# HERBICIDE RESISTANCE IN ANNUAL BLUEGRASS (*Poa annua* L.) AND ITS MANAGEMENT

by

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# TABLE OF CONTENTS

## ABSTRACT

Annual bluegrass (*Poa annua* L.) is the most problematic weed of sports turf, particularly in temperate climates. Repeated use of PRE and POST-emergent herbicides in turf has resulted in evolution of resistance in this weed species. This study investigated the extent of herbicide resistance to six different herbicide modes of action used to control *P. annua* in turf in Australia. The herbicide resistance status was determined in 31 populations (18 from Victoria, 6 from NSW and 7 from SA) collected from golf courses in south-eastern Australia in 2017. The majority of populations were resistant to POST application *ALS* inhibitors (97%), PSII inhibitors (94%), endothall (100%), *ACC*ase inhibitors (94%), and inhibitors of microtubule assembly (81%), whereas, only 7% of populations were resistant to PRE-application of inhibitors of microtubule assembly (propyzamide) and 13% of populations were resistant to POST application of *EPSPS* inhibitor (glyphosate). An additional population (HT) collected from a home garden in Adelaide, South Australia was confirmed resistant to glyphosate only. Four multiple resistant populations were selected for further research (P18, P27, P262-16 and P413-17) to determine the level of resistance, the mechanism of resistance and inheritance of resistance.

A dose-response study showed that these four populations had a high level of resistance to the *ALS* inhibitors rimsulfuron (>19 fold) and foramsulfuron (>56 fold), but medium to low level of resistance to endothall >7-fold, the *ACC*ase inhibitor pinoxaden >4.3-fold, the PSII inhibitor simazine, propyzamide and glyphosate. Two different target-site mutations in the *ALS* gene were identified in these populations, with Pro197Ser substitution in P18 and a Trp574Leu substitution in the other populations (P27, P262-16 and P413-17). A target-site mutation (Ile1781Leu) was identified in the *ACC*ase gene in all four resistant populations of *P. annua*. However, target site mutations were not identified in the *psbA* gene, *EPSPS* or *α-tubulin* gene.

The population HT collected from a home garden in Adelaide, South Australia was 2-fold resistant to glyphosate compared to S due to increased *EPSPS* gene copy number (1.7-9.3 fold) compared to the susceptible (S). *EPSPS* gene expression was also 3.5 to 16.3-fold higher in HT compared to the susceptible. Increase in *EPSPS* gene copy number was not observed in any other glyphosate-resistant population. Other common mechanisms of glyphosate resistance including target site mutations, reduced herbicide absorption or translocation and increased activity of aldo-keto reductase were not detected in any resistant population. The mechanism of resistance remains unknown in four glyphosate-resistant populations other than HT. Cross-pollination between one resistant population and the susceptible (P262-16♂ × S♀) was successful and an investigation of the progeny showed that the inheritance of glyphosate resistance appears to be controlled by a single, nuclear dominant gene.

Cross-pollination was successfully undertaken between two propyzamide resistant populations and a susceptible population. Inheritance of propyzamide resistance in P18 was due to a single dominant gene, but at least two genes contributed to resistance in P413-17. The differences in inheritance pattern between the resistant populations suggest different mechanisms might be involved in propyzamide resistance. No target-site mutation or other resistance mechanisms were identified in the resistant populations. Thus, the resistance mechanism to propyzamide remains unknown.

Field experiments conducted in spring and autumn between 2018 and 2020 on a multiple-herbicide (*ALS* inhibitors, *ACC*ase inhibitors, PSII inhibitors and endothall) resistant *P. annua* population in bermudagrass (*Cynodon dactylon*) turf at a golf course in South Australia. Amicarbazone was identified as the most effective alternative herbicide. Amicarbazone reduced the occurrence of *P. annua* by 98-100% followed by terbuthylazine >80%, indazaflam >63%, and pyroxasulfone >57%. Availability of multiple herbicides with different modes of action will enable greenkeepers to rotate herbicides in their management

program to minimize the risk of resistance. The findings reported here provide improved understanding of the herbicide resistance status of *P. annua* populations in Australia, their resistance mechanisms and has identified some herbicide options that could be used to control multiple resistant populations in turf.

## PUBLICATIONS ARISING FROM THIS THESIS

- Barua R, Boutsalis P, Malone J, Gill G and Preston C, Incidence of multiple herbicide resistance in annual bluegrass (*Poa annua* L.) across southeastern Australia. Weed Science , Volume 68 , Issue 4 , July 2020 , pp. 340–347, DOI https://doi.org/10.1017/wsc.2020.35.

- Barua R, Malone J, Boutsalis P, Gill G and Preston C, Inheritance and mechanism of glyphosate resistance in annual bluegrass (*Poa annua* L.). Pest Management Science. Submitted Paper

- Barua R, Malone J, Boutsalis P, Gill G and Preston C, Inheritance and mechanism of propyzamide resistance in multiple-resistant annual bluegrass (*Poa annua* L.). Pest Management Science. Submitted Paper

- Barua R, Boutsalis P, Kleemann S, Malone J, Gill G and Preston C, Alternative Herbicides for Controlling Herbicide-Resistant Annual Bluegrass (*Poa annua* L.) in Turf. Agronomy 2021, 11, 2148. https://doi.org/10.3390/ agronomy11112148

- Barua R, Boutsalis P, Malone J, Gill G and Preston C, Incidence of multiple herbicide resistance in annual bluegrass (*Poa annua* L.) across south-eastern Australia. Page 220 in the 27th Asia-Pacific Weed Science Society Conference 2019. Kuching, Sarawak, Malaysia.

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## ABBREVIATIONS

| | |
|---|---|
| a.e | acid equivalent |
| a.i | active ingredient |
| AGRF | Australian genome research facility |
| ACCase | acetyl CoA Carboxylase |
| ALS | acetoacetate synthase |
| ANOVA | analysis of variance |
| DAP | day after treatment |
| EPSPS | 5-enolpyruvylshikimate-3-phosphate synthase |
| $GR_{50}$ | herbicide dose required for control 50% growth reduction |
| HAT | hour after treatment |
| $LD_{50}$ | herbicide dose required for control of 50% of the plants |
| LSD | least significance difference |
| NCBI | national centre for biotechnology information |
| PCR | polymerase chain reaction |
| *psb*A | photosystem II |
| PBO | piperonyl butoxide |

| | |
|---|---|
| PRE | pre-emergent |
| POST | post-emergent |
| EPOST | early post-emergent |
| QPCR | quantitative polymerase reaction |
| R | resistant |
| RI | resistance index relative to sensitive biotype |
| S | susceptible |
| SME | standard error of mean |
| SNP | single nucleotide polymorphism |
| *α-tubulin* | alpha-tubulin |
| *β-tubulin* | beta-tubulin |

# CHAPTER 1: LITERATURE REVIEW

## 1.1 Introduction

Turfgrass usually refers to the sod created by plants that can be maintained at a height and density suitable for specific purposes. It can be characterized as visually appealing grassy landscapes that continue to operate as useful groundcover while being subjected to moderate pressure from foot traffic. Turfgrass is widely used for sports fields, golf courses, and lawn. Turfgrass offers several social and environmental benefits, such as reducing air pollution, noise and soil erosion reduction, regulating temperature, improving soil structure, and changing the landscape to a form appropriate for recreational activity (Fang and Ling, 2003; Hedblom et al., 2017; Wang et al., 2016). According to Milesi et al. (2005), 163,800 $km^2$ of turf is grown in the US. The economic importance of the US turfgrass and lawn care industry was estimated in 2005 to be $62.2 billion (Haydu et al., 2006). According to a recent report by Balmoral Group Australia, the economic value of turfgrass production was estimated to be $6.02 billion per year in Greater Melbourne and $5.32 billion per year in Greater Sydney (Anonymous, 2020).

Turfgrasses can be divided into two types based on the growing season: warm season and cool season grasses (De, 2017). Warm season grasses such as couch, kikuyu, buffalo, and zoysia are the most common varieties growing in warmer climatic regions with mild winter like northern Australia (De, 2017). In contrast, cool-season grasses, such as, ryegrass, fescue, Kentucky bluegrass, and bent grass, dominate turfs in Southern Australia where winter maximum temperature sometimes can get quite low (Riesterer et al., 2000). Warm season turfgrasses maintain green colour when the temperature is warm and loose its colour when the temperature is cooler, which is not the case for cool season grasses.

Weeds compete with the desirable turfgrass for moisture, nutrients, light and space and tend to reduce its growth and uniformity. Weeds also decrease the quality of the turf by creating

unsightly appearance from dead or bare patches and resulting in an uneven surface that affects the ball roll in sports turf. Turfgrass weeds can be classified into three types: annual, biennial, and perennial (Landschoot, 2006). Annual weeds are divided into two groups: summer annual (e.g. crabgrass and prostate knotweed etc.) and winter annual (annual bluegrass and common chick weed). Warm-season turfgrasses, such as Bermuda grass and zoysia grass are especially sensitive to annual bluegrass infestation in winter. Various cultural, mechanical, biological and/or chemical techniques are available for controlling weeds in turf; however, chemical control tends to be the most common practice due to the ease of use and low cost. In recent years however, the evolution of herbicide resistance particularly in annual bluegrass has reduced the number of herbicide options available to control this weed in turf.

### 1.2 Annual Bluegrass (*Poa annua*)

*Poa annua* L. (annual meadow grass also known as bluegrass or winter grass), originated from Europe and has spread all over the world. *P. annua* is a weed that occurs in cool-season turfgrasses belonging to the Poaceae family (Carroll et al., 2021). Mao and Huff (2012) and Wagner et al. (1999) stated that it is not only a common weed in golf courses, but also in gardens, lawns, pastures, urban parks, the margin of the streams, roadside, and coastal and wet forests. It is a self-pollinated species, but depending on environmental conditions up to 15% out-crossing can occur (Carroll et al., 2021). According to the survey by Weed Science Society of America (WSSA) conducted in the United States and Canada in 2020, *P. annua* ranked as 1$^{st}$ the most problematic weed in turfgrass systems and ranked as 2$^{nd}$ the most troublesome of all grass crops (Van Wychen, 2021). In Australia, it has been classified as an environmental weed in the states of Victoria, New South Wales and Western Australia (Anonymous, 2021b; Simon and Alfonso, 2011).

### *1.2.1 Biology and ecology of P. annua*

*P. annua* is an allotetraploid (2n=28) that derives from a cross between the diploids *P. supina* and *P. infirma* (Carroll et al., 2021; Darmency and Gasquez, 1997). Although, *P. annua* is usually considered an annual species, perennial biotypes do exist (Carroll et al., 2021; Huff et al., 2003; Yelverton, 2000). It can occur in a wide range of soil types and locations ranging from low altitude grasslands to mountain pastures. It has been identified in the UK at elevations up to 1200 m and at higher altitudes in the tropics (GISD, 2021; Holm et al., 1997). *P. annua* has a slightly creeping fibrous root system. The stem grows 15 to 25 cm long and is usually slightly flattened. The panicle is open and shaped like a triangle. The spikelet is awnless and there are usually 5 to 8 spikelets along the stalk. It blooms in early spring in the northern hemisphere and is typically day-neutral (Carroll et al., 2021). It occurs as a contaminant in grass seed crops and creates a problem for grass seed growers. This species becomes problematic due to its large seed bank. According to Cline (2002), annual biotypes develop seedheads in 46 days, while perennial biotypes need 65 days. Annual biotype seeds have dormancy that permits them to survive in the soil for more than a year (Beard, 1970). However, according to (GISD, 2021), seeds can survive in soil for up to 6 years. Due to seed dormancy, the species can escape environmental stress until conditions are favourable for its growth (Bogart and Beard, 1973; Carroll et al., 2021). The seeds are present as a seed bank in many agricultural soils. In the early 1980s, *P. annua* seed banks on Melbourne golf greens contained an average of 200,000 seeds per square meter (Ford, 2012). A single plant may generate a high number of seeds (1050-2250 seeds/plant), according to Holm et al. (1997). Although *P. annua* is a cool-season grass weed, it can germinate throughout the year if the conditions are favourable. Germination typically occurs from August to early May in the South-eastern United States and from late April to early October in Australia (Kaminski and Dernoeden, 2007). In general germination starts in late summer as soil temperature falls below 20°C (McCarty, 2001).

### *1.2.2 Flowering, photoperiod and vernalisation in P. annua*

The annual types of *P. annua* flower at all day lengths and mature in 3 to 4 months. However, perennial types flower more abundantly in short days and cooler environments (Netland, 1984; Wells, 1974). According to Koshy (1969), *P. annua* possess a remarkable ability to ripen viable seeds on panicles excised from the plant shortly after pollination (1 to 2 days after pollination). Annual biotypes of *P. annua* do not require vernalisation; whereas perennial biotypes do (Heide, 2001; Johnson and White, 1997).

Fig 1. The young seedling (a), flowers (b), and seeds (c) of *P. annua*

### *1.2.3 Introduction and geographical distribution of P. annua in Australia*

*P. annua* originated in Europe and it is now distributed worldwide, including Australia, and ranked in the top five of the most widely distributed plants of the world (Fenner, 2012). *P. annua* can grow in a wide range of climatic conditions from the Mediterranean through temperate to arctic-alpine (Warwick, 1979). It can occur in arable land, disturbed ground, grasslands and lawns. It is widely adapted to southern and eastern Australia, but also occurs in southern and western parts of Western Australia (Figure 1) and is regarded as an environmental weed in Victoria, New South Wales and Western Australia (Anonymous, 2021b; Simon and Alfonso, 2011). It has also been reported from the Northern Territory and some Australian island territories (Lord Howe Island, Norfolk Island, Macquarie Island and McDonald Island).

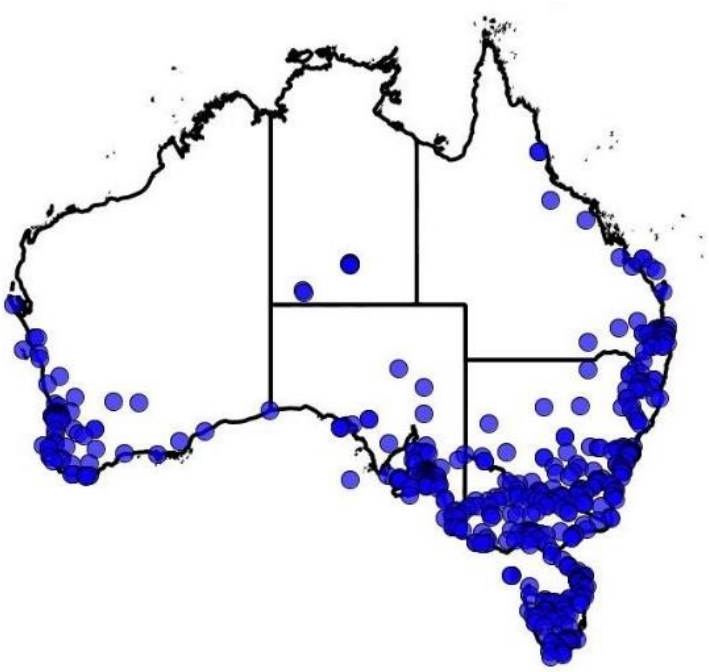

Fig. 2 Distribution of *P. annua* in Australia (https://avh.ala.org.au/occurrences/search?taxa=Poa+annua#tab_mapView)

### *1.2.4 The major problem of P. annua*

In established turfgrass stands, *P. annua* is a significant and problematic weed as it decreases the aesthetic value by forming unsightly seed heads at mowing heights as low as 6 mm. Because of its upright growth habit, which generates an uneven surface, it impairs ball roll in golf, lawn bowls, and other sports. This grass has a shallow root system and is sensitive to high-temperature stress (Hart and McCullough, 2007). During summer it dies, leaving unsightly dead and bare patches that reduce the aesthetic value of turf. However, emerging evidence suggests death is due to disease incidence and not heat stress (Chaves and Mitkowski, 2013). *P. annua* is a weed that not only affects turf but also temperate crops, and considered a weed in 38 crops across 80 countries according to Holm et al. (1997), including vegetables, cereals, sugar beet, potatoes, and orchards. It is frequently described as a significant weed species that can thrive in a variety of crops and environments (Table 1).

