CONYZA BONARIENSIS GLYPHOSATE TOLERANCE, AS AFFECTED BY ORIGIN, TEMPERATURE AND GROWTH STAGE

by

JACOBUS MUNTINGH HAMMAN

Submitted in partial fulfillment of the requirements for the degree

MSc (Agric) Agronomy

In the Faculty of Natural and Agricultural Sciences

University of Pretoria

Supervisor: Prof. C.F. Reinhardt

Co-supervisor: Dr. B.J. Vorster

July 2017

DECLARATION

I hereby certify that this dissertation is my own work, except where duly acknowledged. I also certify that no plagiarism was committed in writing this dissertation.

_____

Jacobus Muntingh Hamman

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Glyphosate was patented as a broad-spectrum, non-selective, systemic herbicide in 1974. In 1996, glyphosate-resistant *Lolium rigidum* (rigid ryegrass) was reported in Australia. Since then 34 other weeds the world over have evolved resistance to this herbicide. The first case of glyphosate resistance in South Africa was in rigid ryegrass in vineyards. Resistance to glyphosate in *Conyza bonariensis* (L.) Cronquist (flax-leaf fleabane) was reported in 2003 in the Breede Valley, Western Cape. Glyphosate resistant *Conyza canadensis* (horseweed fleabane) reportedly becomes sensitive at low temperatures (below 12 ^{o}C). If the resistance mechanism is vacuolar sequestration, low temperatures will prevent glyphosate to be translocated into the vacuole. Tank mixtures with foliar manganese and other foliar-applied nutrient elements, in particular cations such as Mn^{2+}, Zn^{2+}, Fe^{2+}, etc, are known to decrease glyphosate efficacy. The aim of the present study is to determine if growth stage has an influence on glyphosate tolerance in *C. bonariensis*, if highly glyphosate-tolerant *C. bonariensis* plants become sensitive at low temperatures, and to assess if there is an effect between high manganese levels and response to glyphosate, as well as if mancozeb (fungicide containing Mn and Zn) influences the glyphosate-manganese interaction. *C. bonariensis* seed was collected at 12 locations. At the four to six leaf stage six dosages of glyphosate was applied: 0, 0.25, 0.5, 1, 2 and 4 times the recommended dosage (2 L ha^{-1} Roundup Turbo). Fresh mass were measured at 21 days after treatment (DAT). Data was subjected to ANOVA. GR_{50} and Resistant / Sensitive values were calculated. From the screening experiment two highly tolerant, two tolerant and two susceptible populations were identified. The growth stage experiment was conducted in the exact same manner as the screening experiment, with growth stage as an added factor. Plants were treated with glyphosate at two different growth stages (10 – 12 and 16 – 20 leaves). A second screening experiment with four populations from the same area was performed. Plants from a highly tolerant population were grown in the greenhouse up to the four leaf stage and then moved to the temperature gradient table. Plants were exposed to temperature ranges of 8 - 13 ^{o}C, 15 - 20 ^{o}C and 22 - 27 ^{o}C, respectively. Plants were treated with glyphosate at 0, 0.5, 1, 2 and 4 times the recommended dosage and fresh mass measured at 21 DAT. The manganese experiment had three factors: manganese level, location and glyphosate dosage. Seed from a

sensitive and highly tolerant population were planted in a seeding tray. Seedlings were transplanted to a hydroponic system containing three different nutrient solutions with different manganese levels. Plants were treated with glyphosate at the 4-6 leaf stage at 1 and 2 times the recommended dosage. The rest of the materials and methods are the same as for the temperature experiment. The final experiment was performed to examine if mancozeb has an influence on the efficacy of glyphosate when applied to *C. bonariensis.* Mancozeb was applied before and after glyphosate to susceptible *C. bonariensis* plants. For two of the populations in the screening experiment all of the replicates survived the recommended dosage, and hence, they were classified as highly tolerant. Two other populations were classified as tolerant and two as sensitive. There is a clear difference in the sensitivity of the various populations to glyphosate as well as populations from the same area. These six populations were further used in the growth stage experiment where the same results were obtained regarding the sensitivity of the populations at the 10 - 12 leaves growth stage. Plants at the 16 – 20 leave growth stage are much more tolerant to glyphosate. The second screening experiment showed that *C. bonariensis* plants from the same area differ in the tolerance towards glyphosate. Highly glyphosate-tolerant plants did not become susceptible at a low temperature. Susceptibillity to glyphosate increased at the higher temperatures. Therefore, vacuolar sequestration is probably not the mechanism of resistance responsible for the high tolerance to glyphosate. There was no replication of manganese treatments in the manganese experiment and therefore differences could not be tested. The unique methodology employed in this experiment is, however, of value. Mancozeb did not have an influence on glyphosate efficacy when applied either before or after glyphosate. If resistance to glyphosate develops in *C. bonariensis* and in other species on a wider scale than is currently the case in South Africa it will be a big problem for farmers in various cropping systems. Therefore, the label must be followed very strictly to ensure that plants are treated at the correct dosage and growth stage to ensure that populations are not incorrectly referred to as resistant.

INTRODUCTION

According to Holm and Johnson (2009), more time, money and energy have been devoted to weed control throughout the history of agriculture than to any other agricultural activity. Chemical weed control has provided much assistance and relief from the demanding task of mechanical weed control (Pieterse 2010). Chemical weed control has been applied since ancient times when civilizations used acids, common salt, wood ash and heavy metals to control unwanted plants (Holm and Johnson 2009, Pieterse 2010). It was only after World War II that chemical weed control was revolutionized with the introduction of the first selective synthetic herbicides, 2,4-D (Duke and Powles 2008) and MCPA (Pieterse 2010).

In comparison with fungicides and insecticides, herbicides were slow to develop resistance; the first case of resistance to a herbicide (simazine) was reported in 1968 (Ryan 1970). Glyphosate was first introduced to the market in 1974 and due to the slow development of resistance to this herbicide it was speculated that resistance to glyphosate was unlikely to develop (Powles 2008). Glyphosate quickly became the world's most extensively used herbicide (Powles 2008) due to numerous advantages like efficiency, environmental friendliness, no soil activity (allowing flexibility in crop rotations), low human health risks and cost-effectiveness (Boerboom and Owen 2013). Glyphosate-resistant soybean was the first Roundup Ready® crop to be introduced to the market in 1996, which further increased the use of glyphosate due to economics and convenience (Dill 2005).

Glyphosate was, however, used too persistently and the first case of resistance reported was to *Lolium rigidum* in 1996 in Australia (Heap 2014). Since then 34 other glyphosate-resistant weeds have been reported over the world (Heap 2016). According to Heap (2014), the worst herbicide-resistant weeds in the world are species of the genera *Amaranthus, Echinochloa, Lolium* and *Conyza.* Of those weeds, *Conyza canadensis* is the most wide-spread glyphosate-resistant weed in the world (Heap 2014).

In 2003, Cairns reported glyphosate resistance in *Conyza bonariensis* (flax-leaf fleabane) in South African vineyards, as well as in orchards (Pieterse 2010, Heap 2016). De Wet (2005) confirmed glyphosate resistance of *C. bonariensis* in the Breede Valley, Western Cape. *Conyza bonariensis* is the most common and wide-spread weedy *Conyza* in South Africa, and was the weed focused on in this study.

In South Africa no work on glyphosate resistance in *C. bonariensis* outside of the winter rainfall region, specifically the Western Cape, has yet been done. Despite claims by farmers that resistance of *C. bonariensis* occurs in other areas of the country there have been no proven cases to date.

The main aim of the study was to establish if there are glyphosate-resistant populations of *Conyza bonariensis* outside the winter rainfall region in South Africa, and other objectives were to establish if seed source, growth stage, temperature and high manganese levels influence the response of this weed towards glyphosate. The hypotheses tested thus were:

- There are regions other than the Western Cape in South Africa where *C. bonariensis* has developed resistance to glyphosate.
- Differences in the response of *C. bonariensis* towards glyphosate are dependent on the population/seed origin.
- Tolerance of plants to glyphosate increases with growth stage.
- Glyphosate-resistant populations will be more susceptible to glyphosate at low temperatures if vacuolar sequestration is the mechanism of resistance.
- The presence of manganese will increase plant tolerance to glyphosate by complexing with glyphosate in the plant or in the spray tank, thereby causing a reduction in response to the herbicide.
- Mancozeb fungicide, which contains Mn and Zn atoms in the active ingredients' molecular structure, will influence the efficacy of glyphosate.

CHAPTER 1

LITERATURE REVIEW

1.1. *Conyza bonariensis* (L.) Cronquist

Conyza bonariensis (flax-leaf fleabane, hairy fleabane) originated from the temperate regions in South America (Michael 1977), and is also a major weed in South Africa (Wu et al., 2007, Bromilow 2010). It is widely spread throughout the warmer regions of Europe, Africa, Asia, the Caribbean, and Central America. The only place where it is not distributed is Antarctica (Mifsud 2015). *C. bonariensis* is closely related to *Conyza sumatrensis* (L.) Cronquist (tall fleabane) and *Conyza canadensis* (L.) Cronquist (horseweed fleabane). These three species are similar in growth habit and are widely distributed in South Africa (Bromilow 2010). During the early growth stages these species do not differ much in their morphology (Malatji 2013), and definitive identification is often only possible when plants reach maturity.

1.1.1. Taxonomy

Kingdom Plantae

Subkingdom Tracheobionta (vascular plants)

Division Magnoliophyta (flowering plants, angiosperms)

Class Magnoliopsida (dicotyledons)

Subclass Asteridae

Order Asterales

Family Asteraceae (sunflower family)

Genus Conyza (horseweed)

Species *Conyza bonariensis* (L.) Cronquist (Flax-leaf fleabane, Hairy fleabane)

Conyza canadensis (L.) Cronquist (Horseweed fleabane, Canadian fleabane)

Conyza sumatrensis (L.) Cronquist (Tall fleabane)

1.1.2. Morphology and structure

C. bonariensis has long stems that can grow as tall as 100 cm and the plant has a well-developed taproot. Branches form at the base and can grow taller than the main stem (Bromilow 2010, Mifsud 2015). The stems, leaves and flowering parts have many short, white and fine trichomes (leaf hairs). It has been reported that *C. bonariensis* has more trichomes than *C. sumatriensis*, which explains the common name, hairy fleabane (Wu and Zhu 2014). The high density of leaf hairs on *C. bonariensis* can be a reason for the natural tolerance of this weed towards herbicides due to the trichomes' hydrophobic nature, creation of air pockets and droplet interception (Wu and Zhu 2014).

Figure 1.1 *Conyza bonariensis* (Photo by Prof. C.F. Reinhardt)

The leaves of hairy fleabane grow alternately along the stem at a sharp angle in groups of three to six leaves. The leaves are lanceolate to linear, narrow, crinkled and somewhat toothed around the edges, and has a grayish-green color (Mifsud 2015).

Flowers develop only at the end of stems as dense, cone-shaped panicles. A cup-shaped involucre consisting of overlapping green phyllaries encloses the flower. These phyllaries often have characteristic purple tips that form a purple "ring" around the flower head as can be seen in Figure 1.1. The flower head does not have petals. It is made up of yellow disc florets that is attached to a common receptacle and surrounded by white pappii that is enclosed by the green involucre. The disc florets are further modified to filliform florets. Filliform florets has the same structure as disc florets but they have pistils without any fused stamens (Mifsud 2015).

The pappii develop with the fruit and as the fruit matures the pappii become loose and form a feathery structure with persistent hairs. The seeds are small achenes with an unbranched filamentous pappus (Mifsud 2015). The pappus acts like a parachute and disperse the seed away from the plant with the wind. One flowerhead can produce an average of 400 seeds (Kempen and Graf 1981).

1.1.3. Biology and ecology

C. bonariensis is an annual or short-lived perennial weed from the sunflower family (Asteraceae) (Prieur-Richard et al., 2000, Wu et al., 2007). C*. bonariensis* tends to be abundant in conservation agriculture systems and, in particular, in no-till fields due to a micro-environment that promotes this weed's seed germination and survival (Wu et al., 2007). This species can grow almost anywhere: crop fields, roadsides, gardens, water canals, fallow land, forests, pavements, and golf greens (Bromilow 2010, Mifsud 2015). No-till fields have higher surface moisture which favors the emergence of *C. bonariensis* which is very sensitive to soil burial. C*. bonariensis* mainly emerge from the soil surface (0-2 cm), with an optimum depth of 0.5 cm, and no emergence occurring from a planting depth greater than 2 cm (Wu et al., 2007). One of the reasons for the shallow germination requirement is that C*. bonariensis* seed is photoblastic, and therefore, germination is highly stimulated under light (Michael 1977, Wu et al., 2007). Another reason is that *C. bonariensis* seed is very small and the amount of substrate

required for emergence is minimal (Grundy et al., 2003). Surface moisture conditions are likely to stay favorable for longer periods under no-tillage conditions in comparison with conventional tillage (Wu et al., 2007).

In a field experiment, Wu et al. (2007) found that 99 % of seed emerge in late autumn, and early and late winter, while the other 1 % of seed emerged in the middle of spring. *C. bonariensis* germinates at temperatures between 10 and 25 ^{o}C. The optimum temperature for germination is 20 ^{o}C, the base temperature for germination is 4.2 ^{o}C, and no germination occurs at 35 ^{o}C.

C. bonariensis plants can produce between 119 100 and 375 561 wind-dispersed seeds per plant (Kempen and Graf 1981, Wu et al., 2007). The light-weighted seed can be dispersed over very long distances due to a pappus attached to it. Long-distance dissemination takes place by wind and surface run-off (Wu et al., 2007). The combination of prolific seed production, wind and water dissemination imply that *C. bonariensis* can spread very easily across a landscape (Wu et al., 2007).

Flax-leaf fleabane follows a winter or summer annual lifecycle (Bromilow 2010). As previously mentioned, the plants mainly emerge in autumn and early winter. The plants then form a basal rosette, stays in that stage over winter and flower in the following summer or spring (Wu et al., 2007). There is, however, a portion of the plants that germinates in spring and bolts without an overwintering growth stage. The overlap in time of emergence can be explained by the optimum temperatures for germination mentioned above (Wu et al., 2007). Flowering of *C. bonariensis* is favored by long photoperiods (14- hour light periods), however, it flowers all year around (Amsellem et al., 1993).

Despite cold and dry conditions, seedlings that emerged in autumn and early winter grow actively during the winter. Aboveground growth appears to be minimal during the rosette stage, while root growth is strong during this period (Wu et al., 2007). *C. bonariensis* has a taproot that can grow deeper than 35 cm. This strong root system that developed over winter supplies enough food reserves for rapid growth during the following spring. It has been

reported (Urbano et al., 2009) that it is difficult to control these over-wintering plants because, even though the plants are small in appearance, they are old by age (Wu et al., 2007).

Wu et al. (2007) found that buried *C. bonariensis* seed lost its viability in the first year. After three years of burial only 6 % of the seed was still viable. Although 6 % is a small percentage it should not be misleading due to enormous seed production of this plant, and therefore, for effective long-term management of this weed it is imperative that seed set should be prevented.

C. bonariensis can easily be controlled mechanically by means of cultivation or tillage. Tillage is, however, not always an option, such as in no-tillage or conservation agriculture systems. Deep cultivation to bury seeds can be used to deplete the seed bank (Wu et al., 2007). This weed can be controlled chemically by, amongst others, glyphosate, paraquat, diuron, 2,4-D/dicamba, carfentrazone-ethyl and bromoxynil (de Wet 2005, Malatji 2013). Due to the potential difficulty in uptake of foliar herbicides, root-absorbed herbicides should be used in combination with foliar-applied herbicides for better control (Wu and Zhu 2014).

It has been reported that *C. bonariensis* has allelopathic effects (Malatji 2013), and it has developed resistance to the herbicides glyphosate and paraquat in South Africa (de Wet 2005). Herbicide resistance in South Africa is discussed in more detail from page 18 in section 1.4.

1.2. Glyphosate

Henry Martin of a little Swiss pharmaceutical company called Cilag apparently was the first to create the glyphosate molecule [N-(phosphonomethyl)glycine] (Franz et al., 1997, Duke and Powles 2008). In other words, glyphosate is a phosphonomethyl derivative of the amino acid glycine (Nandula 2010). The molecule had no pharmaceutical value and was not tested let alone patented as a herbicide at the time (Duke and Powles 2008, Nandula 2010). Glyphosate for use as an herbicide was first synthesized and tested in 1970 by John E Franz of Monsanto Company (St. Louis, MO, USA). Glyphosate was patented for use as a herbicide soon thereafter in 1974 under the trade name Roundup® (Nandula et al., 2005).

At physiological pH levels, glyphosate is an anionic compound (Duke and Powles 2008). Glyphosate is naturally amphoteric and can therefore easily be dissolved in dilute aqueous bases and strong aqueous acids in order to generate respectively anionic and cationic salts (Nandula 2010). The solubility of glyphosate increases significantly when the free acid of glyphosate is converted to monobasic salts. It is commonly formulated as concentrated water solutions of approximately 30-50 % (Nandula 2010), and as a salt with various cations like trimethylsulfonium, isopropylamine, ammonium, sodium, trimesium and potassium (Baylis 2000, Woodburn 2000). Glyphosate has a low volatility (2.59×10^{-5} Pa at 25 ^{o}C) and high density (1.75 g.cm^{-3}) which shows that it does not easily evaporate or shift through the air to other non-target organisms after it has been applied (Nandula 2010).

Glyphosate is marketed as a post-emergence, broad-spectrum, non-selective and systemic herbicide. It has become the world's most extensively used herbicide because it is efficient, economically viable and environmentally friendly (Nandula et al., 2005, Powles 2008). Since the commercialization of glyphosate, it has been used in crop and non-crop lands. Due to glyphosate's lack in selectivity it was initially only used in pre-plant and post-harvest situations for weed control in grain crops (Dill 2005, Nandula et al., 2005). Glyphosate is also used all over the world for broad-spectrum weed control in and between rows of established perennial crops such as fruit, nut tree and vine crops. Glyphosate is also the worldwide choice for weed control in various environmental situations in urban and industrial areas, national parks and along

roads (Powles 2008). The big reason for glyphosate being so popular is that it gives very effective and economical broad-spectrum control of weeds (Baylis 2000).

While this broad-spectrum action and non-selectivity of glyphosate can be seen as a strength, it was a weakness in annual crops because it could not be used after emergence of the crops as it would kill the crop together with the weed (Baylis 2000). In 1996, Monsanto introduced the first transgenic crop, soybeans, containing a bacterial gene which made the crop resistant to glyphosate (Powles 2008). This development revolutionized crop production and weed control, and resulted in a whole new glyphosate-use pattern. Transgenic crops include soybeans, maize, cotton and canola (Nandula et al., 2005). Glyphosate can now be used to control weeds emerging after the emergence of a glyphosate-resistant crop without damaging the crop. The discovery of glyphosate-resistant crops permitted glyphosate to be used in these crops as a selective herbicide that provides easy, economical and efficient weed control. This introduced other advantages as well, such as earlier seeding and zero-tillage (Powles 2008). In countries where genetically modified crops are grown, glyphosate-resistant crops are exceptional commercial successes.

1.2.1. Mechanism of action

Glyphosate has an unique mechanism of action. It is the only molecule that effectively inhibits the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) of the shikimate pathway (Duke & Powles, 2008). EPSPS is mainly situated in the plastids of plant cells, but a cytoplasmic form also exists (Baylis 2000). EPSPS catalyzes the transfer of the enolpyruvyl moiety of phosphoenolpyruvate (PEP) to shikimate-3-phosphate (S3P). This is an important step in the synthesis of three essential aromatic amino acids, hormones and other critical plant metabolites which include lignins, flavonoids and other phenolic compounds (Dill, 2005). A simple schematic layout of the shikimate pathway, and glyphosate's site of action is presented in Figure 1.2.

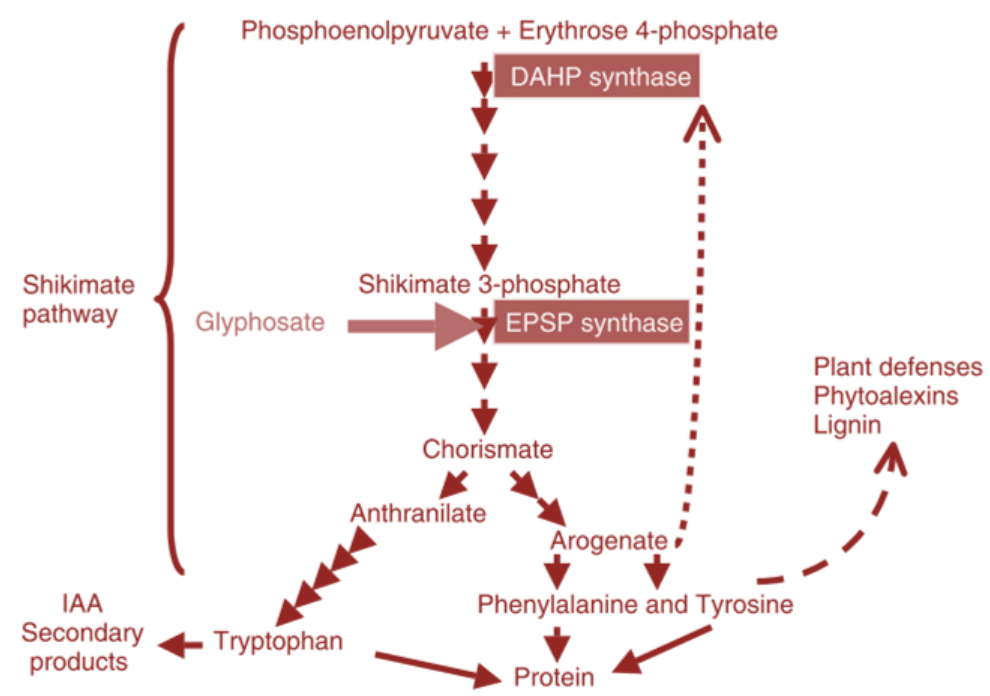

Figure 1.2 The shikimate pathway and the site of inhibition by glyphosate (Duke and Powles 2008)

Glyphosate acts as a transition state analog of phosphoenolpyruvate which is one of the substrates for EPSPS (Dill 2005, Duke and Powles 2008). It was, however, also reported (Sikorski and Gruys 1997) that glyphosate does not act as a transition state inhibitor but that it's binding behavior can be better described as an "adventitious allosteric interaction" where a considerable part of the glyphosate molecule binds outside the EPSPS active site. It is then this conformational change after glyphosate binding that makes the active site unavailable to PEP (Baylis 2000). Regardless of how this inhibition takes place it results in reduced feedback inhibition which leads to vast carbon flow to S3P that is converted to high levels of shikimate in the glyphosate treated plant tissues (Franz et al., 1997).

