A quick test for glyphosate resistance in annual ryegrass

Aaron Preston[1, 2], Joseph Moore[1, 2], Jim Pratley[1, 2] and Gabriel Thelen[1]

[1] School of Agricultural and Wine Sciences, Faculty of Science, Charles Sturt University, Boorooma Street, Wagga Wagga, NSW 2678, apreston@csu.edu.au
[2] Graham Centre for Agricultural Innovation, Charles Sturt University, Pugsley Place, Wagga Wagga, NSW 2650

Abstract
Annual ryegrass is the most economically damaging winter crop weed in Australia. Effective control measures are key to minimising management costs and yield loss. Glyphosate is an effective herbicide used in post-emergent control of annual ryegrass, although continuous use has led to the evolution of herbicide resistance. Early and rapid identification of resistant plants facilitates control measures for resistant populations and helps minimise their spread. Some methodologies have been developed for the quick testing of glyphosate resistance in *Lolium* spp. The aim of this study was to assess a further methodology, using un-germinated and pre-germinated seed, for speed, simplicity and accuracy in determining phenotype. Four biotypes of annual ryegrass, two glyphosate resistant and two susceptible, were selected to evaluate the capability of the test to segregate between resistant and susceptible populations. The performance of the pre-germinated assay was compared with an assay using un-germinated seed. Seeds were placed on filter paper within Petri dishes containing glyphosate concentrations of 0, 2.5, 10 and 40 mg ae/L. Dishes were then placed in a growth incubator. After 7 days, root lengths were measured. Both assays were able to differentiate between resistant and susceptible biotypes. Differences in root length inhibition were greatest amongst samples with lower concentrations of glyphosate, with 2.5mg ae/L providing the greatest segregation for un-germinated seed (43% inhibition for susceptible versus resistant biotypes). The higher dosage of 10mg ae/L provided greater phenotype discrimination in pre-germinated seed (61% inhibition for susceptible versus resistant biotypes). Both pre-germinated and un-germinated seed assays provided a quick and simple method to identify glyphosate resistance in annual ryegrass.

Key words
Herbicide resistance testing, *Lolium rigidum*

Introduction

Herbicide resistance in annual ryegrass (*Lolium rigidum*) is an ongoing agricultural challenge, the species having already evolved resistance to 11 different groups and sub-groups of herbicides (Heap, 2014). The rapid adaptation of ryegrass ensures that we too must adapt in order to maintain effective weed management and long term control. Herbicide resistance testing plays a vital part in ryegrass and herbicide management, helping to determine whether errant ryegrass plants are herbicide survivors or application escapees. The most common methods for herbicide resistance testing involve variations of testing seed or pre-grown tillers in either glasshouse trials or laboratory based assays. Both have advantages and short comings: glasshouse trials are considered to be better representation of field conditions but are resource intensive; laboratory assays (Petri dish, enzymatic determination etc.) are generally faster, but can require extensive expertise and/or costly equipment.

Previous studies have evaluated herbicide resistance assays with simple Petri dish methods for glyphosate, an economically important herbicide, on *Lolium spp*. (Ghanizadeh, Harrington, James, & Woolley, 2014; Neve, Sadler, & Powles, 2004) using un-germinated seed in various media and concentrations. This study evaluated resistance testing annual ryegrass biotypes with both un-germinated and pre-germinated seed on filter paper.

Method and materials:

Annual ryegrass biotypes were selected from Charles Sturt University herbicide resistance testing service seed collection. Four biotypes, two with known glyphosate resistance history and two with known susceptibility were selected. Two assay methods were trialled, using pre-germinated and un-germinated seed.

Un-germinated seed

The surface of the seed was sterilised using a modified version of the procedure used by Sauer (Sauer & Burroughs, 1986). Seed was fully immersed in a 2% bleach solution for 2 min before thoroughly rinsing with deionised water and then dried in a laminar flow. This protocol limits interference from microorganism growth in the bioassay. Ten seeds from each biotype were selected and placed within 90mm plastic Petri dishes lined with filter paper (Advantech No. 2). A concentration range was selected that would capture a discriminating dosage of glyphosate. A similar test on Italian and perennial ryegrass used a dosage range of 0 to 320mg ae/L although differing concentration ranges were used for resistant and susceptible populations (0-320 mg ae/L and 0-40mg ae/L, respectively)(Ghanizadeh, et al., 2014). Adhering to the aim of developing a cheap and simple test, one concentration was range (0- 40mg ae/L) was tested. Four mL of glyphosate solution (0, 2.5, 10 and 40mg ae /L) were applied directly to the filter paper. Each treatment was replicated 4 times and dishes arranged in a randomised block design. Petri dishes were sealed with parafilm and stored in a growth chamber (12hr 15/25°C and day/night cycle). Root length was measured after seven days.

Pre-germinated seed

Pre-germinated seed was treated in the same manner as un-germinated seed, undergoing surface sterilisation and drying before being transferred to a Petri dish lined with filter paper. Four mL of deionised water were added to each Petri dish before being sealed with parafilm and placed in a growth incubator (12hr 15/25°C and day/night cycle) for 5 days. Once germinated, the assay was repeated in the same manner as for the un-germinated seed.

