

Table 3.5. The amount of glufosinate-ammonium and glyphosate required for 50% control of the susceptible, Kesang and Jerantut biotypes of goosegrass. Values are LC_{50} calculated by Probit Analysis on the data from greenhouse experiments.

Treatment	Biotype	LC_{50} (g ai ha^{-1}/g ae ha^{-1})	Resistance Index**
Glufosinate-ammonium	Susceptible	301 (135-523)*	1.00
	Kesang	593 (347-903)	1.97
	Jerantut	2297 (1580-3594)	7.63
Glyphosate	Susceptible	232 (24-621)	1.00
	Kesang	1950 (892-4888)	8.41
	Jerantut	5653 (2588-29618)	24.37

* Values in parentheses represent the 95% confidence intervals.

** The Resistance Index is the ratio of LC_{50} of suspected resistant biotypes to that of the susceptible population.

*** Glyphosate rate is in g ae ha^{-1}

Table 3.6. Differences in control of goosegrass by rates (glufosinate-ammonium and glyphosate) and biotypes for transplanted goosegrass.

Herbicide	Rate (g ai ha^{-1}) / (g ae ha^{-1})**	Biotypes*		
		Susceptible	Kesang	Jerantut
Glufosinate-ammonium	495	aFG	aFG	aH
	990	bF	bG	bH
	1980	cdFG	cdFG	cdH
	3960	cdFG	cdFG	cdH
Glyphosate**	540	abcdFH	abG	acFH
	1080	abcdFH	abG	bcdFH
	2160	abcdFG	cdFG	abcdH
	4320	abcdFG	cdFG	bcdH

* Values followed by the same uppercase letters in a row, and those followed by the same lowercase letters in a column are not significantly different at $p < 0.05$ (Tukey's test).

** Glyphosate rate is in g ae ha^{-1}

3.3 Seed Test on the Kesang, Jerantut and Susceptible Goosegrass Biotypes

Glufosinate-ammonium sprayed on goosegrass grown from seeds collected in the field provided satisfactory control of the weed. At the recommended rate of 450 g ai ha^{-1}, 77% of control on the Kesang biotype was achieved 14 days after treatment. The control of goosegrass increased with the parallel increase in rates, with 8 times more than the recommended rate (450 g ai ha^{-1}) yielded nearly total control of goosegrass (99%) (Table 3.7; Fig. 3.21). However, the efficacy of the herbicide that controls some of the Kesang biotype did not prevail with the Jerantut biotype. The recommended rate

provided a mere control of 15%, with no sign of breakdown with age (Fig. 3.22). The increased in rates improved glufosinate-ammonium efficacy with 61% control of goosegrass at double the initial rate. At four and eight times (1980 and 3960 g ai ha^{-1}) more than the recommended rate, the herbicide provided satisfactory kill of the scourge with 82% and 83% each (Table 3.7; Fig 3.23).

The goosegrass of Kesang and Jerantut biotypes grown from seeds displayed high tolerance towards glyphosate, as evident in Table 3.7. Interestingly, although little control was achieved at the recommended rate and at twice the recommended rate, a large increase in the control of the scourge was observed for the Kesang biotype at 2160 g ae ha^{-1}. This rate-mediated increase, although expected, was surprising as the increase was very high (about 60%) (Fig. 3.24 and Fig. 3.25). However, this significant increase in control of goosegrass was not evident for the Jerantut biotype. As illustrated in Fig. 3.26 and Fig. 3.27, there was still rate-mediated increase over time, but the increase was not significant.

The LC_{50} values together with the resistance index are shown in Table 3.8. The Kesang biotype grown from seeds has a resistant index of 5.604 for glufosinate-ammonium. The parallel figure for the Jerantut biotype also grown from seeds was 30.606. The same Kesang biotype recorded a resistance index of 1.37 for glyhosate and 3.28 for the Jerantut biotype. The seeds of both the Kesang and Jerantut biotypes were grown in the greenhouse.

Table 3.7. Percentage control of goosegrass from seeds by different rates of glufosinate-ammonium and glyphosate 14 days after treatment (DAT).

Treatment	Rate (g ai/ha) / (g ae/ha)*	Percentage Control by Biotype	
		Kesang	Jerantut
Glufosinate-ammonium	495	77	15
	990	88	61
	1980	95	82
	3960	99	83
Glyphosate*	540	12	6
	1080	11	15
	2160	71	18
	4320	87	43

* Glyphosate rate is in g ae ha^{-1}

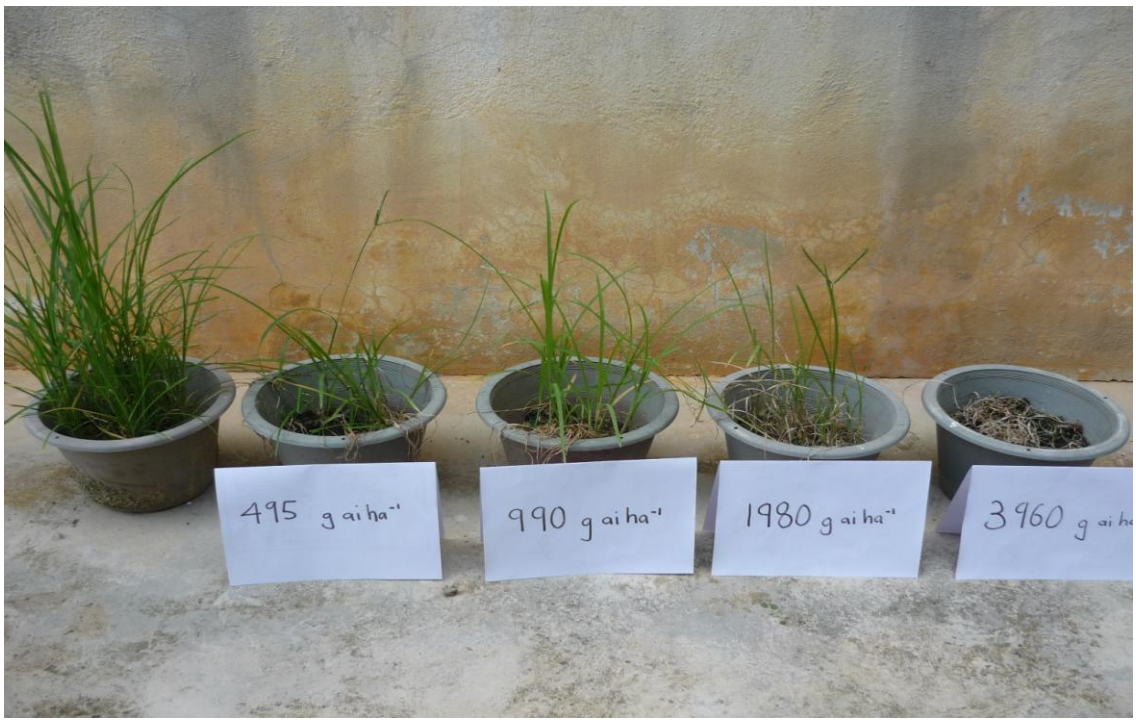

Fig. 3.21. Greenhouse evaluation of goosegrass grown from seed (Kesang biotype) with different rates of glufosinate-ammonium. Plants in the left pot represents the untreated control.

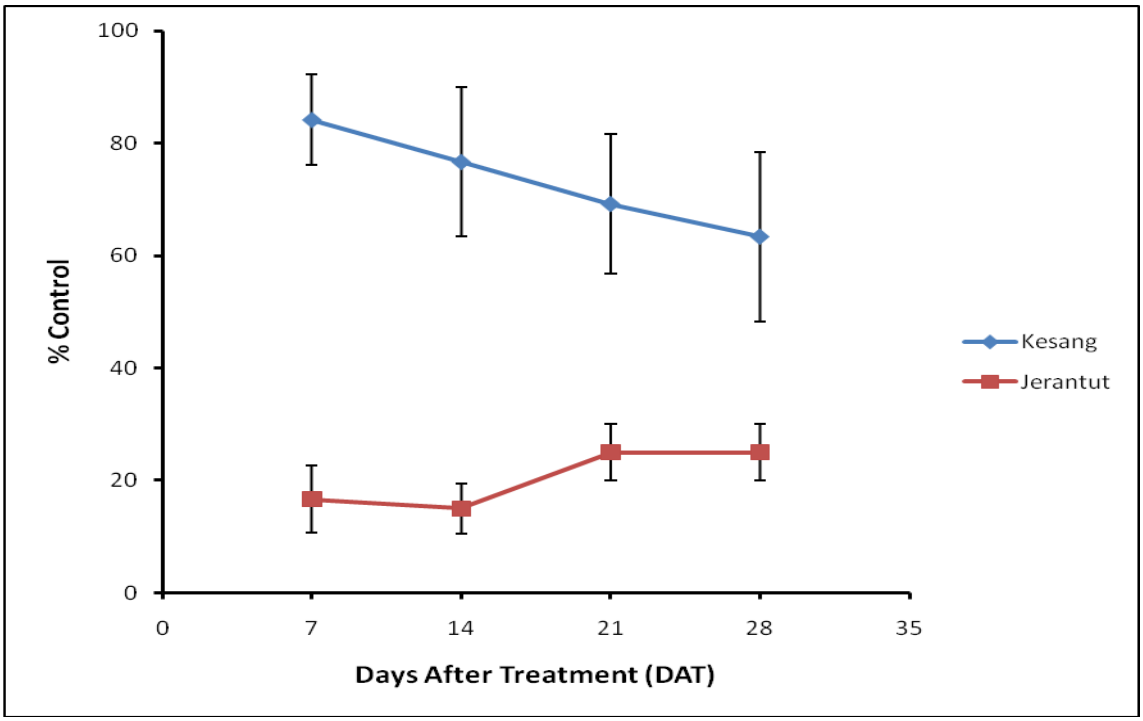

Fig. 3.22. Greenhouse evaluation on the differential responses of the Kesang and Jerantut biotypes grown from seeds to glufosinate-ammonium at 495 g ai ha^{-1}. Bars represent 1±SD values.

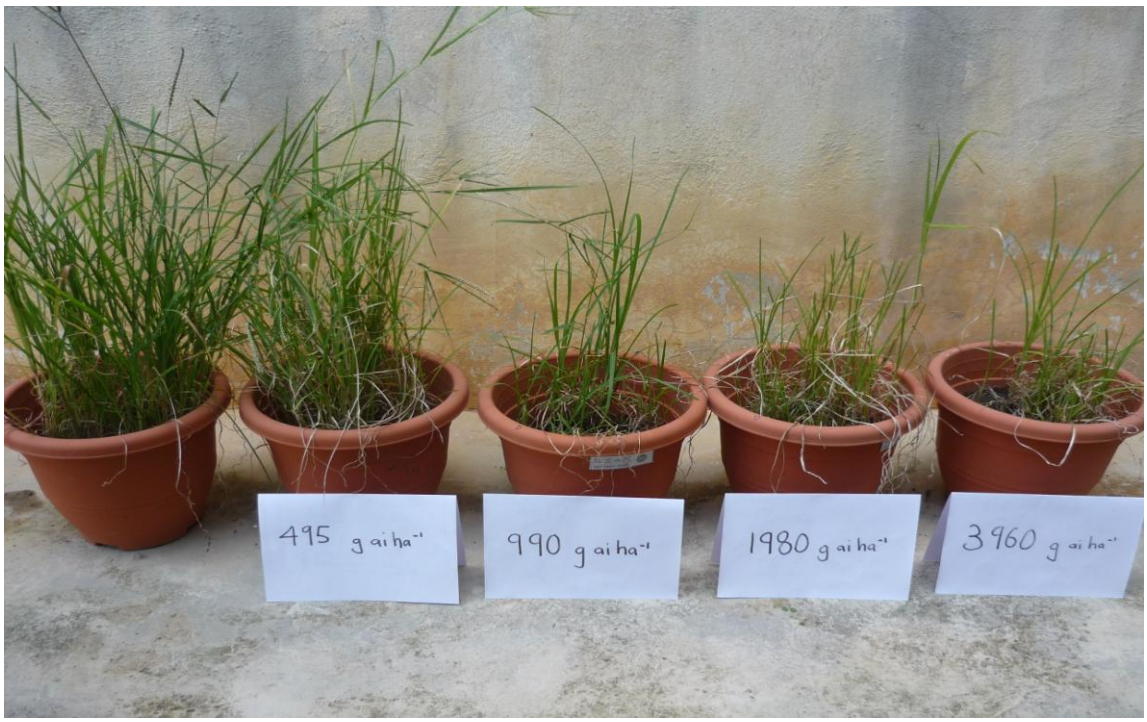

Fig. 3.23. Greenhouse evaluation of goosegrass grown from seed (Jerantut biotype) with different rates of glufosinate-ammonium. Plants in the left pot represents the untreated control.

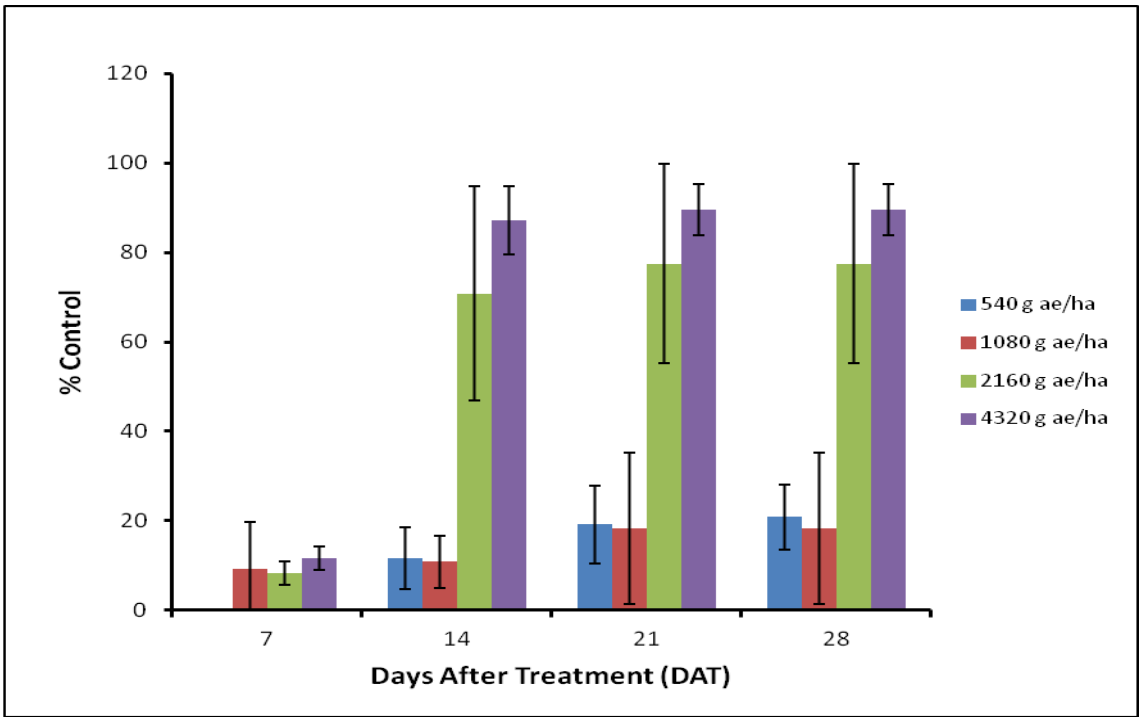

Fig. 3.24. Greenhouse evaluation on the differential responses of the Kesang biotype grown from seeds to glyphosate at 540 to 4320 g ae ha^{-1}. Bars represent 1±SD values.

Fig. 3.25. Greenhouse evaluation of goosegrass grown from seed (Kesang biotype) with different rates of glyphosate. Plants in the left pot represents the untreated control.

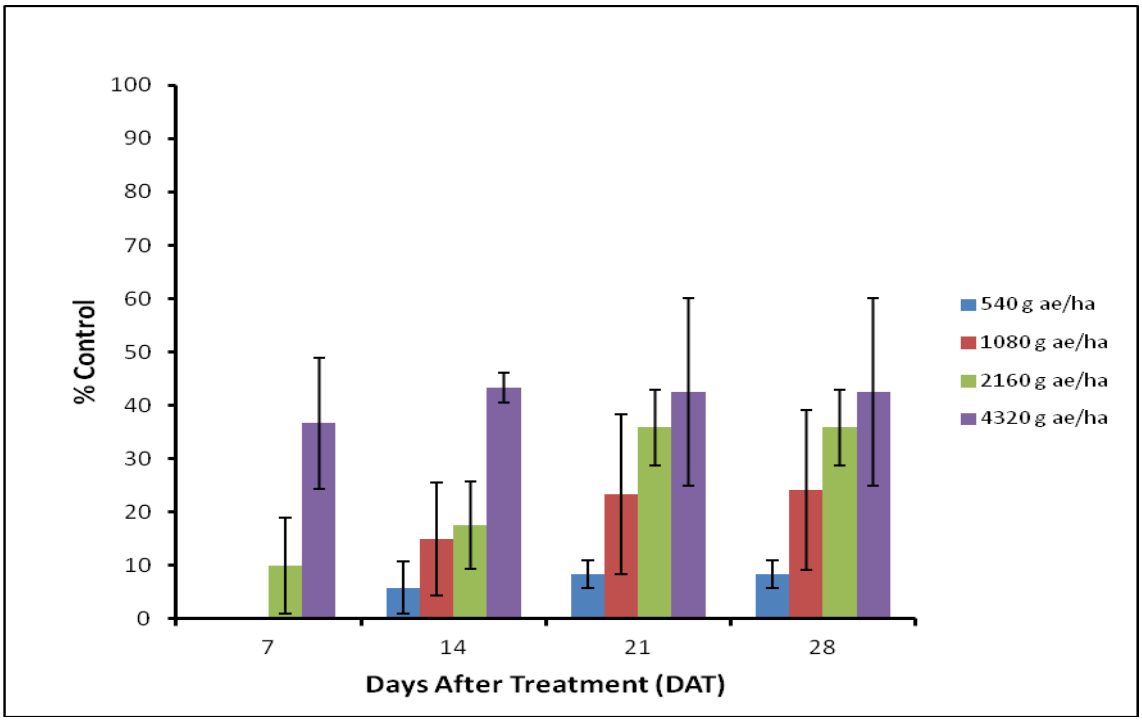

Fig. 3.26. Greenhouse evaluation on the differential responses of the Jerantut biotype grown from seeds to glyphosate at 540 to 4320 g ae ha^{-1}. Bars represent 1±SD values.

Fig. 3.27. Greenhouse evaluation of goosegrass grown from seed (Jerantut biotype) with different rates of glyphosate. Plants in the left pot represents the untreated control.

Table 3.8: The amount of glufosinate-ammonium and glyphosate required for 50% control of the susceptible, Kesang and Jerantut biotypes of goosegrass grown from seeds. Values are LC_{50} calculated by Probit Analysis on the data from greenhouse experiments.

Treatment	Biotype	LC_{50} (g ai ha^{-1})	Resistance Index**
Glufosinate-ammonium	Susceptible	29.8 (0-284)*	1.00
	Kesang	167 (0.18-500)	5.604
	Jerantut	909 (122-2018)	30.606
Glyphosate	Susceptible	1297 (743-2198)	1.00
	Kesang	1775 (1054-3105)	1.37
	Jerantut	4260 (2482-9642)	3.28

* Values in parentheses represent the 95% confidence intervals.

** The Resistance Index is the ratio of LC_{50} of suspected resistant biotypes to that of the susceptible population.

Tukey's test (Table 3.9) showed that there were no significant differences between the rates (495 to 3960 g ai ha^{-1}) in terms of the control achieved for the susceptible biotype. However for the Kesang and Jerantut biotype, each rate tested produced significantly different level of control for both Kesang and Jerantut biotype.

Glyphosate at 540 and 1080 g ae ha^{-1} produced significantly different control on the susceptible biotype. At four and eight times more than the recommended rate, the control achieved were more or less the same (better to state the value). Kesang biotype also registered no significant differences in control between 540 and 1080 g ae ha^{-1}, and between 2160 and 4320 g ae ha^{-1}, but between the two lowest and two highest rates used, there were a marked difference in the control capacity on the weed. Interestingly, the Jerantut biotype showed significant differences in each of the rate tested.

In terms of differences in response between biotypes, treatment with glufosinate-ammonium at 495 g ai ha^{-1} resulted in significant difference between susceptible, the Kesang and the Jerantut biotypes. However at two to eight times (990-3960 g ai ha^{-1}) more than the recommended rate, only the Jerantut biotype showed significant difference. Both the Kesang and the susceptible biotypes had no significant differences in their control.

The case was nearly the same for glyphosate treatment. The only difference is the rate where significant difference was achieved between the susceptible and the Kesang biotypes was at twice than the recommended label rate for glyphosate (1080 g ae ha^{-1}). For the other three rates (540, 2160 and 4320 g ae ha^{-1}), there were no significant difference between the control of goosegrass achieved for susceptible and Kesang biotypes. Again, only the Jerantut biotype had a significant affect from the treatment of glyphosate at these rates (540, 2160 and 4320 g ae ha^{-1}).