According to Duke and Powles (2008) it is not clear how glyphosate-induced inhibition of the shikimate pathway really kills the plant. Although EPSPS is the only recognized enzyme target of glyphosate, it also affects several physiological and physiochemical processes (Baylis 2000). Many believe that the unsatisfactory production of aromatic amino acids to maintain necessary protein synthesis is the main effect. The slow development of symptoms in plants that are

susceptible to glyphosate supports this belief (Duke and Powles 2008). Others suggest that the deregulation of the shikimate pathway by the inhibition of EPSPS, which results in increased carbon flow to the pathway, leads to shortages of carbon for other essential pathways (Servaites et al., 1987).

By determining shikimate levels in plants it can be used as a diagnostic test to establish whether glyphosate was involved where crop injury occurred due to spray drift (Singh and Shaner 1998).

1.2.2. Uptake and translocation

The dose of glyphosate that reaches the symplastic or living portion of the plant is directly responsible for the efficiency of glyphosate as a herbicide (Nandula et al., 2005). The uptake of glyphosate occurs reasonably quickly through plant surfaces (Duke and Powles 2008) although poor rainfastness in comparison to paraquat has been noted as a rare weakness of the herbicide. The most probable mode of transport taking place across the plant cuticle is through the process of diffusion. Due to physiological differences between plant species the leaf uptake rates between species vary greatly and may explain the differences in response to glyphosate that are observed between plant species (Duke and Powles 2008). Glyphosate is enabled by its physiochemical properties to be transported via the phloem to the same plant tissues that are metabolic sinks for sucrose (Siehl 1997). Thus, all actively growing tissues or organs such as young roots, meristems, storage organs and leaves can be reached by phytotoxic levels of glyphosate.

In certain plant species such as sugarbeet, glyphosate acts so fast that its own translocation can be limited when there is a reduction in photosynthesis and sucrose metabolism because of glyphosate phytotoxicity (Geiger et al., 1999). Because glyphosate is transported via the phloem and the efficiency of translocation is linked to plant health and developmental stage, environmental conditions will also have an effect on the efficacy of glyphosate (Nandula et al., 2005).

1.2.3. Toxicity and environmental impacts

Glyphosate is one of the pesticides that are the least toxic to animals (Franz et al., 1997, Duke et al., 2012a). Because of the low toxicity to animals and humans, glyphosate is used all over the world in urban and recreational areas as well as agricultural and industrial land. With an LD_{50} for rats greater than 5 g kg^{-1}, aspirin or sodium chloride is more toxic than glyphosate (Duke and Powles 2008). A number of cationic salts and other formulation materials used with glyphosate are more toxic than the glyphosate anion itself, as a result of which, glyphosate formulations have been adapted over the years. Glyphosate does not have any sub-acute chronic toxicity and it does not cause cancer, reproductive problems, nervous system effects or birth defects (Nandula et al., 2005). When glyphosate is used at its recommended rate and according to label instructions it should not be expected to create a health risk to humans (Williams et al., 2000).

Glyphosate is an environmentally friendly herbicide (Franz et al., 1997). Glyphosate has very little movement to soil and groundwater because it binds tightly to the soil colloidal fractions (Duke et al., 2012a). In soils with obvious preferential flow and macropores, glyphosate can move to the groundwater but cases of this occurring in the field have not been well reported on (Kjær et al., 2005). Glyphosate is degraded by microorganisms in non-sterile water, soil and water systems (Nandula et al., 2005). Indigenous microflora in the soil degrades glyphosate under anaerobic and aerobic conditions. Aminophosphonic acid (AMPA) is the main glyphosate degradation product (Kjær et al., 2005). AMPA or sarcosine (which is another degradation product) is then further degraded by various bacteria to inorganic phosphate, ammonia and carbon dioxide (Franz et al., 1997, Giesy et al., 2000). Because of microbial degradation in the soil, glyphosate has a relatively short half-life. Due to glyphosate not being volatile, the herbicide does not cause any atmospheric contamination and is unlikely to evaporate from untreated surfaces and injure non-target plants (Nandula et al., 2005, Duke and Powles 2008).

Glyphosate basically have no soil activity because it is strongly adsorbed by soil colloids, and it has been reported that root uptake of glyphosate from soil is negligible (Nandula et al., 2005). Therefore, it can only be used as a foliar-applied, post-emergence herbicide (Duke and Powles

2008). The active target site of glyphosate, EPSPS, is only present in green plants, some fungi and certain bacteria species (Duke et al., 2012a). The only non-target organisms the glyphosate molecule has an effect on are some fungi (Franz et al., 1997). There is no evidence of adverse effects in the environment where glyphosate has been applied at commercial rates. Some studies showed that glyphosate at very low levels can stimulate the growth of certain plant species (Baylis 2000).

1.3. Glyphosate-resistant crops

1.3.1. Development of glyphosate-resistant crops

Given the high efficiency and desirable toxicological and environmental properties of glyphosate, the only shortcomings that the herbicide had early on were the lack of glyphosate-resistant crops, and the consequent need for pre-plant and post-harvest applications (Bradshaw et al., 1997). Attempts to develop glyphosate-resistant crops from whole plant and tissue culture selection had limited success, and therefore, led to genetic modification strategies being developed. Three genetic transformation mechanisms were evaluated in order to introduce glyphosate resistance into crop species (Dill 2005), namely: 1) over-expression of the sensitive target enzyme (target site amplification mechanism); 2) detoxification of the glyphosate molecule (metabolic inactivation mechanism), and 3) expression of an insensitive form of the target enzyme (target-site modification mechanism).

The strategy of over-expression of the sensitive target enzyme (EPSPS) in order to escape the herbicidal effects of glyphosate had limited success (Shah et al., 1986, Nandula 2010). There are currently no marketed glyphosate-resistant crop species that use over-expression of native EPSPS as the mechanism of resistance (Dill 2005). Detoxification of the glyphosate molecule, however, has been confirmed via two pathways (CaJacob et al., 2004). The one pathway yields phosphate and sarcosine, whereas the other results in the formation of aminomethylphosphonic acid (AMPA) and glyoxylate, collectively referred to as glyphosate oxidase (GOX). Unfortunately, neither of these mechanisms occurs in higher plants to a considerable extent. GOX is however used in glyphosate-resistant canola in combination with a glyphosate-insensitive EPSPS, since the detoxification mechanism alone provided insufficient resistance (Dill 2005).

The introduction of an insensitive EPSPS was the mechanism that resulted in commercial glyphosate-resistant crops marketed under the Roundup Ready® brand (Dill 2005). A number of approaches have been tried to introduce glyphosate-insensitive EPSPS. All of the commercial glyphosate-resistant crops on the market contain the bacterial EPSPS that was isolated from *Agrobacterium* species. This bacterium was isolated from a waste stream of a glyphosate

manufacturing facility (Nandula 2010). The enzyme is known as CP4 and is insensitive to glyphosate (Bradshaw et al., 1997). The glyphosate-binding region and substrate of sensitive EPSPS found in most plant species is identical to the glyphosate-binding region and substrate of CP4-EPSPS (Sidhu et al., 2000). Figure 1.3 explains how these crops were developed. The CP4-EPSPS protein is 50.1% overall similar and only 23.3% identical to native maize EPSPS. This shows that conformational changes exclude binding of glyphosate. The conformational change is a result of amino acid sequence changes outside the glyphosate/PEP binding region (Dill 2005). CP4-EPSPS has a very high tolerance for glyphosate and high affinity for PEP. This allows plants with the CP4-EPSPS insertion to bypass the endogenous EPSPS system and therefore allowing the shikimate pathway to function normally (Bradshaw et al., 1997).

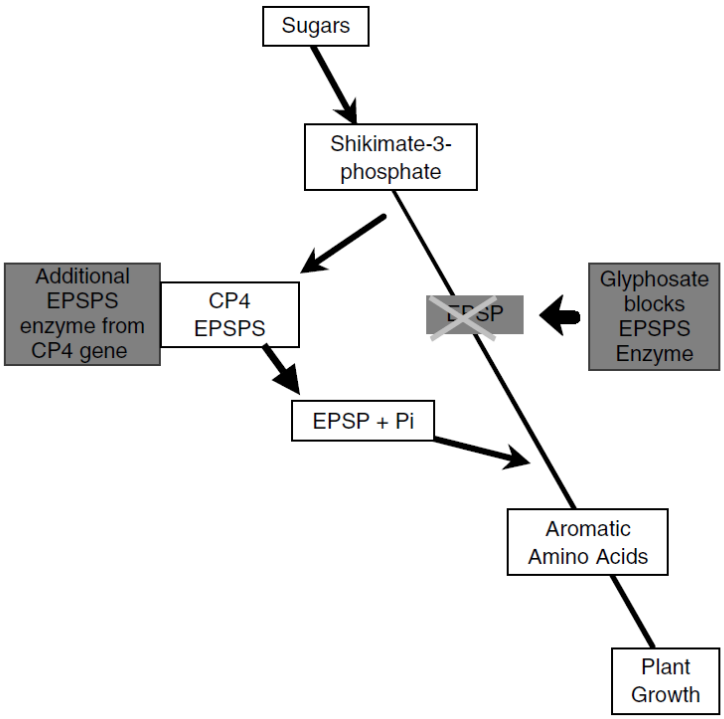

Figure 1.3 Strategy for the development of glyphosate-resistant crops (Dill 2005)

1.3.2. Commercialization

In 1996, glyphosate-resistant soybean was the first crop to be marketed under the Roundup Ready® brand (Dill 2005). Six glyphosate-resistant agronomic crops have been approved to be grown by farmers in the USA but only four are currently commercialized in the USA and other countries. Soybean, cotton, maize and canola are the four glyphosate-resistant crops, while glyphosate-resistant sugarbeet and alfalfa is not planted commercially (Duke and Powles 2008). Farmers were very quick to adopt glyphosate-resistant crops. By 2007, 90% of all the soybeans, 60% of all maize and 91% of cotton grown in the USA were glyphosate-resistant (Powles 2008). The same pattern was observed in canola with 75% of canola planted in the USA by 2005 being glyphosate-resistant. Nearly 100% soybeans grown in Argentina are glyphosate-resistant and the use of glyphosate-resistant crops in Brazil is also growing spectacularly (Duke and Powles 2008). The total maize area in SA was 2.73 million hectares in 2013. 86.6% of this area consisted of biotech maize and of this 71.6 % (1 709 032 ha) were glyphosate-resistant. Glyphosate-resistant soybeans was planted on 478 000 ha in South Africa (92% of total area planted) in 2013. All of the 8 000 ha cotton planted in South Africa in 2013 was glyphosate-resistant (James 2015).

According to Dill (2005), the adoption of glyphosate-resistant soybeans was driven by economics and convenience while in cotton and canola the main driving force was superior weed control in comparison with alternative conventional methods. The slow adoption rate for maize is mainly due to the several competitive and economical weed control strategies available and used successfully (Gianessi 2008). However, the combination of glyphosate and glyphosate resistant crops provides weed management that is generally cheaper, simpler, more flexible and better than the conventional weed management methods (Dill 2005).

Glyphosate-resistant crops also allowed farmers to employ no-tillage practices that can improve the following soil properties: soil organic matter content, water-use efficiency, as well as promote reduction in top soil losses, less fuel requirements, less capital requirements, and ultimately, lower input costs. Another advantage of glyphosate-resistant crops is that row spacing can be changed because the broad-spectrum control glyphosate gives is sufficient to

replace cultivation as a weed control measure. This allows rows to be spaced closer which will result in faster canopy closure and will give the crops a competitive advantage above weeds (Dill 2005). Figure 1.4 shows how no-tillage has increased since the introduction of glyphosate-resistant soybeans in the USA.

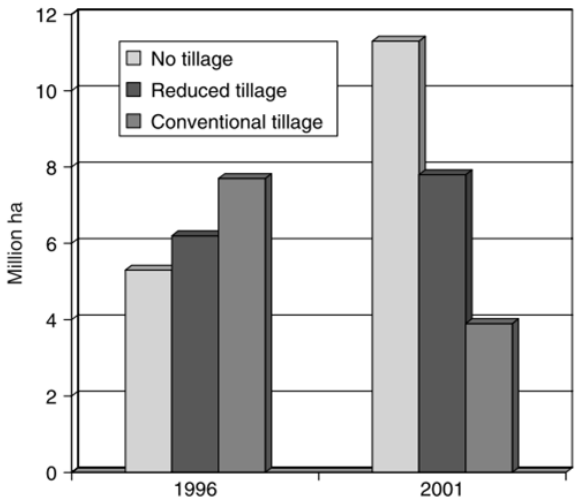

Figure 1.4 Tillage methods of soybean by hectares in the USA in 1996 and 2001 (Duke and Powles 2008)

The safety of glyphosate-resistant crops has been evaluated in many countries and not one of the scientific advisory panels has identified any problems with regards to the safety of these biotechnological derived products (Dill 2005). Researchers analyzed possible differences in the composition of glyphosate-resistant soybeans and a parental line of soybeans and no differences were found in nutrients nor anti-nutrients (Padgette et al., 1996). Glyphosate-resistant maize and soybeans are currently imported to the European Union. There is thus no scientific data to support public opinions on the negative impacts of transgenic crops (Duke and Powles 2008). Transgene flow from glyphosate-resistant crops to weeds is a potential environmental risk. However, this can only happen when the crop and the weed species is very closely related (Duke and Powles 2008).

1.4. Weed resistance

Heap (2016) defined resistance as “the evolved capacity of a previously herbicide-susceptible weed population to withstand the herbicide and complete its life cycle when the herbicide is used at its normal rate in an agricultural situation”. According to the Weed Science Society of America (WSSA) resistance is “the inherited ability of a plant to survive and reproduce following exposure to dose of herbicide normally lethal to the 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” (Heap 2016). Tolerance can be described as “the naturally occurring variability in response to herbicides that exists within a species or larger taxonomic group before first use of the chemical” (Holt 1992).

In 1908, insecticide resistance was reported for the first time and fungicide resistance followed in 1940. This caused scientists to believe that herbicide resistance will also appear shortly after the introduction of herbicides in the 1940s (Heap 2014). This was, however, not the case because plants have much longer lifecycles than fungi and insects and the first case of herbicide resistance was only reported in 1968 (Ryan 1970).

This historical first herbicide resistance case was resistance against the triazine herbicides atrazine and simazine in common *Senecio vulgaris* (groundsel), and was discovered by a conifer nursery owner in Washington after repeated use of the herbicide for many years (Holt 1992, Pieterse 2010, Heap 2014). The afore-mentioned resistance occurred due to a mutation in the chloroplast gene that encodes the herbicide binding protein of photosystem II where numerous photosynthetic inhibitors bind (Radosevich and Devilliers 1976, Holt 1992).

According to Heap (2014), herbicide resistance is a normal and expected result of natural selection. Mutations that allow plants to be resistant to the herbicide exists within a weed population before first application of the herbicide (Heap 2014) and according to Holt (1992) there is no evidence to suggest that herbicide resistance have occurred due to mutations caused by the herbicide. Pieterse (2010) reported that mutation and natural selection are two factors that have a direct influence on the evolution of glyphosate-resistant weeds. The frequency of individual weeds that are naturally resistant to a herbicide is assumed to be more

or less “one in a million” (Maxwell and Mortimer 1994). Therefore, if a naturally resistant weed plant survives the first application of a herbicide and produce progeny there will be much more resistant plants the next season. If selection continues by persistent use of the same herbicide (or a herbicide within the same group) the number of resistant plants will increase and a disproportionate number of resistant progeny will be contributed to the next season (Pieterse 2010).

Selection and the frequency of naturally herbicide-resistant weed individuals is, however, not the only factors that contribute to the rate of herbicide resistance appearing in weed populations (Pieterse 2010). Other factors include the initial frequency of herbicide-resistant individuals, number of treated individuals, the inheritance and fitness of the gene or genes responsible for resistance, as well as the nature and amount of herbicide-use (Gressel 2002, Heap 2014).

The selection pressure for herbicide resistance can be influenced by three factors of herbicide use: frequency of use, the efficiency of the herbicide and the time that the herbicide has an effect (Gressel 2002). The number of individuals treated over time is a very important factor and should not be underestimated (Heap 2014). When an herbicide like difenzoquat is used occasionally on a small area and only acts on a small number of weeds it is improbable that many resistant weeds will be selected even if the initial mutation frequency for resistance was high. On the contrary, when a herbicide like glyphosate is used on a very big area and acts on a lot of weeds the chances are good that many resistant weeds will be selected even if the initial mutation frequency is low. The highest risk for selecting for resistance is when a herbicide is used on a very big area, acts on a wide variety of weeds and the initial mutation frequency is high, as in the case of the ALS inhibitors (Heap 2014). Herbicide groups that have more than one mechanism of action have less chance of weeds developing resistance to herbicides belonging to those groups (Beckie et al., 1999, Heap 2014). Figure 1.5 shows that the ALS inhibitors have selected the most resistant weed species. ALS inhibitors, photosystem II inhibitors, ACCase inhibitors, synthetic auxins, bipyridilliums and glycines are the herbicide groups that have selected the most resistant weeds (Heap 2014).

The worst herbicide-resistant weeds in the world are species of the genera *Amaranthus, Echinochloa, Conyza* and *Lolium* (Heap 2014). Species in these genera are genetically diverse and have proven to easily develop resistance to various herbicide mechanism of action groups (Powles 2008, Heap 2014). *L. rigidum* is the worst herbicide-resistant weed in the world due to its high level of genetic variability, and have evolved resistance to 11 modes of action on more than two million hectares in the world (Heap 2014). *Avena fatua* (common wild oats) have evolved resistance to five herbicide mechanism of action groups, and according to Heap (2014) is probably the herbicide-resistant weed with the greatest economic impact.

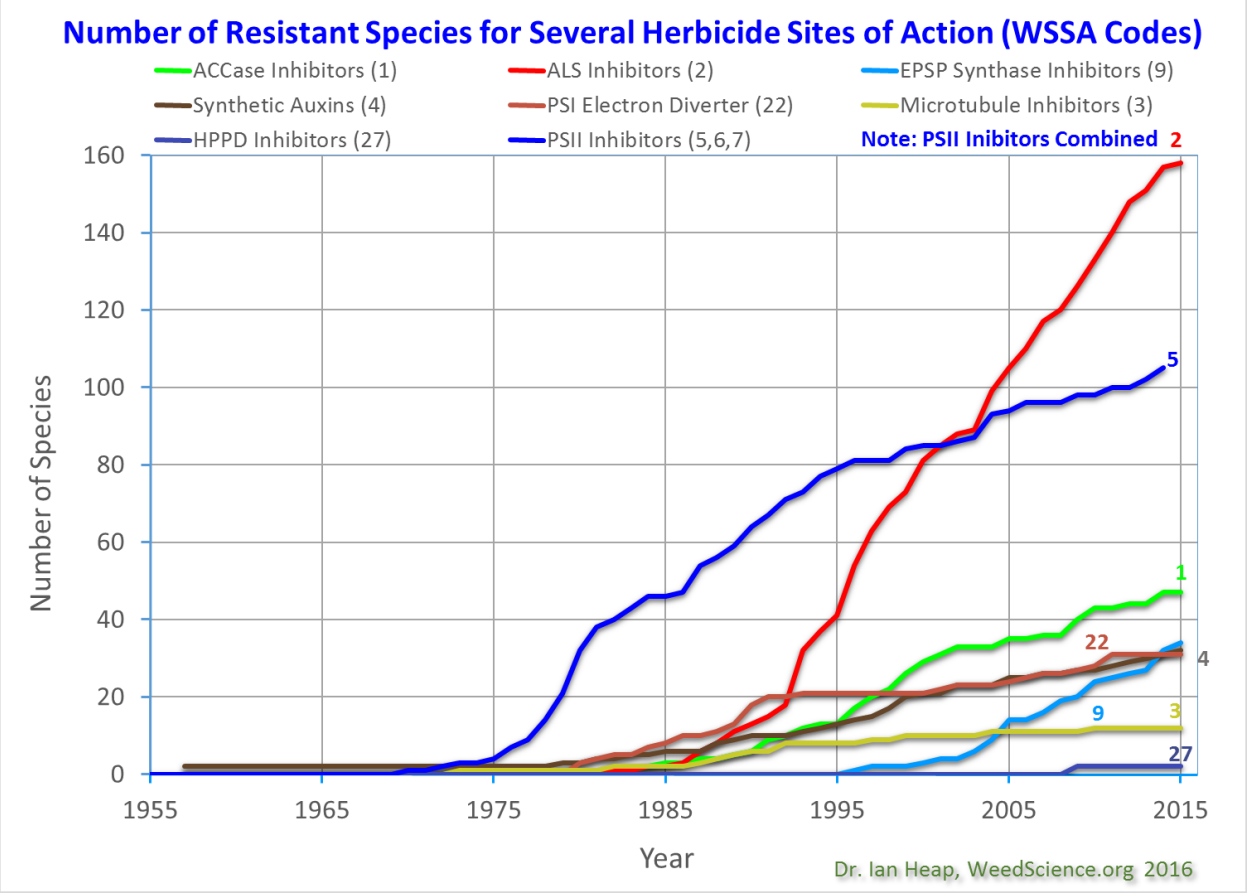

Figure 1.5 Number of resistant species for several herbicide sites of action (Heap 2016)

Currently, there have been 471 unique cases (where a specific weed species is resistant to a specific herbicide mechanism of action) of herbicide resistance reported (Heap 2016). Weeds have evolved resistance to 23 of the 26 known herbicide sites of action with 145 dicots and 105 monocots making up the total of 244 species resistant to herbicides (Heap 2016). In South

Africa there are eight herbicide resistant weed species reported of which three has developed multiple resistance (Heap 2016).