Table 1. Problem with *P. annua* in different countries

| ***P. annua* present as a weed in** | **Country** |
|---|---|
| Sports turf | UK (Raikes et al., 1994), Australia (J Neylan, 2016) and USA (Van Wychen, 2017; Vittum and Tashiro, 1987) |
| Vegetable crops | New South Wales, Australia (Greenhalgh and Michael, 1989) |
| Vines and soft fruit as a weed | The European Union (Clay, 1987) |
| Oilseed rape | North-east Scotland (Whytock and Carnegie, 1990) |
| Orchards | Portugal (Sa et al., 1989) |
| Grazing land | New Zealand (Wardle et al., 1994) |
| Most important monocotyledonous weeds | Pakistan (Hussain and Rashid, 1989; Riaz et al., 2009) |

According to Calhoun (2010), a large percentage of *P. annua* seed sheds and seeds will germinate in the first year following production. To eliminate *P. annua* from turf, the weed has to be completely controlled for several years until the seed bank is fully depleted. For the management of *P. annua*, POST herbicides and plant growth regulators (PGRs) are widely used (Vargas and Turgeon, 2004). The PGRs ethephon [(2-chloroethyl) phosphonic acid] and flurprimidol [a-(1-methylethyl)-a-[4-(trifluoromethoxy) phenyl]-5-pyrimidinemethanol] can inhibit growth and seedhead formation to various degrees, according to McCarty (2008). *P. annua* can also be controlled with dithiopyr and oxadiazon although this mixture can have short-term phytotoxicity to desirable species such as zoysia grass (Vargas and Turgeon, 2004). Glyphosate is a broad-spectrum herbicide that controls *P. annua* during winter when turf grasses are dormant (Velsor et al., 1989).

### 1.3 Major herbicides used for controlling *P. annua* in Australia

Both pre-emergent and post-emergent herbicides are commonly used to control *P. annua* in turf. Pre-emergence herbicides are applied to inhibit weed seed germination or emergence. A post-emergent herbicide treatment can be used to kill plants in spring that have not been

controlled with pre-emergent herbicides. Weed control can be optimised when both pre- and post-emergent herbicides are used in the same season. A list of some commonly used herbicides in Australia for controlling *P. annua* in turf are given in Table 2.

Table 2. List of some commonly used herbicide to control *P. annua* in sports turf in Australia

| **Name of the product** | **Chemical name** | **Application time** | **HRAC group** | **Chemical Company** |
|---|---|---|---|---|
| Tribute | 22.5 g/L Foramsulfuron | POST | 2 | Bayer |
| Nominee | 100 g/L Bispyribac-sodium | POST | 2 | Sumitomo Chemical Australia Pty Ltd |
| Monument | 100 g/L Trifloxysulfuron | POST | 2 | Syngenta |
| Coliseum | 250 g/L Rimsulfuron | POST | 2 | TURF Culture |
| Quali-Pro Negate | 167 g/kg Rimsulfuron<br>200 g/kg Metsulfuron-Methyl | POST | 2 | ADAMA |
| Xtron 700 WG | 700 g/kg Amicarbazone | POST | 5 | Nuturf |
| ClearUp Bio 360 | 360 g/L Glyphosate | POST | 9 | Arbor green |
| Poachek | 175 g/L Endothall | POST | 0 | Campbell (Chemicals) Pty Ltd |
| Pronamide | 500 g/L Propyzamide | PRE, EPOST | 3 | Amgrow |
| Pendi-Pro 22-0-5 | 7.5 g/kg Pendimethalin | PRE | 3 | Nuturf |
| Pendant | 220 g/L Pendimethalin | PRE | 3 | Amgrow |
| Barricade | 480 g/L Prodiamine | PRE | 3 | Syngenta |
| Embargo | 400 g/L Oryzalin | PRE | 3 | TURF Culture |
| OxaMAX | 10 g/kg Oxadiazon | PRE | 14 | Nuturf |
| Ronstar® | 20 g/kg Oxadiazon | PRE | 14 | Bayer |
| Tramat® | 500 g/L Ethofumesate | PRE | 15 | Bayer |
| Meteor | 960 g/L Metolachlor | PRE | 15 | Amgrow |
| Pennmag | 960 g/L Metolachlor | PRE | 15 | Syngenta |
| Specticle | 200 g/L Indaziflam | PRE | 29 | Bayer |

Source: https://nuturf.com.au/problem/wintergrass/

## 1.4 Evolution of herbicide resistance in weeds

Herbicide resistance has been a growing concern in agronomic crops like maize and soybean for decades. Herbicide resistance has developed in turfgrass systems, such as golf courses, sports fields, and sod production, especially in annual bluegrass and goosegrass biotypes. Herbicide resistance is defined by the Weed Science Society of America (WSSA) as a plant's inherited ability to survive and reproduce after being exposed to a dose of herbicide that would normally kill the wild type. Resistant individuals are naturally present in populations at low frequencies and are selected by herbicide use (Powles et al., 1996). The use of a particular herbicide for a long period to control the susceptible genotype will allow resistant genotypes to increase their proportion in the population until the herbicide fails. The first well studied example of a herbicide-resistant weed was the discovery of triazine-resistant *Senecio vulgaris* in 1968 (Ryan, 1970). Since then, resistance to 23 herbicide mechanisms of action has been recorded in at least 521 plant biotypes (Heap, 2021). According the International Survey of Herbicide Resistance Weeds, weeds have evolved resistance to 164 different herbicides in 94 crops in 71 countries (Heap, 2021).

In 1982, the first herbicide-resistant *Lolium rigidum* biotype was identified in Australia (Heap and Knight 1982) and the number of resistant populations has increased significantly over the last three decades. At present, Australia is ranked second in the world, just after the United States, in the number of resistant biotypes (Table 3).

Table 3 The top 10 countries where herbicide resistance weed were found by (Heap, 2021)

| Sl. No. | Country | Total no. of weed biotype | Different site of action | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | **A** | **B** | **C1** | **C2** | **D** | **G** | **K1** | **O** | **Other/ Unknown** |
| 1 | USA | 165 | 15 | 53 | 26 | 11 | 6 | 17 | 6 | 10 | 21 |
| 2 | Australia | 102 | 14 | 27 | 8 | 0 | 11 | 21 | 3 | 4 | 14 |
| 3 | Canada | 68 | 4 | 25 | 13 | 3 | 3 | 6 | 1 | 6 | 7 |
| 4 | France | 56 | 6 | 22 | 22 | 1 | 0 | 3 | 0 | 1 | 1 |
| 5 | Brazil | 52 | 7 | 20 | 4 | 1 | 1 | 11 | 0 | 3 | 5 |
| 6 | China | 45 | 8 | 17 | 1 | 2 | 5 | 2 | 0 | 5 | 5 |
| 7 | Spain | 43 | 2 | 10 | 18 | 3 | 0 | 8 | 0 | 1 | 1 |
| 8 | Israel | 38 | 6 | 14 | 12 | 2 | 0 | 2 | 0 | 0 | 2 |
| 9 | Japan | 36 | 2 | 21 | 1 | 0 | 7 | 3 | 2 | 0 | 0 |
| 10 | Germany | 33 | 5 | 11 | 13 | 3 | 0 | 0 | 0 | 0 | 1 |

Many troublesome herbicide resistant weeds belong to the Poaceae family (Matzrafi et al., 2014). For example, *L. rigidum*, which has evolved resistance to many herbicide groups and develops multiple-resistance as well as cross-resistance to many herbicides (Owen et al., 2007). The herbicide groups with the highest number of resistance cases reported are the ALS inhibitors (159 cases), followed by Photosystem II inhibitors (105 cases) and ACCase inhibitors (48 cases) (Heap, 2021).

### 1.5 Evolution of herbicide resistance in *P. annua*

Herbicide resistance in *P. annua* was first reported in the 1970s, however, these populations were not selected from turfgrass management systems (Darmency and Gasquez, 1983). Herbicide resistance in *P. annua* has been reported more frequently since then, with 48 reports worldwide (Heap, 2021).

### *1.5.1 Acetyl CoA Carboxylase (ACCase)-Inhibitor resistance (HRAC Group 1)*

In managed turfgrass systems, there is limited use of ACCase inhibiting herbicides, as they are only used to control weeds in the rough of golf courses. However, recently haloxyfop resistant annual bluegrass has been confirmed in New Zealand resulting from a to target-site mutation of Ile-2041-Thr (Ghanizadeh et al., 2020). ACCase inhibitor resistance has also been confirmed in turf in other weed species e.g. *Eleusine indica* (McCullough et al., 2017) and *Digitaria ischaemum* (Kuk et al., 1999).

### *1.5.2 Acetolactate Synthase (ALS)-resistance (HRAC Group 2)*

ALS-inhibiting herbicides are used more frequently in turf than any other MOA herbicide, thus resistance is also more frequent in *P. annua* to ALS-inhibiting herbicides than any other MOA herbicides (Heap, 2021). The mechanism of resistance to these herbicides in *P. annua* populations is most often due to target-site mutations, most commonly a Trp-574-Leu substitution on the *ALS* enzyme (McElroy et al., 2013). A different *ALS* mutation (Ala-205-Phe) was reported by Brosnan et al. (2016) in a *P. annua* population from a Tennessee golf course that conferred resistance to imidazolinone, sulfonylurea, triazolopyrimidine, sulfonylamino-carbonyl-triazolinone, and pyrimidinyl (thio) benzoate herbicides. All reported resistance cases in *P. annua* were found in warm season turfgrass, as there are limited uses of ALS inhibiting herbicide in cool season turfgrass, except for bispyribac-sodium. It is challenging to control *P. annua* with these herbicides without causing damage to the desirable cool season turfgrasses (McCullough et al., 2009).

### *1.5.3 Microtubule-inhibitor (MTI) resistance (HRAC Group 3)*

The MTI herbicides are commonly used for pre-emergence control of *P. annua* in turf, however, resistance to different MTI families has occurred (Cutulle et al., 2009). These herbicides are absorbed by both roots and shoots of emerging seedlings with little subsequent translocation. The emerging shoot is the primary absorption and action site in grass species. Pronamide resistance as a POST application was confirmed in *P. annua* in a sod farm in Georgia (USA), however the biotype was susceptible to PRE application of pronamide and the mechanism of resistance was reported as reduced absorption and translocation of foliar applied pronamide (McCullough et al., 2017). Another report by Lowe et al. (2001), identified a target site mutation (Arg241Lys) in *β-tubulin* gene conferring dinitroaniline-resistant in *P. annua*. Besides, populations of *P. annua* were showed resistance to MTI collected from North Carolina, (Isgrigg III et al., 2002) and Alabama (Russell, 2021), and the resistance was found to be attributed to either *α* or *β tubulin* gene mutations. Furthermore, two *P. annua* populations from the golf course of Texas showed resistance at post-application of pronamide, however, the resistance mechanism remained unknown (Singh et al., 2021).

### *1.5.4 Photosystem II (PS II) - inhibitor resistance (HRAC Group 5)*

Resistance to PSII-inhibiting herbicides in *P. annua* was first discovered in 1975 along a roadside in Normandy, France (Darmency and Gasquez, 1983). However, in managed turf, the first simazine resistant *P. annua* population was found in Japan in 1982 (Heap, 2021). In Mississippi, USA several *P. annua* populations from golf courses that had evolved resiatnce to PSII inhibiting herbicides were identified and the resistance mechanism was target-site mutation (Ser-264-Gly) in *psbA* gene (Kelly et al., 1999). The same mutation was reported by Perry et al. (2012) conferring resistance to the POST application of amicarbazone in *P. annua*.

However, a different mutation (Val-219-Ile) conferred metribuzin resistance in *P. annua* in grass seed production fields (Mengistu et al., 2000). Besides, previous research suggested reduced absorption, translocation, and increased metabolism of atrazine confers PS II inhibitor herbicide resistance in *P. annua* where known mutations in the *psbA* gene were lacking (Svyantek et. al 2016).

### *1.5.5 EPSPS inhibitor resistance (HRAC Group 9)*

Glyphosate application in managed turf is very limited and it is only used during the cool season when warm-season turfgrasses becomes dormant. Glyphosate resistance in *P. annua* was first reported in 2010 in the United States (Missouri, Tennessee) (Heap, 2014). Following that, several more populations from US golf courses with resistance to glyphosate have been identified (Binkholder et al., 2011; Breeden et al., 2017; Brosnan et al., 2012; Cross et al., 2015).

### *1.5.6 Protophyrinogen (PPO) inhibitor resistance (HRAC Group 14)*

In turf, PPO inhibiting herbicides are used regularly in the United States. A population of *P. annua* from a Georgia golf course was found resistant to the PPO-inhibiting herbicide flumioxazin when applied as POST (Yu et al., 2018).

### *1.5.7 Lipid synthesis inhibitor resistance (HRAC Group 15)*

Ethofumesate belongs to the benzofuran herbicide family and is use as a PRE and early POST for *P. annua* control. In grass seed producing fields in Oregon, more than 20 ethofumisate resistant biotypes of *P. annua* have been discovered (Heap, 2021) . Because ethofumisate is one of the few effective herbicides for EPOST *P. annua* control in cool-season turfgrass,

resistance to this herbicide would be a major problem. In managed turf no ethofumisate-resistant *P. annua* has been discovered to date (Brosnan et al., 2020).

### *1.5.8 Multiple herbicide resistance in P. annua*

Multiple herbicide-resistance is an emerging threat for weed management. *P. annua* shows a high propensity to evolve herbicide resistance. *P. annua* is ranked third in terms of resistance to different sites of action and has become resistant to ten different herbicide modes of action (Heap, 2021). Despite this, there are only a few reports of multiple resistance in *P. annua* populations, including a biotype resistant to simazine and trifloxysulfuron (Brosnan et al., 2015) and another resistant to prodiamine and glyphosate (Breeden et al., 2017) discovered in Tennessee, USA (Breeden et al., 2017). A third population from a golf course in Texas has been confirmed to have resistance to three different herbicides MOA foramsulfuron/trifloxysulfuron, simazine, and prodiamine (Singh et al., 2020). In Australia, the first case of multiple herbicide resistance in *P. annua* was confirmed in 2017 (Heap, 2021). In the current work, a total of 31 populations were examined and 50% were resistant to more than three herbicide modes of action (Barua et al., 2020).

## 1.7 Mechanisms of herbicide resistance

Herbicide resistance mechanisms are often divided into 2 broad categories: i) Target site resistance and ii) Non-target site resistance (Cobb and Reade, 2011). Target site resistance covers mechanisms that change the amount or sensitivity of the target enzyme for the herbicide (Cobb and Reade, 2011). Non-target site resistance covers all other mechanisms.

### *1.7.1 Target-site Resistance Mechanisms*

Target site resistance mechanisms commonly involve mutations within the gene encoding the target site that reduce or prevent the herbicide from binding to the protein (Heap, 2014). For example, in some glyphosate resistant weed biotypes, the amino acid Pro at site 106, has been replaced by a Ser, Thr, Ala, or Leu amino acid that changes the efficacy of glyphosate binding to the *EPSPS* enzyme (Perez-Jones and Mallory-Smith, 2010; Preston et al., 2009). This is a common resistance mechanism reported in studies of herbicide resistance and which was observed in many weed species including *Eleusine indica* (L.) (Ng et al., 2003), *Digitaria insularis* (L.) (de Carvalho et al., 2012), *Amaranthus tuberculatus* L. (Nandula et al., 2013) and *Echinochloa colona* (L.) (Alarcón-Reverte et al., 2013). Most, but not all, cases of resistance to acetolactate synthase (ALS) inhibitors, Acetyl-Coenzyme A Carboxylase (ACCase) inhibitors, dinitroaniline and triazine herbicides are due to target site mutations. Target site resistance is usually the result of a mutation at a single point in the DNA that is responsible for changing an amino acid in the protein. For example, there are eight distinct amino acid mutations in the *ALS* protein that confer ALS herbicide resistance identified so far. Of these, four provide high resistance to sulfonylurea herbicides and six confer strong resistance to imidazolinone herbicides. Only a few mutations provide resistance to both groups of herbicides (Hatami et al., 2016; Preston, 2014). As various herbicide groups bind differently in the binding pocket of the target site, different mutations can affect only one or both types of herbicides. Target site resistance while widespread, is nonetheless variable to different herbicides with the same mode of action.