Table 3.9: Differences in control of goosegrass by rates (glufosinate-ammonium and glyphosate) and biotypes for goosegrass grown from seeds.

Herbicide	Rate (g ai/ha) / (g ae/ha)**	Biotypes*		
		Susceptible	Kesang	Jerantut
Glufosinate-ammonium	495	abcdF	abG	aH
	990	abcdFG	abcFG	bH
	1980	abcdFG	bcdFG	cdH
	3960	abcdFG	cdFG	cdH
Glyphosate	540	aFG	abFG	abH
	1080	bF	abG	abcH
	2160	cdFG	cdFG	bcdH
	4320	cdFG	cdFG	cdH

* Values followed by the same uppercase letters in a row, and those followed by the same lowercase letters in a column are not significantly different at $p < 0.05$ (Tukey's test).

** Glyphosate rate is in g ae ha^{-1}

3.4 Protein Extraction

Goosegrass shoots of the susceptible, the Kesang and the Jerantut biotypes pulverized under liquid nitrogen produced fine, greenish-coloured powders. Following filtration (two layers of muslin cloth) and centrifugation (12000 rpm, 40 min), the homogenates underwent ammonium sulphate precipitation (up to 80%) before being centrifuged again at 12000 rpm for 10 min. Once dissolved with buffer A (20 mM Tris-HCl, pH 7.5, 1mM DTT with protease inhibitor) the crude homogenates were then filtered through 0.45 μm syringe filter.

The solutions were subjected to Sephadex G-25 column. Figure 3.28 illustrates the elution profile of the susceptible/ resistant goosegrass biotypes. Two distinct peaks of different sizes were resolved. The first peak (peak I) was collected for further analysis while the second peak (peak II) was eluted out along with the salts that were present in the protein solution.

3.5 SDS-PAGE

The collected elutions of the susceptible, the Kesang and the Jerantut biotypes from size filtration chromatography were first concentrated, then subjected to discontinuous SDS-PAGE to visualize the proteins present in each biotype. The proteins were separated based on their molecular weight (Fig. 3.29). Multiple bands were revealed for all three biotypes (the susceptible, the Kesang and the Jerantut biotypes).

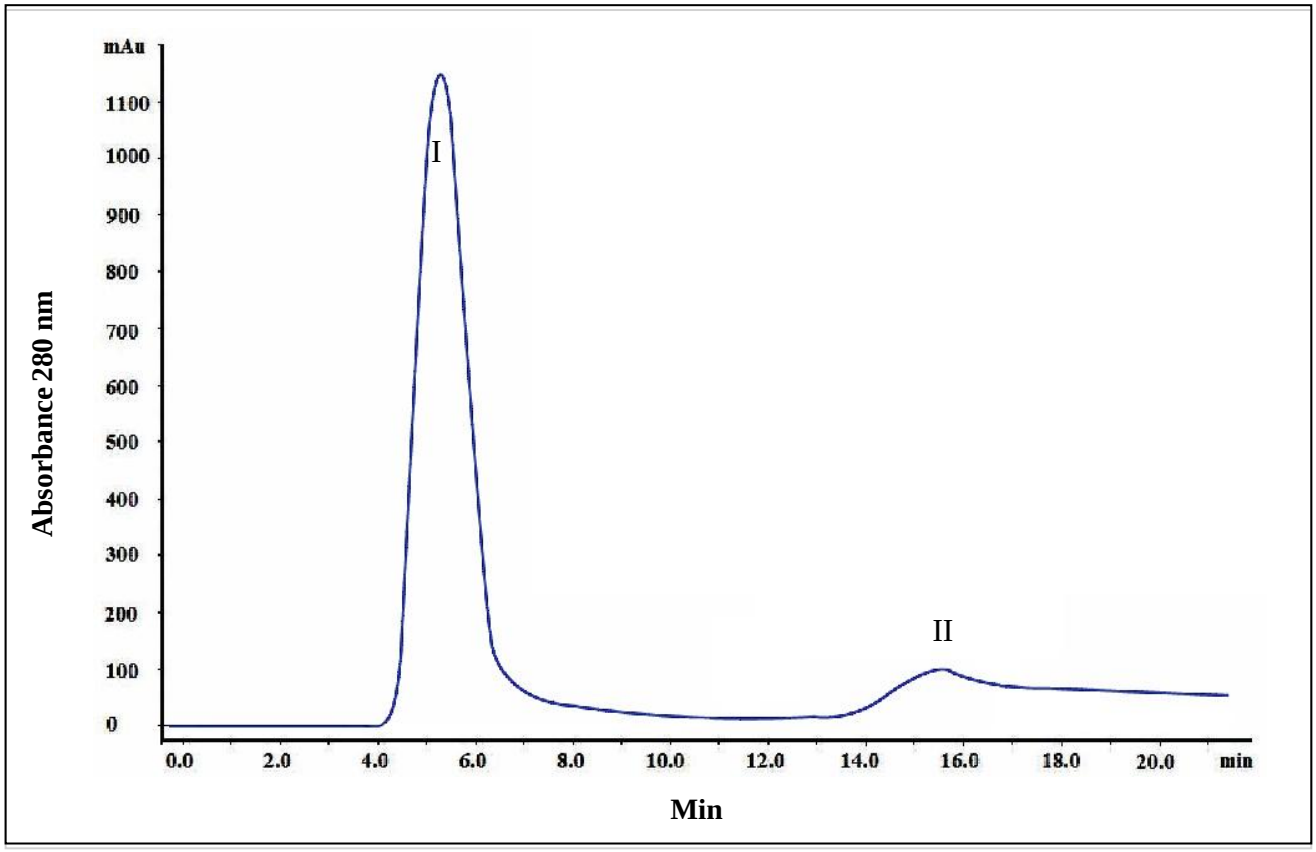

Fig. 3.28. Elution profile of the goosegrass biotypes on Sephadex G-25, equilibrated with 20 mM Tris-HCl, pH 7.5, containing 1mM DTT. Five ml of sample were applied and flow rate was set at 2.5 ml/min. Elution profiles of the Jerantut and Kesang biotypes were omitted for clarity.

3.6 Two-Dimensional (2-D) Gel Electrophoresis

The concentrated protein samples of goosegrass (susceptible, Kesang and Jerantut biotypes) underwent 2-D electrophoresis in order to further separate the proteins according to their isoelectric points and molecular weights. Figures 3.30 illustrates the proteome profile for the susceptible, the Jerantut and the Kesang biotypes respectively.

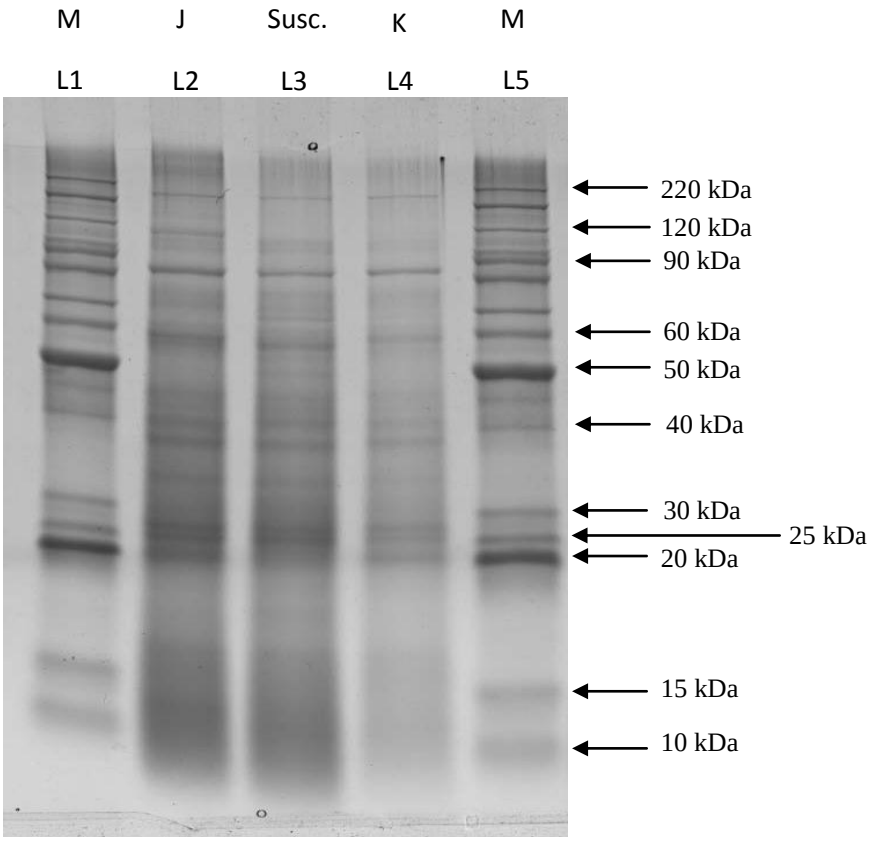

Fig. 3.29. The SDS-PAGE result of the Jerantut, the susceptible and the Kesang biotypes extracts on 12% polyacrylamide gel following gel chromatography on Sepahadex G-25. SDS-PAGE was performed by the method of Laemmli (1970). M = molecular weight marker(s), L = lane, Susc. = susceptible, J = Jerantut biotype, K = Kesang biotype. Molecular weight markers used were BENCHMARK^{TM} from Invitrogen. 10.50 µg of Jerantut biotype sample protein were loaded into lane 2, 15.08 µg of susceptible biotype sample protein were loaded into lane 3and 10.00 µg of Jerantut biotype sample protein were loaded into lane 4. 8 µg of molecular weight markers were used in both lane 1 and lane 5.

3.7 Proteome Analysis

Protein spots revealed by colloidal coomassie staining of the gels in the susceptible, the Kesang and the Jerantut biotypes were then analysed using ImageMaster Platinum 7 software. The susceptible biotype was used as control and its protein spots were matched against the Jerantut and the Kesang biotypes. The protein spots were checked based on their percentage volume and a t-test was carried out on the matched spots in order to determine whether they were significantly expressed or otherwise.

The ImageMaster analysis revealed that there were a total of 82, 113 and 93 protein spots for the susceptible, Jerantut and Kesang biotypes, respectively. Between the susceptible and the Jerantut biotypes, there were 150 matched spots. Out of 150 spots, 45 spots were present in the proteome of both the susceptible and the Jerantut biotypes. However, a student's t-test showed only 4 spots were differentially expressed. Thirty seven spots were present only in the susceptible biotype and 68 spots were present only in the Jerantut biotype. Table 3.10 shows a list of selected spots. The total spots are listed in Appendix D1.

Between the susceptible and the Kesang biotypes, a total of 144 spots were matched. Thirty one spots were present in both the susceptible and the Jerantut biotypes, but only 3 spots were differentially expressed. There were 51 spots that were only available in the susceptible biotype and 62 spots present only in the Kesang biotype. Table 3.11 shows a list of selected spots. The total spots are listed in Appendix D2.

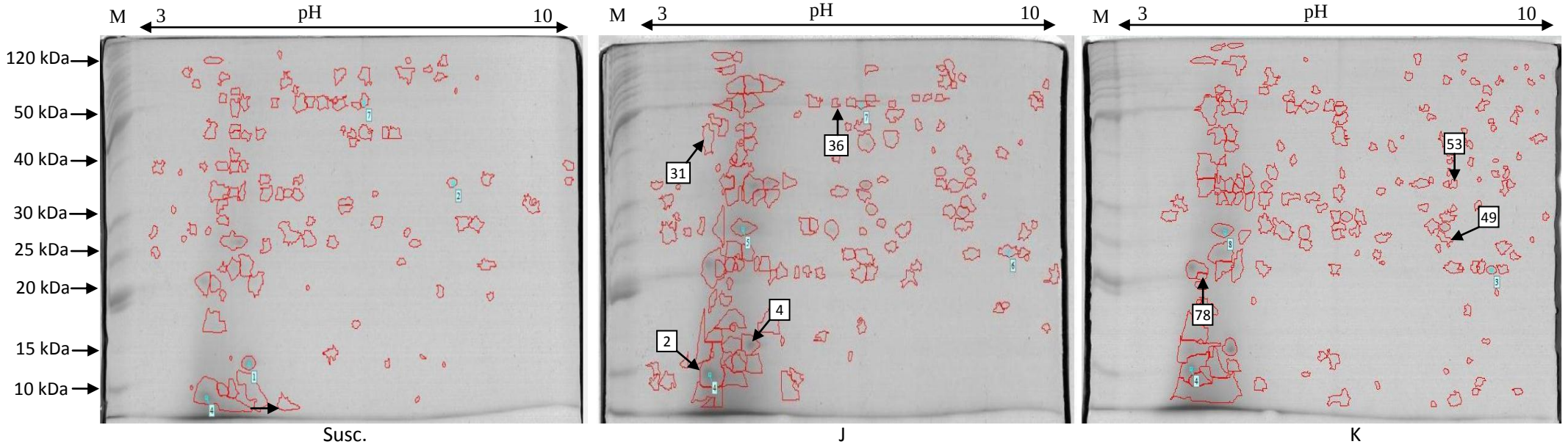

Fig. 3.30. Protein profiles of different biotypes of goosegrass. Proteins were separated by isoelectric points ranging from pH 3-10 (NL) by IEF and resolved on 12% polyacrylamide gel. M = molecular weight markers, Susc.= susceptible, J = Jerantut biotype, K = Kesang biotype. Molecular markers used were BENCHMARK™ from Invitrogen. A total of 150.8 ug, 105 ug and 100 ug of the susceptible (Susc.), the Jerantut (J) and the Kesang (K) biotype sample protein were loaded into their respective gels. The number in the white boxes show the spots that were present in the susceptible and the Jerantut biotypes (spot no. 2, 4, 31 and 36) and in the susceptible and the Kesang biotypes (spot no. 49, 53 and 78). The numbers correspond to Table 3.10 and Table 3.11. The numbers in the blue boxes were from the software (ImageMaster Platinum 7) used in running the analysis.

Table 3.10. Mean volumes of selected spots between the susceptible and the Jerantut biotypes*.

Spot No.	Percentage Volume (mean)		Expression fold	T-test
	Susceptible biotype	Jerantut biotype		$p < 0.05$
2	23.5713	11.6548	-2.02245	0.025726
4	3.56795	1.78848	-1.99496	0.01180
31	0.14538	0.478199	+3.2893	0.04723
36	0.398881	0.153762	-2.59415	0.001961
43	1.1855	-		NA**
69	0.637785	-		NA
73	0.194973	-		NA
164	-	0.319382		NA
165	-	0.136688		NA
167	-	0.216187		NA
172	-	0.854836		NA
183	-	0.425706		NA

*Complete list is in Appendix D1

**NA – not applicable

Table 3.11. Mean volumes of matched spots between the susceptible and the Kesang biotypes*.

Spot No.	Percentage Volume (mean)		Expression fold	T-test
	Susceptible biotype	Jerantut biotype		$p < 0.05$
49	0.556205	0.19911	-2.7934	0.034128
53	0.562996	0.0838862	-6.7114	0.02099
78	1.93845	0.296506	-6.5376	0.03757
29	0.446804	-		NA**
34	0.408136	-		NA
36	0.403388	-		NA
37	0.564688	-		NA
89	-	2.58392		NA
96	-	0.346853		NA
100	-	0.32396		NA
103	-	0.328636		NA
128	-	0.200548		NA

*Complete list is in Appendix D2

**NA – not applicable

3.8 MALDI-TOF Peptide Mass Fingerprinting

A set of 36 protein spots were chosen from the Jerantut biotype proteome profile and sent to the Proteomics International in Perth, Australia, for MALDI-TOF MS analysis. From this set of 36 protein spots, three spots provided poor spectra that could not be used for further analysis and the remaining 33 spots were used for database searches to identify proteins with similar peptide mass fingerprints.

A total 13 of the spectra were matched to peptides with known functions based on genome analysis, functional studies or sequence comparisons with proteins of known functions. A further 16 spectra were hypothetical proteins, mostly based on genome analysis of various plant species. The remaining 4 spectra were of unknown proteins, matched to amino acid of several species deduced by conceptual translation method (Table 3.12).

However, only 6 from the 13 matched spectra were of high confidence, having a Z score of more than 1.65 (Spot no. 202, 4, 161, 28, 174 and 163) . Similarly, protein spot no. 164 and 168, two of the 16 hypothetical proteins identified, produced a Z score in excess of 1.65 above. Another two unknown proteins also had a Z score that is more than 1.65 (Spot no. 24 and 173) (Table 3.13).

Table 3.12. Identification of mass fingerprints using ProFound. Searches were made against Viridiplantae NCBI NR database. Parameters such as one missed cleavage allowed, carbamidomethylation of cysteine, methionine oxidation and an initial mass tolerance of 0.05 Da were keyed in prior to the search. The value of the Z score, probability, and the percentage of the sequence coverage were used as criteria for identification of proteins.

*Spot no.	Identified Protein [Plant Species]	Z score*	Probability	Coverage (%)	Predicted MW/pI
202	chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (*Flaveria vaginata*)	2.21	1.00E+00	26	11.7 / 5.4
2	NADH dehydrogenase subunit J (*Arabidopsis thaliana*)	0.3	6.60E-01	28	14.9 / 5.9
150	Unidentified	-	-	-	-
1	unknown (*Picea sitchensis*)	1.16	1.00E+00	39	15.03 / 6.8
4	peptidyl-prolyl cis-trans isomerase / cyclophilin (CYP2) / rotamase (*Arabidopsis thaliana*)	2.43	1.00E+00	21	18.87 /8.8
155	hypothetical protein SORBIDRAFT_03g016086 (*Sorghum bicolour*)	0.09	1.50E-01	14	18.19/ 5.2
11	hypothetical protein OsJ_20009 (*Oryza sativa Japonica* Group)	1.35	6.60E-01	20	20.57 / 5.9
161	chloroplastic 2-Cys peroxiredoxin BAS1	2.29	1.00E+00	21	23.39 /5.5
11	Os02g0707900 (*Oryza sativa Japonica* Group)	1.14	5.10E-01	16	20.20 / 6.0
13	hypothetical protein SORBIDRAFT_06g001600 (*Sorghum bicolor*)	1.45	1.00E+00	18	24.36/ 5.6
166	Hypothetical protein MICPUN_104759 (*Micromonas* sp. RCC299)	0.43	8.80E-01	13	26.75 / 5.5
83	cytochrome-c oxidase (*Pisum sativum*)	1.44	9.00E-01	8	28.81 / 5.0
83	cytochrome-c oxidase (*Pisum sativum*)	1.34	6.80E-01	8	28.81 / 5.1
26	Os05g0198100 (*Oryza sativa Japonica* Group)	1.41	9.40E-01	11	33.64 / 5.8
175	Os05g0198100 (*Oryza sativa Japonica* Group)	0.88	9.00E-01	11	33.64 / 5.9
172	hypothetical protein VITISV_027126 (*Vitis vinifera*)	0.9	4.20E-01	20	30.2 /5.0
24	unknown (*Arabidopsis thaliana*)	2.43	1.00E+00	15	33.99 / 5.0
173	unknown (*Zea mays*)	2.43	1.00E+00	12	33.7 / 6.7
28	Chain A, Pea FNR Y308s Mutant In Complex With NADP+	2.43	1.00E+00	17	34.99 / 6.5
210	AT-HSFB3; DNA binding / transcription factor (*Arabidopsis thaliana*)	1.56	9.90E-01	18	28.57 / 5.3

*Protein spots are from the Jerantut biotype proteome

Table 3.12 ***(cont.)***

15	ATMKK8 (*Arabidopsis lyrata* subsp. *Lyrata*)	0.16	4.50E-01	7	28.28 / 6.5
174	WD-repeat protein (*Humulus lupulus*)	2.43	1.00E+00	13	38.13/ 4.9
183	hypothetical protein VOLCADRAFT_103197 (*Volvox carteri f. Nagariensis*)	0.47	8.20E-01	10	42.29 / 6.4
25	maturase K (*Succisa pratensis*)	0.62	9.80E-01	14	35.18 / 9.5
171	hypothetical protein SORBIDRAFT_01g032640 (*Sorghum bicolor*)	0.92	5.50E-01	8	32.88 / 6.2
21	phosphoserine phosphatase (*Chlamydomonas reinhardtii*)	1.41	8.30E-01	12	29.32 /6.3
167	Unidentified	-	-	-	-
14	hypothetical protein OsI_07141 (*Oryza sativa Indica* Group)	0.09	2.20E-01	5	25.58 / 7.0
163	granule-bound starch synthase (*Neomicrocalamus prainii*)	1.66	1.00E+00	15	24.05 / 6.2
207	Unidentified	-	-	-	-
164	hypothetical protein VITISV_043600 (*Vitis vinifera*)	2.43	1.00E+00	20	20.10 / 9.5
12	conserved hypothetical protein (*Ricinus communis*)	0.15	1.90E-01	15	20.27 / 9.3
20	predicted protein (*Populus trichocarpa*)	1.58	1.00E+00	18	26.73 / 8.5
168	hypothetical protein ARALYDRAFT_485883 (*Arabidopsis lyrata* subsp. *lyrata*)	2.14	1.00E+00	11	29.17 /9.5
177	unknown (*Picea sitchensis*)	1.25	8.10E-01	8	36.53 / 9.1
178	Os03g0754800 (*Oryza sativa Japonica* Group)	1.24	7.70E-01	8	35.08 / 9.9

*Protein spots are from the Jerantut biotype proteome

Figure 3.31 shows the location of the identified proteins in the gel of the Jerantut biotype proteome. From the ten identified spectra that have a Z score of more than 1.65, three were expressed in both susceptible and Jerantut biotype (spot no. 4, 24 and 28), with only protein spot no. 4 (peptidyl-prolyl cis-trans isomerase) showed significant difference in expression level between the two biotypes (Table 3.10 and Table 3.13). The remaining seven spots were present only in the Jerantut biotype proteome.