The first case of herbicide resistance reported in South Africa was resistance to diclofop-methyl (ACCase inhibitors site of action group) in *A. fatua*. Table 1 shows a summary of the herbicide resistance cases in South Africa. *Amaranthus hybridus* was the first broadleaf weed to be reported resistant in South Africa, with resistance to atrazine reported in 1996 (Pieterse 2010). Resistance in broadleaf weeds to non-selective herbicides was first reported in the Western Cape in vineyards in 2003 (Pieterse 2010) and confirmed by de Wet (2005). *C. bonariensis* was reported to be resistant to glyphosate and paraquat (de Wet 2005).

Table 1 South African weed species with proven resistance to herbicides with various mechanisms of action (Heap 2016)

	Species	**Common name**	**First year reported**	**Herbicide mode of action**
1	*Amaranthus hybridus (syn:quitensis)*	Smooth pigweed	1993	Photosystem II inhibitors
2	*Avena fatua*	Common wild oats	1986	**Multiple resistance: 2 sites of action** ACCASE inhibitors ALS inhibitors
3	*Conyza bonariensis*	Flax-leaf fleabane	2003	PS l electron diverter
4	*Conyza bonariensis*	Flax-leaf fleabane	2003	EPSP Synthase inhibitors
5	*Lolium rigidum*	Rigid ryegrass	1993	**Multiple resistance: 2 sites of action** ACCASE inhibitors ALS inhibitors
6	*Lolium rigidum*	Rigid ryegrass	2001	EPSP Synthase inhibitors
7	*Lolium rigidum*	Rigid ryegrass	2002	PS l electron diverter
8	*Lolium rigidum*	Rigid ryegrass	2003	**Multiple resistance: 3 sites of action**

				ACCASE inhibitors PS I electron diverter EPSP Synthase inhibitors
9	*Phalaris minor*	Little seed canary grass	1999	**Multiple resistance: 2 sites of action** ACCASE inhibitors ALS inhibitors
10	*Plantago lanceolata*	Buckhorn plantain	2003	EPSP Synthase inhibitors
11	*Raphanus raphanistrum*	Wild radish	1997	ALS inhibitors
12	*Stellaria media*	Common chickweed	2002	ALS inhibitors

According to the "International Survey of Herbicide-resistant Weeds" (Heap 2016) there are five criteria that have to be met in order for a weed to be listed on the website as resistant:

1.) Fulfillment of the WSSA and International Survey of Herbicide-Resistant weeds definition of resistance described above.
2.) Data confirmation using acceptable scientific protocols.

The most preferred test for resistance is a dose-response experiment by using whole plants under controlled conditions like in a glasshouse. A range of herbicide dosages are used that include lethal and sub-lethal dosages for both resistant and susceptible populations (Heap 2016). The GR_{50} value is the dosage that is required to reduce shoot weight by 50% relative to untreated plants and is calculated for each population. Resistance can be confirmed if there is a statistical difference between the expected resistant and sensitive populations (Heap 2016). A ratio based on the GR_{50} of the resistant biotype (R) compared to the susceptible biotype (S) is calculated. High level resistance is confirmed if the R/S resistance ratio is greater than 10 fold. Low level resistance can however be easily confused with natural variation in weed populations (Heap 2016). However, the method described above does not take into account the recommended dosage of an herbicide. Two populations might differ significantly in response to a herbicide but it does not mean that the most resistant one will not by killed by the

recommended dosage. Due to natural variation, weed populations from different regions are likely to fluctuate in their GR_{50} values for a herbicide, and in the case of low level resistance populations can not be classified as resistant based on R/S values alone (Heap 2016). This method can be described as the scientific definition of resistance (Heap 2016).

A weed population cannot be classified as resistant if the population survives herbicide application at the recommended dosage under normal field conditions [agricultural field definition of Heap (2016)]. Recommended dosage is a subjective dosage that may vary from region to region and is influenced by crop, specific situation and economics of the herbicide (Heap 2016). A putative resistant weed population in one crop might be sensitive in another. The use of the recommended dosage as a resistance measurement can be misleading without reference to a susceptible control. Environmental conditions may also influence results at the recommended dosage, and therefore, the recommended dosage in growth room conditions is often much more effective than under field conditions.

In order to classify a weed population to be resistant to a herbicide the scientific and agricultural field definitions are combined in a practical definition of resistance (Heap 2016). The scientific definition can be described as the lowest criteria and a population that does not fit into this definition can not be listed as resistant. To demonstrate that the resistant population has a practical impact, the resistant population should have caused a problem in terms of not having been controlled effectively in the field when the herbicide is applied at the recommended field rate (Heap 2016). For low level resistance (resistance factor below 10) one dose-response experiment is not enough to prove resistance. Both greenhouse dose-response experiments and field experiments will be required where susceptible and potentially resistant plants are used.

3.) The resistance must be heritable.

This criteria states that resistance can not be confirmed if the susceptible and potentially resistant plants are removed from the field and tested in greenhouses. The plants may be at different growth stages or may have been already exposed to a herbicide in the field. Testing with collected seed is generally required for sexually propagated species. The testing of second

generation seed from the greenhouse grown plants for resistance is also preferred but not required.

4.) Demonstration of practical field impact.

The weed must be a problem to control for the farmer when a herbicide is used at its recommended rate. Natural variations in the response to a herbicide between weed populations do not justify classification as resistant.

5.) Identification as a problem weed to species level, not the result of deliberate artificial selection.

1.5. Glyphosate-resistant weeds

After 20 years of glyphosate usage, by 1994, there were no cases of evolved glyphosate resistance identified (Dyer 1994). This showed clearly that glyphosate resistance in a weed species does not evolve quickly and led some to believe that glyphosate resistance evolving in weeds was implausible (Powles 2008). Conversely, since glyphosate resistance was first reported in 1996, instances of evolved glyphosate resistance in weed species are increasing, particularly after the introduction of glyphosate-resistant crops, also in 1996 (Powles 2008, Pieterse 2010, Heap 2014).

That first glyphosate-resistant weed, rigid ryegrass (*L. rigidum*), was however not reported in a glyphosate-resistant crop but in an orchard in Australia where glyphosate had been used repeatedly (five to ten times per year) for more than 15 years (Heap 2014). Since then, 34 other glyphosate-resistant weeds have been reported over the world (Heap 2016). Table 2 shows a list of glyphosate-resistant weeds in the world. There are three glyphosate-resistant weeds reported in South Africa (Heap 2016). *Lolium rigidum* was also the first glyphosate-resistant weed reported in South Africa, and it was discovered in vineyards in the Western Cape in 2001. Glyphosate resistance in *C. bonariensis* was first reported in 2003 in South Africa, also in vineyards in the Western Cape. *Plantago lanceolata* is the third glyphosate-resistant weed reported in South Africa, and there is no other place in the world where this species has been reported as resistant to glyphosate (Heap 2016).

Table 2 Glyphosate-resistant species in the world (Heap 2016)

	Species	**Country**	**First year**
1	*Amaranthus hybridus (syn: quitensis)* (Smooth pigweed)	Argentina	2013
2	*Amaranthus palmeri* (Palmer amaranth)	USA (24 states)	2005
3	*Amaranthus spinosus* (Spiny amaranth)	USA (Mississippi)	2012
4	*Amaranthus tuberculatus (=A. rudis)* Tall waterhemp	Canada, USA (16 states)	2005
5	*Ambrosia artemisiifolia* (Common ragweed)	Canada, USA (15 states)	2004
6	*Ambrosia trifida* (Giant ragweed)	Canada, USA (14 states)	2004
7	*Bidens pilosa* (Hairy beggarticks)	Mexico	2014
8	*Brachiaria eruciformis* (Sweet summer grass)	Australia	2014
9	*Bromus diandrus* (Ripgut brome)	Australia	2011
10	*Bromus rubens* (Red brome)	Australia	2014
11	*Chloris elata* (Tall windmill grass)	Brazil	2014
12	*Chloris truncate* (Windmill grass)	Australia	2010
13	*Chloris virgata* Feather fingergrass	Australia (3 states)	2015

14	*Conyza bonariensis* (Flax-leaf fleabane)	South Africa, Spain, Brazil, Israel, Columbia, USA, Australia (3 states), Greece and Portugal	2003
15	*Conyza canadensis* (Horseweed fleabane)	USA (25 states), Brazil, China, Spain, Czech Republic, Canada, Poland, Italy, Portugal and Greece	2000
16	*Conyza sumatrensis* (Samatran fleabane)	Spain, Brazil, France and Greece	2009
17	*Cynodon hirsutus* (Gramilla mansa)	Argentina	2008
18	*Digitaria insularis* (Sourgrass)	Paraguay and Brazil	2005
19	*Echinochloa colona* (Junglerice)	Australia (3 states), USA (California), Venezuela and Argentina	2007
20	*Eleusine indica* (Goosegrass)	Malaysia, Colombia, Bolivia, China, Costa Rica, USA (2 states) and Argentina	1997
21	*Hedyotis verticillata* (Woody borreria)	Malaysia	2005
22	*Kochia scoparia* (Kochia)	USA (10 states), Canada (3 provinces)	2007
23	*Lactuca serriola* (Wild lettuce)	Australia	2015

	Prickly lettuce		
24	*Leptochloa virgate* (Tropical srangletop)	Mexico	2010
25	*Lolium perenne* (Perennial ryegrass)	Argentina, New Zealand and Portugal	2008
26	*Lolium perenne ssp. multiflorum* (Italian ryegrass)	Chile, Brazil, USA (7 states), Spain, Argentina, Italy, Japan and New Zealand	2001
27	*Lolium rigidum* (Rigid ryegrass)	South Africa, Australia (4 states), USA (California), France, Spain, Israel and Italy	1996
28	*Parthenium hysterophorus* (Ragweed parthenium)	Colombia	2004
29	*Plantago lanceolate* (Buckhorn plantain)	South Africa	2003
30	*Poa annua* (Annual blue grass)	USA (3 states)	2010
31	*Raphanus raphanistrum* (Wild radish)	Australia	2010
32	*Salsola tragus* (Russian-thistle)	USA	2015
33	*Sonchus oleraceus* (Annual sowthistle)	Australia	2014
34	*Sorghum halepense* (Johnsongrass)	Argentina and USA (3 states)	2005
35	*Urochloa panicoides* (Liverseedgrass)	Australia	2008

Powles (2008) emphasized the importance of comparing evolved resistance to glyphosate in traditional non-selective glyphosate use patterns versus selective (in-crop) use in glyphosate-resistant crops. The rationale behind this approach is that for 20 years of non-selective herbicide usage no weed resistance was reported and within a few years after the introduction of glyphosate-resistant crops, which led to selective use, numerous weeds have developed resistance to the herbicide all over the world. However, it has to be considered that weed resistance which developed in glyphosate-resistant crops is not the result of glyphosate-resistance technology but rather an increase in persistent glyphosate usage in these situations (Powles 2008), as well as an increased potential for selecting glyphosate-resistant weeds (Boerboom and Owen 2013).

Between 1974 and 1996 glyphosate has been used primarily for broad-spectrum weed control before planting because of its non-selective properties (Bradshaw et al., 1997). As can be expected of such an efficient herbicide it has been used wherever possible with little diversity in chemical weed control options. When glyphosate was only used as a non-selective herbicide in orchards, vineyards, roadsides and for burn-down treatments in crops there were only a few reports of glyphosate-resistant weed populations in these situations despite persistent use of the herbicide (Powles 2008). One of the reasons for this is that glyphosate is neither residual nor active once it comes into contact with the soil (Franz et al., 1997). Glyphosate acts only on emerged plants which results in short and intense selection (Powles 2008).

Before the advent of glyphosate-resistant crops, because weeds emerge throughout the growing season and glyphosate is not applied again once the crop is established, there was less overall selection pressure and a significant fraction of the weed population remained unselected, unlike the case with soil-residual herbicides where selection can take place for a number of months (Powles 2008, Nandula 2010). As a result, weeds emerging after the burndown treatment remain unselected and therefore the population of weeds does not get dominated by resistant individuals.

In addition to decreased selection pressure referred to above, weed control diversity (especially in annual crops) also had a big impact on the slow development of glyphosate-resistant weeds

(Powles 2008). Alternative weed control strategies include rotation of herbicides with different mechanisms of action or using tank mixtures of herbicides with different mechanisms of action, crop rotation, cover crops, , tillage and cultivation (mechanical control), and biological control, which includes taking advantage of the crop's ability to compete with weeds (Nandula et al., 2005, Boerboom and Owen 2013, Owen 2016). According to Powles (2008), herbicide resistance may evolve very slowly or not at all if there is an integrated weed control system in place. Such a system would extend the sustainable use of a valuable herbicide such as glyphosate (Powles and Gaines 2016). The reason why a diverse weed control system is effective in reducing glyphosate-resistance in weeds is that the glyphosate-resistant survivors are killed by other weed control mechanisms such as tillage before offspring can be produced (Dill 2005).

Where glyphosate has been used persistently, glyphosate resistance has evolved in weed populations (Nandula et al., 2005). Typical situations where glyphosate are applied more than once per season are between rows of tree and vine crops, as well as for roadside weed control. Glyphosate-resistance in these situations was first experienced in several species of the genera *Conyza* and *Lolium* (Powles 2008). The common factor responsible for glyphosate-resistance evolving in situations where glyphosate was used non-selectively, was over-reliance on solely glyphosate (Powles 2008).

As mentioned earlier, the use of glyphosate-resistant crops (maize, soybean, cotton and canola) have been adopted spectacularly in the USA (Powles 2008). After the introduction of these crops the use of glyphosate increased dramatically since glyphosate could now be used in agronomic crops as a selective herbicide (Heap 2014). Due to the economy and high efficiency of glyphosate the use of most selective herbicides was replaced by glyphosate (Powles 2008, Boerboom and Owen 2013). Because glyphosate acts on a wide range of weed species and is used on a very large area in glyphosate-resistant crops, it poses a risk for the development of resistance even though glyphosate is considered a low-risk herbicide for selecting for resistance (Heap 2014).

Glyphosate-resistant maize, soybean and cotton are mainly used in rotation on the same fields which leaves little diversity for weed control (Powles 2008, Owen 2016). When glyphosate-resistant crops are adopted by farmers they usually stop using other selective herbicides, reduce tillage and rely almost solely on glyphosate for weed control, thereby creating high glyphosate selection pressure on weeds (Young 2009). It is understandable why farmers tend to do this due to the simplicity combined with the efficiency of applying only glyphosate as well as reduced costs, environmental benefits and flexibility in timing of application (Nandula et al., 2005, Powles 2008).

According to Powles (2008) the introduction of glyphosate-resistant crops could have facilitated an increase in the diversity of herbicides due to the unique mechanism of action of glyphosate. It could have been so easily achieved as glyphosate can be combined with several other herbicides in a tank mixture. The reality is that farmers use glyphosate alone and this is mainly due to economic reasons (Nandula et al., 2005).

The first reported weed that evolved glyphosate-resistance in a glyphosate-resistant crop was *C. canadensis* and it has become the most widespread glyphosate-resistant weed (Heap 2014). Luckily it can be controlled with other inexpensive alternatives and therefore does not pose a great threat (Heap 2014). Of all the glyphosate-resistant weeds *Amaranthus palmerii* in the Southern USA and *Amaranthus tuberculates* in the Mid-West have the greatest economic impact because they are prevalent in glyphosate-resistant crops and have evolved resistance to other herbicides as well (Heap 2014). Various other species of the genera *Ambrosia* and *Amaranthus* also developed resistance against glyphosate and could have much more damaging effects in USA cotton production (Heap 2014). *Sorghum halepense* and *Digitaria insularis* in South America, *L. rigidum* in Australia and South Africa (Pieterse 2010) and *Kochia scoparia* in the USA are the other glyphosate-resistant weeds of economical importance (Heap 2014).

1.5.1. Management strategies for glyphosate-resistant weeds

Farmers should not exclude other weed management options and rely completely on glyphosate-resistant crops in combination with glyphosate to control weeds because this puts tremendous glyphosate selection pressure on weed populations (Nandula et al., 2005, Owen 2016). Weed control by glyphosate can be sustainable if there is sufficient diversity in weed management sytems (Dill 2005, Nandula et al., 2005, Powles 2008, Boerboom and Owen 2013). Herbicides with different mechanisms of action should be alternated, or even better, mixed in the same tank.

Growers manage glyphosate-resistant *C. canadensis* successfully by combining glyphosate and auxin-type herbicides before planting glyphosate-resistant soybeans, and after planting this weed is controlled by combining glyphosate with ALS inhibitors (Dill 2005, Boerboom and Owen 2013). Other control measures should also be employed for diversity in control options (Nandula et al., 2005). The different practices should be adopted depending on the unique situation that each grower experiences (Jussaume Jr and Ervin 2016). For example, under zero-tillage conditions the use of tillage and rotation with cultivars that are not resistant to glyphosate will not be possible (Powles 2008).

It is important to stick to the basic principles of effective weed management that are fundamental to integrated weed management (IWM) programs (Pieterse 2010). In IWM different weed management practices (mechanical-, cultural-, chemical- and biological control) are combined wherever practical and employed throughout the growth season. This will ensure that herbicides remain efficient by reducing the number of resistant plants in the population (Pieterse 2010, Owen 2016). It is important for farmers to become aware of weed species that are likely to develop resistance in order to delay resistance (Pieterse 2010). Resistance can be delayed or even prevented from occurring if farmers scout their fields regularly for early detection of individuals surviving herbicide applications and then killing those individuals by other control measures (Nandula et al., 2005).

Another tactic to control *C. bonariensis* that is hard to control with glyphosate or even resistant to glyphosate is known as the double knock tactic (Walker et al., 2012). This tactic can be

described as the sequential application of glyphosate or a mixture of glyphosate and 2,4-D followed by paraquat and diquat or paraquat alone (Werth et al., 2010). It is very important that the follow-up application of paraquat and diquat or paraquat alone take place within 5-7 days after the glyphosate application. Walker et al. (2012) also reported that this tactic gives 98% control across all weed ages of *C. bonariensis*. Where cross-resistance or multiple resistance occurs this tactic will not be effective.

As early as 1982, it has been reported that in order to manage resistance there has to be sufficient diversity in weed control in order to reduce selection pressure for evolved herbicide-resistant weeds (LeBaron and Gressel 1982). According to Heap (2014) the industry has not brought a new herbicide to the market in more than 30 years. In order for herbicides to remain an effective weed control measure IWM systems should be applied as it is the best way to manage resistance (Pieterse 2010, Heap 2014).

1.5.2. Influence of growth stage on the tolerance of *C. bonariensis* towards glyphosate

It has been reported by various scientists that growth stage influences the response of *Conyza* spp to glyphosate (Shrestha et al., 2007, Urbano et al., 2009, VanGessel et al., 2009, Walker et al., 2012). Other researchers (Koger et al., 2009) reported that the influence of growth stage had little effect on the level of resistance in glyphosate-resistant *C. canadensis*.

Shrestha et al. (2007) reported that at the 5 to 8 leaf stage there were no survivors for a glyphosate-sensitive horseweed population at rates of 111, 238 and 448 g ai ha^{-1} glyphosate treatments. At the 11 to 15 leaf stage however, 10 % of the glyphosate-sensitive plants survived the 111 g glyphosate treatment and at bolting 30 % of these plants survived the same treatment. After bolting when the plants were between 15 and 30 cm tall, 70 % of the glyphosate-sensitive plants survived the 111 g treatment. It was concluded that the level of resistance did vary with growth stage and that plants had to be controlled at an early growth stage before the development of eight true leaves (Shrestha et al., 2007).

In a survey done by Walker et al. (2012) it was found that glyphosate efficacy against glyphosate-susceptible *C. bonariensis* populations was not affected by weed age. It was

however reported that the efficacy of glyphosate was reduced significantly when glyphosate-resistant plants of three months old were treated in comparison with two month old plants (Walker et al., 2012). VanGessel et al. (2009) found that glyphosate-resistant *C. canadensis* was constantly more responsive to glyphosate at the seedling stage in comparison with all later growth stages but that glyphosate-sensitive plants were controlled at all growth stages at commercial rates. Urbano et al. (2007) measured chlorophyll content of *C. bonariensis* plants after glyphosate application and found significant differences in efficacy between seedling, rosette, tillering and flowering growth stages. All individuals of the sensitive population were controlled at the seedling growth stages with a glyphosate rate of 560 and 1120 g ha^{-1}. However, at the tillering and flowering growth stages, 2 240 g ha^{-1} was needed for complete control. For the resistant population, plants at the seedling stage was controlled completely at 4 480 g ha^{-1} while no plants at the other growth stages were controlled at this rate (Urbano et al., 2009).