Gene amplification/overexpression is another type of target site mechanism, which increases the amount of target enzyme. Excess target enzyme can act as a sponge to dilute herbicides (Gaines et al., 2010). This mechanism is most frequent in glyphosate-resistant weeds. Gaines et al. (2010) reported that the activity of *EPSPS* enzyme from resistant and

susceptible plants of *Amaranthus palmeri* was equally inhibited by glyphosate, but resistant plant genomes contained 5-fold to 160-fold more copies of the *EPSPS* gene than susceptible plant genomes. This overexpression of the *EPSPS* gene as a result of gene amplification results in the evolution of glyphosate-resistant weeds (Gaines et al., 2010). The overexpression of the *EPSPS* enzyme is heritable and correlated to the relative copy number of the *EPSPS* gene and the increases in the number of the target site. The excess in target site proteins reduces the herbicidal efficacy to below toxic levels and allows the plant to continue normal physiological function (Powles and Yu, 2010). *EPSPS* gene amplification has been identified in numerous other weed species including *P. annua*. (Brunharo et al., 2019) According to Brunharo et al. (2019) *EPSPS* duplication and target-site mutation at 106 position, confers 18-fold resistant in resistant biotype of *P. annua*.

### *1.7.2 Non-target-site resistance Mechanisms*

Non-target site resistance is a group of mechanisms that result in insufficient herbicide reaching the site of action to cause death of the plant. This can occur due to reduced herbicide absorption, reduced herbicide translocation, increased herbicide sequestration and/or increased herbicide detoxification (Kohler et al., 2004; Preston, 2004).

Herbicide detoxification is the most common non-target site resistance mechanism reported and is where the herbicide is rapidly metabolised inside the plant allowing the plant to survive. All of the enzymes responsible for herbicide degradation are still not fully known. However, evidence points to increased activity of several enzymes members of glutathione transferases (GST), Cytochromes P450 and others rather than a single enzyme (Yuan et al., 2007). Herbicide detoxification is most commonly observed for cereal selective herbicides, such as HRAC Groups 1, 2, 3, 5 and 4. Recently, Pan et al. (2019) suggested overexpression of aldo-keto reductase was responsible for glyphosate resistance in

*Echinochloa colona*. Preston (2014) reported that sometimes plants fail to convert the herbicide into an active compound and as a result, the herbicide is not effective. This mechanism has been found in triallate resistant wild oats in Canada, but not in Australia (Preston, 2014).

Another type of non-target resistance mechanism involves reduction in herbicide translocation (Délye, 2013). This arises when an herbicide is sequestrated in the leaf tips or vacuoles or in cell walls that keeps the herbicide away from the site of action. To date there is little understanding behind the mechanisms involved (Powles and Yu, 2010); however, vacuole sequestration correlated with glyphosate resistance in *Lolium* spp. from Australia, South America and Europe (Ge et al., 2012). Generally, sequestration occurs within the cytomembrane or the vacuoles (Heap and Duke, 2018) due to herbicidal movement into subcellular compartments (Stewart Jr et al., 2010). The activity of tonoplast glyphosate transporters increases when herbicide is trapped into the vacuole, which suggests a primary role in this mechanism (Heap and Duke, 2018). This is the most common type of resistance for HRAC group 9 and 22; however, it also has been seen in group A (Délye, 2013). For example, this is a common mechanism reported in *Lolium* spp. and *Conyza* spp. resistant to glyphosate (Fernández-Moreno et al., 2017). In resistant plants, the amount of glyphosate moved out of the treated leaf to the rest of the plant is much reduced. Reduced translocation is also the most commonly reported mechanism of resistance to the photosystem I inhibitor herbicide paraquat. For paraquat resistance, the situation is more complicated. In the dark, there is only small amounts of herbicide moved, but translocation is dramatically increased in the light. Again, it appears that sequestration of the herbicide in the vacuole is responsible for herbicide resistance. This type of resistance is usually limited to a single herbicide as transporters are specific for the herbicide. However, the exception to this resistance to paraquat where cross resistance to diquat always occurs (Preston, 2014). In few cases, the development of hairy epidermis and waxy cuticles may also limit the availability of herbicide at the target site, which confers

herbicide resistance (Ferreira and Reddy, 2000). Reduced absorption and translocation mechanism confers pronamide-resistant have been reported in *P. annua* (McCullough et al., 2017).

## 1.8 Factors contributing to the evolution of herbicide resistance in weeds

In general, plants evolve resistance to herbicides by natural selection, but outcrossing to weeds from herbicide tolerant crops can contribute in specific situations. The continuous use of herbicides from the same mode of action on weed populations selects for resistant individuals within the population (Jasieniuk et al., 1996). Many factors, such as initial frequency of resistance alleles, selection intensity, inheritance of resistance, gene flow and the fitness of both resistance and susceptible biotypes in the presence and absence of herbicide influence the speed at which evolution of herbicide resistance occurs (Jasieniuk et al., 1996).

### *1.8.1 Genetic variation/ initial frequency of genes*

Genetic variation plays a key role in the survival and adaptability of species. This variation is a precondition for the evolution of herbicide resistance in a susceptible weed population. Mostly, gene mutations are not caused by the application of a herbicide, rather they occur through spontaneous processes (Warwick and Marriage, 1982). According to Preston and Powles (2002), there are low frequencies of resistance alleles residing within susceptible weed populations that are then selected by the use of the herbicide. The size of the weed population is another important factor that influences the probability of selection of a resistance allele (Jasieniuk et al., 1996). For example, it is harder to select for resistance in small populations, due to the rarity of resistance alleles, and this may explain why resistance is not selected in some situations. According to Charmet et al. (1996), inbreeding species are less genetically

variable than out-crossing species and resistance selection may take longer in inbreeding or self-pollinated species.

### *1.8.2 Selection*

The selection pressure produced by herbicide usage is without a doubt the most significant element that influences herbicide resistance in weed populations. According to Moss (2002), selection pressure is the survival rate of a resistant weed biotype in contrast with susceptible biotypes after herbicide application. Therefore, selection pressure can be estimated by the effective kill or percentage mortality of plants for both resistant and susceptible biotypes after the application of an herbicide. Herbicides create intense selection against susceptible plants, as they result in 95-99% death of susceptible individuals (Jasieniuk and Maxwell, 1994). In general, the more intense the selection pressure, the faster the evolution of resistance in weeds (Jasieniuk and Maxwell, 1994). In addition to the herbicide rate, frequency of applications drives selection for resistance. Furthermore, the evolution of resistance in weed populations can be delayed by rotating herbicide modes of action. Resistance is likely to evolve faster with frequent use of herbicides of the same mode of action over a long period of time (LeBaron & McFarland, 1990). For instance, a high number of weed biotypes have become resistant to HRAC herbicide groups 1 and 2 due to their widespread repeated use (Preston et al. 1999).

### *1.8.3 Genetic inheritance of resistance*

According to Rao (2000), inheritance refers to the process where a genetic trait is passed from one generation to the next (parents to offspring) and this heritability of traits is regulated by both nuclear inheritance and cytoplasmic genes. For most herbicides, nuclear genes encode the target protein and mutation of that target site gene can be passed on by both male and female

gametes (Jasieniuk and Maxwell, 1994). However, for target site resistance to Photosystem II inhibiting herbicides the trait is only inherited through ovules (i.e. maternal parent) in cytoplasmic inheritance. In this context, resistant allele movement within and between populations is much quicker for nuclear inherited traits than for cytoplasmic-inherited resistance.

### *1.8.4 Gene flow*

Gene flow is the movement of genes within or between populations. In addition to existing or new mutations, gene flow is a source of resistance alleles for selection of herbicide resistance (Jasieniuk et al., 1996; Jasieniuk and Maxwell, 1994). In general, gene flow is more frequent than novel mutations and, therefore, can accelerate the rate of resistance evolution (Rao, 2000). In cross-pollinated species, gene flow can occur through pollen or seed, whereas it occurs only through seed migration in self-pollinated species (Darmency, 1996). The comparative importance of mutation and gene flow for resistance evolution is still unknown in most cases. While mutation results in the initial appearance of herbicide resistance in a certain geographical region, gene flow is more likely to spread resistance within a region. Studies of isozyme variation in triazine resistant and a susceptible population of *Chenopodium album* support this hypothesis. For example, in France, different isozyme makeup was observed in triazine resistant populations collected from different areas, but the opposite for populations collected from same areas (Gasquez and Compoint, 1981). Similar results were obtained for triazine resistant populations of lambsquarters in Canada (Warwick and Marriage, 1982). From these findings, it is evident that mutation for resistance tends to occur independently in separate geographical regions.

### *1.8.5 Fitness of resistant biotypes*

Fitness refers to the survival and reproduction rate of viable progeny under selection pressure. The spontaneous mutations that give rise to resistance in populations may be associated with a fitness cost (Vila-Aiub et al., 2009), or there may be linkage between resistance genes with one or more loci with a fitness cost. If there is a direct association between the herbicide resistance trait and fitness cost, the frequency of resistance individuals will decline in the absence of herbicide (Jasieniuk et al., 1996). As other traits can influence fitness of individuals, it is necessary to compare near-isogenic lines (NILs) of herbicide resistant and sensitive plants to understand the effect of resistance mutation on fitness (Jasieniuk et al., 1996). Where fitness penalties have been observed, they can be measured as a reduction in growth or a decline in reproductive ability (Vila-Aiub et al., 2009).

## 1.9 Research aims and objectives

The use of herbicides is the most effective option for controlling *P. annua* in different production systems and golf courses. However, the utility of herbicides is being restricted by widespread evolution of herbicide resistance in many weed species. The aim of this study is to identify the extent of herbicide resistance, and mechanism associated with resistance, as well as identify the alternate herbicides effective in controlling herbicide resistant *P. annua*. To fulfil the aims of the project the objectives are as follows:

- To screen *P. annua* populations for resistance to different herbicide modes of action;
- To undertake detailed dose-response experiments to assess the level of resistance in different *P. annua* populations;
- To explore different herbicide resistance mechanisms including mutations that confer resistance by sequencing target enzymes;

- To undertake crosses between resistant and susceptible parents to investigate segregation of resistance; and
- To identify alternate herbicides to control herbicide resistant *P. annua* populations in turf systems.

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# CHAPTER 2: INCIDENCE OF MULTIPLE HERBICIDE RESISTANCE IN ANNUAL BLUEGRASS (*POA ANNUA* L.) ACROSS SOUTHEASTERN AUSTRALIA

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# Incidence of multiple herbicide resistance in annual bluegrass (*Poa annua*) across southeastern Australia

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**Abstract**

Annual bluegrass (*Poa annua* L.) is a problematic annual weed in established turf where the intensive use of herbicides has resulted in the evolution of herbicide resistance. In 2017, 31 populations of *P. annua* suspected to be resistant to herbicides commonly used to control this weed in turf were collected from golf courses across southeastern Australia to check the resistance status to different herbicide groups. All populations were found to be resistant to multiple turf herbicides. Dose–response experiments confirmed resistance to propyzamide, simazine, rimsulfuron, foramsulfuron, endothall, and pinoxaden. Levels of resistance to rimsulfuron (>56-fold), foramsulfuron (>19-fold), endothall (>7-fold), and pinoxaden (>4.3-fold) compared with the susceptible population were high, but levels of resistance to propyzamide (>2-fold) and simazine (>2-fold) were lower. Considerable variation in resistance to endothall and pinoxaden was observed among the populations of *P. annua*. Target-site resistance was confirmed for acetolactate synthase and acetyl-CoA carboxylase inhibitors, but not for photosystem II and microtubule assembly inhibitors. This study documented the extensive resistance to herbicides in *P. annua* from turf in Australia. Three of the populations investigated exhibited multiple resistance to herbicides from five mechanisms of action. The identification of multiple-resistant *P. annua* on several golf courses is a serious concern for turf managers.

## Introduction

Annual bluegrass (*Poa annua* L.; also known as annual meadow grass or winter grass) has been documented on all continents including Antarctica, spanning environments where both warm- and cool-season turfgrasses are grown (Heide 2001). It is a weed of field crops in many countries and is also found in lawns, urban parks, pastures, gardens, roadsides, and forests (Holm et al. 1997; Wagner et al. 1990). It is a serious weed in turf due to its unsightly appearance, competition with desirable species, and upright growth habit, which produces an uneven surface that affects ball roll in golf and other sports (Toler et al. 2007). Prolific seed production (Holm et al. 1997) and ability to germinate under a wide range of environmental conditions (McElroy et al. 2004) make *P. annua* an exceptionally difficult weed to control. Although many herbicides are registered for *P. annua* control in turf, its high fecundity and high genetic diversity create strong selection pressure for herbicide resistance (Brosnan and Breeden 2013). To date, *P. annua* has evolved herbicide resistance more than any other turf weed species (Brosnan and Breeden 2013).

Worldwide, *P. annua* has evolved resistance to inhibitors of photosystem II (PSII; e.g., simazine), acetolactate synthase (ALS; e.g., trifloxysulfuron), enolpyruvylshikimate-3-phosphate synthase (e.g., glyphosate) and mitosis (e.g., dithiopyr) in managed turf systems (Binkholder et al. 2011; McElroy et al. 2013). The occurrence of glyphosate resistance in *P. annua* was first reported in the United States (Missouri, Tennessee) in 2010 (Heap 2014). Similarly, overuse of inhibitors of microtubule assembly (prodiamine) has resulted in the evolution of herbicide resistance in *P. annua* in North Carolina (Isgrigg et al. 2002).

Herbicide resistance is caused by either target-site mutations or alterations in plant metabolism (Délye 2013). So far, both target-site and non–target site based herbicide resistance (NTSR) to various herbicides has been confirmed in *P. annua*. Previous studies have documented that mutations Trp-574 and Ala-205 confer cross-resistance to many ALS inhibitors (Brosnan et al. 2016; McElroy et al. 2013). Similarly, in *P. annua*, Ser-264 was confirmed to provide resistance to atrazine (Perry et al. 2012), Val-219 to metribuzin and diuron (Mengistu et al. 2000), and Pro-106 to glyphosate (Cross et al. 2015). On the other hand, an NTSR-based mechanism causing reduced absorption and translocation was reported as a mechanism of resistance to POST pronomide applications in *P. annua* (McCullough et al. 2017).

**Table 1.** Herbicide used, sources, recommended field rates in Australia, and rates used in the dose–response experiment.

| Chemical name | WSSA group | Trade name | Rates used | Recommended field rate | Source |
|---|---|---|---|---|---|
| | | | g ai ha$^{-1}$ | g ai ha$^{-1}$ | |
| Rimsulfuron | 2 | Coliseum | 0, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 12.5, 25, 50, and 100 | 25 | Turf Culture Pty Ltd, Sunbury, Australia |
| Foramsulfuron | 2 | Tribute® | 0, 0.53, 1.05, 2.11, 4.22, 8.44, 16.88, 33.8, 67.5, and 135 | 33.8 | Bayer CropScience Pty Ltd, Hawthorn East, Australia |
| Iodosulfuron-methyl-Na | 2 | Destiny® | —[a] | 15 | Bayer CropScience Pty Ltd, Hawthorn East, Australia |
| Bispyribac-Na | 2 | Nominee® | —[a] | 50 | Sumitomo Chemical Australia Pty Ltd, Epping, Australia |
| Simazine | 5 | Gesatop® 600 | 0, 13.13, 26.25, 52.5, 105, 210, 420, 840, 1,680, and 3,360 | 840 | Syngenta Australia Pty Ltd, Macquarie Park, Australia |
| Endothall | 27 | Poachek | 0, 65.7, 131.3, 262.5, 525, and 1,050 | 262.5 | Colin Campbell (Chemicals) Pty Ltd, Wetherill Park, Australia |
| Propyzamide | 3 | Kerb® 500 | 0, 125, 250, 500, 1,000, and 2,000 | 500 | Dow AgroSciences Australia Limited, Chatswood, Australia |
| Pinoxaden | 1 | Axial® | 0, 7.5, 15, 30, 60, 120, and 240 | 60 | Syngenta Australia Pty Ltd, Macquarie Park, Australia |

[a] Product only used in preliminary screening experiments.