Table 3.13. Identified proteins that are present in the Jerantut biotype proteome.

Spot No.*	Identified Protein	Z score	Coverage (%)	Expression fold**
4*	peptidyl-prolyl cis-trans isomerase / cyclophilin (CYP2) / rotamase (*Arabidopsis thaliana*)	2.43	21	-1.9949
24*	unknown (*Arabidopsis thaliana*)	2.43	15	+1.679
28*	Chain A, Pea FNR Y308s Mutant In Complex With NADP+	2.43	17	-1.1086
161	chloroplastic 2-Cys peroxiredoxin BAS1	2.29	21	0.8791
163	granule-bound starch synthase (*Neomicrocalamus prainii*)	1.66	15	0.1387
164	hypothetical protein VITISV_043600 (*Vitis vinifera*)	2.43	20	0.3194
168	hypothetical protein ARALYDRAFT_485883 (*Arabidopsis lyrata* subsp. *lyrata*)	2.14	11	0.2239
173	unknown (*Zea mays*)	2.43	12	0.6917
174	WD-repeat protein (*Humulus lupulus*)	2.43	13	0.7438
202	chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (*Flaveria vaginata*)	2.21	26	0.1113

*Indicates spots that are present in both the susceptible and the Jerantut biotype.

**Symbol positive (+) show up-regulation of the protein, while the negative symbol (-) indicates down-regulation of the protein.

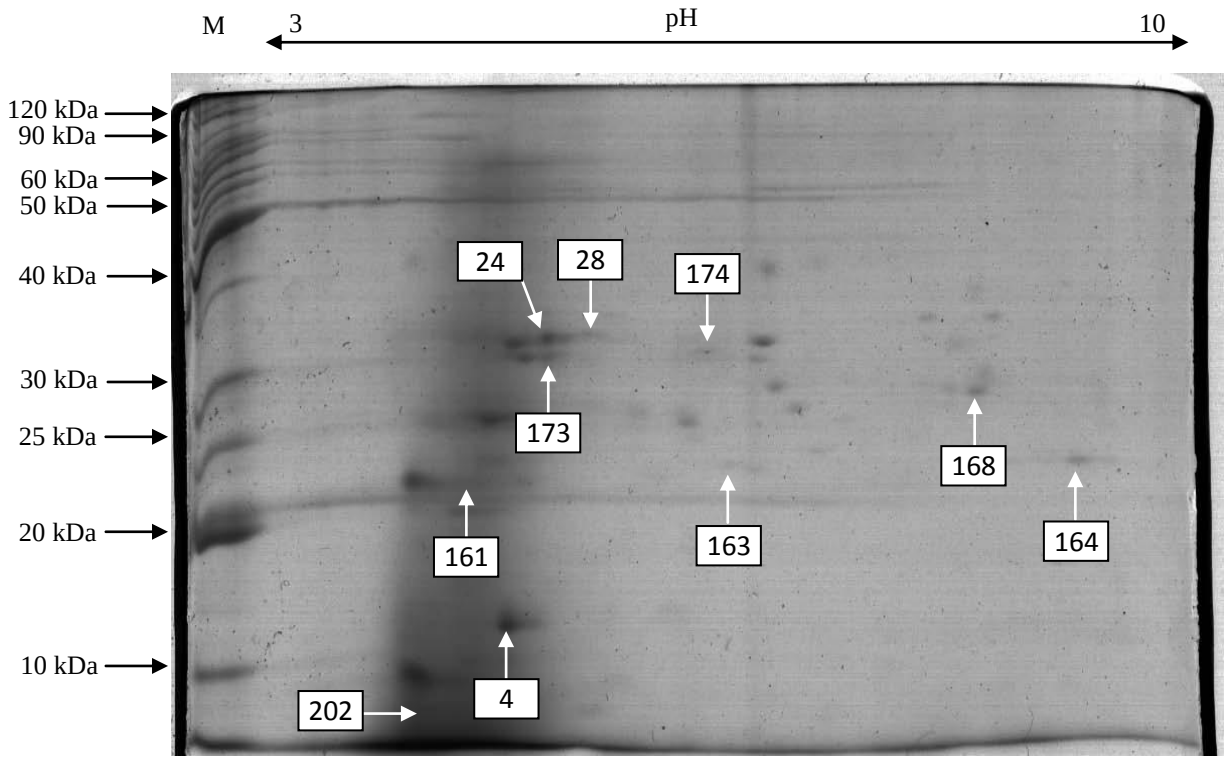

Fig. 3.31. Location of the identified protein from the Jerantut biotype proteome of *Eleusine indica* as listed in Table 3.13. M = molecular weight markers.

CHAPTER 4

GENERAL DISCUSSION

4.1 Herbicide Resistance

Initially this study on goosegrass (*Eleusine indica*) was conducted in response of a much rumoured and suspected, lately, of (new) goosegrass biotypes that are resistant to glufosinate-ammonium. Resistance in goosegrass itself is not a new thing. Since the late 80's, one by one herbicide from different groups, or mode of actions, have been reported to be ineffective in controlling goosegrass. Currently, three major herbicide groups, the bipyridilliums (e.g. paraquat) (Seng *et al*. 2010), the ACCase inhibitors as exemplified by fluazifop-P-butyl (Leach *et al*. 1993), and the glycine (e.g. glyphosate) (Lim and Ngim, 2000) have reported cases of goosegrass resistance in Malaysia.

The Syngenta Quick-Test (QT) was adopted as it was robust, not dependent on seed availability and was not influenced by seed dormancy (Boutsalis 2001). During sampling, goosegrass seeds were mostly non viable for seed test as they were affected either by the earlier herbicide treatment and/or attacked by pests. The QT overcame these problems as it involved cuttings from the whole plant. Additionally it is also applicable to many other graminaceous and dicot weeds. Another major appeal of this QT is the results can be generated within 4 weeks from time of sampling. The feedback from the results should be useful to farmers and enable them to implement other weed management strategies.

Normally for marginal cases of resistance, higher controls of weeds were anticipated under greenhouse studies as the recommended rate is more effective under greenhouse conditions (Heap 2005). This was not the case where lesser percentage of control was achieved by glufosinate-ammonium for both the Kesang and Jerantut biotypes of goosegrass transplanted in our greenhouse studies. We believe this is due to the selection process during sampling, where goosegrass that survived herbicide treatment in the field were collected for the greenhouse trial. It is possible that those

cuttings from the field employed in the greenhouse experiments exhibited a higher level of resistance towards glufosinate-ammonium. However, in the seed test, the level of control of glufosinate-ammonium on the goosegrass was about the same, and in some cases, only slightly higher compared to those achieved in the field trials. This is a total reversal from the results achieved in the greenhouse trial of the transplanted goosegrass biotypes. One explanation for this anomaly is that during seed sampling, seeds were collected over a large area, thus taking in more seeds that represent the true population of goosegrass in respective fields. As such the results of the seed test were similar to the field trials.

Comparing the results from Tables 3.1, 3.3 and 3.7, we can see that glufosinate-ammonium treatment of the Kesang and the Jerantut biotypes of goosegrass generated results at 14 DAT that were somewhat similar to the ones in the field. Intriguingly the treatment of glyphosate on the same biotypes (the Kesang and the Jerantut) produced results at 14 DAT that were more similar, parallel to those results obtained from the transplanted goosegrass (Tables 3.2, 3.4 and 3.7). The possible explanation for this was that the goosegrass populations in both Kesang and Jerantut were still relatively sensitive to glufosinate-ammonium, with the latter populations displaying more resistance than the former populations. We believe there exist goosegrass individuals in both populations that were developing, or has already been resistant to a certain degree, to glufosinate-ammonium but the number of these individuals in both populations were low or minimal compared with the whole population at large. As for the response of the Kesang and the Jerantut biotypes towards glyphosate, it is reasonable to surmise that both populations were homogenous in terms of having developed resistance towards the herbicide. The fact that the transplanted goosegrass and those grown from seeds were affected by glyphosate at nearly the same level following treatment showed that there

was little difference between selectively chosen goosegrass for experimentation and the rest of the population(s).

As such, it was surprising to see differences in resistance index (R.I) values of both types, of goosegrass either transplanted or the ones grown from seeds. By looking at the percentage control by the herbicides on the Kesang and the Jerantut biotypes alone, it would be reasonable to expect the R.I values of the seed-grown goosegrass were lesser than the transplanted goosegrass. Indeed, this was the case for the Kesang and the Jerantut biotypes response to glyphosate. However, this was not the case in their response to glufosinate-ammonium treatments. Surprisingly the resistance index of the Kesang biotype grown from seeds to glufosinate-ammonium was 5.60 compared with 1.97 for the transplanted goosegrass. Meanwhile, the seed grown Jerantut biotype recorded an R.I value of 30.61 compared with 7.63 of the transplanted scourge (Table 3.5 and Table 3.8).

In order to address this problem, we looked into the control capacity of glufosinate-ammonium on susceptible goosegrass used as the control in both the greenhouse (transplanted goosegrass) evaluation and seed test experiments. As illustrated in Fig. 4.1, the time-mediated control of the weed at 495 g ai ha^{-1} of glufosinate-ammonim in the greenhouse experiment were from 68, 66, 50 and 45% at 7, 14, 21 and 28 days after treatment, respectively. However, the percentage control achieved with the same rate of the herbicide in the seed test was very high, ranging from 92 to 97% from the first week to the fourth week after treatment. It was clear that the sensitivity of the susceptible biotype differs greatly in both greenhouse and seed test experiments.

This difference in sensitivity greatly impacted the LC_{50} values of the susceptible biotype. Referring to Tables 3.5 and 3.8, the huge differences in the LC_{50} values resulted

in the major shift of the R.I values for the Kesang biotype from 1.97 (greenhouse transplant experiments) to 5.60 (seed test experiments) and the Jerantut biotype from 7.63 (greenhouse transplant experiments) to 30.61 (seed test experiments).

Moss (2009) had advocated the use of the same herbicide sensitive plant as the control in detecting herbicide resistance. Although the susceptible biotype used in both experiments were of the same origin, it is suspected that these differences in sensitivity was inherent in the plant itself. The susceptible biotypes used in the greenhouse experiments were transplanted, unlike in the seed test where it was grown from seed. This finding could well signify that although goosegrass from urban housing areas were never exposed to any herbicides, it is perhaps due to its exposure from other pollutants, such as the heavy metal, lead, from vehicle exhaust, could confer slight tolerance or otherwise towards herbicides.

Theoretically any resistance index of more than 1 should be considered as resistant. However, Heap (2005) suggested that any resistance index that is less than 10-fold is considered as low level or partial resistance. Taking the resistance index from the seed test experiments, it is reasonable to believe that the Kesang biotype is developing resistance towards glufosinate-ammonium. As it is, the Jerantut biotype poses a more serious threat, most probably already developed resistance to the herbicide.

Despite the differential responses of the three biotypes (the susceptible, the Kesang and the Jerantut) of goosegrass to glufosinate-ammonium, they displayed different degrees of resistance to glyphosate. This was possibly due to the low kill of glyphosate on the Kesang and Jerantut biotypes, including the susceptible counterpart. The low kill of the susceptibly biotype by glyphosate is intriguing, since it has never been previously exposed to glyphosate treatment. After about 10 years since the discovery of goosegrass resistance to glyphosate by Lim and Ngim (2000), there are

possibilities that the resistance genes have escaped from the agricultural environment due to anthropogenic activities. Another possible explanation would be the same as discussed in the paragraph above (tolerance due to exposure to pollutants). However, the actual reason for this resistance to glyphosate remains unknown.

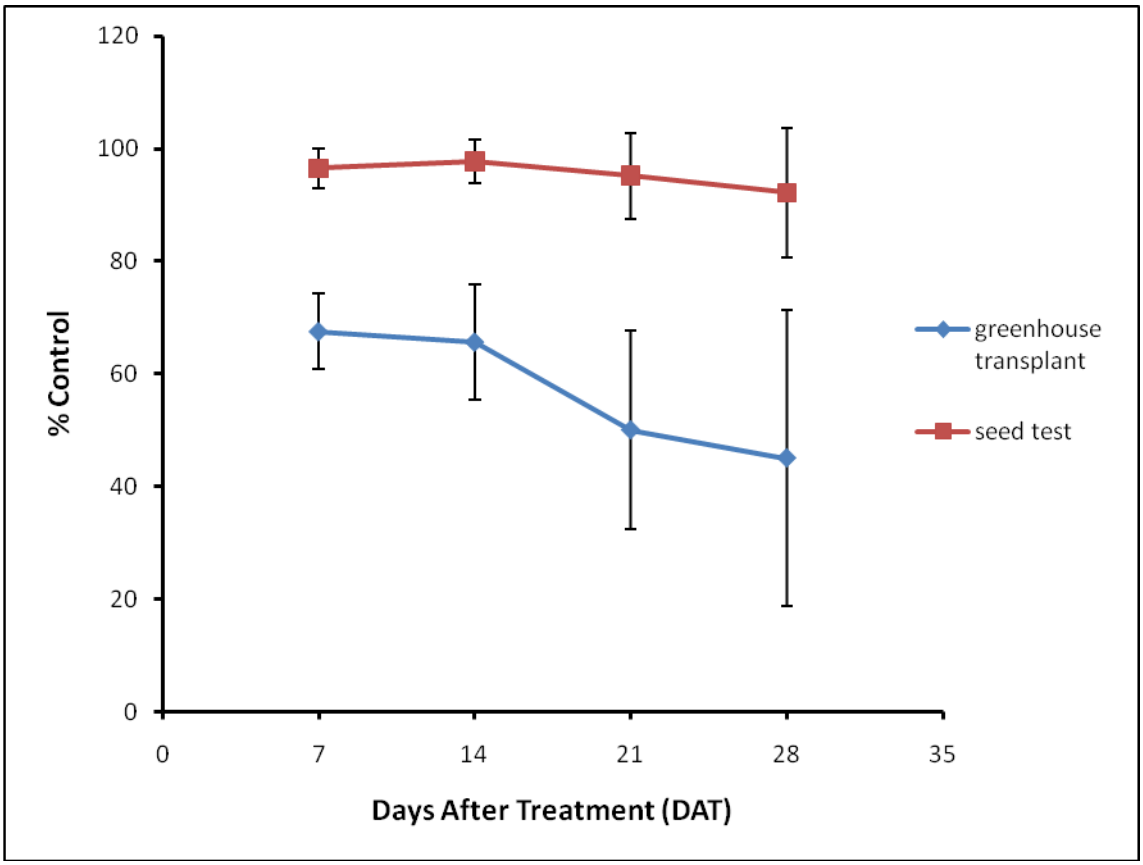

Fig. 4.1. Greenhouse evaluation on differential responses of the susceptible goosegrass biotype in greenhouse evaluation and seed test experiments to glufosinate-ammonium at 495 g ai ha^{-1}. Bars represent 1±SD values.

Treatment history revealed that the vegetable farmer in Kesang, Malacca have only started using glufosinate-ammonium in the past one and half years after the previous glyphosate treatments which failed to control the goosegrass population. In

addition to the chemical control adopted, he ploughed his land each time before a new round of planting. The planters in the palm oil nursery, however, solely rely on glufosinate-ammonium as the only form of weed control for the past 5 years with high intensity of sprays, and there were as many as 24 spray rounds per annum. This high intensity of sprays may have led to selection level leaving only the resistant biotype remaining intact. Further, goosegrass's high fecundity coupled with high selection pressure following repeated sprays with glufosinate-ammonium may have resulted in more resistant gooosegrass populations dominating.

There was clear evidence that the Jerantut biotype was developing resistance to glufosinate (Fig. 3.11, Table 3.8). The Kesang biotype, albeit having a resistance ratio of 5.604, can still be controlled with glufosinate-ammonium, but the ensuing repeated sprays may lead to the build-up of resistance to the herbicide among thriving populations. The control level of goosegrass by glufosinate-ammonium decreased gradually over time, a probable manifestation of age-mediated breakdown of resistance among the treated populations, or reduced efficacy of the herbicide with time, perhaps due to the breakdown of the herbicide. Similar findings were recorded by other workers on age-mediated breakdown or reduction in herbicide resistance by weeds (Baki 1980). The appearance of substantial resistance to glufosinate-ammonium in glufosinate-ammonium selected field populations of goosegrass is truly worrying as this weed species has previously demonstrated resistance to other herbicides such as fluazifop-butyl and glyphosate (Leach *et al.* 1993; Lim and Ngim 2000). Such resistance has now appeared in glufosinate-ammonium-treated field populations of goosegrass in Malacca, Pahang and elsewhere in Peninsular Malaysia (Ngim and Chua 2011, *pers. comms.*).

Previously, there have been reports in the UK and Japan of glufosinate-ammonium-resistant transgenes has been transferred to weedy relatives of experimental

crops (Saji *et al.* 2005; Brown 2005). Recently two reports of goosegrass in Malaysia were reported to be resistance to glufosinate-ammonium (Jalaludin *et. al* 2010; Seng *et. al* 2010) due to selective pressure. Invariably, our data are indicative of being the first case(s) of proven or recorded resistance to glufosinate-ammonium among goosegrass populations in the world in general, and in Malaysia in particular. We advocate that integrated weed management should be adopted by those involved in agricultural practice in order to manage weed resistance problems and to prevent weed resistance to herbicide(s) from escalating.

4.2 Proteome Map of *Eleusine indica*

SDS-PAGE on the extracted protein samples from the susceptible, the Jerantut and the Kesang biotype of goosegrass produced a lot of bands, as evident in Fig. 3.29. These protein bands were separated based on their molecular weights. Although the bands were visible and resolved sharply, there were not many differences noticeable between the gels. However, following two-dimensional gel electrophoresis, the differences between the three goosegrass biotypes were more pronounced, as illustrated in Fig. 3.30. We can easily see differences between the proteome maps of the differing biotypes of goosegrass. Generally more spots can be seen, especially in the 20 – 50 kDa region, whereas the same region in the SDS-PAGE only had not more than 10 bands (at most) that were visible.

The reason behind this is because two-dimensional gel electrophoresis separates proteins based on their isoelectric point (pI) and molecular weight, enabling it to have a very powerful resolving capacity. Furthermore, a single band in the SDS-PAGE does not always translate into a single protein. It could have more than one protein, which is

often the case when resolving complex mixtures of proteins, due to protein isoforms. A single band in an SDS-PAGE could be several spots of proteins in a 2-D gel electrophoresis.

As such, analysis was not carried out on any discrete single bands from SDS-PAGE, as it will most likely reveal multiple proteins. This is true to almost all complex protein samples (Phinney and Thelen 2005). However, PMF (peptide mass fingerprinting) analysis can be carried out from a single SDS-PAGE band if the sample is of low complexity or highly purified samples. Due to this, PMF is most effectively employed in identifying gels on 2-D spots as they are more likely to contain only one prominent protein.

From the proteome map of the susceptible, the Jerantut and the Kesang biotypes, there were major differences in protein spots in the 25 – 50 kDa regions. There were also differences in the 50 kDa region and above and less than 20 kDa region. Most of these differences in abundance were due to the presence or absence of a protein in either the susceptible or the Jerantut and Kesang biotype. This is truly surprising, as often differences in expressed proteins were recorded when the samples (in this case the goosegrass) were exposed to stress such as water deficit, extreme temperature, high salt concentrations, herbicides, etc. (Vincent and Zivy 2007). However the three biotypes used in this study (the susceptible, the Jerantut and the Kesang biotypes) were under the same conditions and were not treated with herbicide prior to pulverization with liquid nitrogen. They were grown from seed and directly processed. These proteins (that were absent in the susceptible biotype) were expressed in low volumes, which could mean that any trigger in stress caused by herbicides could lead to rapid increase in the expression levels of these proteins, which may result in various biochemical pathways involved in resistance towards the herbicides. Perhaps the reason these proteins are

readily expressed was as precautionary measures, a pre-emptive form of protection against herbicides.