The majority of the above-mentioned studies showed that growth stage does have an effect on the tolerance of *Conyza* spp towards glyphosate. One of the known reasons for resistance in horseweed is reduced translocation to the growing points and roots of plants (Feng et al., 2009). When resistant and sensitive horseweed biotypes were compared by Feng et al. (2009) it was found that there was reduced translocation of glyphosate to the roots of resistant plants but not in sensitive plants.

Glyphosate loading to the phloem and xylem is delayed in resistant biotypes and therefore sub-lethal doses reaches other leaves, roots and the crown of the weed plant (Feng et al., 2009). During the seedling and rosette stage, the sink source is the roots (where glyphosate needs to act) and glyphosate moves along with the nutrient stream in the phloem to the roots. When plants start to bolt the roots are no longer the sink but the developing flower organs become the new sink. The nutrient flow (with glyphosate) therefore goes primarily to the developing flowering organs and the doses that reach the roots are not high enough to kill the plant. This might be the reason for increased tolerance at a later growth stage (Shrestha et al., 2007).

1.5.3. Influence of temperature on the tolerance of *C. bonariensis* towards glyphosate

According to Sammons and Gaines (2014), the basic herbicide resistance mechanisms can be categorized as target-site resistance, metabolism, exclusion of the herbicide from the target (physically or physiologically) and avoidance. Avoidance can be explained as the biochemical ability to handle the toxic agent produced by the pesticide and in that way avoid a lethal outcome (Sammons and Gaines 2014). Nandula et al. (2005) reported that the mechanisms for herbicide resistance in weeds are altered herbicide target sites, reduced translocation of the herbicide from the site of absorption to the target site, reduced herbicide absorption and rapid metabolic detoxification of the herbicide.

Glyphosate resistance mechanisms comprise of rapid necrosis response, target-site gene duplication, target-site mutation (Nandula et al., 2005, Powles 2008), limited cellular uptake and active vacuolar sequestration (Sammons and Gaines 2014). It has been reported (Ge et al., 2010) that vacuolar sequestration is the mechanism for glyphosate-resistance in *C. canadensis*.

Further studies (Ge et al., 2011) showed that glyphosate-resistant *C. canadensis* become sensitive to glyphosate at low temperatures (10/8 ^{o}C day/night) when vacuolar sequestration is the mechanism of resistance. Both resistant and sensitive plants were included in the study and this showed that for plants maintained under cold conditions, glyphosate turned out to be less toxic to glyphosate-sensitive plants whereas glyphosate-resistant plants are controlled significantly better than those under warm conditions. The above-mentioned results were measured at 21 days after treatment (DAT). At 41 DAT, however, no resistant plant survived the field-use rate or any higher rates. In another protocol of the same study, plants were moved from the low temperature to a higher temperature (30/20 ^{o}C day/night) at 0, 3, 7 and 14 DAT. In all of the cases where glyphosate-treated plants were moved from cold to warm conditions the plants were saved from the cold-enabled deadly effect of glyphosate. The same results were experienced in a field trial in Columbus, Indiana (Ge et al., 2011).

The same results as mentioned above were found in a study on *C. bonariensis* (Moretti et al., 2013) where a population that showed a five-fold to 20-fold level of resistance to glyphosate in the summer showed no resistance in autumn and had similar mortality as in a sensitive

population. When the same experiment was done in the winter, the resistant population was affected more by glyphosate than the sensitive population when biomass was measured at 21 DAT (Moretti et al., 2013).

Glyphosate is transported across the tonoplast in glyphosate-resistant *C. canadensis* by an ABC transporter and requires ATP to function (Rea 2007). Glyphosate-resistant and glyphosate-sensitive plants both have the ability to pump glyphosate across the tonoplast into the vacuole but the process is far more efficient in glyphosate-resistant biotypes (Ge et al., 2011). It is a possibility that glyphosate-resistant plants over-expresses a glyphosate transporter protein or that a mutation in these plants allows more effective transport of glyphosate across the tonoplast (Ge et al., 2011). Low temperature does not prevent the entry of glyphosate into the plant cell (Devine et al., 1983). The data from field experiments showed that glyphosate-resistant *C. canadensis* can be controlled if the temperature three days before spraying and six days after spraying do not rise significantly above 16 ^{o}C (Ge et al., 2011).

1.5.4. Influence of manganese on the tolerance of *C. bonariensis* towards glyphosate

Hard-water cations (Fe^{2+}, Mg^{2+} and Ca^{2+}) can reduce the efficacy of glyphosate when binding with glyphosate to form salts that cannot be absorbed by plants or bind at the site of action (Thelen et al., 1995, Bernards et al., 2005). Due to the chelating properties of glyphosate, it forms stable complexes with di- and trivalent metal cations (Bernards et al., 2005). By adding an excess of monovalent cations like K^{+}, NH_4^{+} or Na^{+} the antagonistic effect of the hard-water cations can be overcome (Bernards et al., 2005). Ammonium molecules compete for binding sites on the glyphosate molecule and therefore can reverse the interaction if a glyphosate-divalent cation complex have formed (Thelen et al., 1995) That is the reason why it is recommended on the label of Roundup Turbo to add ammonium sulfate at a rate of 2 % before adding Roundup Turbo to the spray solution.

It has also been reported that glyphosate efficacy decreased in tank mixtures containing Mn^{2+} (Bernards et al., 2005, Bailey et al., 2009). In the same way that hard-water cations such as Ca^{2+} complexes with glyphosate and makes the molecule inactive with regards to phytotoxicity, Mn^{2+} can also bind with glyphosate molecules (Bernards et al., 2005). It was further reported

that control of *Abutilon theophrasti* velvetleaf was reduced when the plants were treated with Mn^{2+} before the glyphosate application (2 days, 1 day and 1 hour), and that the antagonism increased as the time period between the Mn^{2+} and glyphosate applications decreased. This finding lead to the suggestion that the antagonism of glyphosate by Mn^{2+} might also take place inside the cytoplasm of the plant and not only in tank mixtures. Other reasons for the observed decreased control are that glyphosate might have bound to Ca^{2+} on the leaf surface (velvetleaf releases calcium-rich substances from chalk glands on the leaf surface), Mn^{2+} may have remained on the leaf hairs and complexed with glyphosate after application of the herbicide, or Mn^{2+} and glyphosate may have entered the plant cuticle together and complexed during the process thereby reducing translocation and absorption of glyphosate (Bernards et al., 2005). The same protocol was performed on *Setaria faberi* (giant foxtail) and *Chenopodium album* (common lambsquaters) but no effect of reduced control due to glyphosate complexing with manganese was observed which suggests that this reaction is species-dependent.

Further research (Bernards et al., 2009) showed that glyphosate binds to Mn^{2+} in a way that is dependent on pH. The greatest interaction takes place at a pH of 7.5 which is similar to the pH in the plant apoplast (xylem) and symplast (phloem) (Bernards et al., 2009). This also suggests that antagonism of glyphosate by manganese takes place inside the cytoplasm and not only in tank mixtures.

The presence of NH_4^+ in solution increases the efficacy, absorption and translocation of glyphosate because the NH_4^+ competes with Mn^{2+} for binding to glyphosate. However, even though NH_4^+ improves the efficiency of glyphosate in the presence of Mn^{2+}, it does not eliminate the antagonistic effects caused by Mn^{2+} (Bernards et al., 2009). Interestingly, it has also been reported that Mn^{2+} ions stimulate plastid and cytoplasmic isozyme pairs of the enzyme 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) synthase that is present in the shikimate pathway (Hrazdina and Jensen 1992).

Several papers were recently published that report glyphosate influences the mineral status of glyphosate-resistant crops (Bott et al., 2008, Zobiole et al., 2010). However, in a comprehensive review by Duke et al. (2012a) it was stated that even though there are contradictory articles on

the effects of glyphosate on mineral nutrition in glyphosate-resistant crops, most of the literature specify that mineral nutrition in glyphosate-resistant crops is not affected by glyphosate application or the glyphosate-resistant trait. The ratio of metal ions to glyphosate molecules in glyphosate-resistant soybean plants treated with glyphosate is 1 000 : 1 (Duke et al., 2012b), therefore, binding of these minerals with glyphosate would not interfere substantially with the mineral status of the plant (Duke et al., 2012a).

CHAPTER 2

MATERIALS AND METHODS

2.1. Screening experiment

In February 2012, *C. bonariensis* seeds were collected from 12 locations in South Africa. The seeds were collected next to the road, basically in a straight line from Pretoria to George as can be seen in Figure 2.1. The rationale of sampling seeds along a main road instead of collecting from crop fields was based on the time constraint related to seed sampling at that time of the year, i.e. we could not avoid collecting seed without first establishing contact with local farmers, because it was expected that most seeds would have been shed by March 2012 (less than one month after seed sampling commenced). Therefore, this initial research activity in the project should be regarded as a preliminary screening of *C. bonariensis* populations with relatively broad geographical distribution, which hopefully, would introduce the potential for genetic diversity.

The 12 locations are: Hatfield experimental farm of the University of Pretoria (-25.751373, 28.258792), Buffelsbaai (-34.083939, 22.960330), De Rust (-33.498030, 22.516054), Oudtshoorn (-33.641487, 22.223834), Wilderness (-33.995523, 22.566407), Gariep (-30.593631, 25.490928), Aberdeen (-32.467747, 24.070924), Edenburg (-29.741936, 25.935518), Ventersdorp (-26.377348, 26.895499), Trompsburg (-30.036878, 25.791766), Middelburg (-25.698407, 29.455821) and Kroonstad (-27.626477, 27.256117). At each location, mature seed, i.e. seed that was ready to be dispersed naturally by wind action, was removed from several plants and placed inside a brown paper envelope. The seeds were stored at room temperature inside the envelopes.

Figure 2.1 Locations in South Africa where 12 populations of *Conyza bonariensis* seed were collected

The experiment was conducted in a greenhouse at the Hatfield experimental farm of the University of Pretoria. Plants were grown under light, day length and temperature conditions prevailing in the greenhouse. No supplemental lighting was provided and temperature control was limited to cooling when necessary. Temperature ranged from a minimum of 7.03 ^{o}C to a maximum of 37.44 ^{o}C, with an average day temperature of between 20 and 25 ^{o}C. The seeds were planted as close to the soil surface as possible at a maximum depth of 0.5 cm in a sand-choir mixture in 12 cm diameter pots. Plants were watered regularly by replenishing the water lost with a minimum 50 ml of tap water per pot. Complete nutrient solution ["Hygroponic" from Hygrotech (Pty) Ltd] was applied twice a week on Mondays and Fridays; 50 ml nutrient solution (1 g Hygroponic mix per 1 L water) was applied to each pot. Fourteen days after planting the seedlings were thinned out to five per pot. After another 14 days the seedlings were thinned out to only one plant per pot.

Label recommendations were strictly followed with regards to glyphosate application. The glyphosate product that was used is Roundup Turbo® from Monsanto. Roundup Turbo is formulated as a potassium salt and contains 450 g glyphosate (glycine) a.e. L^{-1}. Plants were treated with glyphosate at the 4 to 6 leaf stage (eight weeks after planting). Five rates of glyphosate were applied: 0.25, 0.5, 1, 2 and 4 times the recommended dosage. The recommended dosage of glyphosate to control *C. bonariensis* is 2 L ha^{-1} Roundup Turbo

(900 g a.e. L^{-1}). Spraying was performed with an Oxford small-plot precision sprayer at a pressure of 200 kPa by placing pots to be sprayed in a 1 m^2 demarcated area. The label recommended spraying volume is a maximum of 200 L ha^{-1} that was converted to a spraying volume of 20 ml for the 1 m^2 area. ATP AMSUL-50 [ammonium sulfate manufactured by Villa Crop Protection (Pty) Ltd] was added to the spray water in order to attain 2% ammonium sulfate concentration before Roundup Turbo was added. Each treatment combination was replicated five times.

At seven and 14 days after treatment (DAT) the plants were rated on a scale from 1 to 9 in order to establish the progression of the effects of glyphosate. At level "1" a plant is rated as being normal and healthy with no chlorosis or necrosis, and at "9" the plant is completely dead with no green parts (Figure 2.2). Plants were clipped at the soil surface at 21 days after treatment and weighed to obtain fresh mass.

Data were analyzed as for a completely randomized design (CRD) and applying factorial analysis of variance (ANOVA) to test for differences between six doses, 12 locations and the dose x location interaction effects. The residuals were acceptably normal and means were compared using Tukey's least significant difference test at the 5 % level ($p < 0.05$) (Snedecor and Cochran 1980). Data were analyzed using the statistical program GenStat® (Payne 2014).

Dose-response curves were generated using the "drc" package (Ritz and Streibig 2005) of the program R® by using a four-parameter non-linear regression model (Seefeldt et al., 1995). Dose-response curves were used to determine the herbicide rate that causes a mean fresh biomass reduction of 50 % (GR_{50}) in the 12 *C. bonariensis* populations. The above-ground fresh biomass was expressed as a percentage of the mean untreated control.

$$Y = d / (1 + \exp [b (\log x - \log e)]$$

Where:

Y = fresh-biomass expressed as a percentage of the untreated control

d = upper limit

e = GR_{50}

b = slope of the curve at GR_{50}

x = herbicide dose

The relative level of glyphosate-resistance between the 12 populations was determined by calculating the R : S ratio (GR_{50} of resistant biotype / GR_{50} of the average susceptible biotype).

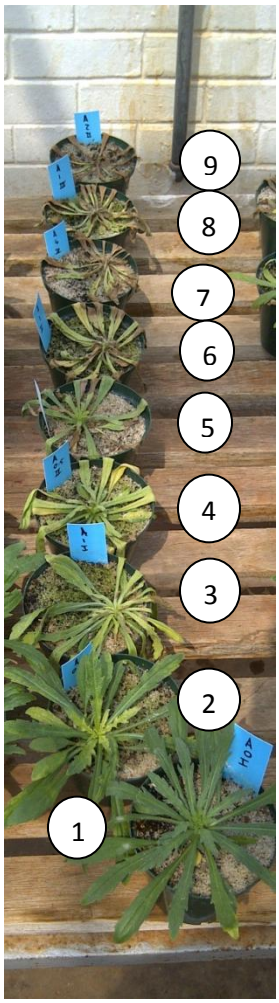

Figure 2.2 Scale to assess the development of symptoms after glyphosate application

2.2 Growth stage experiment

Fresh mass means and visual assessments of the populations from the screening experiment (section 2.1) were used to divide specific populations into three categories. Based on those parameters the populations from De Rust and Oudtshoorn were placed in the “highly tolerant” category, populations from Middelburg and Wilderness in the “tolerant” category and populations from Hatfield and Gariep in the “sensitive” category.

The experiment was conducted in the exact same manner as the screening experiment except for introduction of the growth stage factor with two treatment levels. Glyphosate was applied at two different growth stages to all six of the selected populations at the same dosages as in the screening experiment. The first treatment was at the 10-12 leaf stage and the second treatment at 16-20 leaf stage. Only 21 days separated the growth stages. At the second growth stage certain individual plants started to bolt.

The data were analyzed as for a completely randomized design (CRD) applying factorial analysis of variance (ANOVA) to test for differences between two growth stages, six doses, six locations and the various interaction effects. The residuals were acceptably normal and means were compared using Tukey's least significant difference test at the 5% level ($p < 0.01$) (Snedecor and Cochran 1980). Data were analyzed using the statistical program GenStat® (Payne 2014).

Dose-response curves, GR_{50} values and R/S factors were calculated as described in section 2.1.

2.3 Second screening experiment

Two extra populations from the De Rust area had been collected in February 2013. De Rust 2 population (-33.3080333, 022.4771167) was collected from a lucerne field and De Rust 3 (Symington’s strip no. 1) was collected from an olive orchard. These two populations were screened together with the De Rust (De Rust 1) and Oudtshoorn populations from the first screening experiment. Materials and methods are the same as for the first screening experiment (section 2.1).

Visual assessments, fresh mass measurements and statistical analysis are the same as for the first screening experiment.

Dose response curves, GR_{50} values and R/S factors were calculated as described in section 2.1.

2.4 Temperature experiment

A seed thermogradient table was used for this experiment (Figure 2.3). This table has been developed for seed germination tests in Petri-dishes. In its breadth the table can be set at a minimum temperature of up to -10 ^{0}C on the one side of the table and up to 40 ^{0}C on the other side. Along this gradient there are 10 lanes perpendicular to the temperature gradient, and each lane represents a different temperature that can be measured. The temperature within each lane remains constant along the length of the table.

In previous studies (Ge et al., 2011) it was reported that glyphosate-resistant *C. canadensis* became sensitive to glyphosate at temperatures below 12 ^{0}C. Therefore, the objective was to set the mimimum temperature in this experiment below 12 ^{0}C.

Thermocouples were placed in the growth medium and in the air above the medium inside the container to measure the temperature because in this experiment the table was used for a different application than what it was developed for. After a series of tests to calibrate the temperature setting it was established that a temperature setting of 5 ^{0}C on the table resulted in 8-13 ^{0}C air temperature inside the container. The set maximum temperature was 25 ^{0}C which yielded air temperatures of 22-27 ^{0}C inside the vessels containing *C. bonariensis* seedlings. Only six lanes on the table were used, two on the cold side, two on the warmer side, and two in the middle. The temperature in the middle two lanes varied between 15 and 20 ^{0}C.

Figure 2.3 Seed thermogradient table

Plastic containers with the same diameter as a Petri-dish but with greater depth were used in order to create an individual growth chamber for each experimental unit which consisted of two plants (Figure 2.4). Sand and coir mixture were used as growth medium with a depth of only 3 cm to limit temperature variance between the growth medium and table surface. Seeds from the highly tolerant De Rust population were sown on the surface of the growth medium and lightly tapped down, but not buried. Fourteen days after sowing the plants were thinned out to two plants per container.

The containers were kept in a greenhouse for 35 days after planting. Containers were then placed on the temperature gradient table and allowed to acclimatise for seven days before glyphosate treatment. There were four rates of glyphosate: 0.5, 1, 2 and 4 times the recommended dosage (900 g a.e. L^{-1}) and an untreated control. There were seven replicates for each treatment at each of the three aforementioned temperature ranges.

Figure 2.4 Container with *Conyza bonariensis* seedlings in the temperature experiment

Data were analyzed as a combined split-plot completely randomized design (CRD) replicated three times, and applying factorial analysis of variance (ANOVA) to test for differences between the three temperatures (whole plots), and five doses (sub-plots), as well as the temperature x dose interaction effects. The residuals were acceptably normal and means were compared using Fisher's protected least significant difference test at the 5 % level ($p < 0.05$) (Snedecor and Cochran 1980).

Data were analyzed using the statistical program GenStat® (Payne 2014).

2.5 Hydroponic experiment

A hydroponic system (Figure 2.5) was designed with very specific requirements in order to assess if an interaction exists between manganese levels in the growth medium and response of the plant to glyphosate. Firstly, the system had to allow different nutrient solutions to be applied as treatments. Therefore, three separate units were constructed with its own bulk storage tank in order to have a different nutrient level for each unit.

Each unit consisted of a 25 L plastic bucket that contained the nutrient solution. The nutrient solution was pumped from the bucket through a 12 mm diameter clear flex tube to the one side of six plastic gutters. From the 12 mm tube the solution flowed through smaller GFS tubes connected to the gutters. The GFS tubes had choke taps to control the flow of the solution into the gutters and to ensure that each gutter received the same amount of nutrient solution. The bottoms of the gutters were covered with stones in order to allow equal flow of the solution.

Figure 2.5 Hydroponic system for testing the response of *Conyza bonariensis* seedlings to glyphosate when grown in medium containing different manganese concentrations.

The second requirement in terms of methodology was that the plants had to be moved for spraying, and therefore, could not be transplanted directly into the stones. The solution to this requirement was placing six pots containing the *C. bonariensis* seedlings in each gutter; the pots

could be removed for spraying and returned into position in the gutters. Holes of 10 mm in diameter were drilled into the lower part of pots in order to allow the nutrient solution to flow through stones in the bottom of the pots, thereby ensuring root contact with the circulated solution. At the end of each gutter the solution flowed through a 20 mm hole connected to clear flex tube back into the plastic bucket. From there the nutrient solution was recycled in this closed system as explained above.

Seed from the sensitive Hatfield and highly tolerant De Rust populations were planted in a sand and coir mixture in seeding trays. Seedlings were transplanted to pots in the hydroponic system as soon as the plants were big enough to handle and they had good root growth. Three nutrient solutions with different manganese levels were used. A standard commercial hydroponic fertilizer, Hygroponic mixture from Hygrotech, was used as the control with a manganese concentration of 0.179 mg L^{-1}. For the other two nutrient solutions Hygrotech company prepared specific products for this experiment; the one solution had a manganese concentration of 0 mg L^{-1} while the other solution had a manganese concentration of 0.358 mg L^{-1}, with the rest of the nutrients remaining the same as in the commercial fertilizer. The nutrient solution was replaced once a week to ensure that the concentrations remained constant.

Plants were allowed to grow in the hydroponic system for 28 days before glyphosate treatment was applied. Due to a lack of space in the hydroponic system there were only two glyphosate dosages applied at each of the manganese levels. The two glyphosate dosages were 900 and 1 800 g a.e. L^{-1}. There were five treatment replicates. After harvesting, plant analysis was done by the Soil Sciences laboratory at the University of Pretoria in order to assess the manganese levels in the plants at the different manganese treatments.