Greenskeepers in Australia have reported difficulty in controlling *P. annua* with herbicides. In response to their concerns, 31 purported resistant populations of *P. annua* were collected from golf courses across New South Wales (NSW), Victoria, and South Australia (SA) where herbicides had failed to provide weed control and were tested for resistance to several turf herbicides. Herbicide dose–response pot trials were conducted to investigate resistance to five herbicide mechanisms of action that are commonly used to control this weed on golf courses. The objective of this research was to determine the resistance status of *P. annua* collected from across southeastern Australia and to determine the mechanisms of resistance present.

## Materials and Methods

### Plant Materials

A total of 31 populations of *P. annua* (18 from Victoria, 6 from NSW, and 7 from SA) were collected by turf managers and sent as soil plugs to the Weed Science group of the University of Adelaide (34.9670°S, 138.6360°E). The samples were collected from greens and fairways where turf managers were having trouble controlling *P. annua* with herbicides. One susceptible (S) population collected from non–golf course areas was used as the susceptible control.

### Preliminary Screening for Resistance in Sampled Populations

In December 2017, plants at the 2- to 3-tiller stage and of similar size from the cores were transplanted into 9.5 by 8.5 by 9.5 cm punnet pots (Masrac Plastics, Adelaide, SA) containing standard potting mix (produced by steaming 540 L cocoa peat, 220 L of water, and 60 L of sand for 1 h) (Boutsalis et al. 2012). Each pot had 4 plants of the same population and was replicated three times for each herbicide rate, arranged in a randomized complete block design. Plants were watered daily, and at 1 wk from transplanting, all 31 populations from golf courses and the control population were treated with the 1X rate (field rate of each herbicide) (Table 1) and 3X rate of the ALS inhibitors (WSSA Group 2 [WSSA 2020]) rimsulfuron, iodosulfuron, bispyribac, and foramsulfuron; the PSII inhibitor (WSSA Group 5) simazine; the inhibitor of microtubule assembly (WSSA Group 3) propyzamide, also known as pronamide; and endothall (WSSA Group 27) early POST. Herbicides were applied using a laboratory moving boom sprayer equipped with a twin nozzle (TeeJet® 110° flat fan, Spraying Systems, Wheaton, IL) delivering an output of 118 L ha$^{-1}$ at a pressure of 250 kPa and speed of 1 m s$^{-1}$. Plants exhibiting subsequent growth after 28 d, such as the initiation of new tillers, were classified as survivors, whereas plants with severe stunting or mortality were considered susceptible (Powles et al. 1998). Seeds from nontreated control plants of all populations and from plants surviving the 3X rate of each of the herbicides were collected for further experiments.

A separate screening trial was conducted in June 2018 with pinoxaden (acetyl-CoA carboxylase [ACCase] inhibitor, WSSA Group 1), a herbicide used to control some grass weeds in golf course roughs, applied POST to all 31 populations. Seeds of all populations, collected from nontreated control plants, were germinated in trays (330 by 200 by 50 mm) located outdoors, and 1- to 2-leaf seedlings were transplanted into punnet pots (95 by 85 by 95 mm) containing the previously described standard potting mix, with 4 plants per pot replicated three times. At the 2-leaf stage, plants were treated with herbicides at the 1X and 3X label field rates with three replications (4 plants per replication). The herbicide screening trial with all the herbicides was repeated in July 2018 using seed collected from nontreated plants. Seed were germinated and treated as described earlier, and survival was assessed after 28 d.

Following the initial detection of propyzamide resistance, further experiments were set up in July 2018 to check resistance to propyzamide when used as a PRE treatment. There are no previous reports of propyzamide (inhibitor of microtubule assembly) resistance in any weed species in Australia. Approximately 100 cleaned seeds were measured by volume (0.2 ml) and placed onto the standard potting mix surface, and herbicide was sprayed directly onto the seeds. Immediately after spraying, the seeds were covered with 5 mm of standard potting mix and watered daily as described earlier. Herbicides were applied with the same equipment as described earlier, and the experiment was assessed for seedling survival at 28 d after herbicide treatment. Plants that emerged and grew to the 2-leaf stage were considered survivors.

### Confirming Resistance in Progeny with Dose–Response Experiments

Propyzamide-resistant populations were further investigated with dose–response pot trials to quantify the level of resistance to different

**Table 2.** Primer sequences with fragment size used for amplifying fragments of herbicide target-site genes covering known resistance sites in resistant and susceptible populations of *P. annua*.

| Target gene | Primer name | Primer sequence 5′→3′ | Fragment size | Reference |
|---|---|---|---|---|
| *ALS* | Poa_ALS F574 | TGGGCGGCTCAGTATTACAC | 479 bp | McElroy et al. (2013) |
| | Poa_ALS R574 | ATAGGCAGCACATGCTCCTG | | |
| | Poa ALS_1F | ACCCGCATCAGGTGCTCCACGGT | 454 bp | GenBank accession no. KT346395 |
| | Poa ALS_1R | AGGAGGCGAGGAAGAAGGCTTCCT | | |
| | PoaALS_2F | AAGGGCGCCGACATCCTCGTCGA | 445 bp | GenBank accession no. KT346395 |
| | PoaALS_2R | GCTGCTTGTTCTTGCCAATCTCAGC | | |
| *ACCase* | PoaACCase_ACcp1 F | CAACTCTGGTGCTIGGATIGGCA | 551 bp | Délye et al. (2002b) |
| | PoaACCase_ACcp1 R | GAACATAICTGAGCCACCTIAATATATT | | |
| *psbA* | Psb1F | CTGATGGTATGCCTCTAGGAATCTC | 472 bp | GenBank accession no. KJ716483.1 |
| | TTP2R | AGATTAGCACGGTTGATGATA | | GenBank accession no. M36191.1 |
| α-Tubulin | AW08F | GGAGATTGTTGACCTGTGCCT | 746 bp | GenBank accession no. *E. indica* AJ005599 |
| | AW05R | TGGGTGGCTGGTAGTTGATAC | | (Fleet et al. 2018) |

herbicides with four replications (6 plants per replication). Seeds from survivors of propyzamide in the preliminary screening for the resistant populations were used. These were stored dry for at least 3 mo before use and tested for germination before use. The previously confirmed susceptible population was included as the control for this trial. Dose–response experiments were performed with the Group 2 herbicides rimsulfuron and foramsulfuron, the Group 5 herbicide simazine, the Group 3 herbicide propyzamide, the ACCase inhibitor pinoxaden (WSSA Group 1), and endothall (WSSA Group 27) in August 2018 and repeated in October 2018.

In golf courses, propyzamide is usually applied as both a PRE and early POST herbicide, whereas the other herbicides tested are used POST. Based on this, dose–response experiments with propyzamide were conducted with both PRE and POST treatments, whereas all other herbicides were only evaluated POST at a range of rates (Table 1). Herbicide applications were performed as described earlier. All the experiments were arranged in a completely randomized design with four replications and repeated.

### *Target-Site Mutation Identification*

For resistant populations, tissues were sampled randomly from 5 individual plants of each population that survived the highest rate of herbicide treatment for a given site of action. Tissue samples for the susceptible population were taken from the nontreated controls. Fresh leaf material (0.1 g) was harvested from the youngest fully expanded leaf, snap frozen in liquid nitrogen, and then stored at −80 C until further use. DNA extraction was performed using the ISOLATION II plant DNA extraction kit (Bioline, Alexandrina, NSW, Australia) according to the manufacturer's instructions. A polymerase chain reaction (PCR) was performed to amplify gene sequences that contain reported target-site mutations within each target site tested using ~200 ng of gDNA (genomic DNA) in a standard PCR reaction of 25 µl containing 2× MyFi Mix reaction buffer (containing 0.2 mM of dNTPs and 0.6 mM of $MgCl_2$) and 0.4 µM of each gene-specific primer pair (Table 2). Thermocycling was performed in an automated DNA thermal cycler (Eppendorf Mastercycler Gradient, Eppendorf, Hamburg, Germany) with the following conditions: DNA initial denaturation at 95 C for 30 s followed by 37 cycles of 15 s at 95 C, 15 s at 58 C, and 15 s at 72 C. Samples were electrophoresed in 1× TAE buffer (40 mM Trizma base and 1 mM $Na_2EDTA$, pH to 8 with glacial acetic acid) at 110 V. The amplified fragments were sequenced by the Australian Genome Research Facility, University of Adelaide, Australia, using the same primers used for amplification. Nucleotide sequences were assembled and analyzed using ContigExpress and AlignX programs from the VectorNTi v. 11.5 program suite (Invitrogen, Waverley, VIC, Australia).

### *Statistical Analysis*

The screening trial was subjected to two-way ANOVA for each herbicide, with population and run as variables. There was no difference between runs ($P > 0.05$), so data were pooled. Populations were considered resistant to any herbicide if >50% of the individuals treated survived. Dose–response trials were set up as a completely randomized design and repeated. Data were subjected to three-way ANOVA for each herbicide, with population, rate, and run as variables. For every herbicide, there was a significant effect for rate ($P < 0.0001$) and population ($P < 0.0001$), but not for run, except for simazine ($P < 0.001$). For all herbicides except simazine, data were pooled across experiments. Survival at each rate was converted to mortality, and the data were analyzed using PriProbit v. 1.63 (Sakuma 1998) with the $LD_{50}$ (rate of herbicide that killed 50% of the population) with 95% confidence intervals (CI) determined and resistance ratios calculated as $LD_{50}$ R/$LD_{50}$ S. Population responses were considered different if confidence intervals at the $LD_{50}$ did not overlap.

## Results and Discussion

### *Preliminary Screening*

Out of 31 populations from golf courses, all were resistant to three or more herbicides, with half being resistant to five mechanisms of action (Figure 1). Most populations were resistant to ALS inhibitors (97%), PSII inhibitors (94%), endothall (100%), ACCase inhibitors (94%), and inhibitors of microtubule assembly (81%) POST, but only 7% of populations were resistant to inhibitors of microtubule assembly (propyzamide) PRE (Table 3). Populations with resistance to propyzamide were chosen for additional investigation.

### *Confirmation of Resistance*

#### *Resistance to Group 2 Herbicides*

In turf management, Group 2 herbicides are commonly used for POST control of *P. annua* (Toler et al. 2007). However, this herbicide group is highly prone to the evolution of herbicide resistance, and to date, there are 160 weed species resistant worldwide (Heap 2019). In the dose–response experiments with the Group 2 herbicides foramsulfuron and rimsulfuron, the S population was controlled with foramsulfuron at 2.7 g $ha^{-1}$, and population R1

**Table 3.** Summary of screening result of 31 populations collected from golf courses with different mechanism of action herbicides at the recommended field rate and three times that rate. Recommended field rates are listed in Table 1.

| WSSA group | Chemical name | Application timing | Populations surviving the 1X rate | Populations surviving the 3X rate |
|---|---|---|---|---|
| | | | —%— | —%— |
| 2 | Rimsulfuron | POST | 97 | 84 |
| | Indosulfuron | | 90 | 71 |
| | Bispyribac | | 97 | 87 |
| | Foramsulfuron | | 52 | 38 |
| 5 | Simazine | POST | 94 | 68 |
| 27 | Endothall | POST | 100 | 90 |
| 3 | Propyzamide | POST | 81 | 71 |
| | Propyzamide | PRE | 23 | 13 |
| 1 | Pinoxaden | POST | 94 | 74 |

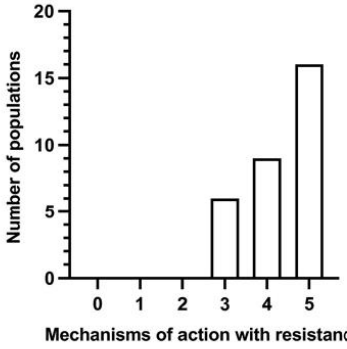


**Figure 1.** Extent of multiple-resistant status of *Poa annua* populations from golf courses across southeastern Australia in response to different herbicide mechanisms of action. There were five herbicide groups used, and each population could be resistant to between 0 and 5 mechanisms of action.

was controlled with 67.5 g ha$^{-1}$ foramsulfuron, whereas no control of the three resistant populations R2, R3, and R4 was observed even at the highest rate used (135 g ha$^{-1}$) (Figure 2A). Population R1 was 9.3-fold more resistant than the S population, and all other resistant populations were >56-fold resistant (Table 4). Similarly, the S population was controlled by 5.37 g ha$^{-1}$ rimsulfuron, whereas no control of any R population was obtained, even at 100 g ha$^{-1}$ (Figure 2B). All the R populations showed >19-fold resistance to rimsulfuron (Table 4). In our studies, populations R2, R3, and R4 exhibited strong resistance to both Group 2 herbicides tested, whereas R1 exhibited lower resistance to foramsulfuron (controlled at 67.5 g ai ha$^{-1}$). Resistance to Group 2 herbicides has previously been observed in *P. annua*. Populations from South Carolina and Georgia were highly resistant to trifloxysulfuron, foramsulfuron, and bispyribac-sodium (Cross et al. 2013), as was a population from Alabama (McElroy et al. 2013). The variable response of R populations to the Group 2 herbicides observed in our study suggests the likelihood of different target-site mutations in ALS or the presence of a different resistance mechanisms (e.g., difference in uptake and translocation) in different populations.

### *Resistance to Group 1 Herbicides*

Recently, pinoxaden was registered at up to 60 g ha$^{-1}$ in the United Kingdom to control ryegrass (*Lolium* spp.) in fine-leaf fescue (*Festuca rubra* and *Festuca ovina*) in turf (Anonymous 2010). *Poa annua* is normally considered to be tolerant to Group 1 herbicides (Herbert et al. 1997); however, some herbicides in this group can provide control (Takahashi et al. 2002). The S population was controlled with 60 g ha$^{-1}$ pinoxaden in our experiments showing it was susceptible to pinoxaden (Figure 2C). In contrast, populations R1, R2, R3, and R4 were not controlled with this rate of pinoxaden. The four R populations exhibited different levels of resistance to pinoxaden (Figure 2C). Population R3 was 4.3-fold resistant to pinoxaden, whereas the other three populations were >4.3-fold resistant (Table 4). To date, 48 weed species have been reported to be resistant to the Group 1 herbicides (Heap 2019).

### *Resistance to Endothall*

Endothall was one of the first herbicides registered to control *P. annua* in turf. Control of the S population was achieved with 525 g ha$^{-1}$, whereas no control of the R populations was observed even at 1,050 g ha$^{-1}$ (Figure 2D). Population R3 exhibited lower resistance (5.5-fold) than the other resistant populations (>7-fold; Table 4). Endothall resistance has not been reported in any weed species other than *P. annua* (Heap 2019). The mechanism of action of endothall is not well understood; however, inhibition of serine/threonine protein phosphatases 1 and 2A are thought to be the targets (Bajsa et al. 2012; Tresch et al. 2011).

### *Resistance to Group 5 Herbicides*

Simazine is a POST herbicide most widely used to control *P. annua* in turf. Simazine at the recommended rate of 840 g ha$^{-1}$ controlled 94% of the S population, but only caused 20% mortality in the four resistant populations, equating to 2- to 8-fold resistance (Figure 2E and F; Table 4). There was a significant effect of run for the simazine dose response, suggesting environmental conditions could affect the response of populations to the herbicide. $LD_{50}$ values were different between the two runs for the S population and populations R3 and R4 (Table 4); however, there was no consistency in these differences. Group 5 resistance was first reported in *P. annua* more than 40 yr ago in France, after 10 yr of repeated exposure to simazine (Darmency and Gasquez 1983). Resistance to Group 5 herbicides has been observed widely in *P. annua* in the United States, having been reported in Oregon (Mengistu et al. 2000), Mississippi (Hutto et al. 2004; Syvantek et al. 2016), Tennessee (Brosnan et al. 2015) Alabama, North Carolina, and Virginia (Heap 2019). Hutto et al. (2004) identified simazine resistance in *P. annua* from 43% of golf courses sampled in Mississippi. This extent of resistance is less than observed in the samples tested here (Table 3). The level of resistance to Group 5 herbicides in the United States (Brosnan et al. 2015; Kelly et al. 1999; Syvantek et al. 2016) appears to be much greater (more than 1,000-fold) than in the Australian populations examined. These populations, while surviving 840 g ha$^{-1}$, the field rate for Australia, could be controlled by the highest rate used, 3.36 kg ha$^{-1}$ (Figure 2E and F). Therefore, these populations may be less of a problem in situations where a higher rate of simazine can be used. As target-site mechanisms typically result in high levels of resistance to Group 5 herbicides (Ashworth et al. 2016), it is likely that NTSR could be responsible for the 2- to 8-fold resistance observed in the populations in Australia.