Between the susceptible and the Kesang biotypes, there were three spots that were present in both biotypes and were differentially expressed, namely spot no. 49, 53 and 78 (Table 3.11; Fig. 3.30). Meanwhile, between the susceptible and the Jerantut biotypes, four spots that were present in both biotypes were found to be differentially expressed, that is spot no. 2, 4, 31 and 36 (Table 3.10; Fig. 3.30). To avoid confusion, it should be noted that the numbers assigned for each spot is exclusive to its own analysis. What this mean is that for example, spot no. 2 in the analysis between the susceptible and the Kesang biotype is not the same with spot no. 2 from the susceptible and the Jerantut biotype analysis.

That being said, although there were significant differences in the expression of several spots between the susceptible and the Kesang biotypes, only spots from the Jerantut biotype were excised and sent for MALDI-TOF analysis. The reason behind the selection of spots exclusively from the Jerantut biotype was because between the Jerantut and the Kesang biotypes, the Jerantut showed a higher level of resistance towards glufosinate-ammonium and glyphosate (Table 3.8 and 3.5 respectively) than the Kesang biotype.

Furthermore, despite the availability of spots with significant differences in expression between the susceptible and the Jerantut biotypes, only 36 spots were excised and sent for MALDI-TOF analysis (Table 3.12). This was because most of the spots, especially the small ones, although were visible through the image analysis software, were barely visible to the naked eye. Due to the manual spot picking, it was very hard to correctly excise the spots, causing a lot of the spots to be overlooked,

including spots of high interest such as spot no. 2, 31 and 36 (spots with significant differences in expression between the susceptible and the Jerantut biotypes). Automated spot picking, where the scanned image of the gel is linked to a machine that excised the spots, can easily overcome this problem and greatly improve the identification process of the protein spots.

Of the 36 protein spots cut out for MALDI-TOF MS, only 10 recorded estimated Z scores of more than 1.65 (95th percentile) with probability values very close to 1 (Table 3.13). These 10 proteins were considered to have a high probability to be the sample proteins. Spots that scored estimated Z values in the 90th percentile were not considered since there is a 10% probability it could be other proteins in the random match population and the 10% probability is just too high. The other proteins identified scored either low Z values (less than 1.65) or low probability or both and as such, were unlikely to be the sample proteins.

Out of the ten highly probable proteins, three were expressed in both the susceptible and the Jerantut biotypes. They are peptidyl-prolyl cis-trans isomerase (spot no. 4), an unknown protein (spot no. 24) and Chain A, pea ferredoxin $NADP^+$ reductase, or FNR (spot no.28; Table 3.13). However, only peptidyl-prolyl cis-trans isomerase had a significant difference in its expression. The other seven highly probable proteins are present only in the Jerantut biotype proteome. They consist of chloroplastic 2-Cys peroxiredoxin, granule bound starch synthase, WD repeat protein, chloroplast RuBisCo small subunit, 2 hypothetical proteins and another unknown protein (Table 3.13).

Two proteins were of high interest, due to their significance in expression level and functions in plants. The two said proteins are peptidyl-prolyl cis-trans isomerase and chloroplastic 2-Cys peroxiredoxin Bas1. Peptidyl-prolyl cis-trans isomerase is

involved in the folding of proteins, where it catalyzes the conversion of *cis* and *trans* isomers of peptide bonds with the amino acid proline. This protein was detected in both the susceptible and the Jerantut biotype, with a reduction of about 2 fold in expression. Apart from the basic role of assisting protein folding, peptidyl-prolyl cis-trans isomerase or cyclophilin is believed to also play an important role in mRNA processing, protein degradation and signal transduction and thus may be crucial during both development and stress responsiveness (Romano *et al.* 2004). Furthermore, Marivet *et al*. (1994) had demonstrated that there were differences in mRNA accumulation profile upon heat and salt stress, further suggesting that cyclophilin might be a stress-related protein. How exactly it contributes to herbicide resistance towards glufosinate-ammonium remains unknown, since its expression is lower in the Jerantut biotype but the fact that cyclophilin could play a role in plants under abiotic stress is worthy of note.

In the case of herbicide-resistant plants and its response towards herbicides, it was observed that proteins involved in the reactive oxygen species (ROS) scavenging mechanisms were often induced. Chloroplastic 2-Cys peroxiredoxin Bas1 is one of those enzymes. This protein was expressed in the Jerantut biotype with no expression detectable in the susceptible variety. 2-Cys peroxiredoxins is a family of enzymes which catalyze the transfer of electrons from sulfhydryl residues to peroxides. They are thiol-specific antioxidant proteins (TSA) which confer a protective role in cells through its peroxidase activity by reducing hydrogen peroxides, peroxynitrite, and organic hydroperoxides.

Netto *et al.* (1996) reported that TSA protects glutamine synthethase from inactivation by a metal-catalyzed oxidation (MCO) system. However TSA is not able to prevent glutamine synthetase and other enzymes from oxidative inactivation if a nonsulfhydryl reducing agent replaces a thiol compound in the reaction mixture. This

protein is mainly expressed in the plastids and chloroplasts of the leaf blade, sheath, basiplast, stem and green spike with maximal expression in young developing shoots segments where cell division and elongation take place, to protect it from oxidative damage and that the damage is reduced by the accumulation of 2-Cys peroxiredoxin (Baier and Dietz 1996; 1999).

Despite the capability of 2-Cys peroxiredoxin to protect glutamine synthethase, it is highly plausible that 2-Cys peroxiredoxin role in the Jerantut biotype is limited to only reducing the ROS. As explained by Netto *et al.* (1996), 2-Cys peroxiredoxin as a TSA only protects glutamine synthethase from oxidative inactivation as long as the reaction mixture does not involve nonsulfhydryl reducing agent. However, in the case of glutamine synthase inhibition by glufosinate-ammonium, it inhibits glutamine synthetase due to the fact that it is an analogue to glutamate.

The other identified proteins belong to various groups of biochemical pathways in plants. For example, the chain A pea FNR was involved in photosynthesis, where it catalyzes the reduction of $NADP^+$ to NADPH. Expressed in both susceptible and the Jerantut biotypes, it is believed that it does not contribute to resistance to the herbicide since it was expressed in both biotypes and the differences were non-significant.

It is interesting to note that the other remaining highly probable proteins were available only in the Jerantut biotype proteome (Table 3.13). Four of the proteins consist of unknown and hypothetical proteins, which make their functions in the Jerantut biotype unknown. The granule-bound starch synthase or GBSS for short are involved in the biosynthesis of cell wall polysaccharides, the addition of N-linked glycans to glycoproteins, and the attachment of sugar moieties to small molecules such as hormones and flavonoids (Keegstra and Raikhel 2001). How they are related or could

play a role to the resistance of goosegrass towards glufosinate-ammonium remains unknown.

The chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (RuBisCO), identified from *Flaveria vaginata,* is another protein that functions in photosynthesis. Unlike the chain A pea FNR, this protein was found to be expressed only in the Jerantut biotype proteome. Although only the small subunit was identified, the possibility of the large subunit present in the proteome should not be ruled out. RuBisCO catalyzes the photosynthetic carbon fixation and photorespiratory carbon oxidation (Mehta *et al.* 1992). Researches have shown that RuBisCO degrades under abiotic stress such as herbicides and drought (Feller *et al.* 2008; Sedigheh *et al.* 2011). It is possible that the overexpression of RuBisCO was to prepare the plant for this very reason.

The WD-repeat protein, on the other hand, is a protein of a wide variety of important biological functions. Its role in plants ranges from signal transduction, transcription regulation , apoptosis, signalling and vision, cell motility, flowering and meristem organization, to name a few (Li and Roberts 2001). Its exact contribution towards glufosinate-ammonium resistance remains to be uncovered, but from its critical involvement in plant's signalling and regulation, it is plausible it could have a hand in the Jerantut biotype's resistance towards glufosinate-ammonium.

The peroxiredoxin identified is a thiol-specific antioxidant while the peptidyl-prolyl cis/trans isomerase could have a role in heat and salt stress. Although the peroxiredoxin was only detected in the Jerantut biotype proteome, the presence or absence of other antioxidants or ROS scavenging enzymes in the susceptible biotype of goosegrass cannot be ruled out as there are still tens of spots unidentified. For the other highly probable proteins that were only expressed in the Jerantut biotype proteome,

their absence from the susceptible biotype is truly intriguing. The fact that there is huge difference in protein abundance between the glufosinate-ammonium-resistant goosegrass (the Jerantut biotype) and the susceptible goosegrass biotype leads to an interesting possibility that hidden in those unidentified spots could be a protein that might well explain the occurrence of resistance in goosegrass towards glufosinate-ammonium.

CHAPTER 5

CONCLUSION

To date, goosegrass in Malaysia have been reported to be resistant towards several herbicides with different modes of action. They include the ACCase inhibitors, the bipydiriliums and the glycines. With this study, it is undoubtful that another class of herbicides are included in that group. Glufosinate-ammonium belongs to the glutamine-synthase inhibitors. This study confirms that there exist populations/ biotypes of goosegrass that are developing and/or have developed resistance towards glufosinate-ammonium. Furthermore another independent study by another Malaysian weed scientist reported the same finding, but with a different population of goosegrass (Seng *et al.* 2010).

The Kesang biotype registered a resistance index (R.I) of 1.97 for transplanted goosegrass and 5.6 for seed-grown goosegrass against glufosinate-ammonium. Against glyphosate, its R.I was 8.41 and 1.37 for transplanted and seed-grown goosegrass, respectively. Meanwhile the Jerantut biotype had an R.I of 7.63 for transplanted goosegrass and 30.6 for goosegrass grown from seeds against glufosinate-ammonium. Against glyphosate, its R.I was 24.37 and 3.28 for transplanted and seed-grown goosegrass, respectively.

Regardless of the difference in the R.I value between the transplanted and seed-grown goosegrass, it is suffice to say that the Kesang biotype is developing resistance towards glufosinate-ammonium. On the other hand, the Jerantut biotype is most likely to have had already developed resistance towards the herbicide. The same can be said for both biotypes against glyphosate, where both the Jerantut and Kesang biotype were resistant towards glyphosate. One of the more interesting revelation is perhaps the control of the susceptible goosegrass biotype by glufosinate-ammonium and glyphosate. The susceptible biotype seems to have acquired resistance towards glyphosate, while still being sensitive towards glufosinate-ammonium treatment.

Despite the increase in reported cases of herbicide-resistant weeds, it is impossible for those involved in agricultural practice to avoid using herbicide as a form of chemical control for scourge plants. It is also impossible to assume that weeds will not become resistant to new or other herbicides with different mode of actions in the near future. Integrated weed management provide both short and long term solution by focusing not just on chemical control techniques, but also physical and biological methods in an integrated manner without excessive reliance on any one method (Powles and Matthews 1992).

The proteomics study approach have revealed the differences in proteins expressed in abundance by the three biotypes (the susceptible, the Jerantut and the Kesang). The Jerantut and the Kesang goosegrass biotypes have many more proteins in abundance compared to the susceptible biotype, even under the absence of herbicide (glufosinate-ammonium) stress.

Although there were ten spots identified from the Jerantut biotype proteome, many more were still unknown. It is imperative to remember that this proteome does not represent the total proteome of the Jerantut biotype of *Eleusine indica*. Size exclusion chromatography was used in order to desalt the sample and to enrich it with high molecular weight proteins, eliminating low molecular weight proteins and those that were eluted along with salts.

The diversity of protein solubilities and plant tissue composition ensure no single protein extraction method is effective enough for all samples. The sheer dynamic range between low and high abundance proteins alone presents an uphill challenge in obtaining total proteome. It was estimated that only 25% of the expected proteome can be observed in 2-D gels (Patterson 2004), and entire proteome coverage is not possible. Any future endeavour in deciphering the resistance mechanism through proteomics may

consider a few aspects, such as different protein extraction methods, anlaysis of the proteome under herbicide stress, isolation of the subproteomes, and combining proteomics with metabolomics studies.

Analysis of subcellular proteins could improve the proteome coverage by several folds, and unmask the low abundance proteins. It could also provide new insights into the functions, regulations and intracellular protein transport of organelles. Combination of proteomic and metabolomic studies will allow better understandings of the integrated plant responses to herbicides, or glufosinate-ammonium in particular.

With the confirmation of this new glufosinate-resistant *Eleusine indica*, the importance of investigating its resistance mechanisms is more pronounced than ever. Proteomics could allow identification of proteins or novel genes, characterisation of their regulation and function and perhaps the very cellular processes involved in the response under herbicide treatment. Better understanding of the resistance mechanisms is vital in order to manage herbicide resistant weeds in the future and protecting our precious cash crops in the economic long run.

PUBLICATIONS

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APPENDICES

APPENDIX A-1

Raw data of field evaluation on Kesang biotype of goosegrass with glufosinate-ammonium.

Treatment	**247.5 g ai/ha**			
DAT	7	14	21	28
% Control	70	75	75	75
% Control	70	70	80	70
% Control	80	85	95	90
Average	73.33333	76.66667	83.33333	78.33333

Treatment	**495 g ai/ha**			
DAT	7	14	21	28
% Control	85	80	80	80
% Control	90	85	85	80
% Control	85	80	90	75
Average	86.66667	81.66667	85	78.33333

Treatment	**990 g ai/ha**			
DAT	7	14	21	28
% Control	80	85	85	85
% Control	90	98	90	90
% Control	95	98	98	98
Average	88.33333	93.66667	91	91

Treatment	**1980 g ai/ha**			
DAT	7	14	21	28
% Control	95	95	98	95
% Control	98	98	98	95
% Control	98	98	100	98
Average	97	97	98.66667	96

APPENDIX A-1, *cont.*

Raw data of field evaluation on Kesang biotype of goossegrass with glyphosate.

Treatment	**1080 g/ha**			
DAT	7	14	21	28
% Control	0	0	5	10
% Control	15	10	10	10
% Control	25	20	10	10
Average	13.33333	10	8.333333	10

Treatment	**2160 g/ha**			
DAT	7	14	21	28
% Control	15	10	5	10
% Control	10	20	5	5
% Control	15	25	10	10
Average	13.33333	18.33333	6.666667	8.333333

Treatment	**4320 g/ha**			
DAT	7	14	21	28
% Control	0	10	5	5
% Control	5	10	5	10
% Control	10	20	15	15
Average	5	13.33333	8.333333	10

APPENDIX A-2

Raw data of field evaluation on Jerantut biotype of goosegrass with glufosinate-ammonium.

Treatment	**495 g ai/ha**			
DAT	7	14	21	28
% Control	10	0	0	0
% Control	10	0	0	0
% Control	15	0	5	10
Average	11.66667	0	1.666667	3.333333

Treatment	**990 g ai/ha**			
DAT	7	14	21	28
% Control	40	20	10	0
% Control	70	65	20	10
% Control	45	50	15	5
Average	51.66667	45	15	5

Treatment	**1980 g ai/ha**			
DAT	7	14	21	28
% Control	75	60	60	40
% Control	85	70	50	30
% Control	80	65	40	20
Average	80	65	50	30

Treatment	**3960 g ai/ha**			
DAT	7	14	21	28
% Control	90	80	75	90
% Control	90	90	70	60
% Control	95	85	70	50
Average	91.66667	85	71.66667	66.66667

APPENDIX A-2, *cont.*

Raw data of field evaluation on Jerantut biotype of goosegrass with glyphosate.

Treatment	**540 g/ha**			
DAT	7	14	21	28
% Control	0	0	0	0
% Control	0	0	0	0
% Control	5	0	5	5
Average	1.667	0	1.6667	1.6667

Treatment	**1080 g/ha**			
DAT	7	14	21	28
% Control	0	0	0	0
% Control	5	0	0	0
% Control	0	0	0	0
Average	1.667	0	0	0

Treatment	**2160 g/ha**			
DAT	7	14	21	28
% Control	0	0	0	0
% Control	5	0	0	0
% Control	10	10	0	0
Average	5	3.333	0	0

Treatment	**4320 g/ha**			
DAT	7	14	21	28
% Control	0	0	0	0
% Control	5	5	0	0
% Control	10	5	5	10
Average	5	3.333	1.667	3.3333

APPENDIX A-3 - Raw data of greenhouse experiment on transplanted Kesang biotype of goosegrass with glufosinate-ammonium.

Treatment	495 g ai/ha			
DAT	7	14	21	28
% Control	20	20	5	10
% Control	40	40	50	70
% Control	40	40	50	40
% Control	60	60	60	30
% Control	40	40	60	30
% Control	15	25	40	40
% Control	80	60	60	20
% Control	75	60	60	40
Average	46.25	43.13	48.13	35.00

Treatment	1980 g ai/ha			
DAT	7	14	21	28
% Control	98	98	85	80
% Control	100	100	100	90
% Control	100	100	100	100
% Control	100	100	100	100
% Control	100	100	100	90
% Control	100	100	100	100
% Control	100	100	100	100
% Control	100	100	100	100
Average	99.75	99.75	98.13	95.00

Treatment	990 g ai/ha			
DAT	7	14	21	28
% Control	95	98	98	90
% Control	80	90	75	30
% Control	60	40	10	10
% Control	75	60	80	70
% Control	80	60	60	10
% Control	80	70	70	10
% Control	75	60	60	10
% Control	80	100	100	100
Average	78.13	72.25	69.13	41.25

Treatment	3960 g ai/ha			
DAT	7	14	21	28
% Control	95	100	100	85
% Control	100	100	100	40
% Control	100	100	100	100
% Control	100	100	100	100
% Control	100	100	100	70
% Control	100	100	100	100
% Control	100	100	100	20
% Control	100	100	100	100
Average	99.38	100.00	100.00	76.88

APPENDIX A-3 (*cont.*) - Raw data of greenhouse experiment on transplanted susceptible biotype of goosegrass with glufosinate-ammonium.

Treatment	495 g ai/ha			
DAT	7	14	21	28
% Control	20	65	40	25
% Control	75	65	40	60
% Control	75	65	60	60
% Control	75	55	40	10
% Control	80	70	90	95
% Control	60	40	10	5
% Control	80	100	100	100
% Control	75	65	20	5
Average	67.50	65.63	50.00	45.00

Treatment	1980 g ai/ha			
DAT	7	14	21	28
% Control	100	100	100	100
% Control	100	100	100	100
% Control	100	100	100	100
% Control	100	100	100	100
% Control	100	100	100	100
% Control	100	100	100	100
% Control	100	100	100	100
% Control	100	100	100	100
Average	100.00	100.00	100.00	100.00

Treatment	990 g ai/ha			
DAT	7	14	21	28
% Control	98	100	100	100
% Control	95	100	100	100
% Control	98	100	100	40
% Control	98	100	100	100
% Control	100	100	100	90
% Control	98	100	100	100
% Control	98	100	100	60
% Control	100	100	100	100
Average	98.13	100.00	100.00	86.25

Treatment	3960 g ai/ha			
DAT	7	14	21	28
% Control	100	100	80	75
% Control	100	100	100	100
% Control	100	100	100	100
% Control	100	100	100	100
% Control	100	100	100	100
% Control	100	100	100	100
% Control	100	100	100	100
% Control	100	100	100	100
Average	100.00	100.00	97.50	96.88

APPENDIX A-3 (*cont.*) - Raw data of greenhouse experiment on Jerantut biotype of goosegrass with glufosinate-ammonium.

Treatment	495 g ai/ha			
DAT	7	14	21	28
% Control	5	5	5	5
% Control	0	0	0	5
% Control	5	5	0	5
% Control	5	5	30	20
% Control	0	0	0	5
% Control	5	0	5	5
% Control	5	5	10	5
% Control	10	5	5	5
Average	4.38	3.13	6.88	6.88

Treatment	1980 g ai/ha			
DAT	7	14	21	28
% Control	60	50	5	5
% Control	5	5	0	5
% Control	50	40	0	0
% Control	100	100	100	100
% Control	5	5	5	5
% Control	5	5	5	5
% Control	5	5	0	5
% Control	20	15	20	5
Average	31.25	28.13	16.88	16.25

Treatment	990 g ai/ha			
DAT	7	14	21	28
% Control	50	50	0	0
% Control	60	60	0	0
% Control	50	40	0	10
% Control	25	15	5	10
% Control	15	10	0	0
% Control	98	100	0	0
% Control	20	10	5	10
% Control	25	10	10	10
Average	42.88	36.88	2.50	5.00

Treatment	3960 g ai/ha			
DAT	7	14	21	28
% Control	60	40	5	0
% Control	60	40	0	5
% Control	60	90	80	10
% Control	60	20	20	10
% Control	100	100	100	100
% Control	100	100	100	100
% Control	60	60	60	40
% Control	60	60	60	60
Average	70.00	63.75	53.13	40.63

APPENDIX A-4 - Raw data of greenhouse experiment on Kesang biotype of goosegrass with glyphosate.