In this trial the 3 doses by 2 locations were randomised within each Manganese (Mn) treatment (nested design), but there were no replications of the Mn treatments, which thus cannot be tested for significance. Therefore, a nested ANOVA was used to test for differences between the dose, location and dose x location interaction, as well as all interactions with Mn effects.

Means were compared using Tukey's LSD test at the 5% level (Snedecor and Cochran 1980). Data were analyzed using the statistical program GenStat® (Payne 2014).

2.6 Mancozeb experiment

Seed from the sensitive Hatfield population was sowed and seedlings cultivated in the exact same manner as in the screening experiment. Six different treatments were applied at the 4-6 leaf stage. The treatments consisted of a control, glyphosate, glyphosate mixed with manganese sulfate ($MnSO_4 \cdot 4H_2O$), glyphosate mixed with zinc sulfate ($ZnSO_4 \cdot 7H_2O$), Mancozeb applied before glyphosate, and mancozeb applied after glyphosate.

Where glyphosate was part of a treatment it was applied as the product Roundup Turbo at the recommended dosage of 2 L ha^{-1} (900 g a.e. L^{-1}). Glyphosate was mixed in a 2 % ammonium sulfate solution as described above, except for the mixtures of glyphosate and manganese sulfate and glyphosate and zinc sulfate.

Mancozeb is the active ingredient in the commercial product Villa Unizeb WP obtained from Villa Crop Protection (Pty) Ltd. Mancozeb was applied at a dosage of 1 000 g ha^{-1} as specified on the label for use against various fungal diseases in vineyards. The spray volume was 500 L ha^{-1}. Ten gram of the product was mixed with 1 000 ml of water. Of this solution 10 ml was mixed with 40 ml water and sprayed onto the plants with the Oxford small-plot precision sprayer in order to attain the equivalent field rate.

The chemical formula of Mancozeb is $[C_4H_6MnN_2S_4]_x\ Zn_y$. Because Mancozeb contains the metals zinc and manganese, the effects of these two metals on the efficacy of glyphosate also needed to be tested separately. For the dosage of manganese sulfate and zinc sulfate, calculations were done in order to apply the same amount of moles manganese and zinc that is contained in Mancozeb when applied at the recommended rate. When 1 000 g of Villa Unizeb WP is applied per hectare, 800 g of the 1 000 g consists of the active ingredient Mancozeb, and of that 800 g only 162.06 g is manganese. An amount of 658.11 g ha^{-1} of manganese sulfate was used to apply the same amount of moles manganese (162.06 g ha^{-1}) as when Mancozeb is applied. Therefore, a bulk solution of 10 g manganese sulfate was mixed with 1 L of water.

From that bulk solution 6.6 ml was mixed with 2 ml glyphosate solution and 41.4 ml of water. This gives 6.58 g of manganese sulfate per m^2, which is the equivalent of 658.11 g ha^{-1} manganese sulfate.

The same formula was used to calculate the required concentration for zinc sulfate. Of the 800 g ha^{-1} active ingredient Mancozeb applied only 192.89 g is zinc. An amount of 848.20 g ha^{-1} zinc sulfate was used to apply the same amount of moles zinc (192.89 g ha^{-1}) as when Mancozeb is applied. Therefore, a bulk solution of 10 g zinc sulfate was mixed with 1 000 ml of water. From that bulk solution 8.5 ml were mixed with 2 ml of the glyphosate solution and 39.5 ml of water, which gave 0.0848 g of zinc sulfate per m^2, which is the equivalent of 848.20 g ha^{-1} zinc sulfate.

For the Mancozeb before and after glyphosate treatments, Mancozeb was applied at the recommended rate as described above. Glyphosate was sprayed within minutes after the Mancozeb treatment. For the glyphosate before Mancozeb treatment, exactly the same rates and procedures were applied as for the above-mentioned “Mancozeb before glyphosate” treatment. It is only the order of the Mancozeb and glyphosate treatments that was reversed.

Data were analyzed as for a completely randomized design (CRD), and applying factorial analysis of variance (ANOVA) to test for differences between six treatments on *C. bonariensis* from one location. Treatments were replicated 10 times. The residuals were acceptably normal and means were compared using Tukey's least significant difference test at the 5% level ($p < 0.01$) (Snedecor and Cochran 1980). Data were analysed using the statistical program GenStat® (Payne 2014).

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Screening experiment

In this experiment the main effects for glyphosate dosage and *C. bonariensis* population, as well as the dosage x population interaction effect were highly significant (ANOVA presented in Appendix A, Table A1).

Significant differences in the fresh mass of untreated (control) *C. bonariensis* plants from different locations can possibly be explained by differences in adaptability to conditions in the experiment (Figure 3.1).

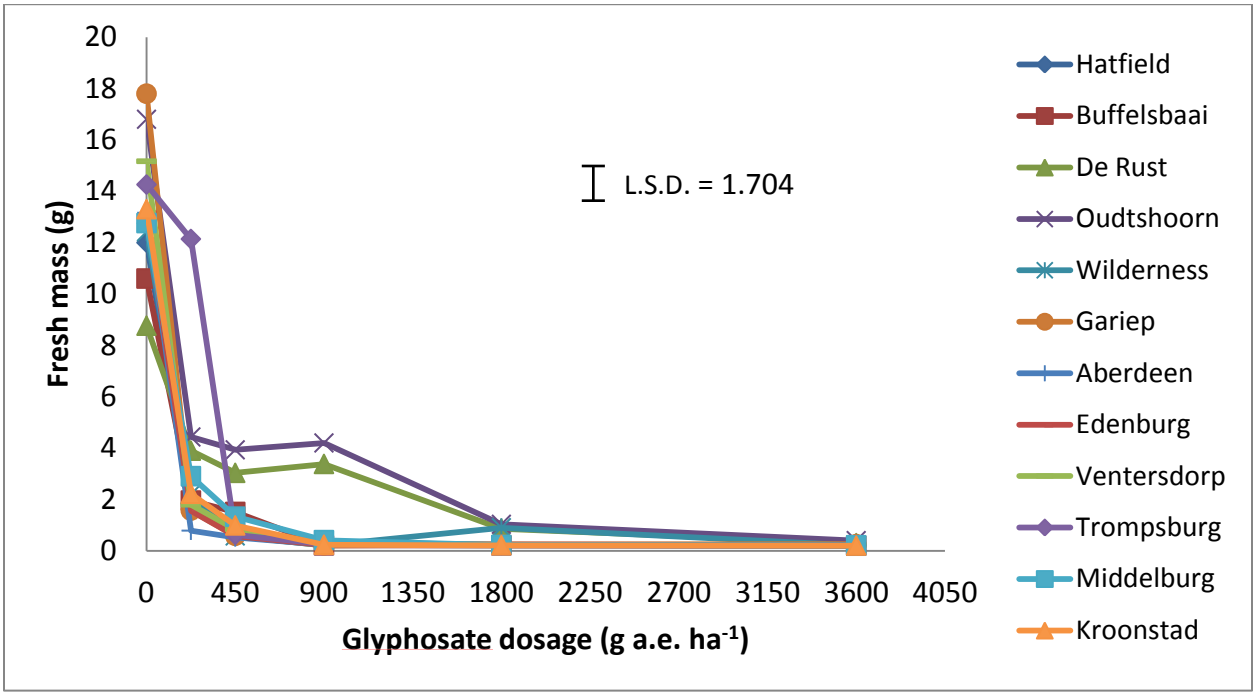

Figure 3.1 Fresh mass of 12 populations of *Conyza bonariensis* at six glyphosate dosages (ANOVA presented in Appendix A, Table A1)

At the untreated control, Gariep population attained the highest fresh mass which differed significantly from De Rust population that attained the lowest fresh mass (Figure 3.1).

At the recommended glyphosate dosage (900 g a.e. ha^{-1}) the fresh biomass of Oudtshoorn population was significantly higher than that of all the other populations except De Rust. While there is no significant difference between De Rust and Oudtshoorn, De Rust differed significantly from the rest of the populations at the recommended dosage. This implies that Oudtshoorn and De Rust populations are at least more tolerant to glyphosate than the rest. For both populations from De Rust and Oudtshoorn, all of the plants survived glyphosate treatment at the recommended dosage (Figure 3.2). Since resistance could not yet be claimed these two populations were classified as highly tolerant.

Figure 3.2 Representatives from each dosage (0, 0.25, 0.5, 1, 2 and 4 times the recommended dosage) for the De Rust population at 14 DAT. Dosage increases from left to right.

No plants of the Middelburg population died at the recommended dosage even though all the plants at the higher dosages (1 800 g a.e. ha^{-1} and 3 600 g a.e. ha^{-1}) died. All the replicates at the recommended dosage and four times the recommended dosage for the Wilderness population died but there was one replicate that survived an application of glyphosate at two times the recommended dosage. Populations from Middelburg and Wilderness were therefore classified as tolerant.

For the Hatfield and Gariep populations there were no survivors at the recommended dosage (Figure 3.3). Even at half the recommended dosage (450 g a.e. ha^{-1}) there were also some dead plants in these two populations, which supports their classification as sensitive towards glyphosate.

Figure 3.3 Representatives from each dosage (0, 0.25, 0.5, 1, 2 and 4 times the recommended dosage) for the Hatfield population at 14 DAT. Dosage increases from left to right.

The six above-mentioned populations that were categorized into susceptible, tolerant and highly tolerant were used in further experiments.

For all of the other populations (Buffelsbaai, Aberdeen, Edenburg, Ventersdorp, Trompsburg and Kroonstad) plants of all the replicates died at the recommended dosage and there were also no survivors at any of the higher rates.

In order to determine whether the Oudsthooorn and De Rust populations are resistant to glyphosate further investigation is required. For this purpose, equations for best-fit dose-response graphs (Figure 3.4) were used to calculate GR_{50} values and resistance factors (Table 3).

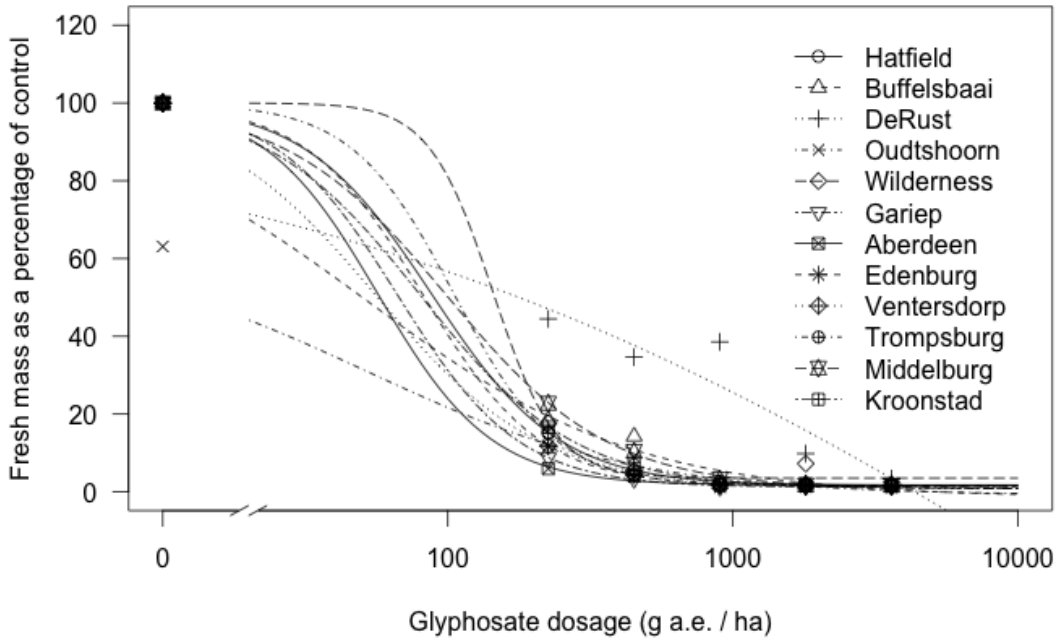

Figure 3.4 Dose-response curves of 12 populations of *Conyza bonariensis*

Table 3 GR_{50} values and R/S factor for 12 populations of *Conyza bonariensis*

Population	GR_{50}	R/S
Hatfield	88	1
Buffelsbaai	52	0.6
De Rust	19575	222.4
Oudtshoorn	52	0.6
Wilderness	143	1.6
Gariep	67	0.8
Aberdeen	57	0.6
Edenburg	82	0.9
Ventersdorp	56	0.6
Trompsburg	105	1.2
Middelburg	99	1.1
Kroonstad	80	0.9

The high GR_{50} value of De Rust population relative to the other populations can be attributed to a very high standard error and therefore this exceptionally high R/S value can not be used to make any conclusions. As the history of the Hatfield population is known in the field to be sensitive to glyphosate, this population was used as the susceptible reference despite not

having the lowest GR_{50} value. The Oudtshoorn population that showed a significant difference in fresh mass compared to the other populations had the lowest GR_{50} value, and consequently, an R/S value below one. Even though there were no dead plants at the recommended dosage, the GR_{50} and R/S values does not support the fresh mass data and it is clear that this population either shows low level resistance or the recommended label dosage needs to be adjusted for the area of Oudtshoorn. As no field trials were performed and no proof of this population not being controlled at the recommended dosage in the field is known, this Oudtshoorn population can not be defined as resistant according to the criteria of the "International survey of herbicide-resistant weeds" (Heap 2016) despite showing signs of low level resistance.

For the rest of the populations the GR_{50} and R/S values correspond to the fresh mass data.

3.2. Growth stage experiment

Figure 3.5 shows that the tolerance of the six populations to glyphosate at the first growth stage (10 – 12 leaves) corresponds to the results from the screening experiment. There were no significant differences between the fresh mass of any of the populations at the untreated control. At the recommended dosage (900 g a.e. ha^{-1}) Oudtshoorn population had the highest fresh mass and differed significantly from Wilderness, Gariep and Hatfield. All plants from the De Rust population at the recommended dosage were still alive and healthy at 21 DAT. Even though the plants appeared a little smaller than the untreated control there were no signs of desiccation or chlorosis.

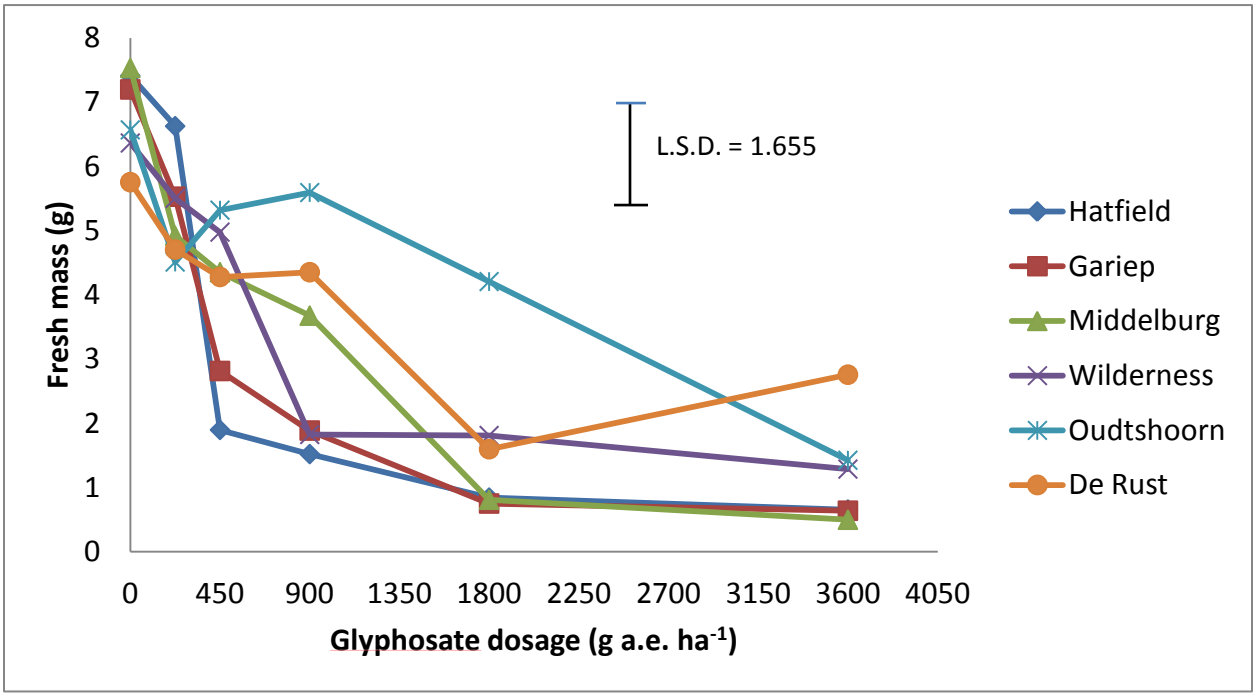

Figure 3.5 Fresh mass of different *Conyza bonariensis* populations at different glyphosate dosages at the first growth stage (10 – 12 leaves). (ANOVA presented in Appendix A, Table A2)

The Oudtshoorn population responded similar to the De Rust population. All of the plants survived the recommended dosage. Except for the one plant that appeared stunted, visually there were no difference between plants at the recommended dosage and the untreated control. This observation corresponds to the fresh mass data in Figure 3.5 as De Rust and Oudtshoorn are the only populations that showed no significant difference between the fresh

mass at the control and the recommended dosage for the particular population (Appendix B, Table B2). At higher dosages (1 800 and 3 600 g a.e. ha^{-1}) the results were variable with healthy and dead plants occurring at both populations. The populations from De Rust and Oudtshoorn held true to its classification of "highly tolerant" with no dead plants at the recommended dosage and variable results at higher dosages. The higher fresh mass at the recommended dosage for De Rust and Oudtshoorn clearly shows that these two populations had a higher tolerance towards glyphosate as there were more survivors than in the case of the other populations.

The results from the two tolerant populations, Wilderness and Middelburg, were variable across all the dosages. For the Wilderness population at 21 DAT only one replicate was completely dead at the recommended dosage while three of the replicates did not seem as if they would survive with a lot of desiccation and chlorosis but with a few green leaves. At two and four times the recommended dosage results were variable with survivors at both dosages even though most of the plants were completely dead. The Middelburg population had no dead plants at 21 DAT at the recommended dosage. Plants of all the replicates survived the glyphosate application. At the higher dosages results were variable, but most plants were dead in the case of both populations. These visual assessments correspond with the fresh mass data in Figure 3.5 as there is a significant difference between the fresh mass of the control and the recommended rate for these two populations (Appendix B, Table B2).

At the recommended dosage, the Hatfield population had the lowest fresh mass despite having the highest fresh mass at the untreated control at 21 DAT. This again shows that the population from Hatfield is very sensitive to glyphosate as there were no survivors at the recommended dosage or any of the higher dosages. Plants of three of the replicates at half the recommended dosage also died. The Gariep population, however, had three replicates where plants survived the recommended dosage at 21 DAT, even though plants of these replicates showed distinct desiccation and chlorosis. No plants of this population survived at two and four times the recommended dosage. There is a significant difference in the fresh mass between the control and the recommended dosage for both these populations (Appendix B, Table B2).

Figure 3.6 shows the dose-response curves of the six populations and it is clear that Oudtshoorn is more difficult to control. Table 4 shows the GR_{50} values and R/S factors for these populations with Oudtshoorn having the highest GR_{50} value. With an R/S factor of 6 for Oudtshoorn, the populations shows low level resistance as it did not reach the critical value of

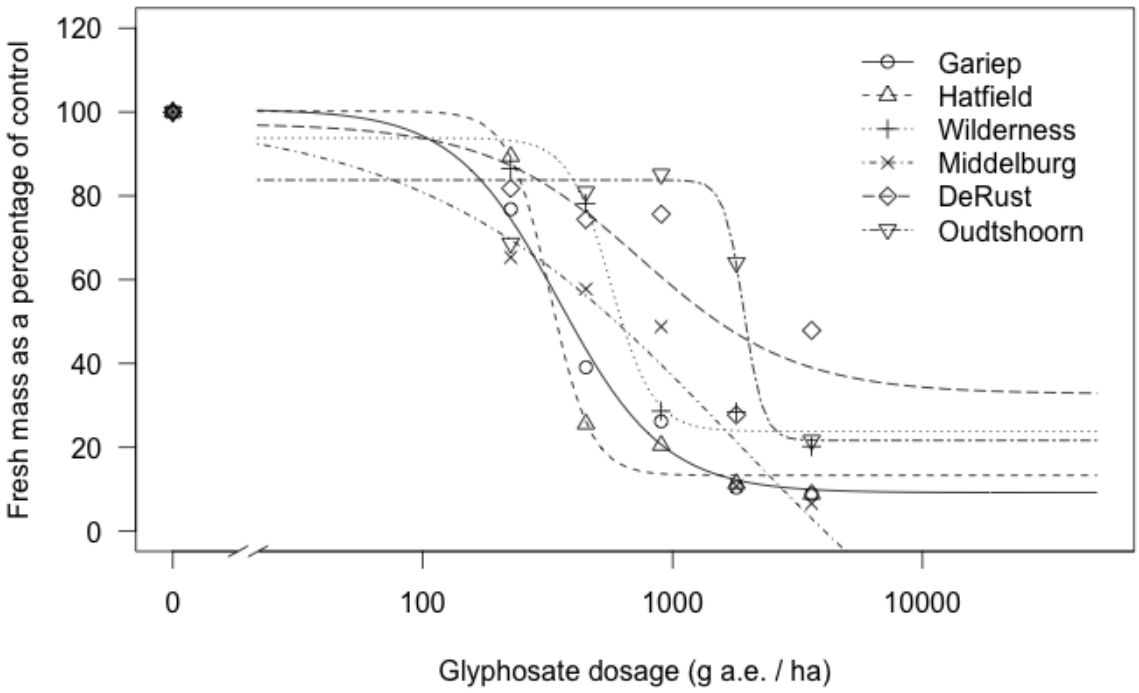

Figure 3.6 Dose-response curves of six populations of *Conyza bonariensis* at the first growth stage (10 – 12 leaves)

Population	GR_{50}	R/S
Gariep	351	1.1
Hatfield	323	1
Wilderness	567	1.8
Middelburg	1790	5.5
De Rust	744	2.3
Oudtshoorn	1926	6

Table 4 GR_{50} values and R/S factors for six populations of *Conyza bonariensis* at the first growth stage (10 – 12 leaves)

10 described in the “International Survey of Herbicide-resistant Weeds”(Heap 2016). This corresponds to fresh mass data and visual assessments as no plants died at the recommended dosage. As no field trials were performed and no proof of this population not being controlled at the recommended dosage in the field is known, this Oudtshoorn population cannot be defined as resistant according to the criteria of the “International survey of herbicide-resistant weeds” (Heap 2016) despite showing signs of low level resistance. The same applies to the populations from Middelburg and De Rust.