### *Resistance to Propyzamide*

Propyzamide, an inhibitor of microtubule assembly, is used for PRE and early POST weed control in golf courses. In the dose–response experiments, complete control of the S biotype was obtained at the recommended rate of 500 g ha$^{-1}$ propyzamide

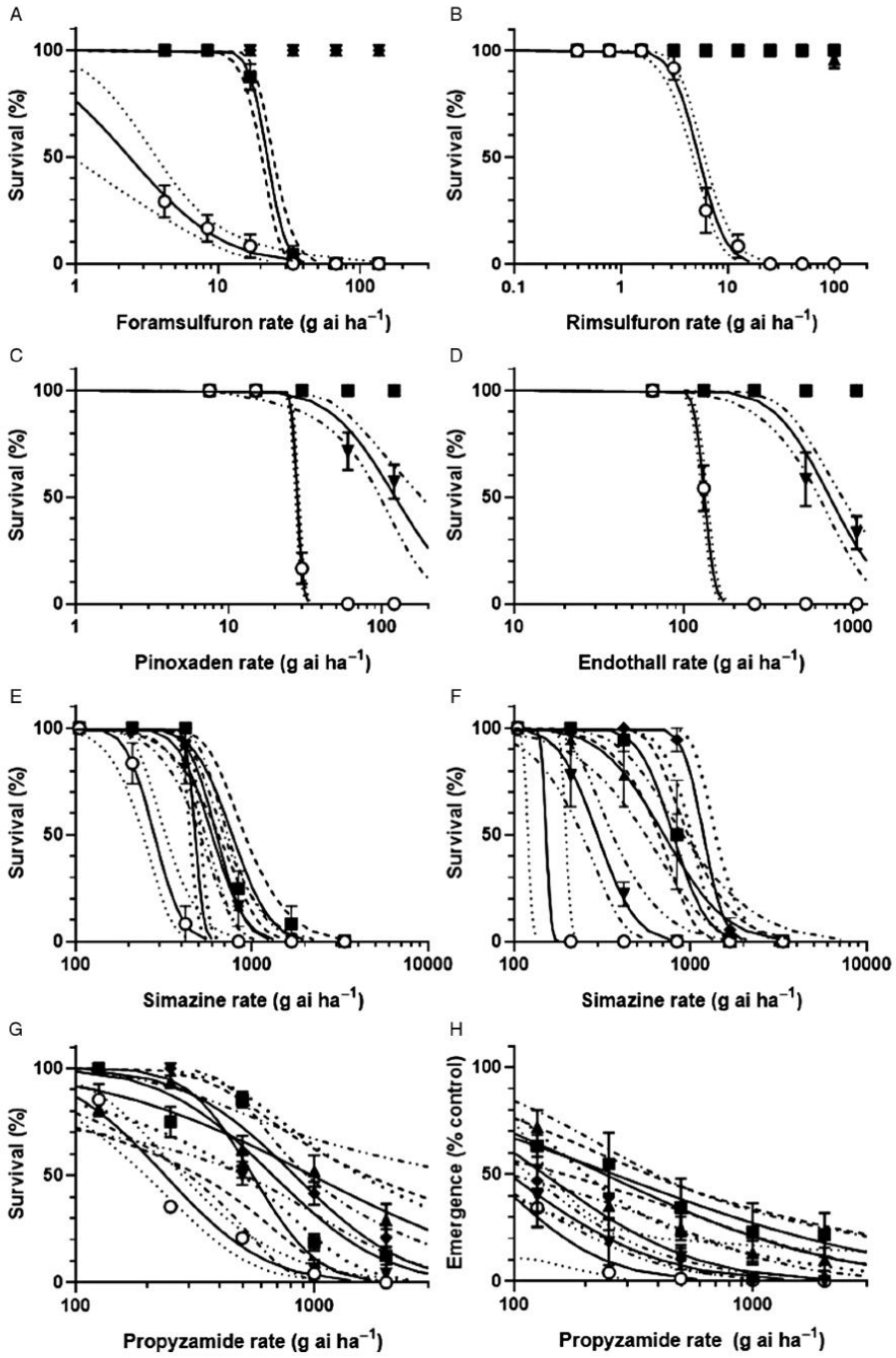


**Figure 2.** Survival after 28 d of susceptible S (○) and resistant R1 (■), R2 (▲), R3 (▼), and R4 (◆) populations of *Poa annua* treated with foramsulfuron (A), rimsulfuron (B), pinoxaden (C), endothall (D), simazine in Experiment 1 (E), simazine in Experiment 2 (F), and propyzamide (G) at POST herbicide timing, and emergence following treatment with propyzamide (H) at PRE herbicide timing. Each point is the mean of eight replications of 6 plants, except for simazine, where it is the mean of four replications of 6 plants for each run. The lines are the probit curves back transformed to percentages with 95% confidence intervals: S (·····), R1 (— — —), R2 (— · —), R3 (— · · —), and R4 (·····). Where no mortality occurred, probit analysis was not conducted and no curve was plotted. Data points are the means of eight replicates (four replicates for each simazine experiment) ± SE.

**Table 4.** The dose required for 50% mortality ($LD_{50}$) of susceptible and resistant *Poa annua* populations in response to various herbicides with 95% confidence intervals (CI) in parentheses.[a]

| | | Population | | | | |
|---|---|---|---|---|---|---|
| Herbicides | | 18 (R1) | 27 (R2) | 262-16 (R3) | 413-17 (R4) | S |
| | | $LD_{50}$ g ai $ha^{-1}$ | | | | |
| Foramsulfuron | | 22.3 (20.3, 24.5) | >135.0 | >135.0 | >135.0 | 2.4 (0.9, 3.7) |
| | R/S | 9.3 | >56 | >56 | >56 | |
| Rimsulfuron | | >100.0 | >100.0 | >100.0 | >100.0 | 5.4 (4.7, 6.1) |
| | R/S | >19 | >19 | >19 | >19 | |
| Pinoxaden | | >120 | >120 | 122.0 (97.3, 180.4) | >120 | 28.0 (27.1, 28.9) |
| | R/S | >4.3 | >4.3 | 4.3 | >4.3 | |
| Endothall | | >1,050.0 | >1,050.0 | 728 (631, 863) | >1,050.0 | 133 (128, 138) |
| | R/S | >7.9 | >7.9 | 5.5 | >7.9 | |
| Simazine | Exp. 1 | 774 (648, 862) | 594 (508, 695) | 631 (545, 729) | 477 (446, 512) | 279 (242, 324) |
| | R/S | 2.8 | 2.1 | 2.3 | 1.7 | |
| | Exp. 2 | 798 (663, 971) | 708 (557, 908) | 297 (243, 362) | 1,190 (1,010, 1,390) | 152 (120, 197) |
| | R/S | 5.3 | 4.7 | 2.0 | 7.8 | |
| Propyzamide POST | | 673 (330, 1,666) | 979 (515, 4,538) | 485.3 (299, 934) | 829 (499, 1,569) | 237 (196, 280) |
| | R/S | 2.8 | 4.1 | 2.0 | 3.5 | |
| Propyzamide PRE | | 262 (162, 366) | 240 (96, 390) | 95.5 (73.3, 116) | 134 (59.3, 200) | 79.5 (0.1, 142) |
| | R/S | 3.3 | 3.0 | 1.2 | 1.7 | |

[a] R/S is the ratio of $LD_{50}$ of resistant and susceptible populations.

**Table 5.** Nucleotide and amino acid changes identified in herbicide target-site genes in resistant *Poa annua* populations.

| Gene name | Amino acid position | Nucleotide and amino acid changes | Population |
|---|---|---|---|
| *ALS* | 574 | TGG (Trp) – TTG (Leu) | R2, R3, and R4 |
| | 197 | CCG (Pro) – TCG (Ser) | R1 |
| *ACCase* | 1781 | ATA (Ile) – TTA (Leu) | R1, R2, R3, and R4 |

applied PRE, whereas 1,000 g $ha^{-1}$ applied POST was required for complete control. Resistance to POST propyzamide was confirmed in populations R1, R2, R3, and R4 (Figure 2G), with resistance ranging from 2.0-fold for population R3 to 4.1-fold for population R2 (Table 4). The level of propyzamide resistance was much lower in the PRE treatment (Figure 2H). Only population R1 had an $LD_{50}$ different from the S population for PRE propyzamide and was 3.3-fold resistant.

To date, *P. annua* is the only weed species in which resistance to propyzamide has been confirmed. In Georgia, USA, McCullough et al. (2017) reported resistance to POST application of propyzamide (>10-fold), whereas in our experiments, resistance PRE and POST resistance was confirmed, but the levels of resistance were less than 5-fold. In a recent study, a biotype of ryegrass (*Lolium* spp.) showed 2.7-fold resistance to propyzamide used PRE (Brunton et al. 2018). Our experiments show that *P. annua* populations from golf courses in Australia have evolved resistance to both PRE and POST applications of propyzamide, which could cause control failures.

## Target-Site Mutation Identification

### ALS Gene Mutations

Target-site resistance to Group 2 herbicides is a result of missense mutations causing amino acid substitutions at any of eight sites in ALS: Ala-122, Pro-197, Ala-205, Asp-376, Arg-377, Trp-574, Ser-653, and Gly-654 (Brosnan et al. 2016; Délye et al. 2002b; Heap 2019; Yu et al. 2008). The *ALS* gene of five individuals, out of the 24 treated, from each population that had survived the field rate of herbicides, was sequenced to investigate the presence of a target-site mutation. An amino acid substitution at position 574 from Trp to Leu was present in all individuals of resistant populations R2, R3, and R4, whereas in population R1, all individuals had a Pro-197 to Ser mutation (Table 5). Population R1 showed variable response to the two Group 2 herbicides investigated (foramsulfuron and rimsulfuron). A Trp-574 to Leu amino acid substitution was reported to confer cross-resistance to multiple Group 2 herbicides in *P. annua* (McElroy et al. 2013), and an Ala-205 to Phe mutation provided resistance to imidazolinone, sulfonylurea, triazolopyrimidines, sulfonylamino-carbonyl-triazolinones, and pyrimidinyl (thio) benzoate herbicides (Brosnan et al. 2016). Furthermore, the Pro-197 to Ser mutation has been identified as providing resistance to sulfonylurea herbicides in other weed species, including rigid ryegrass (*Lolium rigidum* Gaudin) (Yu et al. 2008).

### ACCase Gene Mutations

Seven ACCase amino acid substitutions have been confirmed to confer resistance in weed species: Ile-1781-Leu, Ile-2041-Asn, Ile-2041-Val, Trp-2027-Cys, Gly-2096-Ala, Asp-2078-Gly, and Trp-1999-Cys (Délye et al. 2002a, 2003, 2005; Liu et al. 2007; Zhang and Powles 2006b). An amino acid substitution at position 1781 from Ile to Leu was identified in all five individuals from each of the four resistant populations (R1, R2, R3, and R4) (Table 5). However, population R3 was not as resistant to pinoxaden as the other populations (Figure 2), which suggests other mechanisms may play a role. The Ile-to-Leu substitution at position 1781 has been identified in other grass species resistant to Group 1 herbicides (Zhang and Powles 2006a). This mutation has previously been identified in *P. annua* (Délye and Michel 2005). Those authors considered the mutation to be the likely source of the inherent tolerance of *P. annua* to some Group 1 herbicides. However, the susceptible populations in our study had Ile at position 1781. This suggests there may be different lineages of *P. annua* with and without this mutation. Further research would be required to confirm this.

### psbA Gene Mutations

Previous studies have confirmed that resistance to Group 5 herbicides in many weed species is conferred by mutations in the highly conserved chloroplast gene *psbA* (Lu et al. 2019). Seven mutations resulting in amino acid substitutions at six positions (Ser-264-Gly,

Ser-264-Thr, Val-219-Ile, Asn-266-Thr, Ala-251-Val, Phe-255-Ile, and Leu-218-Val) in *psbA* have been shown to confer resistance to Group 5 herbicides (Thiel and Varrelmann 2014). The most common of these mutations, Ser-264-Gly, confers high levels of resistance to the triazine herbicide atrazine in *P. annua* and other weed species (Perry et al. 2012). Another report confirmed that the Val-219-Ile mutation provides resistance to *psbA* (metribuzin and diuron) in *P. annua* (Mengistu et al. 2000). However, in our study, no mutations within the *psbA* gene were found in any of the populations (Table 5). Therefore, resistance to simazine observed in this study is likely due to a non–target site based mechanism or another mutation not reported previously in the literature. This is consistent with the moderate levels of resistance in the *P. annua* populations compared with the much higher levels of resistance due to target-site mutation reported for Group 5 resistance in *P. annua* populations elsewhere (Perry et al. 2012).

### *α-Tubulin Gene Mutations*

Genetic evidence suggests that the herbicidal effects of the dinitroanilines amiprophos-methyl and propyzamide are related to microtubule function (Schibler and Huang 1991). However, no mutation in the α-tubulin gene was identified in plants from either of the resistant populations that had survived either PRE or POST propyzamide. It is therefore likely that the low level resistance identified in *P. annua* is non–target site based. McCullough et al. (2017) reported that resistance to POST propyzamide in a population of *P. annua* from a golf course in Georgia, USA, was associated with reduced absorption and translocation.

Even though resistance to nine mechanisms of action has been confirmed in *P. annua* globally, there have only been two confirmed reports of multiple resistance, a biotype resistant to simazine and trifloxysulfuron (Brosnan et al. 2015) and another resistant to prodiamine and glyphosate (Breeden et al. 2017). In this study, 31 populations of *P. annua* from golf courses that were not being adequately controlled with herbicides were tested for herbicide resistance. Resistance to multiple mechanisms of action was confirmed in all populations. Dose–response experiments conducted with four of these populations confirmed resistance to propyzamide, simazine, rimsulfuron, foramsulfuron, endothall, and pinoxaden. High levels of resistance to rimsulfuron, foramsulfuron, endothall, and pinoxaden and lower levels to propyzamide and simazine were confirmed. Only a single susceptible population from an area where herbicides had not been applied was used in this study. It is possible that this population may not have been representative of the susceptibility of *P. annua* to herbicides. This means that the resistance ratios reported here could be different if another susceptible population was used. This would not affect the conclusion that the populations were resistant to the Group 1, Group 2, or Group 27 herbicides, but could do so for resistance to the Group 3 and Group 5 herbicides. Known target-site mutations were identified in ALS and ACCase, but not in PSII or α-tubulin. Therefore, future research should aim to explore mechanisms of NTSR in these populations and response to herbicides from different site-of-action groups. *Poa annua* is primarily a self-pollinated species (Ellis 1973), so multiple resistance may have arisen from sequential selection with multiple herbicides over an extended period. The turf managers indicated that most of the sites had received multiple applications of different herbicides each year for at least the past 10 yr, showing a high intensity of selection for multiple resistance. However, Ellis (1973) suggests up to 15% outcrossing can occur in this species, which provides the possibility of accumulation of herbicide-resistance traits through cross-pollination. The extensive amount of herbicide resistance present and the limited nonchemical methods available means that management of multiple-resistant *P. annua* in turf will be challenging.

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# CHAPTER 3: INHERITANCE AND MECHANISM OF GLYPHOSATE RESISTANCE IN ANNUAL BLURGRASS (*POA ANNUA* L.)

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| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. | | |
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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# Inheritance and mechanism of glyphosate resistance in annual bluegrass (*Poa annua* L.)

**Short running title:** Glyphosate resistance in *Poa annua*

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**ABSTRACT**

**BACKGROUND:** In initial screening, glyphosate was ineffective in controlling five *Poa annua* populations. These populations were tested for resistance, and studies undertaken to determine resistance mechanisms and inheritance pattern.

