# A new method for determination of herbicide resistance: using Diversity Array Technology to determine annual ryegrass resistance to trifluralin (Group D herbicide)

Aaron Preston[1,2], Jim Pratley[1], Joseph Moore[1,2] and Andrzej Kilian[3]
[1] Graham Centre for Agricultural Innovation, Locked Bag 588, Wagga Wagga, NSW 2678, Australia
[2] School of Agricultural and Wine Sciences, Faculty of Science, Charles Sturt University, Boorooma Street, Wagga Wagga, NSW 2678, Australia
[3] Diversity Arrays Technology, Building 3 Level D, University of Canberra, Kirinari Street, Bruce, ACT 2617, Australia
(apreston@csu.edu.au)

**Summary** Annual ryegrass (*Lolium rigidum* Gaud.) is considered the worst winter crop weed of Australia. This is due in part to its propensity to evolve resistance to herbicides, having evolved resistance to 11 different herbicide groups. As levels of herbicide resistance in Australia are increasing, new methods are needed to assist in the early identification and treatment of resistant weeds. Conventional methods for the determination of resistance involve glasshouse pot trials, Petri dish assays or enzymatic determination, all of which consume considerable time and resources. A pilot study was conducted to determine the feasibility of utilising diversity arrays technology (DArT) as a faster and cheaper method for herbicide resistance testing. DArT is a high throughput genotyping technology which utilises microarrays for the discovery of genetic markers. The aim was to identify genetic markers in resistant and susceptible biotypes of annual ryegrass and to assess the ability of DArT to discriminate between herbicide resistant phenotypes.

**Keywords** Annual ryegrass, *Lolium rigidum*, herbicide resistance, trifluralin.

## INTRODUCTION

Annual ryegrass (*Lolium rigidum* Gaud.) is the most economically damaging weed in southern Australian cropping systems (Yu *et al.* 2004) costing $139.7 million in yield losses, and is a significant factor in the $3.7 billion spent on herbicides and herbicide application in Australia each year (ABS 2008, Jones *et al.* 2005). It was introduced into Australia in the late 18th century as a pasture plant (Kloot 1983). It rapidly adapted to Australian conditions and became a dominant plant in the southern landscape. As it has the ability to out compete many crop species, several methods are utilised in its control, the foremost being herbicide application. This is problematic as *L. rigidum* has exhibited an acute ability to evolve resistance to some herbicides within four years. Consequently, annual ryegrass has resistance to 11 different herbicide modes of action in Australia (Heap 2014).

Several tests have been developed for the determination of herbicide resistance. The predominant methods fall into three categories: glasshouse trials, Petri dish analysis or enzymatic determination. The conventional method is resistance testing by glasshouse trials, in which seed is collected from plants with suspected herbicide resistance. Once seed dormancy is broken, the seed is either sprayed with herbicide immediately (pre-emergent) or is allowed germinate and develop (post-emergent), depending upon the activity of the herbicide (Boutsalis and Broster 2006). This process can take up to nine months, consuming valuable time and resources. Time is a critical factor, as fast herbicide resistance detection allows for earlier control and more effective control options to be employed. A quicker method for resistance testing was developed by Syngenta, which utilises whole plants collected from affected fields. Plants are then trimmed, separated into tillers and grown in pots. After one week, these plants are sprayed with the selected herbicide, and assessed after 21 days. This method reduced the analysis time to approximately four weeks, however it is not suitable for pre-emergent herbicides (Boutsalis 2001).

Petri dish analysis and enzymatic determination are faster methods for the determination of resistance. A Petri dish method developed by Syngenta utilises collected seedlings and plates them on herbicide enriched agar. This resulted in a 10 day determination of resistance status to Group A and B herbicides (ACCase and ALS inhibitor herbicides) (Kaundun *et al.* 2011).

Enzymatic determination typically relies on the extraction of an enzyme or enzyme by-product affected by herbicides. Analysis of the concentration of these enzymes within the plant tissue indicate the resistance status. Rapid diagnostic methods for the determination of resistance using enzymatic response have been developed for Group B herbicides, with a reported turnaround of 2 hours (Gerwick *et al.* 1993).

Herbicide resistance is due to two mechanisms, target site (TS) and non-target site (NTS) resistance. TS resistance is the results of a modification of

the activity site of the plant that prevents herbicide interaction. NTS resistance is due to the evolution of a metabolic or translocation processes that limits herbicide interaction. Herbicide resistant plants can have either or mixture of these mechanisms.

Fast methods for the diagnosis of resistance have been determined, although high costs or the laborious nature of some tests make them unsuitable for large-scale application. These tests provide limited information about the type of resistance present. The highly selective nature of these tests means that they lack the ability to determine the resistance of the plant to the non-selected herbicides. Exact determination of the form of resistance can be difficult; a difference in a single nucleotide substitution can cause wide variation of the efficacy of herbicides within the same group (Corbett *et al.* 2006).