Results at the second growth stage were completely different from results at the first growth stage (Figure 3.5) and the screening experiment (Figure 3.1). Three weeks separated the growth stages. No plants from De Rust, Oudtshoorn, Middelburg or Gariep died at the recommended dosage or any of the higher dosages at 21 DAT. No plants from Hatfield and Wilderness were dead at the recommended dosage or two times the recommended dosage while both populations had one dead plant at four times the recommended dosage.

Figure 3.7 confirms what was visually observed as there is not a significant difference between the fresh mass of the control and the recommended dosage for the Hatfield population or any of the other populations (Appendix B, Table B3). At the first growth stage (Figure 3.5) the sensitive Hatfield population had the lowest fresh mass at the recommended dosage while at the second growth stage (Figure 3.7) this population had the highest fresh mass at the recommended dosage. This shows that a very sensitive population of *C. bonariensis* can become tolerant at a later growth stage and then be wrongly labeled as resistant.

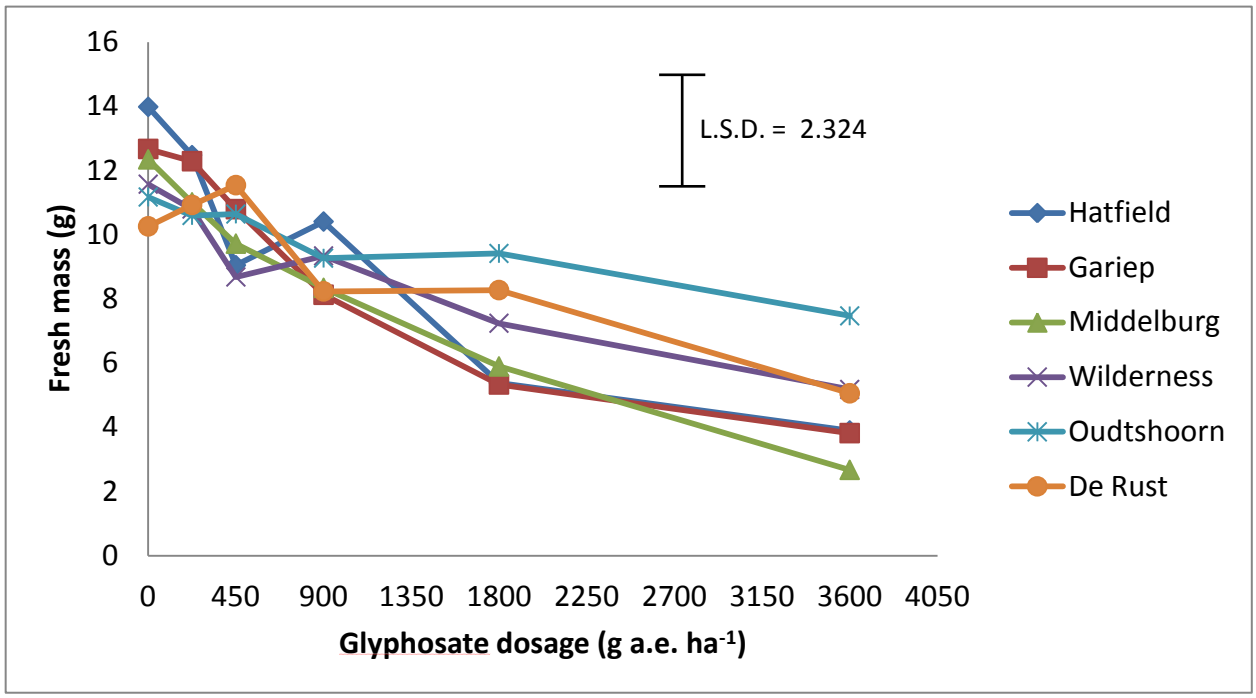

Figure 3.7 Fresh mass of different *Conyza bonariensis* populations at different glyphosate dosages at the second growth stage (16 – 20 leaves) (ANOVA) presented in Appendix A, Table A3)

The dose response curves (Figure 3.8), GR_{50} values and R/S factors (Table 5) support visual assessments and observations in the fresh mass data (Figure 3.7). This finding that the level of glyphosate resistance changes with growth stage is supported by various studies (Shrestha et al., 2007, Urbano et al., 2009, VanGessel et al., 2009, Walker et al., 2012).

Table 5 GR_{50} values and R/S factors for six populations of *Conyza bonariensis* at the second growth stage (16 – 20 leaves)

Population	GR_{50}	R/S
Gariep	936	0.4
Hatfield	2473	1
Wilderness	20698	8.4
Middelburg	9407	3.8
De Rust	3119	1.3
Oudtshoorn	31862	12.9

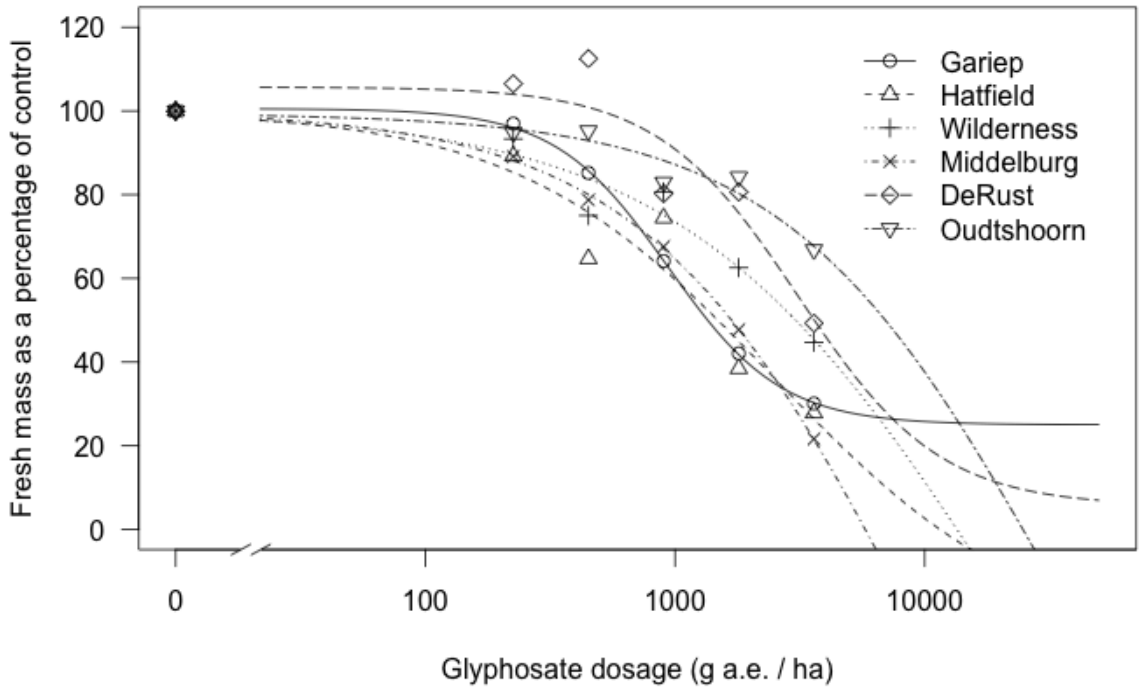

Figure 3.8 Dose-response curves of six populations of *Conyza bonariensis* at the second growth stage (16 – 20 leaves)

3.3 Second screening experiment

As can be seen on Figure 3.9 the fresh mass of the De Rust 2 population differed significantly from the fresh mass of the other three populations (Appendix B, Table B4 and Table B5). This was especially apparent at the recommended dosage and the higher dosages.

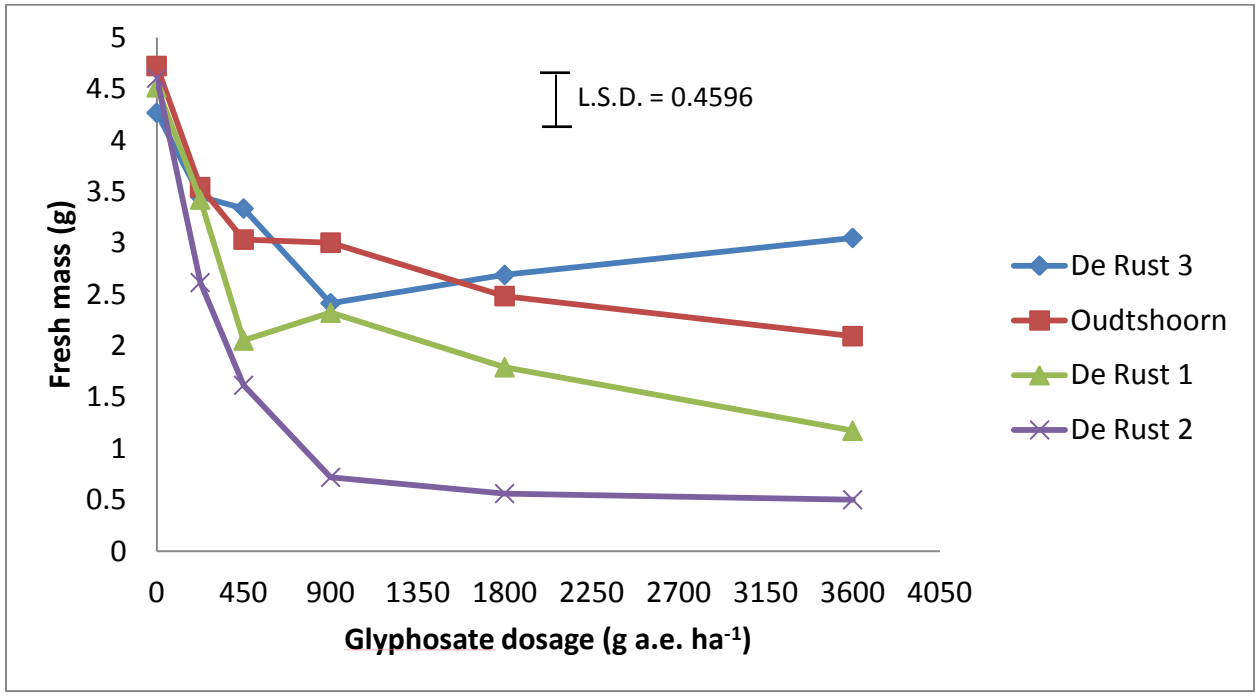

Figure 3.9 Mean fresh mass of different *Conyza bonariensis* populations at different glyphosate dosages at 21 DAT (ANOVA presented in Appendix A, Table A4)

The fresh mass data support visual assessments as plants of three out of the five replicates died at the recommended dosage of the De Rust 2 population and no plants of this population survived higher dosages. For the De Rust 1, De Rust 3 and Oudtshoorn populations there were no dead plants at the recommended dosage and variable sensitivity at higher dosages with few dead plants at higher dosages as can be seen in Figure 3.10.

Unfortunately, the spraying history of the fields in which seed from these populations have been sampled is unknown. It does, however, make sense that no glyphosate has been sprayed on the lucerne field as there are no glyphosate-resistant lucerne cultivars in South Africa. Therefore, there was no selection pressure for this population (De Rust 2).

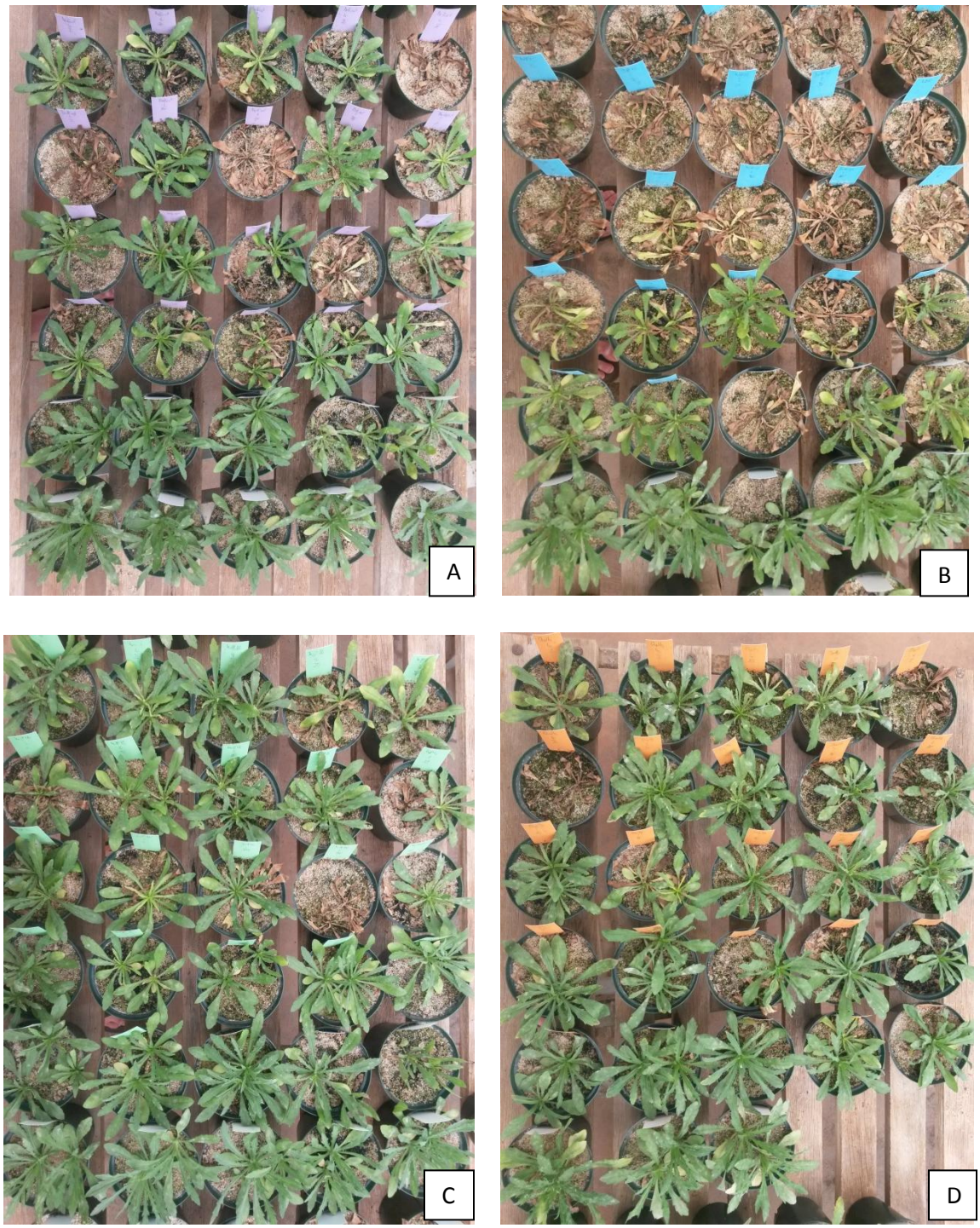

Figure 3.10 Pictures of the second screening experiment at 21 DAT. **A**: De Rust 1 population, **B**: De Rust 2 population, **C**: De Rust 3 population, **D**: Oudtshoorn population. In each picture glyphosate dosage increases from the bottom row upwards to four times the recommended rate. Each row represents a dosage: 0, 0.25, 0.5, 1, 2 and 4 times the recommended rate.

The De Rust 3 population was collected from an olive orchard (where frequent glyphosate use is a big possibility) and thus might have been under huge glyphosate selection pressure.

This experiment clearly shows that there is a big difference in the tolerance of one weed species towards glyphosate in the same area.

3.4 Temperature experiment

The interaction effect between dosage and temperature is highly significant. Even though the difference between the fresh mass means at the recommended dosage is not significant (Appendix B, Table B6) there is a strong tendency that the fresh mass is lower for the treated plants at the recommended and higher dosages at the temperature of 22 – 27 ^{o}C as can be seen in Figure 3.11. This tendency supports visual assessments that showed many more dead plants at the high temperature range (22 – 27 ^{o}C) than at the recommended dosage (Figure 3.12). The De Rust population used in this temperature study was highly tolerant towards glyphosate in all of the previous experiments with no dead plants at the recommended dosage.

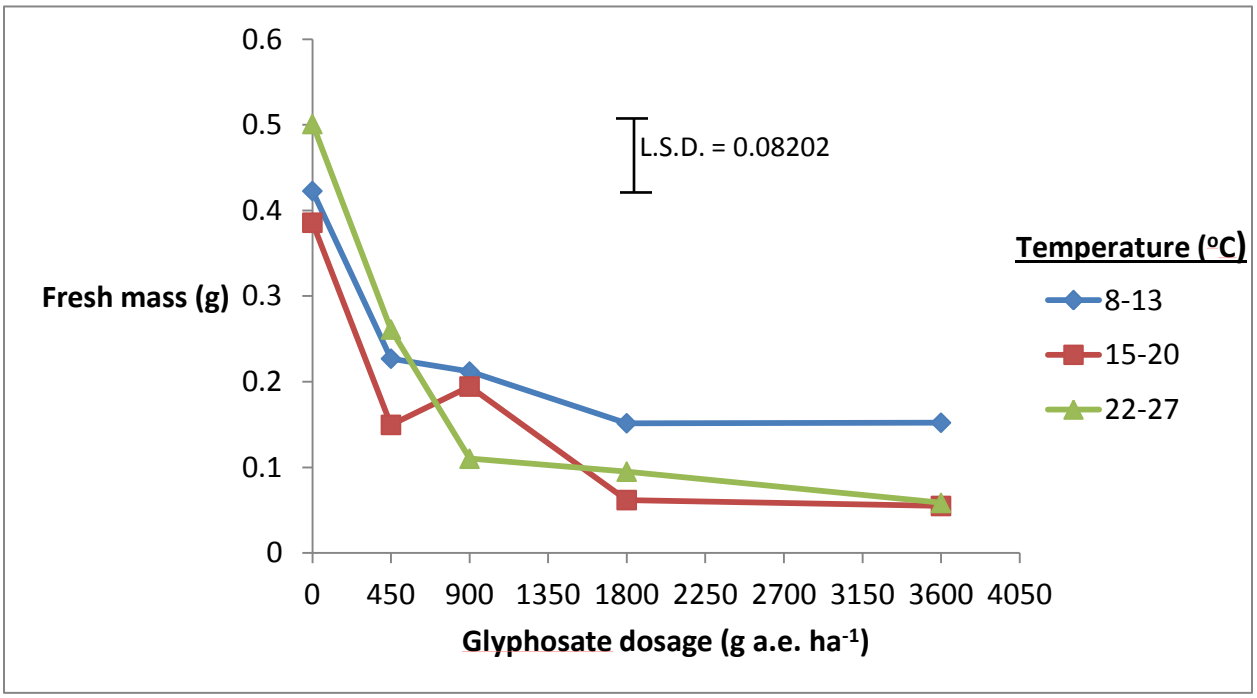

Figure 3.11 Fresh mass of *Conyza bonariensis* at different glyphosate dosages and temperatures (ANOVA presented in Appendix A, Table A5)

The tendency that was observed whereby *Conyza bonariensis* plants that are highly tolerant towards glyphosate are better controlled at higher temperatures is in contrast with the literature (Ge et al., 2011). Ge et al. (2011) found that glyphosate-resistant *Conyza canadensis* can become sensitive to glyphosate at temperatures below 12 ^{o}C if vacuolar sequestration is the mechanism of resistance.