**RESULTS:** Temperature (high and low) has been documented to affect the level of glyphosate resistance in different weed species. Dose response studies conducted at 16/12 °C and 27/20 °C on the five putative resistant populations showed low-level resistance (1.4-2.5 fold) to glyphosate. There was a tendency for higher survival and larger $LD_{50}$ values for all populations at high temperature (27/20 °C) compared with low temperature (16/12 °C). Shikimic acid accumulation in response to glyphosate confirmed differences among the populations, with more shikimic acid accumulated by the susceptible population. *EPSPS* gene copy number was 1.7-9.3 fold greater in one resistant population (HT) compared to the susceptible (S), but not in the others. *EPSPS* gene expression was 3.5 to 16.3-fold higher in HT, compared to the susceptible. Target site mutations, differences in glyphosate absorption or translocation or altered expression of Aldo-keto reductase (*AKR*) were not identified in any of the resistant populations. Crosses were successful between one resistant population and the susceptible (P262-16♂ × S♀) and inheritance of glyphosate resistance appears to be controlled by a single, nuclear dominant gene in this population.

**CONCLUSION:** Our study identified *EPSPS* gene amplification in a South Australian glyphosate resistant *P. annua* population (HT). This mechanism of resistance was not identified in the other four glyphosate resistant populations, and other common mechanisms were excluded. While the resistance mechanism in some *P. annua* populations remains unknown, inheritance studies with one population suggest the involvement of a single dominant gene.

***Keywords:*** Annual bluegrass, *Poa annua*, Glyphosate, *EPSPS* Gene amplification, Inheritance.

## 1. INTRODUCTION

Glyphosate [(*N*-phosphonomethyl)glycine] is used for control of *Poa annua* L. in dormant seasonal turf grass such as zoysia grass (*Zoysia* spp.)[1], where the actively-growing *P. annua* can be controlled with minimal damage to the dormant turf. However, the continuous use of glyphosate in turf has resulted in the evolution of herbicide resistant *P. annua*. [2]

Glyphosate inhibits the 5-enolpyruvylshikimate 3 phosphate synthase (*EPSPS*) enzyme in the shikimic acid pathway, responsible for biosynthesis of the aromatic acids tyrosine, tryptophan, and phenylalanine. [3] Glyphosate resistance can be conferred by non-target site resistance (reduction of absorption, reduction in translocation, metabolism of glyphosate or vacuolar sequestration), [4-7] and/or target site resistance (alteration of the target enzyme, *EPSPS* duplication/overproduction). [2, 8-10]

Reduced glyphosate translocation, identified in several weed species, can enable plants to survive higher herbicide doses compared with target-site resistance mechanisms. [11] To date there is little understanding behind the mechanism involved, [9] however, vacuole sequestration correlated with glyphosate resistance in *Lolium* spp. from Australia, South America and Europe. [4] A report on a glyphosate-resistant horseweed population suggested the involvement of the ABC-transporter genes (M10 and M11) enhance glyphosate sequestration into the vacuole. [12, 13] Other non-target site mechanisms responsible for resistance in *Echinochloa colona* include an increase in the activity of aldo-keto reductase (*AKR*). [6]

Target site mutations at $Pro_{106}$ to Ser, Ala, Leu or Thr have been reported to confer glyphosate resistance in 14 different weed species, including *P. annua*. [2, 10, 14] Additional target site mutations at $Thr_{102}$ to Ile and Ser have been identified in five species and $Ala_{103}Val$ has been identified in one species. [2] The first report of glyphosate resistance in *P. annua* was due to an amino acid substitution (Pro106 to Ala), that provided 4.4-fold ($LD_{50}$ = 810 g a.e. $ha^{-1}$) resistance in a resistant biotype. [10]

Gene amplification of *EPSPS* refers to the process where additional copies of the gene are created from a segment of DNA in the genome of an organism. [15] The extra *EPSPS* gene copies result in the overproduction of the EPSPS enzyme, which is the target enzyme inhibited by glyphosate. [8] *EPSPS* gene amplification was first identified in *Amaranthus palmeri*, [16] and has subsequently been identified in numerous other weed species including *P. annua*. [14] According to Brunharo et al., [14] *EPSPS* duplication and target-site mutation at position 106, confers 18-fold ($LD_{50}$ = 540 g a.e. $ha^{-1}$) resistance in resistant biotypes of *P. annua*.

The inheritance pattern of glyphosate resistance has been examined in many weed species, but not in *P. annua*. In *Lolium rigidum*, [17, 18] *Erigeron canadensis* [19] and in *Eleusine indica* [20] a single, semi dominant nuclear gene with no influence from maternal effects was responsible for resistance. However, the involvement of multiple genes in glyphosate resistance has also been identified in *L. rigidum.* [21]

The aim of this research was to investigate glyphosate resistance in populations of *P. annua* that glyphosate application had failed to control, as well as to explore possible mechanisms associated with resistance and inheritance pattern.

## 2. MATERIALS AND METHODS

### 2.1 Plant materials

Initial screening was conducted on 31 *P. annua* populations that were obtained from different golf courses in the south-eastern part of Australia (Barua et al. 2020) and a population (hereafter called HT), collected from a residential area in South Australia (SA) where glyphosate was used to control weeds in the garden. From the 32 populations, five populations (four from golf turf and one from a residential area) of *P. annua* were selected for further study because of their varying responses to glyphosate, as described in the next section. Populations P18, P27 and P413-17 were collected from golf courses of Victoria and population P262-16 from New

South Wales (NSW), Australia. All four populations were previously confirmed as multiple resistant to five other herbicide modes of action (ALS inhibitors, ACCase inhibitors, PSII inhibitors, microtubule assembly inhibitors and inhibitor of serine-threonine protein phosphatase) by Barua et al. [22] However, HT population from a residential area in SA was found to be resistant only to glyphosate. The susceptible population used in this study was collected from a residential lawn in Adelaide, South Australia, where no glyphosate had been used.

### 2.2 Preliminary Screening for Resistance in Sampled Populations

Initial screening of the 32 populations was conducted in December 2017 and repeated in June 2018. Seeds were sown in germination trays (330 mm × 200 mm × 50 mm) and at the one-leaf stage, seedlings were transplanted into punnet pots (9.5 cm × 8.5 cm × 9.5 cm) (Masrac Plastic, Adelaide, SA) using a standard potting mix. [23] The experiment was performed in a randomised block design with 3-replications with five plants per pot. At the 4-leaf stage, plants were treated with glyphosate (WSSA Group 9) (Clear up Bio, 360 g a.e. $ha^{-1}$, BARMAC Pty Ltd, NSW, Australia) at 180 g $ha^{-1}$ and 540 g $ha^{-1}$. The greenkeeper's typical use rate for glyphosate of 180 g $ha^{-1}$ is lower than the label rate to avoid damage to turf. [24] Spraying was performed using a laboratory moving boom twin nozzle (TeeJet® 110 ° flat fan, Spray System, Wheaton, IL, USA) sprayer with an output of 118 $Lha^{-1}$ at the pressure of 250 kPa and speed of 1 $ms^{-1}$. Plants were returned outdoors after the spray and herbicide efficacy was determined at 28 days after treatment (DAT), where plants with new tillers and subsequent growth were considered as survivors and plants with complete mortality or severe stunting were considered as dead. Seeds from both untreated control plants and plants surviving the 1x and 3x rate of the herbicide were collected for further experiments and stored dry at 10 °C.

### 2.3 Dose-response experiments at low (16/12 °C) and high (27/20 °C) temperatures

From initial screening, five resistant (R) populations (P18, P27, P262-16, P413-17 and HT) were selected and used for further studies. Temperature (high 30 °C and low 20 °C) has been documented to affect the level of glyphosate resistance, such as in *E. colona*. [25] Therefore, a dose-response study was conducted at two different temperatures to evaluate the impact of temperature on glyphosate resistance. The experiments were performed in two different controlled environment chambers: one set at 16/12 °C day/night and the other at 27/20 °C day/night with a photoperiod of 12 hours at 553-μmol$m^{-2}$ $s^{-1}$ light intensity. Seeds were germinated from all the resistant and the S population and at the one-leaf stage transplanted into pots as described above. Plants at 3 to 4-leaf stage were treated with glyphosate using various rates (0, 45, 90, 180 and 360 g a.e. $ha^{-1}$) using a laboratory moving boom sprayer as described previously. Mortality data were analysed using PriProbit (v. 1.63) [26] and the concentration causing 50% mortality ($LD_{50}$) estimated. Curves were back-transformed for plotting using the GraphPad (GraphPad Inc., San Diego, CA, USA) software. For each population, the resistance index (R/S) was calculated as the ratio of $LD_{50}$ of the resistant population to $LD_{50}$ of the S population. The experiment was repeated once.

### 2.4 Whole plant shikimic acid assay

Shikimate accumulation was measured following the method of Wakelin et al. [27] and Shaner et al. [28] with modification. After germination, plants were grown in controlled-environment chambers at two different temperature regimes as described above. At the 3-4 leaf stage, plants were treated with 180 g $ha^{-1}$ glyphosate using the laboratory spray cabinet described above. Plant shoots were harvested at 0, 6, 12, 24, 48, 72 and 96 hrs after herbicide application and fresh weight (fw) measured. The shoots (0.02 to 0.2 g) were cut into pieces and transferred into 10 mL tubes then 2 mL of 0.25 N HCl added to each tube to digest the shoots. The sample

tubes were frozen at -80 °C and then incubated for 1.5 h at 60 °C followed by centrifugation for 10 minutes. This process of freezing and thawing was repeated until the plant tissue turned brown. A supernatant sub-sample of 1 mL was transferred into a 2.0 mL microcentrifuge tube and stored at -20 °C and kept for a maximum period of one week before analysis.

A flat-bottom microplate were used to analyse the samples. Into each well of the plate, an aliquot of 25 µL of the sub-sample was added to 100 µL of 0.25% (w/v) periodic acid and 0.25% (w/v) sodium meta-periodate and incubated at room temperature for 60 min. Finally, 100 µL (0.6 mol $L^{-1}$ NaOH, 0.22mol $L^{-1}$ Na2SO3) of quench buffer was added to each well and the absorption read immediately at 380 nm using a microplate manager (Benchmark Pols Bio-Red Laboratories, Inc. Hercules, CA, USA). Shikimate standards in the 0-1000 µM range (Sigma, Castle Hill, NSW, Australia) were used to develop a standard curve. The shikimate content was calculated as µmoles shikimate $g^{-1}$ fw.

### 2.5 *EPSPS* gene sequencing

From all resistant and the susceptible populations, 0.1 g tissue was collected from fully expanded leaves of five individual plants and snap-frozen in liquid nitrogen. DNA extraction was carried out using Isolate II Plant DNA extraction kit following the manufacturer's instructions (Bioline, Alexandria, New South Wales, Australia). Using a standard protocol followed by Adu-Yeboah et al., [29] a conserved area of the *EPSPS* gene (374 bp section) was amplified by polymerase chain reaction (PCR) using the primers previously reported by Cross et al. [10] (Table 1). The PCR products were sequenced by the Australian Genome Research Facility, Adelaide University, Australia with the same primers used for amplification. In order to align and evaluate nucleotide sequences, ContigExpress and the AlignX programmes of the VectorNTi (11.5) software suite (Invitrogen Waverley, Victoria, Australia) were used.

Table 1. The primers and probes used to identify the target-site mutation and determine the genomic copy number of *EPSPS* and *ALS* using quantitative real-time PCR

| Primer Name | Primers/probes sequences 5′ –3′ | Reference |
|---|---|---|
| ***EPSPS* Target-site mutation** | | |
| *EPSPS* F | TGTCCGAGGGAACAACTGTG | Cross et al. [10] |
| *EPSPS* R | ACGAACAGGTGGGCAGTTAG | |
| ***ALS* Target-site mutation** | | |
| *Poa_ALS* F574 | TGGGCGGCTCAGTATTACAC | McElory et al. [48] |
| *Poa_ALS* R574 | ATAGGCAGCACATGCTCCTG | |
| *Poa_ALS* 1F | ACCCGCATCAGGTGCTCCACGGT | Barua et al. [22] |
| *Poa_ALS* 1R | AGGAGGCGAGGAAGAAGGCTTCCT | |
| ***EPSPS* gene copy number** | | |
| *EPSPS* F | GGCAGGTTCCCGATTGAAA | |
| *EPSPS* R | TCCACCAGCAGCTACTACA | |
| *EPSPS* Probe | AGGATGCCAAAGAGGAAGTGCAGCTC + *FAM* | |
| *ALS* F | CCAACCCAGGTGTCACAGT | |
| *ALS* R | ATGCGAATCAGTGCCAACTCC | |
| *ALS* Probe | TGACATTGATGGAGATGGTAGCTTCC + *TET* | |
| **Aldo-keto expression** | | |
| *AKR* F | GACGCTGCTAAAGCTCGTG | |
| *AKR* R | GCAAGCAAGTCACCCAGTT | |
| *AKR* probe | TGGTGTGAGTAACCTTGCATCGAA + *FAM* | |

### 2.6 *EPSPS* gene copy number and expression analysis

*EPSPS* gene copy number and expression relative to a reference *ALS* (*acetolactate synthase*) gene were estimated from quantitative PCR (qPCR) procedure as describe by Adu-Yeboah et al. [29] Genomic DNA from 10 single plants from five resistant and the susceptible populations of *P. annua* was extracted as mentioned above. The resistant populations were sampled from those treated with glyphosate and the susceptible from non-treated. The primer and probes used are listed in Table 1. Using the dual label BHQ FRET probes (Bioresearch Technologies, Petaluma, CA, USA), a KAPA PROBE FAST Universal (KAPA Biosystems, MA, USA) assay was performed using a Rotor-Gene- thermal cycler (RG3000). The primers were tested on a dilution series (1×, 1/2×, 1/4× and 1/8×) of the gDNA of the susceptible population to generate a standard curve for primer efficiency. The $R^2$ values were >98 for both primer pairs amplification efficiency. There were three technical replicates run for each sample. The results from the qPCR experiments were analysed using a modified version of the $\Delta C_T$ ($2 = {}^{-\Delta CT}$) technique following Adu-Yeboah et al. [29] The results were reported as the relative copy number of *EPSPS* copy level in comparison to *ALS*.

Since HT showed increased *EPSPS* copy number, relative *EPSPS* expression was investigated in this population. The same qPCR method above was used except with cDNA. Total RNA was extracted using an Isolate II Plant RNA kit (Bioline) according to manufacturer's instruction from the leaf tissue of five individual plants of both HT and S populations to determine their gene expression. An additional DNase treatment was performed using the RNAse free DNAse set (Qiagen) to ensure no DNA contamination in the RNA. A Tetro cDNA synthesis kit (Bioline) was used as per manufacturer's instructions to synthesize cDNA. The qPCR was performed as mentioned above. There were three technical replicates in each run. The relative quantification calculation mentioned above was used for fold increase in *EPSPS* expression.

### 2.7 Aldo-keto reductase (*AKR*)

Aldo-keto reductase (*AKR*) expression was measured with qPCR following the method described above for *EPSPS* gene copy number. Seeds from the resistant and susceptible populations were germinated and at the one-leaf stage, plants were transplanted into punnet pots (9.5 cm × 8.5 cm × 9.5 cm) with nine plants per pot with six replicated pots. After transplanting, one set of plants (with three replications) were moved into the growth chamber at a low temperature (16/12°C day/night) regime and the other set of plants at a high temperature (27/20 °C day/night) regime growth chamber. Both chambers were set at 12 h photoperiod at 553 μmol $m^{-2}$ $s^{-1}$. At 3-4 leaf stage, the plants were treated with 180 g a.e. $ha^{-1}$ glyphosate as previously described. Fresh leaf tissues were collected at 0 and 48 hrs after herbicide application.

Total RNA was extracted from five individual plants of each population prior to and after glyphosate application using the RNeasy Mini Kit (Qiagen, Melbourne, Victoria). cDNA was synthesized using the Tetro cDNA synthesis kit (Bioline) according to the manufacturer's instructions. The *AKR* gene expression relative to *ALS* control gene was measured using the *AKR* gene primers and probes and the *ALS* control primers and probes with different fluorophores (Table 1). *ALS* was previously found not to have significant expression changes in *Festuca arundinacea* following glyphosate application. [30] Amplification of cDNA templates (~20 ng) was performed using the RG3000 Rotor-Gene real-time thermal cycler technique and conditions as detailed previously for the *EPSPS* gene amplification study. The experiment was conducted twice with 5 individuals of each population in the first run and 4 individuals in the second run with 3 technical replicates in each run. Data was pooled across experiments. Relative *AKR* gene expression prior to glyphosate application and after glyphosate application was calculated as described for *EPSPS* copy number above. Data from prior to glyphosate application were analysed by two way ANOVA with population and temperature as factors.