Treatment	540 g/ha			
DAT	7	14	21	28
% Control	0	0	0	0
% Control	0	0	0	5
% Control	0	0	10	10
% Control	0	0	0	0
% Control	0	0	70	25
% Control	0	0	60	40
% Control	0	0	70	30
% Control	0	0	70	70
Average	0	0	35	22.5

Treatment	2160 g/ha			
DAT	7	14	21	28
% Control	15	75	100	90
% Control	25	85	98	100
% Control	20	70	98	100
% Control	10	60	100	100
% Control	10	80	98	95
% Control	5	15	60	100
% Control	15	80	100	80
% Control	60	100	100	95
Average	20	70.625	94.25	95

Treatment	1080 g/ha			
DAT	7	14	21	28
% Control	0	5	25	60
% Control	0	10	15	25
% Control	0	10	40	100
% Control	5	10	25	20
% Control	5	15	20	30
% Control	10	15	20	5
% Control	5	10	40	30
% Control	5	5	60	20
Average	3.75	10	30.625	36.25

Treatment	4320 g/ha			
DAT	7	14	21	28
% Control	60	95	100	100
% Control	100	100	100	100
% Control	60	98	100	80
% Control	85	100	100	95
% Control	65	90	95	90
% Control	30	80	98	100
% Control	60	98	100	100
% Control	20	90	98	100
Average	60	93.875	98.875	95.625

APPENDIX A-4 (*cont.*) - Raw data of greenhouse experiment on susceptible biotype of goosegrass with glyphosate.

Treatment	540 g/ha			
DAT	7	14	21	28
% Control	60	95	100	100
% Control	60	90	90	75
% Control	50	75	95	95
% Control	50	85	100	95
% Control	60	95	100	100
% Control	50	70	95	30
% Control	60	95	100	100
% Control	60	80	95	75
Average	56.25	85.625	96.875	83.75

Treatment	2160 g/ha			
DAT	7	14	21	28
% Control	35	95	100	80
% Control	60	100	100	100
% Control	60	95	100	100
% Control	40	80	100	100
% Control	25	90	100	100
% Control	0	60	100	80
% Control	60	100	100	100
% Control	100	100	100	80
Average	47.5	90	100	92.5

Treatment	1080 g/ha			
DAT	7	14	21	28
% Control	20	80	100	80
% Control	60	98	100	100
% Control	10	98	100	100
% Control	80	100	100	90
% Control	60	100	100	100
% Control	60	100	100	100
% Control	60	85	95	90
% Control	60	90	95	90
Average	51.25	93.875	98.75	93.75

Treatment	4320 g/ha			
DAT	7	14	21	28
% Control	75	100	100	100
% Control	75	100	100	95
% Control	75	100	100	100
% Control	65	100	100	100
% Control	5	90	100	95
% Control	95	100	100	100
% Control	75	100	100	95
% Control	80	95	98	95
Average	68.125	98.125	99.75	97.5

APPENDIX A-4 (*cont.*) - Raw data of greenhouse experiment on Jerantut biotype of goosegrass with glyphosate.

Treatment	540 g/ha			
DAT	7	14	21	28
% Control	5	0	0	0
% Control	0	0	0	0
% Control	10	5	10	5
% Control	0	0	40	15
% Control	5	0	10	15
% Control	10	5	10	10
% Control	5	0	5	10
% Control	0	0	5	10
Average	4.375	1.25	10	8.125

Treatment	1080 g/ha			
DAT	7	14	21	28
% Control	0	15	20	5
% Control	5	40	100	100
% Control	0	5	10	10
% Control	0	60	95	100
% Control	10	35	100	100
% Control	0	0	5	5
% Control	5	10	70	95
% Control	0	10	10	20
Average	2.5	21.875	51.25	54.375

Treatment	2160 g/ha			
DAT	7	14	21	28
% Control	20	100	100	100
% Control	10	70	98	100
% Control	0	15	95	100
% Control	0	0	0	0
% Control	5	5	10	100
% Control	0	0	0	0
% Control	5	5	5	0
% Control	5	5	10	60
Average	5.625	25	39.75	57.5

Treatment	4320 g/ha			
DAT	7	14	21	28
% Control	5	5	10	10
% Control	10	15	100	100
% Control	10	15	100	100
% Control	5	5	5	100
% Control	0	5	95	100
% Control	30	90	100	0
% Control	5	10	5	5
% Control	0	5	5	100
Average	8.125	18.75	52.5	64.375

APPENDIX A-5 - Raw data of greenhouse experiment on Kesang biotype of goosegrass grown from seed with glufosinate-ammonium.

Treatment	495 g ai/ha			
DAT	7	14	21	28
% Control	80	80	60	40
% Control	90	85	85	80
% Control	85	60	60	60
% Control	70	60	60	60
% Control	90	90	85	80
% Control	90	85	65	60
Average	84.16667	**76.66667**	**69.16667**	**63.33333**

Treatment	1980 g ai/ha			
DAT	7	14	21	28
% Control	90	95	80	70
% Control	98	100	100	95
% Control	95	95	85	85
% Control	90	80	60	55
% Control	95	98	85	75
% Control	98	100	100	100
Average	94.33333	94.66667	85	80

Treatment	990 g ai/ha			
DAT	7	14	21	28
% Control	95	95	80	70
% Control	80	70	65	55
% Control	95	95	65	55
% Control	80	80	65	55
% Control	98	95	80	75
% Control	98	95	80	70
Average	91	88.33333	72.5	63.33333

Treatment	3960 g ai/ha			
DAT	7	14	21	28
% Control	100	100	100	100
% Control	100	100	100	100
% Control	100	100	100	100
% Control	98	98	85	80
% Control	98	98	85	80
% Control	100	100	100	100
Average	99.33333	99.33333	95	93.33333

APPENDIX A-5 (*cont.*) - Raw data of greenhouse experiment on susceptible biotype of goosegrass grown from seed with glufosinate-ammonium.

Treatment	495 g ai/ha			
DAT	7	14	21	28
% Control	98	100	100	100
% Control	100	100	100	100
% Control	98	98	98	98
% Control	95	98	95	90
% Control	98	100	98	95
% Control	90	90	80	70
Average	96.50	97.67	95.17	92.17

Treatment	1980 g ai/ha			
DAT	7	14	21	28
% Control	100	100	98	85
% Control	100	100	95	85
% Control	100	100	100	100
% Control	100	100	100	100
% Control	100	100	98	100
% Control	98	90	85	80
Average	99.67	98.33	96.00	91.67

Treatment	990 g ai/ha			
DAT	7	14	21	28
% Control	100	100	100	90
% Control	100	100	100	100
% Control	100	100	98	85
% Control	100	100	100	100
% Control	100	100	100	100
% Control	100	100	85	75
Average	100	100	97.17	91.67

Treatment	3960 g ai/ha			
DAT	7	14	21	28
% Control	100	100	100	100
% Control	100	100	85	80
% Control	100	100	100	95
% Control	100	100	95	80
% Control	100	100	100	100
% Control	100	100	100	95
Average	100	100	96.67	91.67

APPENDIX A-5 (*cont.*) - Raw data of greenhouse experiment on Jerantut biotype of goosegrass grown from seed with glufosinate-ammonium.

Treatment	495 g ai/ha			
DAT	7	14	21	28
% Control	10	10	10	10
% Control	10	10	10	10
% Control	15	20	40	40
% Control	20	15	30	30
% Control	20	20	30	30
% Control	25	15	30	30
Average	16.66667	15	25	25

Treatment	990 g ai/ha			
DAT	7	14	21	28
% Control	50	75	60	55
% Control	40	70	55	50
% Control	65	80	65	60
% Control	40	70	60	50
% Control	65	60	10	10
% Control	30	10	10	15
Average	48.33333	60.83333	43.33333	40

Treatment	1980 g ai/ha			
DAT	7	14	21	28
% Control	98	100	100	100
% Control	98	100	100	100
% Control	65	60	45	40
% Control	65	80	65	80
% Control	65	80	80	60
% Control	65	70	60	40
Average	76	81.66667	75	70

Treatment	3960 g ai/ha			
DAT	7	14	21	28
% Control	90	90	85	100
% Control	98	100	100	80
% Control	75	80	60	95
% Control	75	80	60	80
% Control	60	70	50	100
% Control	70	80	40	95
Average	78	83.33333	65.83333	91.66667

APPENDIX A-6 - Raw data of greenhouse experiment on Kesang biotype of goosegrass grown from seed with glyphosate.

Treatment	540 g/ha			
DAT	7	14	21	28
% Control	0	10	35	35
% Control	0	5	20	20
% Control	0	20	15	15
% Control	0	5	20	20
% Control	0	20	15	20
% Control	0	10	10	15
Average	0	11.67	19.17	20.83

Treatment	1080 g/ha			
DAT	7	14	21	28
% Control	0	10	5	5
% Control	30	20	10	10
% Control	5	5	5	5
% Control	5	10	10	10
% Control	5	5	40	40
% Control	10	15	40	40
Average	9.17	10.83	18.33	18.33

Treatment	2160 g/ha			
DAT	7	14	21	28
% Control	10	90	95	95
% Control	5	85	90	90
% Control	10	85	90	90
% Control	10	85	90	90
% Control	5	40	60	60
% Control	10	40	40	40
Average	8.33	70.83	77.50	77.50

Treatment	4320 g/ha			
DAT	7	14	21	28
% Control	15	95	85	85
% Control	10	85	98	98
% Control	10	85	85	85
% Control	10	98	95	95
% Control	10	80	85	85
% Control	15	80	90	90
Average	11.67	87.17	89.67	89.67

APPENDIX A-6 (*cont.*) - Raw data of greenhouse experiment on susceptible biotype of goosegrass grown from seed with glyphosate.

Treatment	540 g/ha			
DAT	7	14	21	28
% Control	0	20	10	15
% Control	0	5	60	80
% Control	0	20	25	30
% Control	0	15	25	40
% Control	0	10	25	25
% Control	0	20	30	30
Average	0	15	29.17	36.67

Treatment	1080 g/ha			
DAT	7	14	21	28
% Control	0	20	100	100
% Control	5	40	75	70
% Control	0	10	80	95
% Control	0	5	20	20
% Control	10	60	40	40
% Control	15	25	70	70
Average	5	26.67	64.17	65.83

Treatment	2160 g/ha			
DAT	7	14	21	28
% Control	20	85	98	98
% Control	10	98	98	98
% Control	20	90	98	98
% Control	80	100	100	100
% Control	10	60	90	90
% Control	10	60	80	80
Average	25	82.17	94	94

Treatment	4320 g/ha			
DAT	7	14	21	28
% Control	20	85	100	100
% Control	40	95	100	100
% Control	20	98	98	98
% Control	15	98	98	98
% Control	90	100	100	100
% Control	40	98	100	100
Average	37.5	95.67	99.33	99.33

APPENDIX A-6 (*cont.*) - Raw data of greenhouse experiment on Jerantut biotype of goosegrass grown from seed with glyphosate.

Treatment	540 g/ha			
DAT	7	14	21	28
% Control	0	0	10	10
% Control	0	0	10	10
% Control	0	10	10	10
% Control	0	10	10	10
% Control	0	10	5	5
% Control	0	5	5	5
Average	0	5.83	8.33	8.33

Treatment	1080 g/ha			
DAT	7	14	21	28
% Control	0	10	5	5
% Control	0	25	0	5
% Control	0	15	30	30
% Control	0	40	40	40
% Control	0	0	15	15
% Control	0	0	50	50
Average	0	15	23.33	24.17

Treatment	2160 g/ha			
DAT	7	14	21	28
% Control	20	25	50	50
% Control	40	10	10	10
% Control	0	5	5	5
% Control	0	20	45	45
% Control	0	20	45	45
% Control	0	25	60	60
Average	10	17.5	35.83	35.83

Treatment	4320 g/ha			
DAT	7	14	21	28
% Control	100	30	100	100
% Control	0	0	5	5
% Control	10	0	15	15
% Control	60	5	60	60
% Control	25	0	30	30
% Control	25	5	45	45
Average	36.67	43.33	42.5	42.5

APPENDIX B-1

Probit Analysis (transplant, glyphosate)

Parameter Estimates

Parameter			Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
PROBIT[a]	Dose		.867	.057	15.232	.000	.755	.978
	Intercept[b]	A	-7.069	.437	-16.160	.000	-7.507	-6.632
		B	-6.565	.428	-15.329	.000	-6.993	-6.137
		C	-4.722	.403	-11.726	.000	-5.124	-4.319
		D	-7.488	.444	-16.875	.000	-7.931	-7.044

a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 2.718 logarithm.)

b. Corresponds to the grouping variable Biotype.

Confidence Limits

	Biotype	Probability	95% Confidence Limits for Dose			95% Confidence Limits for log(Dose)[b]		
			Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT[a]	A	0.01	238.172	13.512	620.726	5.473	2.604	6.431
		0.02	326.211	26.708	783.286	5.788	3.285	6.663
		0.03	398.264	40.949	912.344	5.987	3.712	6.816
		0.04	462.778	56.290	1026.671	6.137	4.031	6.934
		0.05	522.888	72.730	1133.085	6.259	4.287	7.033
		0.06	580.164	90.257	1234.986	6.363	4.503	7.119
		0.07	635.523	108.862	1334.384	6.454	4.690	7.196
		0.08	689.558	128.531	1432.612	6.536	4.856	7.267
		0.09	742.680	149.252	1530.635	6.610	5.006	7.333
		0.1	795.189	171.012	1629.198	6.679	5.142	7.396
		0.15	1.055E3	294.918	2148.973	6.961	5.687	7.673
		0.2	1.321E3	442.460	2752.563	7.186	6.092	7.920
		0.25	1.602E3	611.126	3490.206	7.379	6.415	8.158
		0.3	1.905E3	798.287	4419.542	7.552	6.682	8.394
		0.35	2.237E3	1001.846	5613.822	7.713	6.910	8.633

	0.4	2.605E3	1220.826	7171.015	7.865	7.107	8.878
	0.45	3.018E3	1455.728	9227.533	8.012	7.283	9.130
	0.5	3.489E3	1708.714	11980.636	8.157	7.443	9.391
	0.55	4.033E3	1983.795	15726.646	8.302	7.593	9.663
	0.6	4.674E3	2287.212	20929.443	8.450	7.735	9.949
	0.65	5.442E3	2628.288	28350.841	8.602	7.874	10.252
	0.7	6.390E3	3021.135	39318.065	8.762	8.013	10.579
	0.75	7.598E3	3488.184	56327.098	8.936	8.157	10.939
	0.8	9.214E3	4068.026	84587.035	9.129	8.311	11.346
	0.85	1.154E4	4835.469	136750.006	9.353	8.484	11.826
	0.9	1.531E4	5966.282	252112.489	9.636	8.694	12.438
	0.91	1.639E4	6270.893	292535.574	9.704	8.744	12.586
	0.92	1.765E4	6617.090	343950.875	9.779	8.797	12.748
	0.93	1.915E4	7017.072	411137.030	9.860	8.856	12.927
	0.94	2.098E4	7489.080	502025.356	9.951	8.921	13.126
	0.95	2.328E4	8062.062	630781.909	10.055	8.995	13.355
	0.96	2.630E4	8785.886	825370.862	10.177	9.081	13.624
	0.97	3.056E4	9757.258	1149647.754	10.328	9.186	13.955
	0.98	3.732E4	11203.070	1788161.532	10.527	9.324	14.397
	0.99	5.111E4	13896.727	3595671.102	10.842	9.539	15.095
B	0.01	133.085	4.596	389.030	4.891	1.525	5.964
	0.02	182.279	9.169	486.337	5.206	2.216	6.187
	0.03	222.541	14.171	561.996	5.405	2.651	6.331
	0.04	258.589	19.624	627.785	5.555	2.977	6.442
	0.05	292.177	25.535	687.953	5.677	3.240	6.534
	0.06	324.181	31.912	744.594	5.781	3.463	6.613
	0.07	355.115	38.760	798.924	5.872	3.657	6.683
	0.08	385.308	46.085	851.729	5.954	3.830	6.747
	0.09	414.992	53.897	903.560	6.028	3.987	6.806
	0.1	444.333	62.202	954.825	6.097	4.130	6.862
	0.15	589.583	111.460	1212.111	6.379	4.714	7.100
	0.2	738.195	174.488	1487.896	6.604	5.162	7.305
	0.25	895.212	252.465	1800.975	6.797	5.531	7.496
	0.3	1.064E3	346.388	2171.205	6.970	5.848	7.683

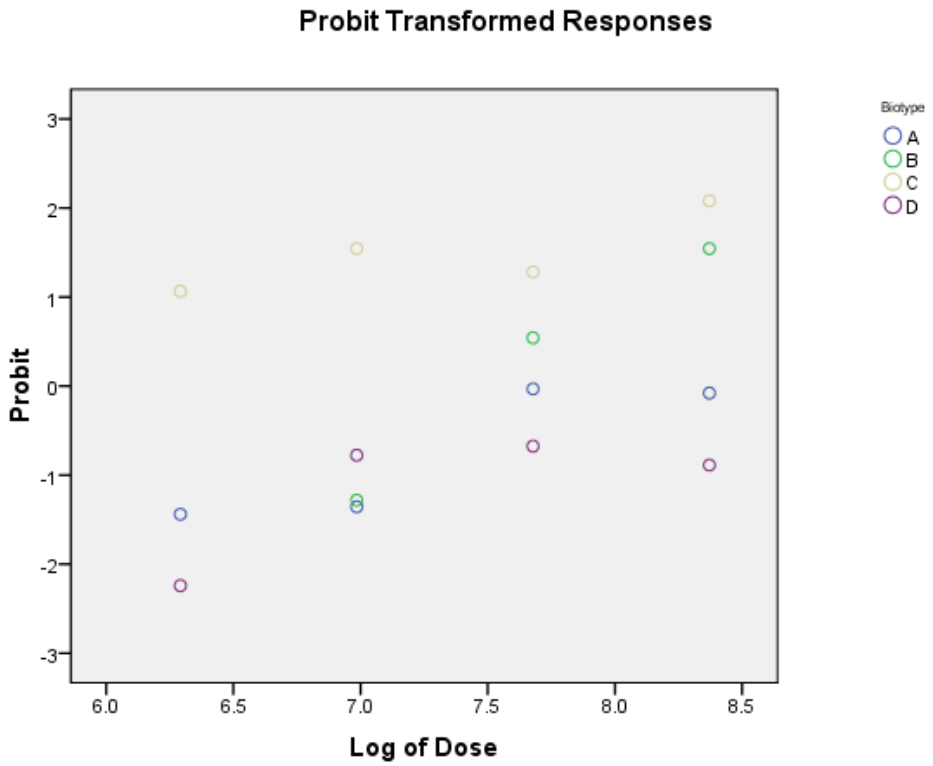
	0.35	1.250E3	456.967	2623.631	7.131	6.125	7.872
	0.4	1.455E3	584.630	3192.131	7.283	6.371	8.068
	0.45	1.686E3	729.695	3924.208	7.430	6.593	8.275
	0.5	1.950E3	892.711	4888.392	7.575	6.794	8.495
	0.55	2.254E3	1074.960	6186.825	7.720	6.980	8.730
	0.6	2.612E3	1279.083	7977.979	7.868	7.154	8.984
	0.65	3.041E3	1509.876	10520.225	8.020	7.320	9.261
	0.7	3.570E3	1775.531	14261.375	8.180	7.482	9.565
	0.75	4.246E3	2089.939	20040.590	8.354	7.645	9.906
	0.8	5.149E3	2477.752	29604.456	8.546	7.815	10.296
	0.85	6.446E3	2987.366	47185.103	8.771	8.002	10.762
	0.9	8.554E3	3732.963	85895.773	9.054	8.225	11.361
	0.91	9.159E3	3933.053	99427.314	9.122	8.277	11.507
	0.92	9.864E3	4160.167	116621.893	9.197	8.333	11.667
	0.93	1.070E4	4422.236	139068.254	9.278	8.394	11.843
	0.94	1.172E4	4731.115	169401.784	9.369	8.462	12.040
	0.95	1.301E4	5105.618	212326.633	9.473	8.538	12.266
	0.96	1.470E4	5578.147	277122.751	9.595	8.627	12.532
	0.97	1.708E4	6211.532	384965.042	9.746	8.734	12.861
	0.98	2.085E4	7153.160	596998.557	9.945	8.875	13.300
	0.99	2.856E4	8905.331	1196106.479	10.260	9.094	13.995
C	0.01	15.865	.073	83.676	2.764	-2.617	4.427
	0.02	21.729	.147	103.936	3.079	-1.920	4.644
	0.03	26.529	.228	119.412	3.278	-1.479	4.783
	0.04	30.826	.317	132.651	3.428	-1.147	4.888
	0.05	34.830	.415	144.571	3.550	-.879	4.974
	0.06	38.645	.522	155.620	3.654	-.650	5.047
	0.07	42.332	.637	166.056	3.746	-.450	5.112
	0.08	45.932	.762	176.042	3.827	-.272	5.171
	0.09	49.470	.896	185.691	3.901	-.109	5.224
	0.1	52.968	1.041	195.082	3.970	.040	5.273
	0.15	70.283	1.925	239.908	4.253	.655	5.480
	0.2	87.999	3.128	283.757	4.477	1.140	5.648
	0.25	106.716	4.728	328.704	4.670	1.554	5.795