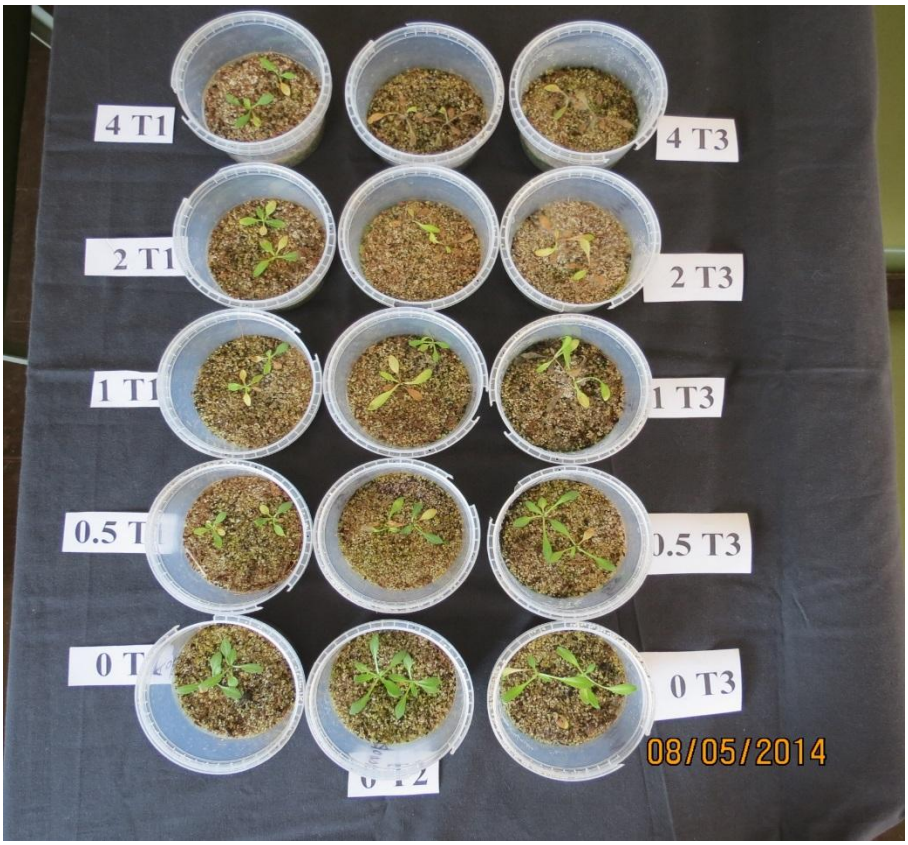

Figure 3.12 Representative plants for each glyphosate treatment at each temperature. Column on the left (T1) represents temperature of 8 – 13 ^{o}C, T2 in the middle represents temperature of 15 – 20 ^{o}C and T3 on the right represents the high temperature range of 22 - 27 ^{o} C. Glyphosate dosage increases from the bottom row (control) upwards up to four times the recommended rate.

In our temperature experiment the tolerance towards glyphosate remained the same at the lower temperature range (8 – 13 ^{o}C) as there were no dead plants at the recommended dosage at this temperature. Based on the finding of Ge et al. (2011), the resistance mechanism for the De Rust population probably is not vacuolar sequestration. A possible explanation for the plants dying at higher temperatures might be the higher humidity inside the containers. Humidity was not measured but was close to 100 % as the condensation was taking place inside these containers.

No information of resistance studies done on a temperature thermogradient table could be found. A new technique has thus been established for studying the effect of temperature on

the efficiency of herbicides. This technique may prove useful where space is limited and/or growth chambers are not available. For this particular study three growth chambers would have been needed.

3.5 Hydroponic experiment

There was no replication of the manganese treatments, therefore differences between manganese effects could not be tested as it is like a blocking effect. The doses and locations were randomized within each manganese treatment so these effects and interactions could be tested. This is, however, of no value as the sensitivity of the populations from Hatfield and De Rust towards glyphosate are already well known from previous experiments. In order to test differences between manganese treatments, the whole experiment has to be repeated a few times (Personal communication, Marie Smith, statistician).

Soil analyses was done on a sample from a vineyard near Worcester in the Western Cape and this showed that the high manganese level in this experiment (3.58 mg L^{-1}) was too low as levels in the soil can be much higher. The manganese level in this soil is 300.325 mg kg^{-1} (EDTA extraction). Mancozeb has been used for decades in vineyards and might have attributed to high manganese levels.

Visual assessments at 21 DAT showed that the two populations reacted to glyphosate application in the same manner across the different manganese treatments as in the other experiments. All of the Hatfield plants died at the two glyphosate dosages except for one survivor at the recommended glyphosate dosage and the high manganese level. The De Rust population had varying results at the two glyphosate dosages with alive and dead plants as was the case in other experiments with this population.

Plant analysis showed that the plants from the high manganese level had a manganese content of 111.83 mg / kg, plants from the control (standard commercial hydroponic fertilizer) had a manganese content of 122.8 mg / kg and plants from the no manganese treatment had a manganese content of 51.49 mg / kg. It would be of value to repeat this experiment with higher manganese levels since there was no increase in the manganese content of plants from the higher manganese nutrient solution in comparison with the standard manganese content solution.

However, the technique that was developed to test the influence of specific nutrient deficiencies or abundances on the efficiency of herbicides could be useful in future studies on this aspect.

3.6 Mancozeb experiment

The results for treatments where mancozeb was applied either before or after glyphosate did not differ significantly from glyphosate applied alone (Figure 3.8). The mixtures of respectively manganese sulfate and zinc sulfate with glyphosate did differ significantly from the treatment where glyphosate was applied alone (Appendix B, Table B7) which shows that the zinc and manganese ions probably inactivated the glyphosate. This was, however, expected as it is known that these cations complex with glyphosate and reduces its efficiency (Nilsson 1985, Thelen et al., 1995, Bernards et al., 2005, Nandula 2010). Even though the efficiency of glyphosate is impacted severely by these mixtures the growth of these plants was still suppressed significantly in comparison with the untreated control.

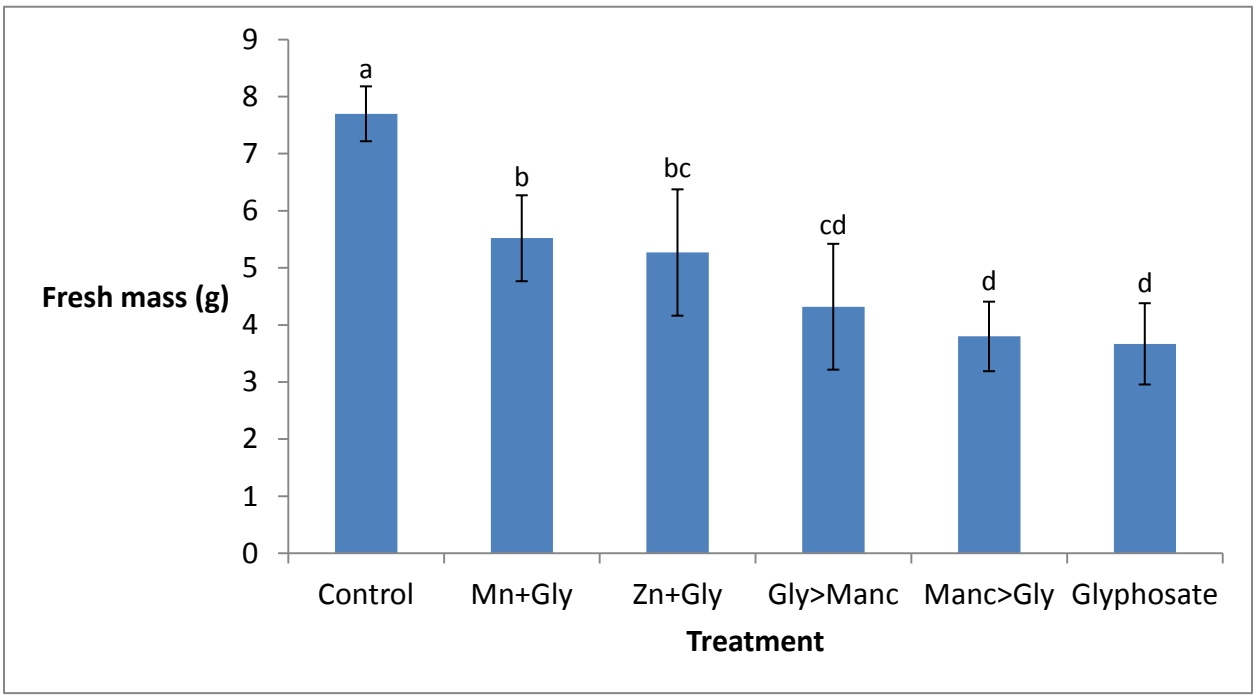

Figure 3.13 Mean fresh mass of glyphosate-sensitive *Conyza bonariensis* from Hatfield at different glyphosate treatment mixtures (Means with the same letters do not differ significantly at P = 0.05; ANOVA presented in Appendix A, Table A6)

This finding underlines the importance of not mixing any foliar fertilizers with glyphosate, especially not without adding ammonium sulfate to glyphosate spraying solutions, in order to prevent antagonism from occurring (Nandula 2010).

CHAPTER 4

CONCLUSIONS

4.1 Screening experiment

There are distinct differences in the tolerance of different *C. bonariensis* populations towards glyphosate. Populations from Gariep, Hatfield, Buffelsbaai, Aberdeen, Edenburg, Ventersdorp, Trompsburg and Kroonstad were classified as sensitive. Due to the varying results for the Middelburg and Wilderness populations they were classified as tolerant. Because all the plants from the Oudtshoorn and De Rust populations survived glyphosate treatment at the recommended dosage, as well as varying results at higher dosages these two populations were classified as being highly tolerant. GR_{50} values and R/S factors showed that Oudtshoorn, Middelburg and De Rust are possible cases of resistance but the populations could not be defined as proven resistant because not all the criteria of the "International Survey of Herbicide-resistant Weeds" could be met (Heap 2016).

4.2 Growth stage experiment

Results at the first growth stage (10 – 12 leaves) corresponded to the results of the screening experiment. The Hatfield population was again very sensitive towards glyphosate as all plants of all the replicates died at the recommended dosage (900 g a.e. ha^{-1}) and some plants even died at half the recommended dosage (450 g a.e. ha^{-1}). At this growth stage all of the other populations also reacted the same as in the screening experiment and remains classified as sensitive, tolerant and highly tolerant, respectively.

Three weeks later, at the second growth stage (16 - 20 leaves), when certain plants started to bolt no plants from any of the populations (the very sensitive Hatfield population included) died at both the recommended and double the recommended dosage. This reduced sensitivity towards glyphosate at a later growth stage clearly shows that *C. bonariensis* becomes more

tolerant towards glyphosate as growth stage increases. This finding supports the results of Shrestha et al. (2007), Urbano et al. (2009), VanGessel et al. (2009) and Walker et al. (2012).

If resistance to glyphosate develops, it will be a big problem for farmers in various cropping systems. Therefore, the label must be followed very strictly to ensure that plants are treated at the correct rate and growth stage to ensure that populations are not incorrectly referred to as resistant.

4.3 Second screening experiment

This experiment showed that there can be variability within a geographic area (De Rust) with regards to glyphosate tolerance in populations of a particular weed species. However, assumptions for a whole area or region can not be made based on one resistant population from one particular field. Cropping practice together with herbicide use history can determine the tolerance of a weed population towards glyphosate, and other herbicides for that matter.

4.4 Temperature experiment

The highly tolerant De Rust population was found to be more susceptible towards glyphosate at higher temperatures (22 – 27 ^{o}C). This finding is contrary to the literature perused (Ge et al., 2011) where glyphosate-resistant *C. canadensis* was found to be sensitive towards glyphosate at temperatures below 12 ^{o}C if vacuolar sequestration is the mechanism of resistance.

The mechanism of resistance for the De Rust *C. bonariensis* population is probably not vacuolar sequestration, since the tolerance of *C. bonariensis* towards glyphosate did not decline at temperatures below 12 ^{O}C. The technique that was developed to use a thermogradient table in resistance assessment studies is, however, proposed as a valuable tool for studies of this nature.

3.5 Hydroponic experiment

The highest manganese level used in this experiment was probably not high enough. The populations used in this experiment reacted in the same manner as in previous screening experiments. It will be of value to repeat this experiment with a wider range of manganese levels. The technique that was developed in this experiment is unique and can be used in further studies of this nature.

As there was no replication of the manganese treatments the hypothesis could not be accepted nor rejected.

3.6 Mancozeb experiment

Mancozeb applied either directly before or after glyphosate did not influence the efficacy of glyphosate significantly.

Existing knowledge about the roles of the cations, zinc and manganese, which are known to inactivate the glyphosate molecule, was confirmed. For this reason it is important that mixtures of glyphosate and foliar fertilizers should not be prepared. Warnings to this effect do appear on labels of glyphosate-containing products, but is apparently ignored at times by certain users. It remains important to add ammonium sulfate to water before adding glyphosate in order to prevent antagonism of glyphosate.

SUMMARY

Conyza bonariensis (flaxleaf fleabane) originated from the temperate regions in South America (Michael 1977) and is also a major weed in South Africa (Wu et al., 2007, Bromilow 2010). Glyphosate [N-(phosphonomethyl) glycine] was first synthesized and tested as a herbicide in 1970 by John E Franz of Monsanto company and was first patented for use as an herbicide in 1974. The herbicide is marketed as a post-emergence, non-selective, broad-spectrum and systemic herbicide. Due to the herbicide being so effective, environmentally friendly and economically viable it quickly became the world's most extensively used herbicide. Glyphosate has a unique mode of action. It inhibits the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (called EPSPS) of the shikimate pathway.

The first glyphosate-resistant case (resistance in *Lolium rigidum*) in the world was reported in 1996, 22 years after glyphosate was introduced to the market. *L. rigidum* was also the first weed to be reported resistant to glyphosate in South Africa in 2001. *C. bonariensis* was reported to be resistant to glyphosate in South Africa in 2003 in the Western Cape. The other glyphosate resistant weed in the country is *Plantago lanceolata.*

The majority of studies with regards to the influence of growth stage on the tolerance of *Conyza* sp. towards glyphosate show that plants become more tolerant at a later growth stage. Reduced translocation is a known mechanism of resistance in *Conyza canandensis*. Another known mechanism of resistance in *C. canadensis* is vacuolar sequestration. Glyphosate-resistant *C. canadensis* plants become sensitive to glyphosate under low temperatures where vacuolar sequestration is the mechanism of resistance. The efficacy of the glyphosate molecule can be reduced when hard-water cations like Fe^{2+}, Mg^{2+} and Ca^{2+} bind to glyphosate. Tank mixtures with glyphosate and manganese fertilizers also reduce the efficacy of glyphosate. It is possible that the antagonism of glyphosate by Mn^{2+} might also take place inside the plant.

C. bonariensis seeds was collected from 12 locations in South Africa. A screening experiment was performed for glyphosate tolerance at 0, 0.25, 0.5, 1, 2 and 4 times the recommended

dosage with five replicates for each treatment. The experiment was performed in the glasshouse. The fresh mass was measured at 21 days after treatment (DAT). The statistical program GenStat® was used to apply factorial analysis of variance (ANOVA) and means were compared using Tukey's least significant difference test at the 5% level. Dose-response curves were generated using the "drc" package of the program R® by using a four-parameter non-linear regression model. GR_{50} values and Resistant / Sensitive values were calculated from the dose-response curves.

Results from the screening experiment were used to divide six of the populations into three categories. Populations from De Rust and Oudsthoorn were "highly tolerant", Middelburg and Wilderness were "tolerant" and Hatfield and Gariep were "sensitive". These six populations were used in a growth stage experiment using the same materials and methods as for the screening experiment with growth stage added as a factor. The first application was at the 10-12 leaf stage and the second application at the 16-20 leaf stage. Statistical analysis was the same as for the screening experiment.

A second screening experiment was performed with the above mentioned De Rust (De Rust 1) and Oudtshoorn populations as well as two more populations from De Rust (De Rust 2 and De Rust 3).

A seed thermogradient table was used to do a temperature experiment. This table was initially developed for seed germination tests in Petri-dishes. Plants were grown in closed transparent plastic containers that were placed on the table. The glyphosate response of the De Rust population were tested at three temperature ranges, 8 – 13 ^{0}C, 15 – 20 ^{0}C and 22 – 27 ^{0}C. The highly tolerant population from De Rust were used in this experiment since the literature revealed that resistant *C. Canadensis* becomes sensitive at temperatures below 12 ^{0}C. Plants were grown in the greenhouse for 35 days after planting and were allowed to acclimatise for seven days on the thermogradient table before glyphosate was applied. Glyphosate was applied at dosages of 0.5, 1, 2 and 4 times the recommended dosage and compared to an untreated control with seven replicates for each treatment. The statistical program GenStat® was used to apply factorial analysis to the data as a combined split-plot completely randomized

design replicated three times. Fisher's protected least significant difference test at the 5% level ($p < 0.05$).

A hydroponic experiment was carried out to assess if manganese levels in the growth medium can have an effect on the susceptibility of *C. bonariensis* towards glyphosate. Seed from the sensitive Hatfield and highly tolerant De Rust populations were planted in seeding trays and transplanted to pots filled with stones in the hydroponic system. The hydroponic system had trays with nutrient solutions containing different manganese levels. The three different manganese levels were 0 mg L^{-1}, 0.179 mg L^{-1} and 0.358 mg L^{-1} with the rest of the nutrients remaining the same for all three solutions. Two glyphosate dosages (one and two times the recommended rate) were applied with five treatment replicates. Plant analysis was done and fresh mass measured at 21 DAT. Because there was no replication of the manganese treatments the data could not be tested for significance.

The final experiment was done in order to examine if the application of Mancozeb has an influence on the efficacy of glyphosate when applied to *Conyza bonariensis*. The Mancozeb (Villa Unizeb WP) and glyphosate (Roundup Turbo) treatments were applied at the recommended label rates of 500 L ha^{-1} and 2 L ha^{-1} separately. Six treatments consisted of glyphosate, mancozeb, glyphosate before mancozeb and glyphosate after mancozeb, glyphosate mixed with zinc sulfate and glyphosate mixed with manganese sulfate. ANOVA was applied to test for differences between treatments and means were compared using Tukey's least significant differences test at the 5 % level ($p < 0.01$).

In the screening experiment all replicates from De Rust and Oudtshoorn survived glyphosate application at the recommended dosage (900 g a.e. ha^{-1}). Both populations had survivors at double and four times the recommended dosage and were classified as being highly tolerant. Populations from Hatfield and Gariep were classified as susceptible. The populations from Wilderness and Middelburg were classified as tolerant.

Results for the growth stage experiment at the first growth stage (10 – 12 leaves) were the same as for the screening experiment. Hatfield was the most sensitive population with the lowest fresh mass at the recommended dosage while De Rust and Oudtshoorn were highly

tolerant. Three weeks later at the second growth stage (16 – 20 leaves) the sensitive Hatfield population had the highest fresh mass at the recommended dosage and not one plant from any of the populations died at two and four times the recommended dosage. Sensitive *C. bonariensis* plants become tolerant to glyphosate at later growth stages.

The second screening experiment showed that the De Rust 2 population is significantly more sensitive to glyphosate than the De Rust 1, De Rust 3 and Oudtshoorn populations. The sensitivity of *C. bonariensis* towards glyphosate differs in the same area depending on the history of glyphosate usage in the specific field.

In the temperature experiment there was no significant difference in the fresh mass of the plants at the different temperatures at the recommended glyphosate dosage. There is however a strong tendency that the fresh mass of treated plants at the high temperature (22 – 27 ^{o}C) is lower than at the low temperature range of 8 – 13 ^{o}C. There were also more dead plants at the high temperature at the recommended glyphosate dosage. The mechanism of resistance for this De rust population is not vacuolar sequestration then. The technique can be used in future temperature studies.

There was no replication of the manganese treatments, therefore differences between manganese effects could not be tested as it is like a blocking effect. To test for differences between manganese treatments the experiment had to be repeated a few times. The manganese levels used in this experiment was also too low. The technique that was developed for this experiment to test the influence that specific nutrient deficiencies or abundances have on the efficiency of herbicides is of great value and can be used in future studies.

The mancozeb experiment showed that mancozeb did not have a significant effect on glyphosate efficacy when applied before or after glyphosate. The cations of zinc and manganese do, however, influence the efficiency of glyphosate in tank mixtures.

References

Amsellem Z, Jansen MA, Driesenaar AR, Gressel J. 1993. Developmental variability of photooxidative stress tolerance in paraquat-resistant Conyza. *Plant Physiology* 103:1097-106.

Bailey WA, Poston DH, Wilson HP, Hines TE. 2009. Glyphosate interactions with manganese1. *Weed Technology* 16:792-9.

Baylis AD. 2000. Why glyphosate is a global herbicide: strengths, weaknesses and prospects. *Pest Management Science* 56:299-308.

Beckie HJ, Thomas AG, Légère A, Kelner DJ, Van Acker RC, Meers S. 1999. Nature, occurrence, and cost of herbicide-resistant wild oat (*Avena fatua*) in small-grain production areas. *Weed Technology*:612-25.

Bernards ML, Thelen KD, Penner D. 2005. Glyphosate Efficacy is Antagonized by Manganese 1. *Weed Technology* 19:27-34.

Bernards ML, Thelen KD, Penner D, Muthukumaran RB, McCracken JL. 2009. Glyphosate interaction with manganese in tank mixtures and its effect on glyphosate absorption and translocation. *Weed Science* 53:787 - 94.

Boerboom C, Owen M. Glyphosate-Resistant Weeds. 2013.

Bott S, Tesfamariam T, Candan H, Cakmak I, Römheld V, Neumann G. 2008. Glyphosate-induced impairment of plant growth and micronutrient status in glyphosate-resistant soybean (Glycine max L.). *Plant and Soil* 312:185-94.

Bradshaw LD, Padgette SR, Kimball SL, Wells BH. 1997. Perspectives on glyphosate resistance. *Weed Technology*:189-98.

Bromilow C, editor. *Problem plants and alien weeds of South Africa*. Pretoria: Briza Publications; 2010.

CaJacob CA, Feng PC, Heck GR, Alibhai MF, Sammons RD, Padgette SR. 2004. Engineering resistance to herbicides. *Handbook of Plant Biotechnology*.

De Wet H. Paraquat and glyphosate resistance in *Conyza bonariensis* in the Western Cape province in the Republic of South Africa. Stellenbosch: University of Stellenbosch; 2005.