Data were log transformed prior to analysis. Data after application was calculated as the ratio of relative *AKR* gene expression following glyphosate treatment to relative *AKR* gene expression prior to treatment for each individual. The ratios were log transformed prior to two way ANOVA as described above.

### 2.8 Effect of temperature on glyphosate absorption and translocation

Glyphosate absorption and translocation was determined following the method of Wakelin et al. [31] with modification. Seeds of all the five resistant and S populations were sown in trays and at one-leaf stage, transplanted into a Black Plastic Tub (26 cm by 19 cm by 9 cm), containing 2 L of modified Hoagland nutrient Solution [32] and 1 kg of black polypropylene beads, with 15 seedlings of each population. Plants were transferred to a growth camber set at 27/20 °C as described above. Solution losses due to evaporation were replaced daily. Plants were treated with 45 g a.e. $ha^{-1}$ glyphosate at the 3 to 4-leaf stage using the laboratory spray cabinet as described above. Immediately after spraying, plants were transferred back to the growth chamber and 1 µL of $^{14}C$ glyphosate (including 0.5 kBq of radiation) was applied to the second completely expanded leaf of each seedling. The $^{14}C$ glyphosate (phosphonomethyl-$^{14}C$) (American Radiolabelled Chemicals, St Louis, MO, USA) had a specific activity 0.185 GBq $mmol^{-1}$. The application of radioactive material was completed within 30 min of herbicide application and the treated leaf was marked for identification during sample collection. Plants were harvested as three sections, the treated leaf, rest of the shoot and roots at 24, 48 and 72 hrs after treatment. The treated leaf was washed in 5 mL of 0.1% Triton X-100 (Sigma-Aldrich, Castle Hill, NSW) to remove non-absorbed radioactivity. The plant parts were dried separately for one week at 40 $^{o}C$ in tissue paper. The dried plant parts were combusted in a bio-oxidizer for 1 min (Sample Oxidizer 307, Perkin Elmer, Shelton, CT, USA). $^{14}CO_2$ was trapped in Carbo Sorb E (Canberra Packard, Groningen, The Netherlands), added to an equal volume of

Permaflour E+ (Canberra Packard, Groningen, The Netherlands) and the radioactivity quantified by liquid scintillation spectroscopy (Beckman Coulter, Fullerton, CA, USA). To quantify radioactivity in the wash solution, 5 mL Ultima Gold XR (Canberra Packard) fluid was added to the wash solution and radioactivity measured using scintillation spectroscopy. The amount of $^{14}$C-glyphosate in each plant part was expressed as a percentage of the amount absorbed. The experiment was repeated once. Before statistical analysis, the data was square root transformed. Two-way analysis of variance (ANOVA) analysis showed the two experimental runs were not statistically different so the data were pooled and analysed by ANOVA (Genstat 19th edition; VSN International, Hemel Hempstead, UK). The means were separated by Fisher's protected LSD multiple comparisons at P = 0.05 (GenStat 19th).

### 2.9 Inheritance of resistance

In a previous study by Barua et al. , [22] populations P18, P27, P262-16 and P413-17 were found to be resistant to *ALS*-inhibiting herbicides due to *ALS* Trp574Leu mutation in P27, P262-16 and P413-17 and Pro197Ser mutation in P18. These mutations were used as a marker in the crossing programme to confirm successful crossing. Population HT did not have a mutation in *ALS* and was not included in the crossing program.

*P. annua* is typically self-pollinated with up to 15% outcrossing. [33] Since the flowers are very small they may be damaged by emasculation, therefore an alternative approach to $F_1$ seed development was undertaken following the method followed by Preston and Malone [34] with modifications. Seeds previously collected from glyphosate survival of the four resistant populations and one S from the untreated plants were germinated in different trays and five seedlings transplanted into a 17 cm diameter pots containing standard potting mix, with a single plant per pot and watered as required. At flowering every morning, pollen from each resistant plant was dusted on to the flowers of susceptible plant and the flowers bagged. Individual

flowers of the both resistant and susceptible plant were also bagged to self-pollinate. Matured seeds of the resistant, S and suspected $F_1$ were collected, dried and, stored in 4 °C for further use.

Seeds of each suspected $F_1$ and resistant parent and sensitive parents were sown into 40 cm by 30 cm by 5 cm trays using the potting mix as described above. At one-leaf stage, plants were treated with rimsulfuron (Turf Culture Pty Ltd, Sunbury, Australia) at 25 g a.e. $ha^{-1}$ with the laboratory herbicide cabinet described above to identify potential crosses. None of the plants from selfed susceptible flowers survived rimsulfuron treatment, but for crosses 8 plants (<1%) survived from one pair of crosses (262-16♂ × S♀). The survivors were rescued for seed multiplication. Thus, the $F_2$ seeds were obtained from the selfing of successful $F_1$.

To confirm the $F_1$ crosses, about 0.1 g of leaf material was harvested from the putative eight $F_1$ plants in addition to a selection of $F_2$ plants, and snap-frozen in liquid nitrogen. *ALS* gene amplification and sequencing was carried out as described above, using *ALS* gene-specific primers (Table 1). All the $F_1$ plants contained the *ALS* Trp574Leu mutation found in the parent thus confirmed as a successful cross.

A detailed dose-response experiment was conducted on the $F_2$ population. Seeds of $F_2$, P262-16 parent and susceptible parent were germinated and at the one-leaf stage, the seedlings were transplanted with nine seedlings per 9.5 by 8.5 by 9.5 cm pot (Masrac Plastics, Adelaide, SA) with three replications. At the 3 to 4-leaf stage, glyphosate was applied at various rates between 0 to 360 g a.e. $ha^{-1}$ using the laboratory pesticide applicator as mention above. Plants were returned outdoors and survival evaluated 28 days after treatment. The mortality data for the parents was analysed by PriProbit (v. 1.63) [26] and the rate providing 50% mortality calculated ($LD_{50}$). Back-transformed curves were plotted.

Two-way ANOVA indicated no difference between the two experimental runs so the data were pooled. Inheritance was tested using an exact binomial test that was two-sided and

used the method of small P values [35] with expected survival values corrected for mortality of the P262-16 population and survival of the S population at the dose chosen.

## 3. RESULTS AND DISCUSSION

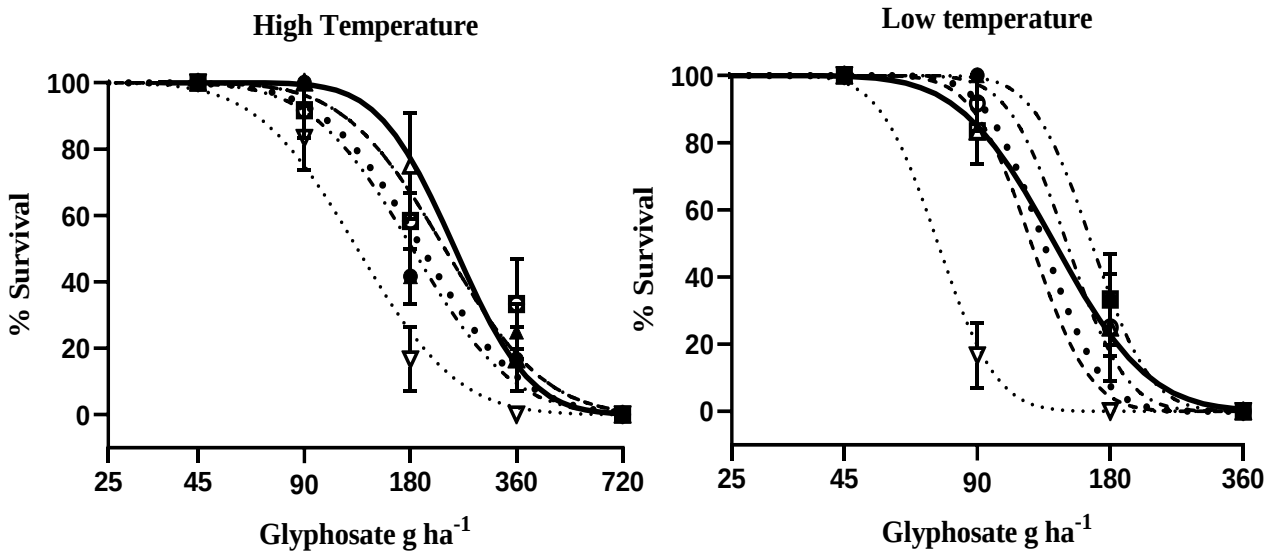
### 3.1 Screening for Resistance

Out of 32 *P. annua* populations examined, five populations were found to be resistant. Three populations (P262-16, P413-17 and HT) survived (100%) glyphosate at 180 g $ha^{-1}$; and, two populations (P18 and P27) survived (>70%) at 540 g $ha^{-1}$. Therefore, further experiments were conducted with these five populations.

### 3.2 Effect of temperature on resistance to glyphosate

Detailed dose-response experiments conducted with five resistant populations confirmed a low level of resistance to glyphosate. Greater survival to glyphosate was identified in all populations at high temperature (27/20 °C) compared with low temperature (16/12 °C) (Figure 1 and Table 2). At both temperatures, the $LD_{50}$ (104.6 to 512 g $ha^{-1}$) for the resistant populations was between 1.3 and 2.5-fold that of the susceptible population. This is a relatively low level of resistance, but sufficient for *P. annua* to survive the low rate of glyphosate used by green keepers. The effects of temperature on glyphosate efficacy in the current study were similar to those observed for *L. rigidum*, [36] *E. colona*, [25] and *Sorghum halepense* [37] where resistance was greater at higher temperatures.

*Experiment 1*




*Experiment 2*

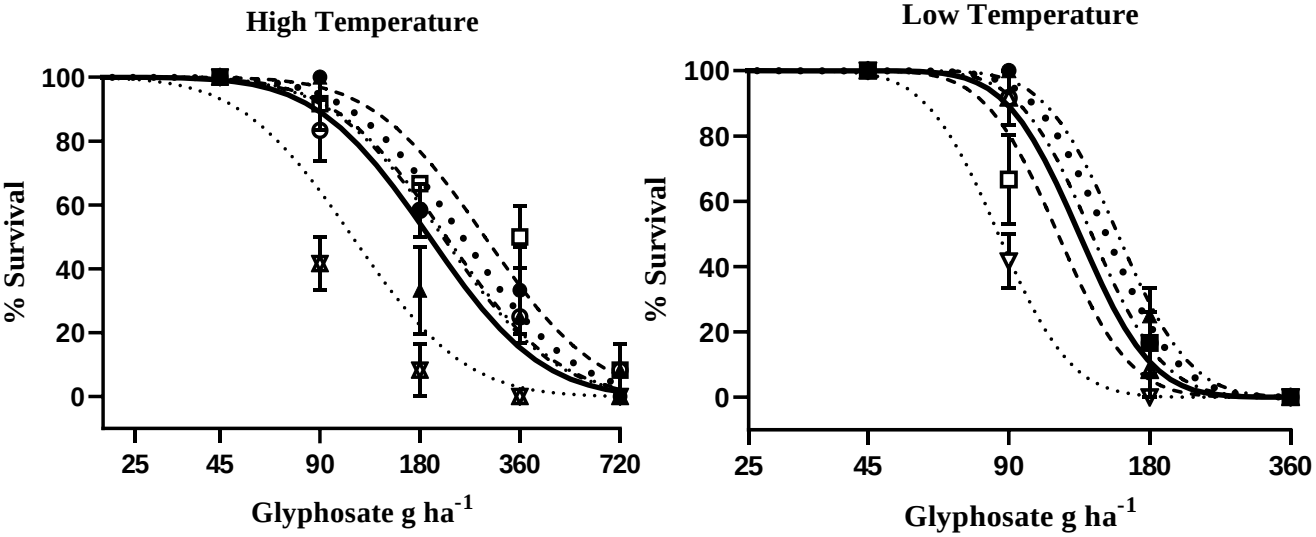



Figure 1: Response of susceptible S (▽), and resistant P18 (●), P27 (▲), P262-16 (○), P413-17 (▢), HT (Δ) populations of *P. annua* after 28 days treated with glyphosate at POST herbicide timing in two different temperature condition. Data were pooled for the two experimental run. Each point is the mean of 48 plants (8 replications of 6 plants). The lines are the probit curves back transformed to percentages with 95% confidence intervals: S (.....), P18 (▪▪▪▪▪ ), P27 (— · · — ), P262-16 (– · –), P413-17 (– – –) and, HT (———).

Table 2. The dose required for 50% mortality ($LD_{50}$) of susceptible and resistant *P. annua* populations with 95% confidence intervals (CI) in parentheses. RI (resistance index) is the ratio of $LD_{50}$ of resistant and susceptible population.

| | Experiment 1 | | | | Experiment 2 | | | |
|---|---|---|---|---|---|---|---|---|
| Population | High Temp. | | Low Temp. | | High Temp. | | Low Temp. | |
| | $LD_{50}$ | RI | $LD_{50}$ | RI | $LD_{50}$ | RI | $LD_{50}$ | RI |
| P18 | 194.4 (149.9, 512.0) | 1.5 | 127.3 (103.9, 155.8) | 1.7 | 241.7 (182.1, 321.8) | 2.1 | 145.2 (118.7, 176.8) | 1.7 |
| P27 | 182.4 (140.3, 236.7) | 1.4 | 163.4 (136.5, 193.9) | 2.2 | 210.7 (159.7, 277.6) | 2.0 | 153.8 (126.2, 186.5) | 1.8 |
| P262-16 | 225.1 (106.6, 475.6) | 1.8 | 143.6 (120.5, 170.0) | 2.0 | 216.1 (164.8, 283.4) | 2.0 | 135.3 (114.7, 168.7)) | 1.6 |
| P413-17 | 225.1 (106.6, 475.6) | 1.8 | 119.3 (100.2, 142.7) | 1.6 | 282.7 (215.4, 371.7) | 2.5 | 114.6 (95.9, 136.8) | 1.3 |
| HT | 240.7 (186.7, 310.6) | 1.9 | 134.6 (104.5, 173.5) | 1.8 | 192.0 (143.3, 256.9) | 1.7 | 127.3 (104.6, 154.9) | 1.5 |
| S | 127.2 (97.5, 165.8) | - | 73.3 (60.5, 87.7) | - | 112.7 (83.9, 151.2) | - | 85.5 (70.5, 103.5) | - |

### 3.3 Shikimate accumulation upon treatment with glyphosate

Shikimate accumulation was measured in whole shoots at 0, 6, 12, 24, 48, 72 and 96 hrs after glyphosate application. Basal levels of shikimate (0 hr) were similar for low temperature (0.12 – 0.16 µmoles $g^{-1}$ FW) and high temperature (0.10 – 0.20 µmoles $g^{-1}$ FW) for all 5 resistant populations and the S population (Fig. 2). Shikimate accumulation was observed in all populations 6 hours after treatment, and at 48 hours after treatment, all populations showed the maximum accumulation of shikimate. Shikimate accumulation reduced from 48 hrs after herbicide application with the exception of the susceptible population at low temperature. The two resistant populations P18 and P27 consistently had the least accumulation of shikimate despite the fact that these two populations were not more resistant than the other resistant populations. This suggests different mechanisms may be contributing to resistance among the various populations. The difference in patterns of shikimate accumulation between the resistant populations compared to the susceptible population indicate that *EPSPS* is initially inhibited by glyphosate in the resistant populations and then recovers, whereas the susceptible population does not recover. [38] Shikimate accumulation is an indication of whether the shikimate pathway is inhibited, the over-expression in HT population is obviously not sufficient to keep the pathway at full capacity.