Methods for the precise diagnosis of herbicide resistance have been explored utilising genetic technologies. An advantage of genetic testing is it allows for the evaluation of the type of mutation present, allowing for an exact determination of the type of resistance. These tests identify fragments of DNA (genetic markers) that are linked to resistance. The majority of these tests use restriction fragment length polymorphism (RFLP), polymerase chain reaction (PCR) and DNA sequencing (Corbett *et al.* 2006). RFLP and PCR have been used successfully for TS resistance for multiple herbicides groups. Due to the complexity and diversity of modes of resistance, a method to detect all forms of resistance has yet to be developed (DéLye *et al.* 2011, Jaccoud *et al.* 2001).

**Diversity Arrays Technology (DArT)** DArT is a genetic technology that allows for the rapid identification of thousands of genetic markers. Although it has been successfully developed for wheat, rice and barley, it has not previously been employed on weed species. As it utilises a genome-wide profiling system it allows for broad analysis of complex polygenic traits, like herbicide resistance. DArT also offers a high-throughput analysis with low cost marker development, without expensive DNA sequencing, providing greater genetic information, faster and cheaper than other methods.

The aim of this study was to identify genetic markers linked to resistance to herbicides in biotypes of annual ryegrass, and to assess the ability of DArT to discriminate between herbicide resistant and susceptible phenotypes.

## MATERIALS AND METHODS

Biotypes resistant to Group D (microtubule formation inhibition) herbicides were selected from Charles Sturt University's Herbicide Resistance Testing Services' seed store. To capture a wide genetic spread, nine biotypes were collected: five from South Australia, two from Western Australia and two from Victoria. A biotype with no herbicide application history was selected to represent a Group D susceptible population.

To confirm Group D resistance, seeds from resistant biotypes were placed on soil in pots and sprayed with trifluralin, a Group D herbicide. Trifluralin was applied at 384 g a.i. $ha^{-1}$ with a laboratory moving boom sprayer equipped with T-jet fan nozzles at a speed of 1 $ms^{-1}$. Output from the sprayer was calibrated at 106 L $ha^{-1}$ at a pressure of 250 kPa. After spraying, the seed was covered with a thin layer of soil to a depth of 5 mm. Pots were placed in a temperature controlled glasshouse and watered as required. After 21 days any emerged plants were deemed resistant. Plants were allowed to develop until sufficient leaf material was available for DNA extraction.

Fresh leaf material was collected from each plant and ground by mortar and pestle under liquid nitrogen before undergoing DNA extraction using a DNeasy Plant Mini Kit (Qiagen, Australia). Extracted DNA was submitted to DArT for DNA complexity reduction, genetic marker development and proprietary data analysis.

## RESULTS

Using DArT, genetic marker associations were developed using the resistance data from the known resistant and susceptible biotypes. Two variants of DArT marker data were utilised, binary scoring and continuous array intensity. When continuous array data on all 7680 array probes were applied, nearly 100 % of the phenotypic value (resistance) was explained by the continuous array signal. In contrast, only 30% of the phenotype could be explained by binary scores.

Analysis was also completed to check how 'complex' the resistance phenotype was by plotting cumulative value of PAVE (a measure of the significance of the marker for the character) for the models of increasing complexity (in terms of number of markers). The continuous array intensity scores provided 70–90% discriminatory power for phenotype with very few array features (<100). Binary scores failed to provide discriminatory power greater than 30% of the phenotype (Figure 1).

## DISCUSSION

DArT provided discrimination between resistant and susceptible biotypes sufficient to establish proof of concept for the use of DArT as a tool for the rapid identification of Group D resistance in annual ryegrass. This analysis was completed in three weeks, providing a fast and accurate method for the determination