Results

Un-germinated assay

Root length was measured and calculated as a percentage of the growth of the control. All biotypes experienced greater root length inhibition as glyphosate dosage increased. An exception to this is the response of the resistant biotypes, which had an increase of root length at 2.5mg ae/L. This response may be the results of hormesis, an effect common in sub-lethal herbicide application (Belz & Duke, 2014). The two resistant biotypes had root lengths inhibited the least of the four biotypes tested. The most informative dosage (dosage that provided the greatest difference in root inhibition) was 2.5mg ae/L (Figure 1A). At this dosage, there is 43% difference in root inhibition between resistant and susceptible biotypes.

Pre-germinated assay

Root length inhibition was calculated in the same manner as the un-germinated assay. Resistant biotypes had the lowest amount of root inhibition of the four biotypes tested. The greatest difference in root inhibition between resistant and susceptible biotypes was at 10 mg ae/L glyphosate (Figure 1B). At this dosage, there is 61% difference in root inhibition between resistant and susceptible biotypes.

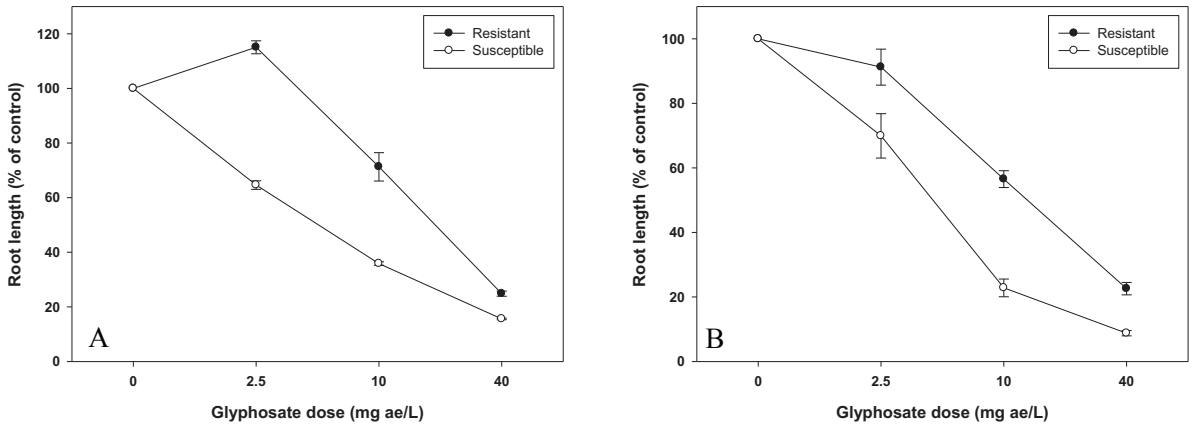

Figure 1: Effect of glyphosate concentration on root length inhibition (as percentage of control). Resistant biotypes were inhibited less than susceptible, A) Un-germinated seed assay, greatest difference between biotypes at 2.5mg ae /L. B) Pre-germinated seed assay, greatest difference between biotypes at 10mg ae /L. Data are the means of biotypes with 4 replicates and 10 seeds per replicates, measured after 7 days, error bars are standard error of the mean.

Discussion

This study set out to determine whether a simple Petri dish assay could provide a quick, simple, cheap, accurate and more informative method of herbicide resistance testing on annual ryegrass using pre-germinated seed. Biotype root inhibition was assessed across four biotypes with known resistance or susceptibility to glyphosate with two assay methods. Differences between biotypes of the same phenotype were not significant ($P>0.05$) and so data were arranged as the mean of the two biotypes. Previously reported resistant biotypes displayed less inhibition of root length than susceptible biotypes in both assays. Susceptible biotypes were more sensitive at low to midrange concentrations of glyphosate in both assays, providing a greater root length differential between phenotypes. A significant difference between resistant and susceptible biotypes was found for both assays ($P<0.05$). Un-germinated seed showed greater phenotype separation at 2.5 mg ae/L dosage with a 43% difference in root inhibition, whereas the higher dosage 10mg ae/L provided greater difference in pre-germinated seed with 61% difference in root inhibition between resistant and susceptible biotypes. An analysis of variance between both assays showed that there was no significant difference of root inhibition between biotypes in un-germinated and pre-germinated assays although it is likely that seed is more vulnerable to the inhibitory effects of glyphosate at the earlier growth stage in the un-germinated seed due to a decreased metabolic rate, resulting in a more pronounced effect at the lower dose. The observed hormesis response in the resistant biotypes in the un-germinated assay may be due to the earlier exposure of a biotype with resistance mechanisms triggered at this low dose responding more robustly than a more developed organism.

Each of the methods trialled had inexpensive materials and labour costs, totalling approximately $40 per biotype, and was completed in 1-2 weeks. This is significantly cheaper than the cost of glasshouse trials which can be in excess of $200, and required few specialised equipment or training. Conventional glasshouse trials do provide an accurate assessment of resistance representative of field conditions, and have the ability to test additional herbicides and different application methods. For a particular herbicide such a test may prove valuable for earlier diagnosis. Further refinement of methodology and additional testing would assess the veracity of these results.

Conclusion

Two seed assays were trialed for detection of glyphosate resistance in annual ryegrass. Both methods were able to segregate biotypes into their respective phenotypes consistent with glasshouse trials. Overall each assay method provided accurate results about individual biotype herbicide tolerance, although additional testing is required. Each method offers a cheap, low tech and simple test for glyphosate resistance.

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