Devine MD, Bandeen JD, McKersie BD. 1983. Temperature effects on glyphosate absorption, translocation, and distribution in quackgrass (Agropyron repens). *Weed Science*:461-4.

Dill GM. 2005. Glyphosate-resistant crops: history, status and future. *Pest Management Science* 61:219-24.

Duke SO, Lydon J, Koskinen WC, Moorman TB, Chaney RL, Hammerschmidt R. 2012a. Glyphosate effects on plant mineral nutrition, crop rhizosphere microbiota, and plant disease in glyphosate-resistant crops. *Journal of Agricultural and Food Chemistry* 60:10375-97.

Duke SO, Powles SB. 2008. Glyphosate: a once-in-a-century herbicide. *Pest Management Science* 64:319-25.

Duke SO, Reddy KN, Bu K, Cizdziel JV. 2012b. Effects of glyphosate on the mineral content of glyphosate-resistant soybeans (glycine max). *Journal of Agricultural and Food Chemistry* 60:6764-71.

Dyer W. 1994. Resistance to glyphosate. *Herbicide Resistance In Plants: Biology And Biochemistry*:353.

Feng PC, Tran M, Chiu T, Sammons RD, Heck GR, CaJacob CA. 2009. Investigations into glyphosate-resistant horseweed (Conyza canadensis): retention, uptake, translocation, and metabolism. *Weed Science*:498-505.

Franz JE, Mao MK, Sikorski JA. 1997. *Glyphosate: a unique global herbicide.*. American Chemical Society.

Ge X, d'Avignon DA, Ackerman JJ, Duncan B, Spaur MB, Sammons RD. 2011. Glyphosate-resistant horseweed made sensitive to glyphosate: low-temperature suppression of glyphosate vacuolar sequestration revealed by 31P NMR. *Pest Management Science* 67:1215-21.

Ge X, d'Avignon DA, Ackerman JJ, Sammons RD. 2010. Rapid vacuolar sequestration: the horseweed glyphosate resistance mechanism. *Pest Management Science* 66:345-8.

Geiger DR, Shieh W-J, Fuchs MA. 1999. Causes of self-limited translocation of glyphosate in Beta vulgaris plants. *Pesticide Biochemistry and Physiology* 64:124-33.

Gianessi LP. 2008. Economic impacts of glyphosate-resistant crops. *Pest Management Science* 64:346-52.

Giesy JP, Dobson S, Solomon KR. 2000. *Ecotoxicological risk assessment for Roundup® herbicide*. Springer.

Gressel J. 2002. *Molecular Biology of Weed Control*ed. CRC Press.

Grundy A, Mead A, Burston S. 2003. Modelling the emergence response of weed seeds to burial depth: interactions with seed density, weight and shape. . *Journal of Applied Ecology*:757 - 70.

Heap I. 2014. Global perspective of herbicide-resistant weeds. *Pest Management Science* 70:1306-15.

Heap I. The International Survey of Herbicide Resistant Weeds. 2016 [cited 2016 Monday, 8 August, 2016]; Available from: www.weedscience.org.

Holm F, Johnson EN. 2009. The history of herbicide use for weed management on the prairies. *Prairie Soils Crops J* 2:1-10.

Holt JS. 1992. History of identification of herbicide-resistant weeds. *Weed Technology*:615-20.

Hrazdina G, Jensen RA. 1992. Spatial organization of enzymes in plant metabolic pathways. *Annual Review of Plant Biology* 43:241-67.

James C. Biotech facts and trends 2014. 2015; Available from: http://www.isaaa.org/.

Jussaume Jr RA, Ervin D. 2016. Understanding weed resistance as a wicked problem to improve weed management decisions. *Weed Science* 64:559-69.

Kempen H, Graf J, editors. Weed seed production. Proceedings of the Western Society of Weed Science; 1981.

Kjær J, Olsen P, Ullum M, Grant R. 2005. Leaching of glyphosate and amino-methylphosphonic acid from Danish agricultural field sites. *Journal of Environmental Quality* 34:608-20.

Koger CH, Poston DH, Hayes RM, Montgomery RF. 2009. Glyphosate-Resistant Horseweed (Conyza canadensis) in Mississippi1. *Weed Technology* 18:820-5.

LeBaron HM, Gressel J. 1982. *Herbicide resistance in plants*ed. John Wiley & Sons.

Malatji MW. Allelopathic potential of *Conyza bonariensis*. Pretoria: University of Pretoria; 2013.

Maxwell BD, Mortimer A. 1994. Selection for herbicide resistance. *Herbicide Resistance in Plants: Biology and Biochemistry*:1-25.

Michael P, editor. Some weedy species of Amaranthus (amaranths) and Conyza-Erigeron (fleabanes) naturalized in the Asian-Pacific region. Proceedings of the Sixth Asian-Pacific Weed Science Society Conference, Jakarta, Indonesia; 1977.

Mifsud S. Flax-leaved fleabane. 2015 [cited 2015 2015-04-27]; Available from: www.maltawildplants.com/ASTR/Conyza_bonariensis.php.

Moretti ML, Hanson BD, Hembree KJ, Shrestha A. 2013. Glyphosate resistance is more variable than paraquat resistance in a multiple-resistant hairy fleabane (Conyza bonariensis) population. *Weed Science* 61:396-402.

Nandula VK. 2010. *Glyphosate resistance in crops and weeds: history, development, and management*. John Wiley & Sons.

Nandula VK, Reddy KN, Duke SO, Poston DH. 2005. GLYPHOSATE-RESISTANT WEEDS: CURRENT STATUS AND FUTURE OUTLOOK. *Outlooks on Pest Management*:183-7.

Nilsson G. 1985. Interactions between glyphosate and metals essential for plant growth. *Herbicide glyphosate/edited by E Grossbard, D Atkinson*.

Owen MD. 2016. Diverse Approaches to Herbicide-Resistant Weed Management. *Weed Science* 64:570-84.

Padgette SR, Biest Taylor N, Nida DL, Bailey MR, MacDonald J, Holden LR, Fuchs RL. 1996. The composition of glyphosate-tolerant soybean seeds is equivalent to that of conventional soybeans. *Journal of Nutrition* 126:702-16.

Payne R. GenStat for Windows, 17th Edn., Introduction. Hertfordshire, UK.2014; Available from: http://www.genstat.co.uk.

Pieterse P. 2010. Herbicide resistance in weeds–a threat to effective chemical weed control in South Africa. *South African Journal of Plant and Soil* 27:66-73.

Powles SB. 2008. Evolved glyphosate-resistant weeds around the world: lessons to be learnt. *Pest Management Science* 64:360-5.

Powles SB, Gaines TA. 2016. Exploring the Potential for a Regulatory Change to Encourage Diversity in Herbicide Use. *Weed Science* 64:649-54.

Prieur-Richard AH, Lavorel S, Grigulis K, Dos Santos A. 2000. Plant community diversity and invasibility by exotics: invasion of Mediterranean old fields by Conyza bonariensis and Conyza canadensis. *Ecology Letters* 3:412-22.

Radosevich S, Devilliers O. 1976. Studies on the mechanism of s-triazine resistance in common groundsel. *Weed Science*:229-32.

Rea PA. 2007. Plant ATP-binding cassette transporters. *Annu Rev Plant Biol* 58:347-75.

Ritz C, Streibig JC. 2005. Bioassay analysis using R. *Journal of Statistical Software* 12:1-22.

Ryan G. 1970. Resistance of common groundsel to simazine and atrazine. *Weed Science*:614-6.

Sammons RD, Gaines TA. 2014. Glyphosate resistance: state of knowledge. *Pest Management Science* 70:1367-77.

Seefeldt SS, Jensen JE, Fuerst EP. 1995. Log-logistic analysis of herbicide dose-response relationships. *Weed Technology*:218-27.

Servaites JC, Tucci MA, Geiger DR. 1987. Glyphosate effects on carbon assimilation, ribulose bisphosphate carboxylase activity, and metabolite levels in sugar beet leaves. *Plant Physiology* 85:370-4.

Shah DM, Horsch RB, Klee HJ, Kishore GM, Winter JA, Tumer NE, Hironaka CM, Sanders PR, Gasser CS, Aykent S. 1986. Engineering herbicide tolerance in transgenic plants. *Science* 233:478-81.

Shrestha A, Hembree K, Va N. 2007. Growth stage influences level of resistance in glyphosate-resistant horseweed. *California Agriculture* 61:67-70.

Sidhu RS, Hammond BG, Fuchs RL, Mutz J-N, Holden LR, George B, Olson T. 2000. Glyphosate-tolerant corn: The composition and feeding value of grain from glyphosate-tolerant corn is equivalent to that of conventional corn (Zea mays L.). *Journal of Agricultural and Food Chemistry* 48:2305-12.

Siehl D. 1997. Inhibitors of EPSP synthase, glutamine synthetase and histidine synthesis. *Reviews in Toxicology* 1:37-68.

Sikorski JA, Gruys KJ. 1997. Understanding glyphosate's molecular mode of action with EPSP synthase: evidence favoring an allosteric inhibitor model. *Accounts of Chemical Research* 30:2-8.

Singh BK, Shaner DL. 1998. Rapid determination of glyphosate injury to plants and identification of glyphosate-resistant plants. *Weed Technology*:527-30.

Snedecor G, Cochran W. Statistical Methods. *The Iowa State University Press, Ames*; 1980. p. 507.

Thelen KD, Jackson EP, Penner D. 1995. The basis for the hard-water antagonism of glyphosate activity. *Weed Science*:541-8.

Urbano JM, Borrego A, Torres V, Leon JM, Jimenez C, Dinelli G, Barnes J. 2009. Glyphosate-resistant hairy fleabane (Conyza bonariensis) in Spain. *Weed Technology* 21:396-401.

VanGessel MJ, Scott BA, Johnson QR, White-Hansen SE. 2009. Influence of glyphosate-resistant horseweed (Conyza canadensis) growth stage on response to glyphosate applications. *Weed Technology* 23:49-53.

Walker S, Boucher L, Cook T, Davidson B, McLean A, Widderick M. 2012. Weed age affects chemical control of Conyza bonariensis in fallows. *Crop Protection* 38:15-20.

Werth J, Walker S, Boucher L, Robinson G. 2010. Applying the double knock technique to control Conyza bonariensis. *Weed Biology and Management* 10:1-8.

Williams GM, Kroes R, Munro IC. 2000. Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. *Regulatory Toxicology and Pharmacology* 31:117-65.

Woodburn AT. 2000. Glyphosate: production, pricing and use worldwide. *Pest Management Science* 56:309-12.

Wu H, Walker S, Rollin MJ, Tan DKY, Robinson G, Werth J. 2007. Germination, persistence, and emergence of flaxleaf fleabane (Conyza bonariensis [L.] Cronquist). *Weed Biology and Management* 7:192-9.

Wu H, Zhu X. Who's who in the fleabane (Conyza spp.) family? Nineteenth Australasian Weeds Conference 2014.

Young BG. 2009. Changes in Herbicide Use Patterns and Production Practices Resulting from Glyphosate-Resistant Crops.

Zobiole LHS, Junior RSdO, Kremer RJ, Muniz AS, Junior AdO. 2010. Nutrient accumulation and photosynthesis in glyphosate-resistant soybeans is reduced under glyphosate use. *Journal of Plant Nutrition* 33:1860-73.

APPENDIX A

Table A1. Abbreviated ANOVA table for the screening experiment (Figure 3.1)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Dose	5	4663.195	932.639	802.20	<.001
Location	11	104.009	9.455	8.13	<.001
Dose.Location	55	239.550	4.355	3.75	<.001
Residual	144	167.415	1.163		
Total	215	5174.169			

Variate: Fresh mass

d.f. - Degrees of freedom

s.s - Sums of squares

m.s. – Mean squares

v.r. - Variance ratio or F value

F pr. - F probabillity

Table A2. Abbreviated ANOVA table for the growth stage experiment at the first growth stage (Figure 3.5)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Dose	5	686.519	137.304	78.34	<.001
Location	5	44.306	8.861	5.06	<.001
Dose.Location	25	156.139	6.246	3.56	<.001
Residual	144	252.378	1.753		
Total	179	1139.342			

Variate: Fresh mass

Table A3. Abbreviated ANOVA table for the growth stage experiment at the second growth stage (Figure 3.7)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Dose	5	1159.559	231.912	67.10	<.001
Location	5	34.028	6.806	1.97	0.087
Dose.Location	25	213.322	8.533	2.47	<.001
Residual	144	497.719	3.456		
Total	179	1904.628			

Variate: Fresh mass

Table A4. Abbreviated ANOVA table for the second screening experiment (Figure 3.9)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Dose	5	1159.559	231.912	67.10	<.001
Location	5	34.028	6.806	1.97	0.087
Dose.Location	25	213.322	8.533	2.47	<.001
Residual	144	497.719	3.456		
Total	179	1904.628			

Variate: Fresh mass

Table A5. Abbreviated ANOVA table for the temperature experiment repeated three times (Figure 3.11)

Source of variation	d.f.(m.v.)	s.s.	m.s.	v.r.	F pr.
Exp stratum	2	2.64425	1.32212	58.64	
Exp.TEMP stratum					
Temperature	2	0.21462	0.10731	4.76	0.088
Residual	4	0.09018	0.02254	1.38	
Exp.TEMP.Rep.DOSE stratum					
Dose	4	4.95735	1.23934	75.84	<.001
Dose.Temperature	8	0.40664	0.05083	3.11	0.002
Residual	292 (2)	4.77196	0.01634		
Total	312 (2)	12.88356			

Variate: Fresh mass

Table A6. Abbreviated ANOVA table for the mancozeb experiment (Figure 3.13)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	5	112.9153	22.5831	32.93	<.001
Residual	54	37.0340	0.6858		
Total	59	149.9493			

Variate: Fresh mass

APPENDIX B

Table B1. Tukey's 95% confidence interval for the screening experiment. Dose x location effect. Means with the same letters do not differ significantly

Dose	Location	Mean	
0	Gariep	17.797	a
0	Oudtshoorn	16.8	ab
0	Ventersdorp	15.167	abc
0	Trompsburg	14.26	abcd
0	Kroonstad	13.3	bcd
0	Aberdeen	13.16	bcd
0	Edenburg	13.143	bcd
0	Middelburg	12.76	cd
0	Wilderness	12.42	cde
0	Hatfield	12	cde
0	Buffelsbaai	10.6	de
0	DeRust	8.767	e
225	Oudtshoorn	4.433	f
900	Oudtshoorn	4.2	fg
450	Oudtshoorn	3.933	fgh
225	DeRust	3.9	fgh
900	DeRust	3.383	fgh
450	DeRust	3.037	fgh
225	Middelburg	2.907	fgh
225	Kroonstad	2.223	fgh
225	Wilderness	2.2	fgh
225	Trompsburg	2.14	fgh
225	Buffelsbaai	1.957	fgh

225	Hatfield	1.833	fgh
225	Ventersdorp	1.797	fgh
225	Gariep	1.567	fgh
225	Edenburg	1.563	fgh
450	Buffelsbaai	1.513	fgh
450	Middelburg	1.33	fgh
1800	Oudtshoorn	1.033	fgh
450	Kroonstad	0.99	fgh
1800	Wilderness	0.903	fgh
1800	DeRust	0.863	fgh
450	Ventersdorp	0.84	fgh
225	Aberdeen	0.787	fgh
450	Hatfield	0.667	gh
450	Trompsburg	0.623	gh
450	Gariep	0.587	gh
450	Wilderness	0.58	gh
450	Edenburg	0.57	gh
450	Aberdeen	0.527	gh
900	Middelburg	0.417	h
3600	Oudtshoorn	0.4	h
900	Hatfield	0.333	h
3600	DeRust	0.3	h
900	Ventersdorp	0.28	h
900	Trompsburg	*0.26*	*h*
3600	Gariep	0.26	h
1800	Edenburg	0.257	h
1800	Gariep	0.25	h
900	Edenburg	0.243	h

900	Kroonstad	0.24	h
900	Aberdeen	0.24	h
1800	Ventersdorp	0.237	h
1800	Hatfield	0.233	h
1800	Trompsburg	0.23	h
1800	Middelburg	0.23	h
3600	Edenburg	0.223	h
900	Gariep	0.22	h
1800	Aberdeen	0.22	h
3600	Trompsburg	0.22	h
1800	Kroonstad	0.22	h
3600	Ventersdorp	0.217	h
3600	Middelburg	0.217	h
900	Wilderness	0.213	h
900	Buffelsbaai	0.213	h
3600	Wilderness	0.213	h
1800	Buffelsbaai	0.213	h
3600	Aberdeen	0.21	h
3600	Kroonstad	0.207	h
3600	Hatfield	0.2	h
3600	Buffelsbaai	0.2	h

L.S.D. = 1.7401

Table B2. Tukey's 95% confidence interval for the growth stage experiment at the first growth stage. Dose x location effect. Means with the same letters do not differ significantly

Dose	Location	Mean	
0	Middelburg	7.532	a
0	Hatfield	7.41	ab
0	Gariep	7.198	ab
225	Hatfield	6.626	abc
0	Oudtshoorn	6.568	abc
0	Wilderness	6.364	abc
0	DeRust	5.754	abcd
900	Oudtshoorn	5.592	abcd
225	Gariep	5.528	abcd
225	Wilderness	5.502	abcd
450	Oudtshoorn	5.318	abcd
450	Wilderness	4.976	abcde
225	Middelburg	4.92	abcde
225	DeRust	4.704	abcdef
225	Oudtshoorn	4.51	abcdefg
450	Middelburg	4.352	abcdefg
900	DeRust	4.352	abcdefg
450	DeRust	4.278	abcdefg
1800	Oudtshoorn	4.206	bcdefg
900	Middelburg	3.676	cdefgh
450	Gariep	2.814	defgh
3600	DeRust	2.756	defgh
450	Hatfield	1.896	efgh
900	Gariep	1.886	efgh
900	Wilderness	1.824	efgh

1800	Wilderness	1.81	efgh
1800	DeRust	1.596	fgh
900	Hatfield	1.52	fgh
3600	Oudtshoorn	1.424	fgh
3600	Wilderness	1.286	gh
1800	Hatfield	0.842	h
1800	Middelburg	0.81	h
1800	Gariep	0.75	h
3600	Hatfield	0.656	h
3600	Gariep	0.638	h
3600	Middelburg	0.498	h

L.S.D. = 1.655

Table B3. Tukey's 95% confidence interval for the growth stage experiment at the second growth stage. Dose x location. Means with the same letters do not differ significantly

Dose	Location	Mean	
0	Hatfield	13.984	a
0	Gariep	12.672	ab
225	Hatfield	12.478	ab
0	Middelburg	12.348	ab
225	Gariep	12.29	ab
0	Wilderness	11.574	abc
450	DeRust	11.544	abc
0	Oudtshoorn	11.174	abc
225	Middelburg	11.008	abc
225	DeRust	10.928	abc
225	Wilderness	10.8	abc
450	Gariep	10.796	abc

450	Oudtshoorn	10.644	abc
225	Oudtshoorn	10.598	abc
900	Hatfield	10.41	abcd
0	DeRust	10.26	abcd
450	Middelburg	9.722	abcde
1800	Oudtshoorn	9.416	abcdef
900	Wilderness	9.34	bcdef
900	Oudtshoorn	9.268	bcdef
450	Hatfield	9.048	bcdef
450	Wilderness	8.682	bcdef
900	Middelburg	8.344	bcdefg
1800	DeRust	8.274	bcdefg
900	DeRust	8.234	bcdefg
900	Gariep	8.128	bcdefg
3600	Oudtshoorn	7.47	cdefg
1800	Wilderness	7.24	cdefgh
1800	Middelburg	5.894	defgh
1800	Hatfield	5.376	efgh
1800	Gariep	5.332	efgh
3600	Wilderness	5.178	efgh
3600	DeRust	5.058	fgh
3600	Hatfield	3.898	gh
3600	Gariep	3.816	gh
3600	Middelburg	2.672	h

L.S.D. = 2.324

Table B4. Tukey's 95% confidence interval for the second screening experiment. Location effect. Means with the same letters do not differ significantly

Location	Mean	
DeRust3	3.201	a
Oudtshoorn	3.147	a
DeRust1	2.622	a
DeRust2	1.77	b

L.S.D. = 0.4596

Table B5. Tukey's 95% confidence interval for the second screening experiment. Dose effect. Means with the same letters do not differ significantly

Dose	Mean	
0	4.527	a
225	3.258	b
450	2.51	bc
900	2.092	c
1800	1.882	c
3600	1.84	c

L.S.D. = 0.5629

Table B6. Fisher's protected least significance test for the temperature experiment. Dose x temperature interaction. Means with the same letters do not differ significantly

Dose	Temperature (^{o}C)	Mean	
0	22-27	0.5011	a
0	8-13	0.4228	a
0	15-20	0.3858	ab
225	22-27	0.2611	bc
450	8-13	0.227	cd
900	8-13	0.212	cd
900	15-20	0.1945	cde
3600	8-13	0.1522	cdef
1800	8-13	0.1516	cdef
450	15-20	0.1497	cdef
900	22-27	0.1102	def
1800	22-27	0.0951	def
1800	15-20	0.0619	ef
3600	22-27	0.0586	ef
3600	15-20	0.055	f

L.S.D. = 0.08202

Table B7. Tukey's 95% confidence interval for the mancozeb experiment. Means with the same letters do not differ significantly

Treatment	Mean	
Control	7.7	a
Mn_Gly	5.52	b
Zn_Gly	5.27	bc
GlyMan	4.32	cd
ManGly	3.8	d
Glyphosate	3.67	d

L.S.D. = 0.743