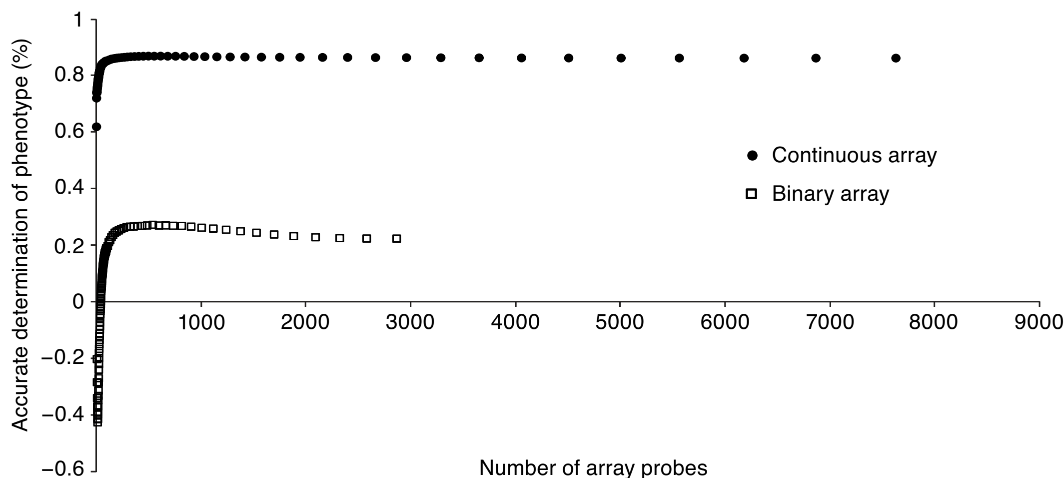


**Figure 1.** Accuracy of phenotype determination for Continuous array (black circle) and Binary array (white square) vs number of array probes used.

of resistance. In practice, this technology could be used to verify whether emerging ryegrass plants are resistant to trifluralin or escaped application. This would allow for informed management strategies to be employed such as the need to rotate herbicides for the following season.

Further analysis with greater numbers of samples capturing a wide genomic range would enhance the discriminatory power of the analysis. The exact mechanism of resistance (TS/NTS) of the plants was not determined. Future study could examine whether the identified markers can discriminate between TS and NTS resistance. This could be achieved by analysing biotypes with known TS and NTS resistance mechanisms. Markers developed from these samples would provide this discrimination and assist in the characterisation of resistance. Due to the complexity of NTS resistance, such analysis would likely require greater marker density and DNA sequencing. This could be achieved with DArTseq, a technology similar to DArT that has the ability to sequence markers. Future studies could also examine the differences in the detection of resistance between DArT and traditional testing methods, examining costs and ease of use and accuracy.

Analysis of biotypes with resistance to other herbicide groups may allow for the identification of genetic markers linked to additional modes of action. This would allow for simultaneous resistance testing to multiple forms of herbicide resistance using DArT. Markers identified as significant could be sequenced allowing greater understanding of genetic networks underlying the evolution of herbicide resistance in *L. rigidum*.

## ACKNOWLEDGMENTS

We wish to thank GRDC for scholarship top-up funding and Dr John Broster for his advice and assistance.

## REFERENCES

ABS (2008). Natural resource management report on Australian Farms. http://www.abs.gov.au/ausstats/abs@.nsf/cat/4620.0 (retrieved 21 June 2013).

Boutsalis, P. (2001). Syngenta Quick-Test: a rapid whole-plant test for herbicide resistance. *Weed Technology* 15, 257-63.

Boutsalis, P. and Broster, J (2006). Herbicide resistance testing of *Lolium rigidum* by commercial institutions. Proceedings of the 15th Australian Weeds Conference, eds C. Preston, J.H. Watts and N.D. Crossman, pp. 448-90. (Weed Management Society of South Australia, Adelaide).

Corbett, C.A. and Tardif, F.J. (2006). Detection of resistance to acetolactate synthase inhibitors in weeds with emphasis on DNA-based techniques: a review. *Pest Management Science* 62, 584-97.

DéLye, C., Pernin, F. and Michel, S. (2011). 'Universal' PCR assays detecting mutations in acetyl-coenzyme A carboxylase or acetolactate synthase that endow herbicide resistance in grass weeds. *Weed Research* 51, 353-62.

Gerwick, B.C., Mireles, L.C. and Eilers, R.J. (1993). Rapid diagnosis of ALS/AHAS-resistant weeds. *Weed Technology* 7, 519-24.

Heap, I. (2014). The international survey of herbicide resistant weeds. www.weedscience.com (retrieved 20 June 2013).

Jaccoud, D., Peng, K., Feinstein, D. and Kilian, A. (2001). Diversity arrays: a solid state technology for sequence information independent genotyping. *Nucleic Acids Research* 29.

Jones, R.E., Vere, D.T., Alemseged, Y. and Medd, R.W. (2005). Estimating the economic cost of weeds in Australian annual winter crops. *Agricultural Economics* 32, 253-65.

Kaundun, S.S., Hutchings, S.J., Dale, R.P., Bailly, G.C. and Glanfield, P. (2011). Syngenta 'RISQ' test: a novel in-season method for detecting resistance to post-emergence ACCase and ALS inhibitor herbicides in grass weeds. *Weed Research* 51, 284-93.

Kloot, P. (1983). The genus *Lolium* in Australia. *Australian Journal of Botany* 31, 421-35.

Yu, Q., Cairns, A. and Powles, S.B. (2004). Paraquat resistance in a population of *Lolium rigidum*. *Functional Plant Biology* 31, 247-54.