	0.3	126.895	6.834	376.182	4.843	1.922	5.930
	0.35	148.985	9.586	427.507	5.004	2.260	6.058
	0.4	173.492	13.177	484.125	5.156	2.578	6.182
	0.45	201.032	17.868	547.819	5.303	2.883	6.306
	0.5	232.401	24.023	620.952	5.448	3.179	6.431
	0.55	268.665	32.162	706.841	5.593	3.471	6.561
	0.6	311.314	43.043	810.391	5.741	3.762	6.698
	0.65	362.522	57.808	939.259	5.893	4.057	6.845
	0.7	425.630	78.248	1106.182	6.054	4.360	7.009
	0.75	506.112	107.315	1334.108	6.227	4.676	7.196
	0.8	613.764	150.232	1669.019	6.420	5.012	7.420
	0.85	768.472	217.221	2218.189	6.644	5.381	7.704
	0.9	1.020E3	332.040	3300.983	6.927	5.805	8.102
	0.91	1.092E3	365.365	3658.611	6.996	5.901	8.205
	0.92	1.176E3	404.158	4103.435	7.070	6.002	8.320
	0.93	1.276E3	449.990	4671.694	7.151	6.109	8.449
	0.94	1.398E3	505.198	5422.851	7.243	6.225	8.598
	0.95	1.551E3	573.464	6461.815	7.346	6.352	8.774
	0.96	1.752E3	661.097	7992.900	7.469	6.494	8.986
	0.97	2.036E3	780.276	10475.609	7.619	6.660	9.257
	0.98	2.486E3	959.459	15214.311	7.818	6.866	9.630
	0.99	3.404E3	1295.353	28108.624	8.133	7.167	10.244
D	0.01	385.903	33.093	948.778	5.956	3.499	6.855
	0.02	528.550	64.165	1220.542	6.270	4.161	7.107
	0.03	645.297	96.805	1444.790	6.470	4.573	7.276
	0.04	749.826	131.114	1650.115	6.620	4.876	7.409
	0.05	847.221	167.030	1847.041	6.742	5.118	7.521
	0.06	940.023	204.463	2040.931	6.846	5.320	7.621
	0.07	1.030E3	243.314	2235.052	6.937	5.494	7.712
	0.08	1.117E3	283.485	2431.658	7.019	5.647	7.796
	0.09	1.203E3	324.884	2632.452	7.093	5.783	7.876
	0.1	1.288E3	367.419	2838.811	7.161	5.907	7.951
	0.15	1.710E3	594.363	3991.892	7.444	6.387	8.292
	0.2	2.141E3	839.299	5432.408	7.669	6.733	8.600

	0.25	2.596E3	1097.151	7278.018	7.862	7.000	8.893
	0.3	3.087E3	1366.002	9669.026	8.035	7.220	9.177
	0.35	3.624E3	1646.385	12788.694	8.195	7.406	9.456
	0.4	4.220E3	1940.648	16888.287	8.348	7.571	9.734
	0.45	4.890E3	2252.611	22324.277	8.495	7.720	10.013
	0.5	5.653E3	2587.547	29618.215	8.640	7.858	10.296
	0.55	6.535E3	2952.500	39558.579	8.785	7.990	10.586
	0.6	7.573E3	3357.020	53383.626	8.932	8.119	10.885
	0.65	8.818E3	3814.522	73130.192	9.085	8.247	11.200
	0.7	1.035E4	4344.821	102350.076	9.245	8.377	11.536
	0.75	1.231E4	4979.142	147727.060	9.418	8.513	11.903
	0.8	1.493E4	5771.079	223217.548	9.611	8.661	12.316
	0.85	1.869E4	6824.530	362736.301	9.836	8.828	12.801
	0.9	2.480E4	8383.779	671670.264	10.119	9.034	13.418
	0.91	2.656E4	8804.775	779987.399	10.187	9.083	13.567
	0.92	2.860E4	9283.627	917790.528	10.261	9.136	13.730
	0.93	3.103E4	9837.308	1097903.343	10.343	9.194	13.909
	0.94	3.400E4	10491.199	1341611.587	10.434	9.258	14.109
	0.95	3.772E4	11285.594	1686936.508	10.538	9.331	14.338
	0.96	4.262E4	12289.914	2208938.776	10.660	9.417	14.608
	0.97	4.952E4	13638.809	3079030.148	10.810	9.521	14.940
	0.98	6.046E4	15648.283	4792641.256	11.010	9.658	15.383
	0.99	8.281E4	19395.851	9644538.820	11.324	9.873	16.082

a. A heterogeneity factor is used.

b. Logarithm base = 2.718.



APPENDIX B-2

Probit Analysis (glufosinate,transplant)

Parameter Estimates

	Parameter		Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
PROBIT[a]	Dose		1.307	.074	17.640	.000	1.162	1.452
	Intercept[b]	A	-8.295	.507	-16.362	.000	-8.802	-7.788
		B	-8.347	.507	-16.473	.000	-8.853	-7.840
		C	-7.458	.492	-15.160	.000	-7.950	-6.966
		D	-10.117	.561	-18.019	.000	-10.678	-9.556

a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 2.718 logarithm.)

b. Corresponds to the grouping variable Biotype.

Confidence Limits

			95% Confidence Limits for Dose			95% Confidence Limits for log(Dose)[b]		
	Biotype	Probability	Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT[a]	A	0.01	96.140	23.875	192.893	4.566	3.173	5.262
		0.02	118.432	32.936	226.825	4.774	3.495	5.424
		0.03	135.185	40.356	251.628	4.907	3.698	5.528
		0.04	149.332	46.992	272.217	5.006	3.850	5.607
		0.05	161.925	53.165	290.322	5.087	3.973	5.671
		0.06	173.477	59.035	306.780	5.156	4.078	5.726
		0.07	184.282	64.695	322.064	5.216	4.170	5.775
		0.08	194.526	70.205	336.472	5.271	4.251	5.819
		0.09	204.336	75.607	350.208	5.320	4.326	5.859
		0.1	213.803	80.931	363.414	5.365	4.394	5.896
		0.15	257.899	107.037	424.521	5.553	4.673	6.051
		0.2	299.345	133.274	481.763	5.702	4.892	6.177
		0.25	340.171	160.436	538.382	5.829	5.078	6.289
		0.3	381.558	189.050	596.338	5.944	5.242	6.391
		0.35	424.392	219.568	657.192	6.051	5.392	6.488
		0.4	469.475	252.441	722.464	6.152	5.531	6.583
		0.45	517.647	288.169	793.853	6.249	5.664	6.677
		0.5	569.878	327.343	873.441	6.345	5.791	6.772
		0.55	627.379	370.706	963.958	6.442	5.915	6.871
		0.6	691.753	419.227	1069.175	6.539	6.038	6.975
		0.65	765.237	474.243	1194.573	6.640	6.162	7.086
		0.7	851.143	537.691	1348.569	6.747	6.287	7.207
		0.75	954.699	612.579	1544.974	6.861	6.418	7.343
		0.8	1.085E3	703.985	1808.556	6.989	6.557	7.500
		0.85	1.259E3	821.574	2189.754	7.138	6.711	7.692
		0.9	1.519E3	987.535	2814.542	7.326	6.895	7.943
		0.91	1.589E3	1030.842	2995.007	7.371	6.938	8.005
		0.92	1.670E3	1079.395	3206.118	7.420	6.984	8.073
		0.93	1.762E3	1134.657	3457.806	7.474	7.034	8.148
		0.94	1.872E3	1198.789	3765.273	7.535	7.089	8.234
		0.95	2.006E3	1275.181	4153.307	7.604	7.151	8.332

	0.96	2.175E3	1369.598	4665.952	7.685	7.222	8.448
	0.97	2.402E3	1493.037	5391.877	7.784	7.309	8.593
	0.98	2.742E3	1670.768	6549.305	7.917	7.421	8.787
	0.99	3.378E3	1986.372	8936.127	8.125	7.594	9.098
B	0.01	100.033	25.472	198.275	4.606	3.238	5.290
	0.02	123.228	35.136	233.171	4.814	3.559	5.452
	0.03	140.660	43.048	258.686	4.946	3.762	5.556
	0.04	155.380	50.123	279.872	5.046	3.914	5.634
	0.05	168.483	56.704	298.508	5.127	4.038	5.699
	0.06	180.502	62.959	315.453	5.196	4.142	5.754
	0.07	191.745	68.990	331.194	5.256	4.234	5.803
	0.08	202.403	74.860	346.037	5.310	4.316	5.847
	0.09	212.611	80.614	360.191	5.359	4.390	5.887
	0.1	222.461	86.284	373.803	5.405	4.458	5.924
	0.15	268.343	114.069	436.841	5.592	4.737	6.080
	0.2	311.467	141.962	495.980	5.741	4.956	6.207
	0.25	353.947	170.802	554.570	5.869	5.141	6.318
	0.3	397.010	201.141	614.648	5.984	5.304	6.421
	0.35	441.579	233.446	677.848	6.090	5.453	6.519
	0.4	488.488	268.181	745.772	6.191	5.592	6.614
	0.45	538.610	305.855	820.219	6.289	5.723	6.710
	0.5	592.956	347.069	903.397	6.385	5.850	6.806
	0.55	652.786	392.576	998.208	6.481	5.973	6.906
	0.6	719.767	443.360	1108.661	6.579	6.094	7.011
	0.65	796.227	500.778	1240.581	6.680	6.216	7.123
	0.7	885.612	566.806	1402.907	6.786	6.340	7.246
	0.75	993.362	644.515	1610.301	6.901	6.468	7.384
	0.8	1.129E3	739.114	1889.042	7.029	6.605	7.544
	0.85	1.310E3	860.531	2292.624	7.178	6.758	7.737
	0.9	1.580E3	1031.600	2954.654	7.365	6.939	7.991
	0.91	1.654E3	1076.209	3145.942	7.411	6.981	8.054
	0.92	1.737E3	1126.215	3369.738	7.460	7.027	8.123
	0.93	1.834E3	1183.123	3636.577	7.514	7.076	8.199
	0.94	1.948E3	1249.160	3962.584	7.574	7.130	8.285

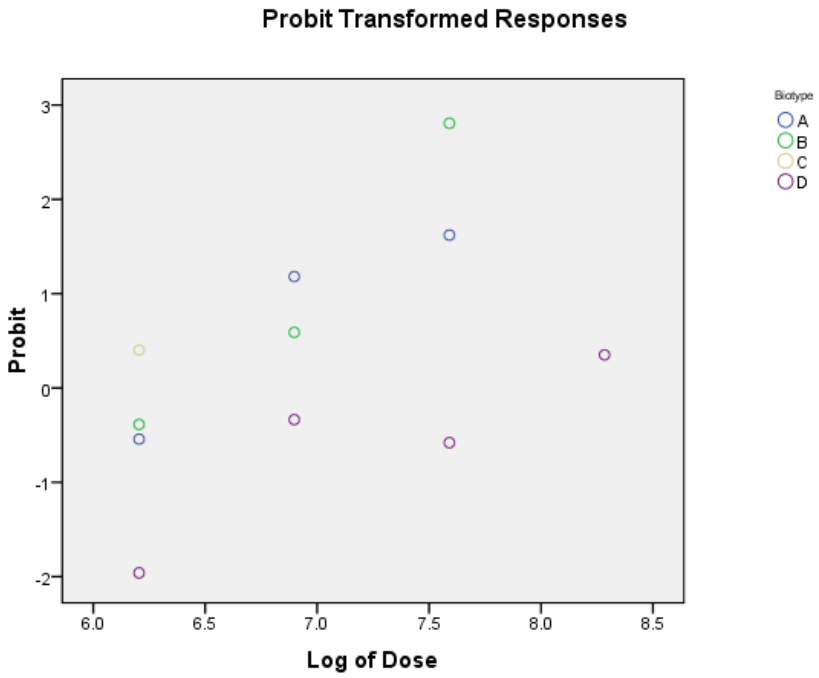
	0.95	2.087E3	1327.819	4374.058	7.643	7.191	8.383
	0.96	2.263E3	1425.039	4917.726	7.724	7.262	8.501
	0.97	2.500E3	1552.150	5687.671	7.824	7.347	8.646
	0.98	2.853E3	1735.193	6915.461	7.956	7.459	8.842
	0.99	3.515E3	2060.305	9447.916	8.165	7.631	9.154
C	0.01	50.687	9.704	117.248	3.926	2.273	4.764
	0.02	62.439	13.366	138.087	4.134	2.593	4.928
	0.03	71.272	16.362	153.329	4.267	2.795	5.033
	0.04	78.731	19.041	165.981	4.366	2.947	5.112
	0.05	85.370	21.532	177.104	4.447	3.070	5.177
	0.06	91.460	23.901	187.211	4.516	3.174	5.232
	0.07	97.156	26.185	196.591	4.576	3.265	5.281
	0.08	102.557	28.409	205.429	4.630	3.347	5.325
	0.09	107.729	30.591	213.847	4.680	3.421	5.365
	0.1	112.721	32.741	221.934	4.725	3.489	5.402
	0.15	135.969	43.305	259.242	4.912	3.768	5.558
	0.2	157.820	53.960	293.977	5.061	3.988	5.684
	0.25	179.344	65.047	328.076	5.189	4.175	5.793
	0.3	201.164	76.800	362.673	5.304	4.341	5.894
	0.35	223.747	89.434	398.628	5.411	4.493	5.988
	0.4	247.515	103.170	436.746	5.511	4.636	6.079
	0.45	272.912	118.268	477.890	5.609	4.773	6.169
	0.5	300.449	135.042	523.091	5.705	4.906	6.260
	0.55	330.765	153.897	573.674	5.801	5.036	6.352
	0.6	364.704	175.376	631.444	5.899	5.167	6.448
	0.65	403.446	200.236	699.001	6.000	5.299	6.550
	0.7	448.737	229.584	780.317	6.106	5.436	6.660
	0.75	503.334	265.144	881.880	6.221	5.580	6.782
	0.8	571.981	309.819	1015.300	6.349	5.736	6.923
	0.85	663.902	369.114	1204.172	6.498	5.911	7.094
	0.9	800.828	455.588	1507.284	6.686	6.122	7.318
	0.91	837.931	478.574	1593.851	6.731	6.171	7.374
	0.92	880.189	504.513	1694.709	6.780	6.224	7.435
	0.93	929.118	534.225	1814.465	6.834	6.281	7.504

	0.94	986.987	568.923	1960.165	6.895	6.344	7.581
	0.95	1.057E3	610.512	2143.280	6.964	6.414	7.670
	0.96	1.147E3	662.225	2384.161	7.045	6.496	7.777
	0.97	1.267E3	730.228	2723.702	7.144	6.593	7.910
	0.98	1.446E3	828.690	3262.320	7.276	6.720	8.090
	0.99	1.781E3	1004.470	4365.968	7.485	6.912	8.382
D	0.01	387.503	138.868	658.399	5.960	4.934	6.490
	0.02	477.354	190.758	777.513	6.168	5.251	6.656
	0.03	544.880	232.893	865.637	6.301	5.451	6.763
	0.04	601.903	270.297	939.569	6.400	5.600	6.845
	0.05	652.660	304.840	1005.229	6.481	5.720	6.913
	0.06	699.221	337.457	1065.481	6.550	5.821	6.971
	0.07	742.770	368.691	1121.952	6.610	5.910	7.023
	0.08	784.060	398.892	1175.673	6.664	5.989	7.070
	0.09	823.602	428.299	1227.343	6.714	6.060	7.113
	0.1	861.760	457.082	1277.463	6.759	6.125	7.153
	0.15	1.039E3	595.274	1515.458	6.946	6.389	7.323
	0.2	1.207E3	729.108	1748.299	7.096	6.592	7.466
	0.25	1.371E3	862.306	1988.650	7.223	6.760	7.595
	0.3	1.538E3	996.931	2245.082	7.338	6.905	7.716
	0.35	1.711E3	1134.488	2525.164	7.445	7.034	7.834
	0.4	1.892E3	1276.369	2836.796	7.546	7.152	7.950
	0.45	2.086E3	1424.102	3189.141	7.643	7.261	8.068
	0.5	2.297E3	1579.543	3593.634	7.739	7.365	8.187
	0.55	2.529E3	1745.087	4065.353	7.835	7.465	8.310
	0.6	2.788E3	1923.963	4625.185	7.933	7.562	8.439
	0.65	3.084E3	2120.690	5303.516	8.034	7.659	8.576
	0.7	3.431E3	2341.894	6147.041	8.141	7.759	8.724
	0.75	3.848E3	2597.897	7232.505	8.255	7.862	8.886
	0.8	4.373E3	2906.134	8697.758	8.383	7.975	9.071
	0.85	5.076E3	3299.733	10824.073	8.532	8.102	9.290
	0.9	6.122E3	3854.623	14315.484	8.720	8.257	9.569
	0.91	6.406E3	3999.696	15324.648	8.765	8.294	9.637
	0.92	6.729E3	4162.550	16505.486	8.814	8.334	9.711

	0.93	7.103E3	4348.185	17913.688	8.868	8.378	9.793
	0.94	7.546E3	4564.001	19634.555	8.929	8.426	9.885
	0.95	8.084E3	4821.616	21807.256	8.998	8.481	9.990
	0.96	8.766E3	5140.783	24679.250	9.079	8.545	10.114
	0.97	9.683E3	5559.221	28749.108	9.178	8.623	10.266
	0.98	1.105E4	6163.680	35245.124	9.310	8.726	10.470
	0.99	1.362E4	7241.403	48664.807	9.519	8.888	10.793

a. A heterogeneity factor is used.

b. Logarithm base = 2.718.



APPENDIX B-3 (seed test, glyphosate)

Probit Analysis

Parameter Estimates

Parameter			Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
PROBIT[a]	Dose		1.113	.063	17.792	.000	.990	1.235
	Intercept[b]	1	-7.977	.459	-17.397	.000	-8.435	-7.518
		2	-8.325	.468	-17.784	.000	-8.794	-7.857
		3	-9.300	.489	-19.007	.000	-9.789	-8.810

a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 2.718 logarithm.)

b. Corresponds to the grouping variable Biotype.

Confidence Limits

	Biotype	Probability	95% Confidence Limits for Dose			95% Confidence Limits for log(Dose)[b]		
			Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT[a]	1	0.01	160.370	29.261	344.980	5.077	3.376	5.843
		0.02	204.886	44.072	415.661	5.322	3.786	6.030
		0.03	239.338	57.057	468.611	5.478	4.044	6.150
		0.04	269.023	69.218	513.376	5.595	4.237	6.241
		0.05	295.864	80.936	553.349	5.690	4.394	6.316
		0.06	320.811	92.403	590.179	5.771	4.526	6.380
		0.07	344.408	103.733	624.807	5.842	4.642	6.437
		0.08	367.006	114.999	657.831	5.905	4.745	6.489
		0.09	388.843	126.252	689.659	5.963	4.838	6.536
		0.1	410.091	137.531	720.584	6.016	4.924	6.580
		0.15	511.144	195.135	867.931	6.237	5.274	6.766
		0.2	608.938	256.059	1012.625	6.412	5.545	6.920
		0.25	707.620	321.426	1162.502	6.562	5.773	7.058
		0.3	809.797	392.052	1323.235	6.697	5.971	7.188
		0.35	917.604	468.675	1500.242	6.822	6.150	7.313
		0.4	1.033E3	552.064	1699.606	6.940	6.314	7.438
		0.45	1.159E3	643.106	1928.808	7.055	6.466	7.565

	0.5	1.297E3	742.907	2197.604	7.168	6.611	7.695
	0.55	1.452E3	852.936	2519.296	7.281	6.749	7.832
	0.6	1.629E3	975.259	2912.794	7.396	6.883	7.977
	0.65	1.834E3	1112.906	3406.248	7.514	7.015	8.133
	0.7	2.078E3	1270.524	4043.936	7.639	7.147	8.305
	0.75	2.378E3	1455.628	4900.521	7.774	7.283	8.497
	0.8	2.764E3	1681.275	6114.215	7.924	7.427	8.718
	0.85	3.293E3	1972.790	7977.505	8.099	7.587	8.984
	0.9	4.104E3	2389.168	11257.295	8.320	7.779	9.329
	0.91	4.328E3	2498.997	12249.747	8.373	7.824	9.413
	0.92	4.586E3	2622.750	13433.918	8.431	7.872	9.506
	0.93	4.886E3	2764.404	14876.620	8.494	7.925	9.608
	0.94	5.246E3	2929.866	16682.060	8.565	7.983	9.722
	0.95	5.688E3	3128.452	19023.761	8.646	8.048	9.853
	0.96	6.256E3	3376.080	22217.648	8.741	8.124	10.009
	0.97	7.032E3	3703.326	26918.589	8.858	8.217	10.201
	0.98	8.214E3	4181.065	34799.544	9.014	8.338	10.457
	0.99	1.049E4	5046.640	52321.851	9.259	8.526	10.865
2	0.01	219.435	44.561	453.932	5.391	3.797	6.118
	0.02	280.346	66.992	547.962	5.636	4.205	6.306
	0.03	327.487	86.597	618.707	5.791	4.461	6.428
	0.04	368.105	104.910	678.740	5.908	4.653	6.520
	0.05	404.832	122.513	732.530	6.003	4.808	6.597
	0.06	438.966	139.697	782.251	6.084	4.939	6.662
	0.07	471.255	156.637	829.147	6.155	5.054	6.720
	0.08	502.176	173.443	874.010	6.219	5.156	6.773
	0.09	532.055	190.191	917.377	6.277	5.248	6.822
	0.1	561.128	206.939	959.640	6.330	5.332	6.867
	0.15	699.399	291.868	1162.780	6.550	5.676	7.059
	0.2	833.212	380.593	1365.186	6.725	5.942	7.219
	0.25	968.238	474.543	1577.842	6.875	6.162	7.364
	0.3	1.108E3	574.644	1809.032	7.010	6.354	7.501
	0.35	1.256E3	681.683	2066.879	7.135	6.525	7.634
	0.4	1.414E3	796.489	2360.602	7.254	6.680	7.767

	0.45	1.586E3	920.073	2701.557	7.369	6.824	7.902
	0.5	1.775E3	1053.786	3104.529	7.482	6.960	8.041
	0.55	1.987E3	1199.526	3589.635	7.595	7.090	8.186
	0.6	2.229E3	1360.038	4185.460	7.709	7.215	8.339
	0.65	2.510E3	1539.387	4934.594	7.828	7.339	8.504
	0.7	2.844E3	1743.796	5904.130	7.953	7.464	8.683
	0.75	3.254E3	1983.252	7207.411	8.088	7.592	8.883
	0.8	3.782E3	2274.977	9054.550	8.238	7.730	9.111
	0.85	4.505E3	2652.197	11890.680	8.413	7.883	9.384
	0.9	5.615E3	3192.074	16883.884	8.633	8.068	9.734
	0.91	5.922E3	3334.686	18395.111	8.686	8.112	9.820
	0.92	6.275E3	3495.471	20198.463	8.744	8.159	9.913
	0.93	6.686E3	3679.624	22395.807	8.808	8.211	10.017
	0.94	7.178E3	3894.864	25146.042	8.879	8.267	10.132
	0.95	7.783E3	4153.359	28713.812	8.960	8.332	10.265
	0.96	8.560E3	4475.910	33581.024	9.055	8.406	10.422
	0.97	9.621E3	4902.472	40746.811	9.172	8.497	10.615
	0.98	1.124E4	5525.672	52764.250	9.327	8.617	10.874
	0.99	1.436E4	6655.762	79497.264	9.572	8.803	11.283
3	0.01	526.665	148.363	997.382	6.267	5.000	6.905
	0.02	672.856	219.712	1222.244	6.512	5.392	7.108
	0.03	785.999	280.625	1396.698	6.667	5.637	7.242
	0.04	883.486	336.390	1548.524	6.784	5.818	7.345
	0.05	971.635	389.016	1687.637	6.879	5.964	7.431
	0.06	1.054E3	439.513	1818.877	6.960	6.086	7.506
	0.07	1.131E3	488.473	1945.029	7.031	6.191	7.573
	0.08	1.205E3	536.277	2067.873	7.094	6.285	7.634
	0.09	1.277E3	583.185	2188.633	7.152	6.369	7.691
	0.1	1.347E3	629.387	2308.203	7.205	6.445	7.744
	0.15	1.679E3	853.782	2907.885	7.426	6.750	7.975
	0.2	2.000E3	1073.033	3542.257	7.601	6.978	8.173
	0.25	2.324E3	1291.744	4240.364	7.751	7.164	8.352
	0.3	2.659E3	1513.012	5026.234	7.886	7.322	8.522
	0.35	3.013E3	1739.591	5925.031	8.011	7.461	8.687

	0.4	3.393E3	1974.346	6966.578	8.129	7.588	8.849
	0.45	3.805E3	2220.533	8188.785	8.244	7.706	9.011
	0.5	4.260E3	2482.073	9642.154	8.357	7.817	9.174
	0.55	4.770E3	2763.912	11396.626	8.470	7.924	9.341
	0.6	5.350E3	3072.580	13552.872	8.585	8.030	9.514
	0.65	6.023E3	3417.119	16262.207	8.703	8.137	9.697
	0.7	6.825E3	3810.742	19764.356	8.828	8.246	9.892
	0.75	7.811E3	4274.064	24465.643	8.963	8.360	10.105
	0.8	9.076E3	4842.106	31120.745	9.113	8.485	10.346
	0.85	1.081E4	5581.945	41330.191	9.288	8.627	10.629
	0.9	1.348E4	6648.958	59296.926	9.509	8.802	10.990
	0.91	1.421E4	6932.054	64734.509	9.562	8.844	11.078
	0.92	1.506E4	7251.727	71223.459	9.620	8.889	11.174
	0.93	1.605E4	7618.439	79130.640	9.683	8.938	11.279
	0.94	1.723E4	8047.739	89028.386	9.754	8.993	11.397
	0.95	1.868E4	8564.152	101870.034	9.835	9.055	11.531
	0.96	2.054E4	9209.604	119391.877	9.930	9.128	11.690
	0.97	2.309E4	10064.667	145194.368	10.047	9.217	11.886
	0.98	2.698E4	11316.205	188479.688	10.203	9.334	12.147
	0.99	3.446E4	13590.459	284810.681	10.448	9.517	12.560

a. A heterogeneity factor is used.

b. Logarithm base = 2.718.

APPENDIX B-4 (seed test, glufosinate)

Probit Analysis

Parameter Estimates

	Parameter		Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
PROBIT[a]	Dose		.668	.073	9.169	.000	.525	.811
	Intercept[b]	1	-2.266	.520	-4.354	.000	-2.787	-1.746
		2	-3.416	.508	-6.723	.000	-3.924	-2.908
		3	-4.550	.528	-8.625	.000	-5.078	-4.023

a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 2.718 logarithm.)

b. Corresponds to the grouping variable Biotype.

Confidence Limits

	Biotype	Probability	95% Confidence Limits for Dose			95% Confidence Limits for log(Dose)[b]		
			Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT[a]	1	0.01	.914	.000	26.716	-.090	-25.400	3.285
		0.02	1.375	.000	34.463	.318	-23.503	3.540
		0.03	1.781	.000	40.576	.577	-22.302	3.703
		0.04	2.164	.000	45.926	.772	-21.399	3.827
		0.05	2.536	.000	50.831	.930	-20.665	3.929
		0.06	2.902	.000	55.446	1.065	-20.042	4.015
		0.07	3.266	.000	59.862	1.184	-19.495	4.092
		0.08	3.631	.000	64.137	1.289	-19.006	4.161
		0.09	3.998	.000	68.312	1.386	-18.562	4.224
		0.1	4.368	.000	72.415	1.474	-18.153	4.282
		0.15	6.305	.000	92.490	1.841	-16.463	4.527
		0.2	8.440	.000	112.818	2.133	-15.125	4.726
		0.25	10.840	.000	134.256	2.383	-13.980	4.900
		0.3	13.571	.000	157.467	2.608	-12.955	5.059
		0.35	16.712	.000	183.118	2.816	-12.009	5.210
		0.4	20.363	.000	211.991	3.014	-11.114	5.357
		0.45	24.653	.000	245.085	3.205	-10.251	5.502
		0.5	29.756	.000	283.753	3.393	-9.406	5.648
		0.55	35.916	.000	329.920	3.581	-8.566	5.799
		0.6	43.482	.000	386.467	3.772	-7.716	5.957
		0.65	52.981	.001	457.933	3.970	-6.845	6.127
		0.7	65.246	.003	551.972	4.178	-5.934	6.313
		0.75	81.686	.007	682.641	4.403	-4.962	6.526
		0.8	104.910	.020	878.997	4.653	-3.896	6.779
		0.85	140.439	.069	1212.578	4.945	-2.681	7.101
		0.9	202.707	.299	1919.332	5.312	-1.206	7.560
		0.91	221.495	.423	2168.915	5.400	-.861	7.682
		0.92	243.885	.611	2491.512	5.497	-.492	7.821
		0.93	271.124	.910	2924.139	5.603	-.094	7.981
		0.94	305.157	1.405	3533.003	5.721	.340	8.170

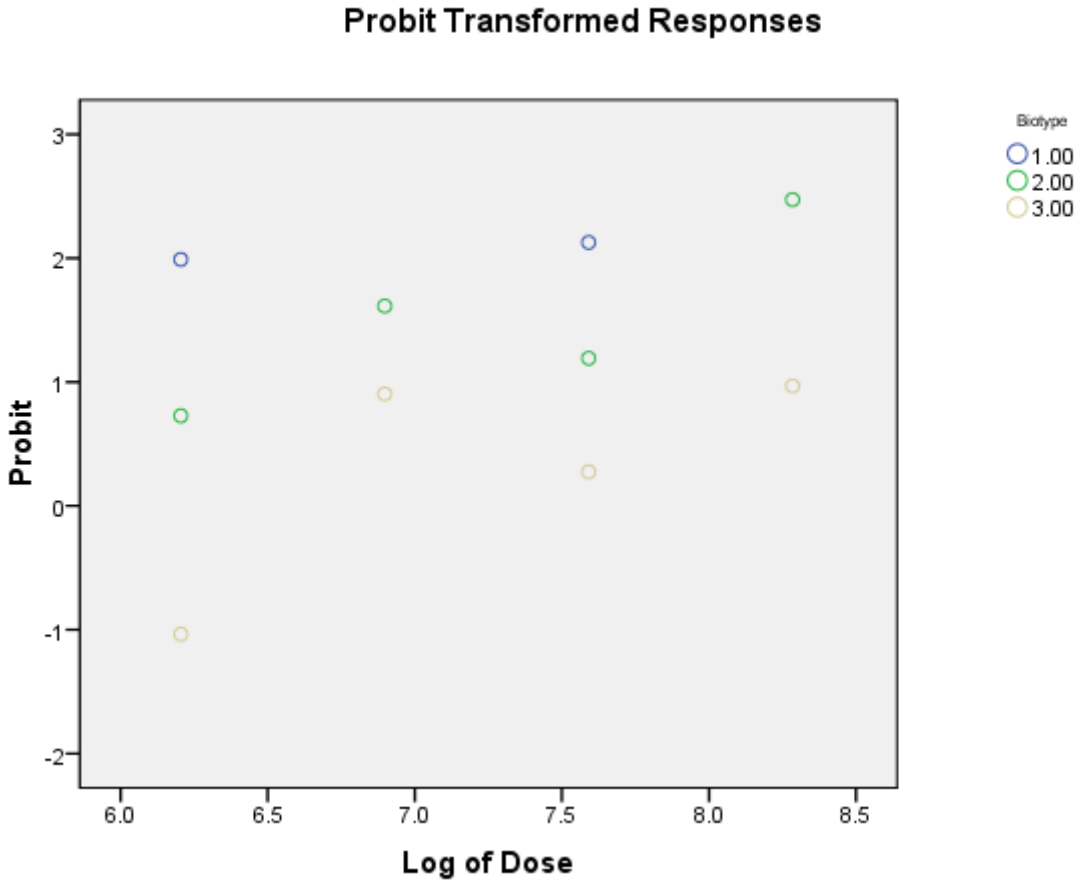
	0.95	349.217	2.271	4448.095	5.856	.820	8.400
	0.96	409.175	3.907	5959.667	6.014	1.363	8.693
	0.97	497.173	7.344	8851.534	6.209	1.994	9.088
	0.98	644.119	15.880	16024.021	6.468	2.765	9.682
	0.99	968.758	45.281	48289.015	6.876	3.813	10.785
2	0.01	5.113	.000	57.510	1.632	-17.900	4.052
	0.02	7.690	.000	72.955	2.040	-15.987	4.290
	0.03	9.962	.000	84.915	2.299	-14.774	4.442
	0.04	12.105	.000	95.238	2.494	-13.862	4.556
	0.05	14.183	.000	104.593	2.652	-13.120	4.650
	0.06	16.231	.000	113.311	2.787	-12.490	4.730
	0.07	18.269	.000	121.582	2.905	-11.937	4.801
	0.08	20.309	.000	129.526	3.011	-11.442	4.864
	0.09	22.362	.000	137.228	3.107	-10.992	4.922
	0.1	24.435	.000	144.747	3.196	-10.579	4.975
	0.15	35.268	.000	180.894	3.563	-8.868	5.198
	0.2	47.212	.001	216.595	3.855	-7.510	5.378
	0.25	60.636	.002	253.477	4.105	-6.349	5.535
	0.3	75.913	.005	292.720	4.330	-5.309	5.679
	0.35	93.487	.013	335.471	4.538	-4.348	5.816
	0.4	113.910	.032	383.060	4.735	-3.439	5.948
	0.45	137.907	.077	437.218	4.927	-2.564	6.080
	0.5	166.454	.181	500.381	5.115	-1.707	6.215
	0.55	200.910	.425	576.225	5.303	-.857	6.356
	0.6	243.234	.999	670.706	5.494	.000	6.508
	0.65	296.371	2.390	794.295	5.692	.871	6.677
	0.7	364.980	5.880	967.475	5.900	1.772	6.875
	0.75	456.940	15.037	1236.425	6.125	2.711	7.120
	0.8	586.856	40.214	1728.500	6.375	3.694	7.455
	0.85	785.601	110.879	2915.981	6.666	4.708	7.978
	0.9	1.134E3	295.683	7564.538	7.033	5.689	8.931
	0.91	1.239E3	355.415	10040.399	7.122	5.873	9.214
	0.92	1.364E3	425.246	13939.508	7.218	6.053	9.542
	0.93	1.517E3	506.884	20432.479	7.324	6.228	9.925

	0.94	1.707E3	602.992	32031.426	7.442	6.402	10.374
	0.95	1.953E3	718.080	54752.240	7.577	6.577	10.911
	0.96	2.289E3	860.387	105329.061	7.736	6.757	11.565
	0.97	2.781E3	1046.569	241728.497	7.931	6.953	12.396
	0.98	3.603E3	1316.748	752104.105	8.190	7.183	13.531
	0.99	5.419E3	1810.330	4700750.574	8.598	7.501	15.363
3	0.01	27.936	.000	166.289	3.330	-11.058	5.114
	0.02	42.015	.000	211.037	3.738	-9.145	5.352
	0.03	54.434	.000	245.805	3.997	-7.933	5.505
	0.04	66.140	.001	275.920	4.192	-7.022	5.620
	0.05	77.496	.002	303.315	4.350	-6.281	5.715
	0.06	88.685	.004	328.943	4.485	-5.652	5.796
	0.07	99.817	.006	353.357	4.603	-5.100	5.867
	0.08	110.966	.010	376.910	4.709	-4.606	5.932
	0.09	122.183	.016	399.848	4.806	-4.158	5.991
	0.1	133.507	.024	422.352	4.894	-3.746	6.046
	0.15	192.701	.130	532.371	5.261	-2.043	6.277
	0.2	257.962	.498	644.937	5.553	-.698	6.469
	0.25	331.305	1.565	766.964	5.803	.448	6.642
	0.3	414.780	4.331	905.754	6.028	1.466	6.809
	0.35	510.800	10.966	1071.765	6.236	2.395	6.977
	0.4	622.389	25.956	1282.834	6.434	3.256	7.157
	0.45	753.504	57.958	1573.431	6.625	4.060	7.361
	0.5	909.480	121.801	2018.050	6.813	4.802	7.610
	0.55	1.098E3	237.091	2794.378	7.001	5.468	7.935
	0.6	1.329E3	416.535	4356.147	7.192	6.032	8.379
	0.65	1.619E3	650.285	7904.933	7.390	6.477	8.975
	0.7	1.994E3	916.351	16808.936	7.598	6.820	9.730
	0.75	2.497E3	1207.718	41683.465	7.823	7.096	10.638
	0.8	3.206E3	1540.183	122203.761	8.073	7.340	11.713
	0.85	4.292E3	1954.469	447931.948	8.365	7.578	13.012
	0.9	6.196E3	2545.302	2379438.170	8.732	7.842	14.682
	0.91	6.770E3	2703.228	3574455.833	8.820	7.902	15.089
	0.92	7.454E3	2882.640	5568044.533	8.917	7.966	15.533

	0.93	8.287E3	3090.068	9075428.200	9.022	8.036	16.021
	0.94	9.327E3	3335.314	1.568E7	9.141	8.112	16.568
	0.95	1.067E4	3634.011	2.930E7	9.276	8.198	17.193
	0.96	1.251E4	4013.244	6.114E7	9.434	8.297	17.929
	0.97	1.520E4	4525.918	1.514E8	9.629	8.418	18.835
	0.98	1.969E4	5297.357	5.062E8	9.888	8.575	20.042
	0.99	2.961E4	6760.852	3.408E9	10.296	8.819	21.949

a. A heterogeneity factor is used.

b. Logarithm base = 2.718.



APPENDIX C-1

Protein Extraction Buffers

Extraction buffer (100 mM Tris-HCl, pH 7.5 with 2mM EDTA, 1.5% (w/v) PVP and 5 mM DTT): 12.114 g of Tris, 1.5845 g of EDTA and 0.7713g of DTT were dissolved in 800 ml of distilled water. The pH was adjusted to 7.5 and the volume was made up to 1 L with distilled water. 5 ml of extraction buffer was added for each gram powder and 50 µl of protease inhibitor cocktail were added for every 5 ml of extraction buffer.

Buffer A (20 mM Tris-HCl, pH 7.5 containing 1 mM DTT): 2.4228 g of Tris and 0.1543 g of DTT were dissolved in distilled water. Its pH was adjusted to 7.5 and the final volume was made up to 1 L with distilled water.

APPENDIX C-2

Protein Content Determination

Bradford reagent: Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml 95% ethanol. To this solution 100 ml 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 liter. Final concentrations in the reagent were 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid.

Protein concentrations in samples were determined as described by Bradford (1976). Each time protein estimation was carried out, a standard curve was constructed. Figure A1 is one example of a standard curve based on the following straight line equation:

$$(\text{Absorbance})_{595\ nm} = 0.0048\ (\text{Amount of protein, mg}) + 0.0019 \quad \textbf{(1)}$$

Absorbance of diluted sample(s) was taken and concentration of sample(s) was determined using equation 1. The amount generated was then multiplied with the dilution factor.

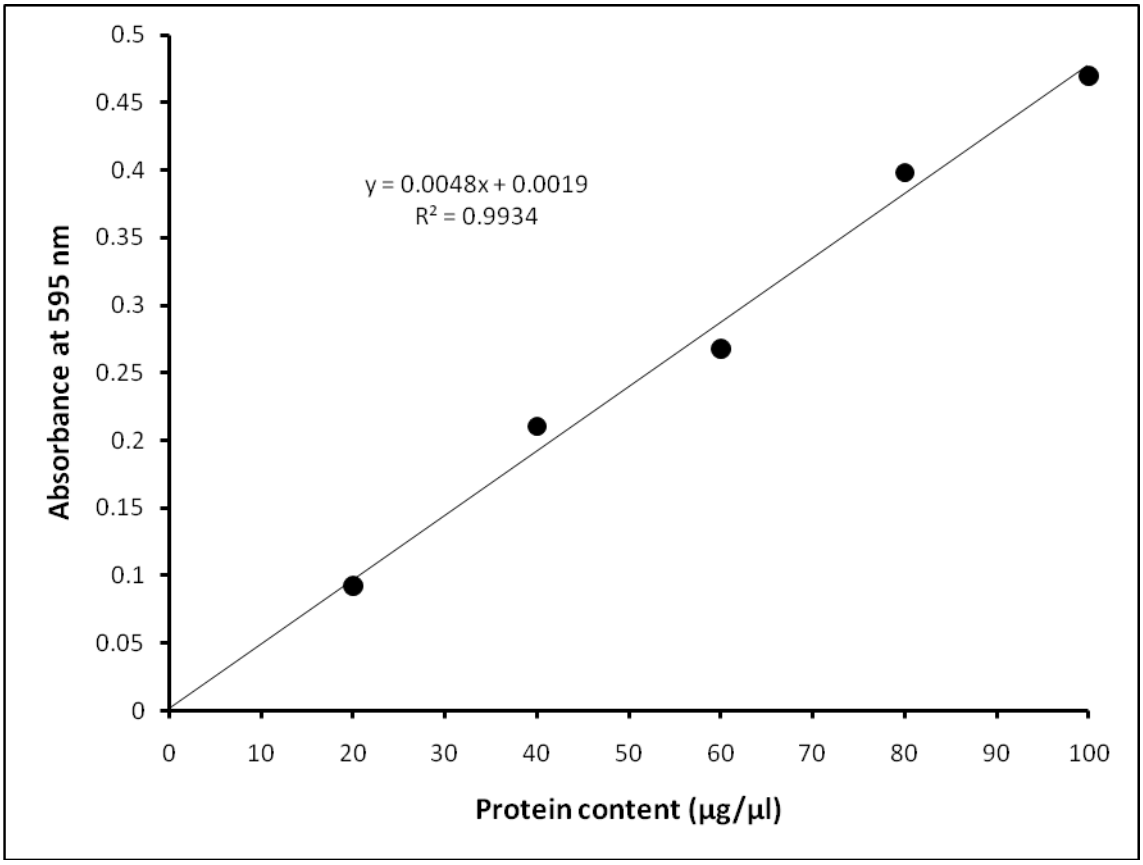


Fig. A1. Standard curve for the determination of protein content based on the method of Bradford (1976).

APPENDIX C-3

Laemli Discontinuous SDS-PAGE

Reagents and Buffers

10% (w/v) SDS solution: 10 g of SDS was dissolved in 50 ml of water with gentle shaking. The final volume was then made to 100 ml.

10% (w/v) APS solution: 10 mg of SDS were dissolved in1 ml of distilled water. The solution was prepared fresh just before gel casting.

Overlay solution: 100 µl of 10% (w/v) was mixed with 900 µl of distilled water.

Running buffer: 10X Tris /Glycine/SDS buffer (stock) was diluted according to the manufacturer's instruction, with 1:9 ratio of running buffer to distilled water.

Sample buffer: 1.25 ml of 0.5 M Tris-HCl, pH 6.8, 2.5 ml of glycerol, 2.0 ml of 10 (w/v) SDS, 0.2 ml of 0.5% (w/v) bromophenol blue (BPB) and 3.55 ml of distilled water were mixed. This stock solution was kept at room temperature. To prepare a 1 ml sample buffer, 50 µl of 2-mercaptoethanol was added to 950 µl of (stock) sample buffer. Sample was diluted with sample buffer at a 1:4 ratio. The sample was then heated at 95 °C for 5 minutes.

APPENDIX C-4

Two-Dimensional (2-D) Gel Electrophoresis

Reagents

Rehydration buffer (8 M Urea, 15 mM DTT, 30 mM Thiourea, 0.5% (v/v) Ampholyte, pH 3-10, 2% (w/v) CHAPS, traces of BPB): 0.48 g of urea was dissolved in 500 µl deionized water in 1.5 ml Eppendorf tube. 0.0015 g of DTT, 0.017 g of thiourea and 0.02 g of CHAPS were added and the mixture was vortexed. 5 µl of Ampholyte was then added. The volume was made up to 1 ml and traces of BPB were mixed to give the solution a pale blue colour.

Equilibration buffer (50 mM Tris-HCl pH 8.0, 6 M Urea, 30% (v/v) glycerol, 2% (w/v) SDS: A stock solution was made by dissolving 7.207 g of urea and 0.4 g of SDS in 5 ml of deionized water. Then, 6.9 ml of glycerol and 0.67 ml of 50 mM Tris-HCL, pH 8.0 was added. The volume was made up to 20 ml.

APPENDIX D-1

Matched spots between the susceptible and the Jerantut biotype.

Spot ID	% Vol.	Susceptible biotype	Jerantut biotype	t-test
0	0.777476	0.777476	0.349157	0.4152
1	3.47347	3.47347	2.16243	0.4996
2	23.5713	23.5713	11.6548	0.0257
3	1.98355	0.20583	1.98355	0.2342
4	3.56795	3.56795	1.78848	0.0118
5	0.549682	0.549682	0.02281	0.0818
6	0.169784	0.169784	0.139955	0.8848
7	0.13177	0.106298	0.13177	0.7295
8	1.83558	1.83558	0.188748	0.1304
9	0.369888	0.246066	0.369888	0.1374
10	0.251769	0.251769	0.031573	0.0727
11	3.86004	2.82172	3.86004	0.3561
12	0.357706	0.357706	0.14987	0.3822
13	1.06803	0.615712	1.06803	0.6609
14	0.228067	0.228067	0.17282	0.7515
15	0.420831	0.420831	0.358855	0.8074
16	0.0336701	0.032089	0.03367	0.9745
17	0.241738	0.140395	0.241738	0.2907
18	0.226036	0.090451	0.226036	0.4797
19	0.336304	0.336304	0.131751	0.4449
20	0.504141	0.504141	0.162173	0.0503
21	0.292433	0.17248	0.292433	0.5634
22	0.19157	0.19157	0.085288	0.3914
23	0.409253	0.409253	0.096195	0.1176
24	1.04282	0.620831	1.04282	0.0712
25	0.383942	0.362036	0.383942	0.9356
26	1.63719	0.172716	1.63719	0.1436
27	0.558285	0.558285	0.45648	0.8785
28	0.28701	0.28701	0.258885	0.8914
29	0.446094	0.446094	0.086331	0.065
30	0.619552	0.037589	0.619552	0.404
31	0.478199	0.14538	0.478199	0.0472
32	0.572029	0.572029	0.039967	0.2506
33	0.155701	0.155701	0.034595	0.4188
34	0.408419	0.408419	0.179204	0.4144
35	0.474109	0.474109	0.365254	0.0654
36	0.398881	0.398881	0.153762	0.002
37	0.554108	0.554108	0.295537	0.3037
38	0.87737	0.169897	0.87737	0.0615

39	0.577387	0.577387	0.410405	0.2951
40	0.280563	0.280563	0.100545	0.5888
41	0.349928	0.349928	0.063076	0.3494
42	0.114223	0.114223		
43	1.18552	1.18552		
44	0.172918	0.172918		
45	0.633054	0.633054		
46	0.534022	0.534022		
47	0.745236	0.745236		
48	0.615736	0.615736		
49	0.552834	0.552834		
50	0.211378	0.211378		
51	0.352117	0.352117		
52	0.406215	0.406215		
53	0.560807	0.560807		
54	0.37867	0.37867		
55	0.547418	0.547418		
56	0.179358	0.179358		
57	0.287168	0.287168		
58	0.718016	0.718016		
59	0.182315	0.182315		
60	0.376906	0.376906		
61	0.124069	0.124069		
62	0.161562	0.161562		
63	0.733316	0.733316		
64	0.352763	0.352763		
65	0.0656824	0.065682		
66	0.076312	0.076312		
67	0.0916142	0.091614		
68	0.831091	0.831091		
69	0.637785	0.637785		
70	0.32457	0.32457		
71	0.462934	0.462934		
72	0.161546	0.161546		
73	0.194973	0.194973		
74	0.240687	0.240687		
75	0.262685	0.262685		
76	0.301074	0.301074		
77	0.265108	0.265108		
81	3.2767	3.2767	1.99731	0.3831
82	1.30995	1.30995	1.00748	0.3458
83	2.17083	1.51598	2.17083	0.7819
148	0.179578		0.179578	
149	0.32951		0.32951	
150	1.38374		1.38374	
151	0.0658553		0.065855	

152	0.0961924		0.096192	
153	0.665318		0.665318	
154	0.604238		0.604238	
155	0.951644		0.951644	
156	0.0602126		0.060213	
157	0.0634799		0.06348	
158	1.00902		1.00902	
159	0.134997		0.134997	
160	0.0943541		0.094354	
161	0.879124		0.879124	
162	1.22048		1.22048	
163	0.138746		0.138746	
164	0.319382		0.319382	
165	0.136688		0.136688	
166	1.65785		1.65785	
167	0.216187		0.216187	
168	0.22388		0.22388	
169	0.189011		0.189011	
170	0.159509		0.159509	
171	0.395177		0.395177	
172	0.854836		0.854836	
173	0.691733		0.691733	
174	0.743769		0.743769	
175	1.90018		1.90018	
176	0.0701215		0.070122	
177	0.192507		0.192507	
178	0.101854		0.101854	
179	0.151094		0.151094	
180	0.266113		0.266113	
181	0.311789		0.311789	
182	0.212034		0.212034	
183	0.425706		0.425706	
184	0.23583		0.23583	
185	0.365403		0.365403	
186	0.419214		0.419214	
187	0.288003		0.288003	
188	0.056273		0.056273	
189	0.0410239		0.041024	
190	0.248369		0.248369	
191	0.872486		0.872486	
192	0.432613		0.432613	
193	0.156524		0.156524	
194	0.209551		0.209551	
195	0.0472487		0.047249	
196	0.632398		0.632398	
197	1.55399		1.55399	

198	0.484333		0.484333	
199	0.356359		0.356359	
200	0.101441		0.101441	
201	0.239461		0.239461	
202	6.11131		6.11131	
203	0.0972767		0.097277	
204	0.484609		0.484609	
205	0.130624		0.130624	
206	0.0577062		0.057706	
207	0.259432		0.259432	
208	0.113947		0.113947	
209	0.0663607		0.066361	
210	0.302774		0.302774	
211	0.0732257		0.073226	
212	0.162969		0.162969	
213	0.159893		0.159893	
214	0.109892		0.109892	
215	0.0911249		0.091125	
216	0.214592	0.214592		

APPENDIX D-2

Matched spots between the susceptible and the Kesang biotype.

Spot ID	% Vol.	Susceptible biotype	Kesang biotype	t-test
0	0.789004	0.789004		
1	2.14009	2.14009	0.508146	0.1069
2	23.7643	23.7643	12.3276	0.0577
3	0.212487	0.212487		
4	3.59147	3.59147	3.25961	0.6290
5	0.549898	0.549898		
6	0.175276	0.175276		
7	0.108111	0.108111	0.045751	0.4622
8	4.69505	1.85691	4.69505	0.2695
9	0.225409	0.225409		
10	0.251578	0.251578		
11	4.37614	2.84301	4.37614	0.4279
12	0.362014	0.362014		
13	4.16536	4.16536	3.08214	0.1078
14	0.058891	0.058891		
15	0.185684	0.185684		
16	0.033127	0.033127		
17	0.142474	0.142474	0.073895	0.5439
18	0.093376	0.093376		
19	0.337749	0.337749		
20	0.509227	0.509227		
21	0.182456	0.178058	0.182456	0.9839
22	0.146025	0.134754	0.146025	0.9023
23	0.415842	0.415842		
24	0.626254	0.626254		
25	0.360881	0.360881	0.063446	0.2446
26	0.31896	0.178302	0.31896	0.5209
27	1.35146	0.576342	1.35146	0.3101
28	0.086312	0.086312		
29	0.446804	0.446804		
30	0.127465	0.127465		
31	0.147757	0.147757		
32	0.575682	0.575682		
33	0.159761	0.159761		
34	0.408136	0.408136		
35	0.479362	0.479362		
36	0.403388	0.403388		
37	0.564688	0.564688		
38	0.175392	0.175392	0.153021	0.9281
39	0.581043	0.581043		

40	0.289637	0.289637		
41	0.3511	0.3511		
42	0.141921	0.117028	0.141921	0.8354
43	1.23986	1.19561	1.23986	0.9298
44	0.174671	0.174671		
45	0.120001	0.120001		
46	0.537799	0.537799	0.151956	0.3373
47	0.568937	0.568937	0.395604	0.4218
48	0.458464	0.458464	0.238401	0.6799
49	0.556205	0.556205	0.19911	0.0341
50	0.212453	0.212453	0.197148	0.2300
51	0.196964	0.196964		
52	0.645187	0.645187		
53	0.562996	0.562996	0.083886	0.0210
54	0.379264	0.379264	0.182692	0.2066
55	0.548805	0.548805	0.250361	0.5233
56	0.232429	0.232429		
57	0.289247	0.289247		
58	0.723926	0.723926		
59	0.078283	0.078283		
60	0.152117	0.152117		
62	0.114811	0.114811		
63	0.739666	0.739666		
64	0.362036	0.362036		
65	0.066469	0.066469		
66	0.077048	0.077048	0.016797	
67	0.09655	0.092348	0.09655	0.9369
68	1.07229	1.07229		
69	0.765927	0.765927		
70	0.326769	0.326769		
71	0.669644	0.669644	0.237087	0.2544
72	0.164542	0.164542		
73	0.384888	0.384888		
74	0.242492	0.242492		
75	0.266963	0.266963		
76	0.301602	0.301602		
77	0.270944	0.270944	0.076535	0.3945
78	1.93845	1.93845	0.296506	0.0376
79	2.98339	2.94338	2.98339	0.9638
80	0.215008	0.215008	0.106643	0.6830
84	0.217653	0.217653		
85	0.714228	0.714228		
86	19.2744		19.2744	
87	0.394305		0.394305	
88	0.096252		0.096252	
89	2.58392		2.58392	

90	0.327165		0.327165	
91	7.27634		7.27634	
92	0.154254		0.154254	
93	0.072633		0.072633	
94	0.288429		0.288429	
95	0.167423		0.167423	
96	0.346853		0.346853	
97	0.101181		0.101181	
98	0.413472		0.413472	
99	0.214949		0.214949	
100	0.32396		0.32396	
101	0.191349		0.191349	
102	0.16849		0.16849	
103	0.328636		0.328636	
104	0.192053		0.192053	
105	0.17179		0.17179	
106	0.412928		0.412928	
107	2.09359		2.09359	
108	0.078575		0.078575	
109	0.131838		0.131838	
110	0.256803		0.256803	
111	0.407044		0.407044	
112	0.3312		0.3312	
113	0.35454		0.35454	
114	0.541301		0.541301	
115	0.045075		0.045075	
116	0.047177		0.047177	
117	3.16638		3.16638	
118	0.058262		0.058262	
119	1.33663		1.33663	
120	0.277147		0.277147	
121	0.167905		0.167905	
122	0.04197		0.04197	
123	0.168306		0.168306	
124	0.087019		0.087019	
125	0.145676		0.145676	
126	0.216321		0.216321	
127	0.232391		0.232391	
128	0.200548		0.200548	
129	0.162192		0.162192	
130	0.277195		0.277195	
131	0.248594		0.248594	
132	0.399927		0.399927	
133	0.178415		0.178415	
134	0.165267		0.165267	
135	0.168856		0.168856	

136	0.056019		0.056019	
137	0.196867		0.196867	
138	0.092131		0.092131	
139	0.040787		0.040787	
140	0.27746		0.27746	
141	0.283044		0.283044	
142	0.280497		0.280497	
143	0.037384		0.037384	
144	0.426322		0.426322	
145	0.402865		0.402865	
146	0.220544		0.220544	
147	0.08002		0.08002	

APPENDIX E-1

Identification of spots listed in Table 3.12 using ProFound

ProFound - Search Result Summary							
Protein Candidates							
Rank	Probability	Est'd Z	Protein Information and Sequence Analyse Tools (T)	%	*pI*	kDa	R
+1	1.0e+000	2.21	gi|321273474|gb|ADW80737.1| chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit [Flaveria vaginata]	26	5.4	11.70	
1	6.6e-001	0.30	gi|164453462|gb|ABY57490.1| NADH dehydrogenase subunit J [Arabidopsis thaliana]	28	5.9	14.90	
1	1.0e+000	1.16	gi|148908879|gb|ABR17544.1| unknown [Picea sitchensis]	39	6.8	15.03	
+1	1.0e+000	2.43	gi|15226467|ref|NP_179709.1| peptidyl-prolyl cis-trans isomerase / cyclophilin (CYP2) / rotamase [Arabidopsis thaliana]	21	8.8	18.67	
1	1.5e-001	0.09	gi|242057419|ref|XP_002457855.1| hypothetical protein SORBIDRAFT_03g016086 [Sorghum bicolor]	14	5.2	18.19	
1	6.6e-001	1.35	gi|222634899|gb|EEE65031.1| hypothetical protein OsJ_20009 [Oryza sativa Japonica Group]	20	5.9	20.57	
+1	1.0e+000	2.29	gi|2499477|sp|Q96468.1|BAS1_HORVU RecName: Full=2-Cys peroxiredoxin BAS1, chloroplastic; AltName: Full=Thiol-specific antioxidant protein; Flags: Precursor	21	5.5	23.39	
1	5.1e-001	1.14	gi|115448199|ref|NP_001047879.1| Os02g0707900 [Oryza sativa Japonica Group]	16	6.0	20.20	
1	1.0e+000	1.45	gi|242072310|ref|XP_002446091.1| hypothetical protein SORBIDRAFT_06g001600 [Sorghum bicolor]	18	5.6	24.36	

1	8.8e-001	0.43	gi|255072661|ref|XP_002500005.1| Hypothetical protein MICPUN_104759 [Micromonas sp. RCC299]	13	5.5	26.75	
1	9.0e-001	1.44	gi|56675440|emb|CAA37047.2| cytochrome-c oxidase [Pisum sativum]	8	5.0	28.81	
1	6.8e-001	1.34	gi|56675440|emb|CAA37047.2| cytochrome-c oxidase [Pisum sativum]	8	5.0	28.81	
1	9.4e-001	1.41	gi|297723807|ref|NP_001174267.1| Os05g0198100 [Oryza sativa Japonica Group]	11	5.8	33.64	
1	9.0e-001	0.88	gi|297723807|ref|NP_001174267.1| Os05g0198100 [Oryza sativa Japonica Group]	11	5.8	33.64	
1	4.2e-001	0.90	gi|147791081|emb|CAN68019.1| hypothetical protein VITISV_027126 [Vitis vinifera]	20	5.0	30.20	
+1	1.0e+000	2.43	gi|21593527|gb|AAM65494.1| unknown [Arabidopsis thaliana]	15	5.0	33.99	
+1	1.0e+000	2.43	gi|219888599|gb|ACL54674.1| unknown [Zea mays]	12	6.7	33.70	
+1	1.0e+000	2.43	gi|4930119|pdb|1QFY|A Chain A, Pea Fnr Y308s Mutant In Complex With Nadp+	17	6.5	34.99	
1	9.9e-001	1.56	gi|15227413|ref|NP_181700.1| AT-HSFB3; DNA binding / transcription factor [Arabidopsis thaliana]	18	5.3	28.57	
1	4.5e-001	0.16	gi|297829148|ref|XP_002882456.1| ATMKK8 [Arabidopsis lyrata subsp. lyrata]	7	6.5	28.28	
+1	1.0e+000	2.43	gi|310897866|emb|CBK62755.1| WD-repeat protein [Humulus lupulus]	13	4.9	38.13	
1	8.2e-001	0.47	gi|302830410|ref|XP_002946771.1| hypothetical protein VOLCADRAFT_103197 [Volvox carteri f. nagariensis]	10	6.4	42.29	
1	9.8e-001	0.62	gi|226534275|gb|ACO71420.1| maturase K [Succisa pratensis]	14	9.5	35.18	
1	5.5e-001	0.92	gi|242035489|ref|XP_002465139.1| hypothetical protein SORBIDRAFT_01g032640 [Sorghum bicolor]	8	6.2	32.88	
1	8.3e-001	1.41	gi|159486427|ref|XP_001701241.1| phosphoserine phosphatase [Chlamydomonas reinhardtii]	12	6.3	29.32	
1	2.2e-001	0.09	gi|218190702|gb|EEC73129.1| hypothetical protein OsI_07141 [Oryza sativa Indica Group]	5	7.0	25.58	
+1	1.0e+000	1.66	gi|85680944|gb|ABC72667.1| granule-bound starch synthase [Neomicrocalamus prainii]	15	6.2	24.05	
+1	1.0e+000	2.43	gi|147780183|emb|CAN75527.1| hypothetical protein VITISV_043600 [Vitis vinifera]	20	9.5	20.10	
1	1.9e-001	0.15	gi|255620495|ref|XP_002540120.1| conserved hypothetical protein [Ricinus communis]	15	9.3	20.27	

1	1.0e+000	1.58	gi|224074567|ref|XP_002304391.1| predicted protein [Populus trichocarpa]	18	8.5	26.73	
+1	1.0e+000	2.14	gi|297820232|ref|XP_002877999.1| hypothetical protein ARALYDRAFT_485883 [Arabidopsis lyrata subsp. lyrata]	11	9.5	29.17	
1	8.1e-001	1.25	gi|116782595|gb|ABK22565.1| unknown [Picea sitchensis]	8	9.1	36.53	
1	7.7e-001	1.24	gi|115455415|ref|NP_001051308.1| Os03g0754800 [Oryza sativa Japonica Group]	8	9.9	35.08	

APPENDIX E-2

Identification of standard control used in MALDI-TOF using ProFound

ProFound - Search Result Summary							
Protein Candidates							
Rank	Probability	Est'd Z	Protein Information and Sequence Analyse Tools (T)	%	*pI*	kDa	R
1	1.0e+000	2.34	gi|229552|prf||754920A albumin	16	5.8	67.78	