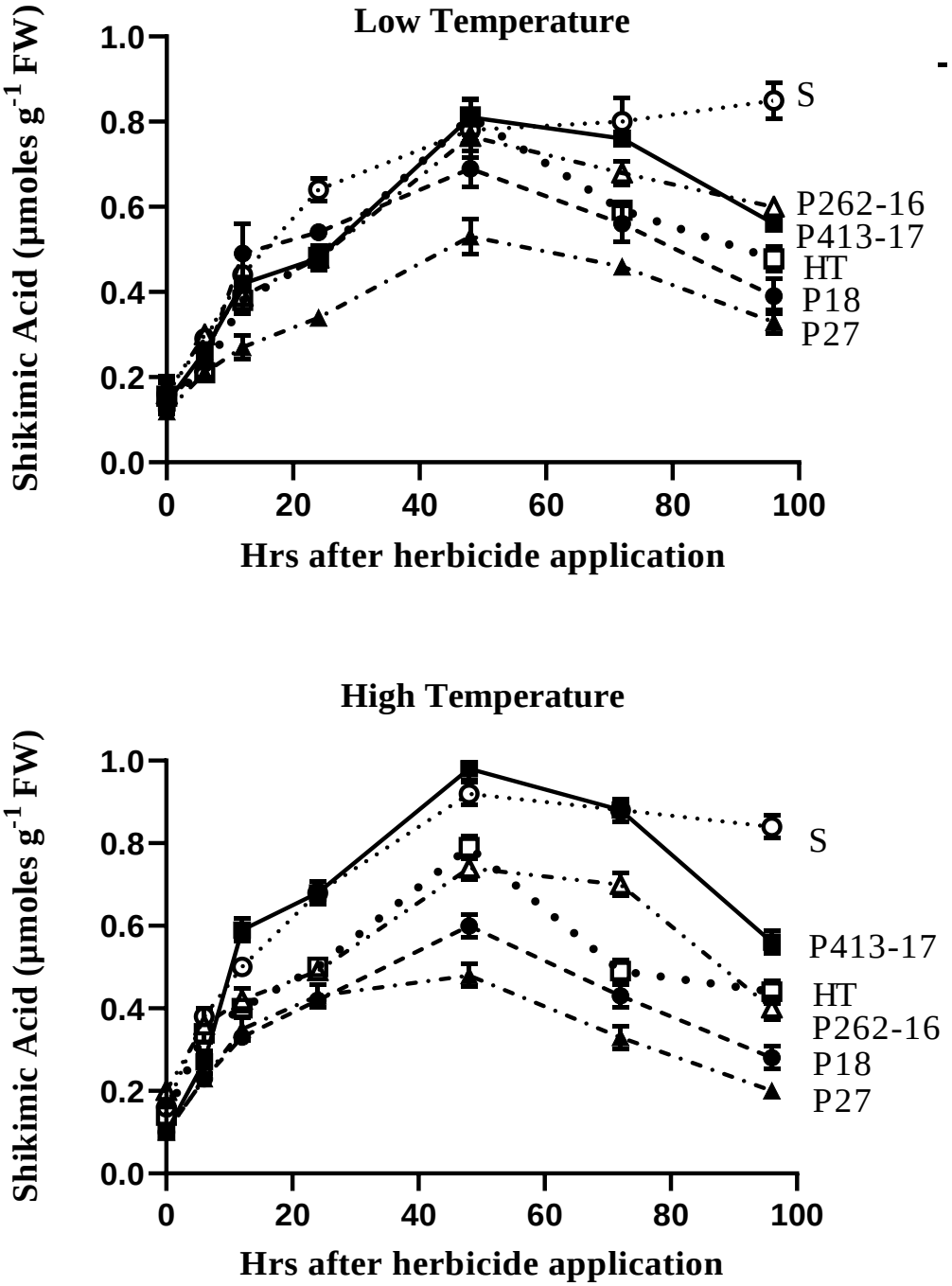



Figure 2. Time course of shikimic acid accumulation in resistant populations P18 (●), P27 (▲), P262-16 (Δ), P413-17 (■), HT (□) and, S (o) population treated with glyphosate (180 g $ha^{-1}$) at two different temperatures. Each data presented the mean amount of shikimic acid ± SE. The data presented the average of ten replication with similar result.

### 3.4 Target-site mutation identification in *EPSPS*

Glyphosate resistance can result from a mutation in the *EPSPS* gene at codon 106 (Pro106) [27, 39, 40] and this was previously found in *P. annua*. [10, 14] However, according to Yu et al., [41] a rare mutation at codon 102 (Thr102) combined with Pro106 confers a higher level of resistance in *E. indica*. Besides, a novel triple substitution (Thr102, Ala103 and Pro106) in *A. hybridus* (syn: quitensis) was identified. [42] To investigate if Pro106 target site mutations were present in the resistant populations, a 374-bp region surrounding the Pro106 codon of the *EPSPS* gene was amplified using primers previously described by Cross et al. [10] No differences were identified between any of the resistant populations and S population at nucleotide positions Pro106 and Thr102 (data not shown). However, a missense mutation at amino acid position Val85Ile was observed in all the resistant populations, which was previously reported by Cross et al. [10] There is no evidence this missense mutation confers glyphosate resistance.

### 3.5 *EPSPS* gene copy number and expression analysis

The relative genomic copy number for the *EPSPS* gene relative to *ALS* was similar in four of the resistant (P18, P27, P262-16 and P413-17) populations and the susceptible population with a mean copy number of 0.8 to 1.5 *EPSPS* relative to *ALS* (Fig. 3) despite these resistance populations being also resistant to ALS inhibiting herbicides.[22] However, population HT had a higher mean copy number for *EPSPS* of 5.0 (1.5-9.3) fold relative to *ALS*. There was considerable variability in the copy number in this population suggesting it may consist of a mixture of susceptible and resistant individuals and this may explain the relatively low level of resistance. Previous research by Brunharo et al. [14] showed that a seven-fold increase in *EPSPS* copy number relative to *ALS* was observed in the resistant *P. annua* population. Besides, an increase in *EPSPS* copy number was reported in other weed species: in *Bassia scoparia* (L.)

A.J.Scott 3-to 9-fold, [43] *A. palmeri* 1.6-to 8.5-fold [44] and *Hordeum glaucum* Stued. 9-to 11-fold [29] more than the susceptible population.

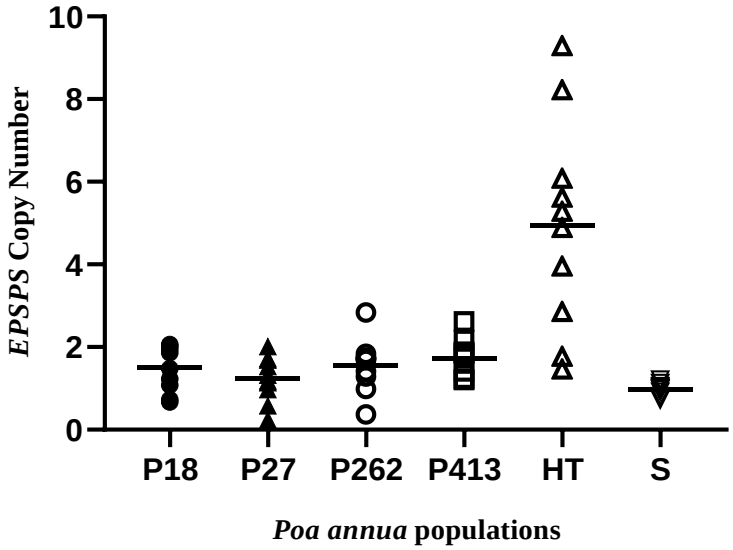



Figure 3. *EPSPS* gene copy number relative to *ALS* for glyphosate resistant and susceptible (S) *P. annua* populations.

Given the higher number of *EPSPS* gene copy number in HT, an additional *EPSPS* relative to *ALS* gene expression analysis was conducted on cDNA using qPCR. This showed that the level of gene expression of the HT population was 3.5 to 16.3-fold higher compared to that of the susceptible population and a positive correlation was identified between the genomic copy number and cDNA expression in individuals of this resistant population (Fig. 4). This type of positive correlation between genomic *EPSPS* copy number and cDNA expression level (relative to *ALS*) was found in *A. palmeri*. [44] In contrast, positive correlation between genomic *EPSPS* copy number and cDNA *EPSPS* expression level (relative to *ALS*) was not found in *H. glaucum*. [29]

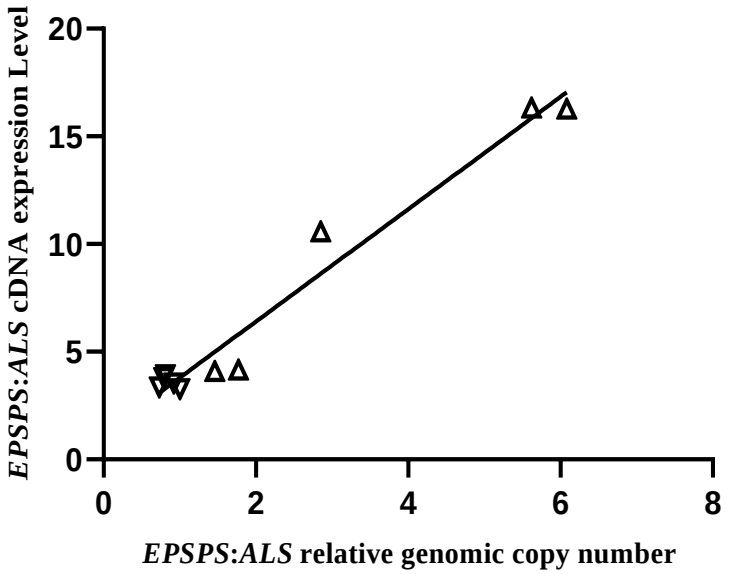



Figure 4. The linear relationship between *EPSPS*:*ALS* genomic copy number and *EPSPS*:*ALS* gene expression of HT (Δ) and susceptible (▽) population. The increase *EPSPS* gene copy number was positively correlated ($y = 2.6078x + 1.1851$, $R^2 = 0.96$) with gene expression.

### 3.6 Aldo-keto reductase (*AKR*) and expression analysis

*AKR* gene expression relative to *ALS* was also investigated using qPCR on cDNA. *ALS* gene expression was not reported to significantly change in *Festuca arundinacea* following glyphosate treatment. [30] At both low and high temperatures, mean *AKR* gene expression ranged from 0.9 to 2.0-fold expression of *ALS* for the different populations in the absence of glyphosate treatment (data not shown) with no significant differences between populations ($P = 0.36$). There was a significant effect of temperature ($P = 0.004$) with relative expression at low temperature (1.6) being greater than at high temperature (1.1). The ratio of relative *AKR* gene expression after glyphosate application to relative *AKR* gene expression prior to glyphosate application varied for population ($P < 0.001$), but not for temperature ($P = 0.51$) (Fig 5). The S

population had a significantly greater increase in *AKR* gene expression compared to the resistant populations P18, P27 and P413-17, but not for P262-16 or HT. The resistant population P262-16 had a larger increase in *AKR* gene expression compared to populations P18 and P27. Population HT also had a larger increase in *AKR* gene expression compared to these populations, as well as P413-17. Metabolic degradation of glyphosate is a rare, but possible mechanism of resistance in plants. Pan et al., [6] found EcAKR4-1 gene overexpression conferred glyphosate resistance in *E. colona*. In the current study, there does not appear to be a relationship between *AKR* gene expression and glyphosate resistance in these populations of *P. annua.*

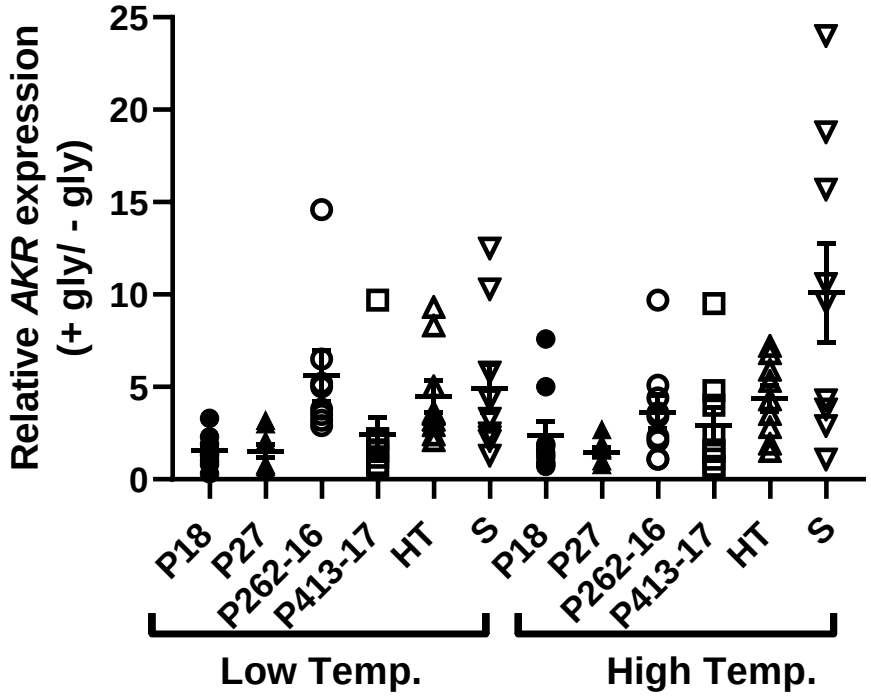



Figure 5. Relative *AKR* gene expression following glyphosate application at low and high temperatures to 5 resistant and one susceptible population of *P. annua*. Values are the ratio of relative expression of *AKR* after application of 180 g $ha^{-1}$ glyphosate compared to relative expression prior to glyphosate application for each individual plant.

### 3.7 $^{14}$C-glyphosate absorption and translocation

Glyphosate absorption and translocation play a key role in the efficacy of this herbicide and a decrease in translocation is known to result in resistance. [38] There were slight variations in the amount of glyphosate absorbed among the populations, with population P27 having significantly less glyphosate absorbed than resistant population P262-16 at 72 hours of treatment (Table 3). However, glyphosate absorption for other resistant populations (P18, P262-16, P413-17 and HT) were not significantly different to the susceptible population. About a third of the absorbed herbicide remained in the treated leaf with the rest translocating to the rest of the shoot and the roots in about equal proportions (Table 3). There was no significant difference between populations in the proportion of absorbed herbicide remaining in the treated leaf or the shoots.

Table 3. $^{14}$C glyphosate absorption and translocation in glyphosate susceptible and resistant populations of *P. annua* at 72 hours after treatment (HAT).

| | | $^{14}$C glyphosate present at 72 HAT | | | |
|---|---|---|---|---|---|
| Populations | *n* | Absorbed | Treated leaf | Rest of shoot | Roots |
| P18 | 10 | 64.9 ab | 30.4 a | 27.3 a | 41.3 b |
| P27 | 10 | 56.2 a | 37.3 a | 28.3 a | 35.5 ab |
| P262-16 | 10 | 70.8 b | 32.9 a | 31.1 a | 36.1 ab |
| P413-17 | 10 | 60.3 ab | 33.7 a | 31.0 a | 35.3 ab |
| HT | 10 | 58.2 ab | 39.4 a | 35.0 a | 25.6 a |
| S | 10 | 59.3 ab | 41.1 a | 32.9 a | 25.9 a |

Mean followed by the same letter are not statistically different at $P \leq 0.05$. *n*, number of plants from each population used to calculate the statistics (Fisher's protected LSD). The data were presented as the representative of six population. P18, P27, P262-16, P413-17, HT were the resistant population, S, susceptible. HAT, hours after treatment.

However, population HT and the susceptible population had significantly less glyphosate in the roots than P18. Where resistance is due to reduced translocation, glyphosate tends to accumulate in high amounts in the treated leaves. [17, 27] The small differences in absorption or translocation of glyphosate observed in this study appear insufficient to explain resistance. Therefore, we can rule out this mechanism for glyphosate resistance in those populations.

### 3.8 Inheritance of resistance to glyphosate

Only one cross producing eight (<1%) possible $F_1$ (P262-16 x S) plants was successful. The $F_1$ plants were confirmed because all eight individuals had the same *ALS* mutation (Arg574Leu) as the resistant parent. [22] The $F_2$ population in the dose-response experiments showed an intermediate response between the P262-16 and S parents. The dose-response curve mostly fitted a model for a single dominant allele calculated (dotted line) for $F_2$ (P262-16 $\times$ S) (Fig. 6). This suggests that a single, nuclear, dominant gene confers resistance. The data were analysed using an exact binomial test for a single-gene-model with a dominant allele and with a recessive allele using the survival values for 180 and 270 g $ha^{-1}$. The survival data fitted the 3:1 ratio for a single dominant allele at both rates and failed to fit the 1:3 ratio for a single recessive allele (Table 4). The similar type of a single, nuclear dominant inheritance pattern was reported in *E. colona* population that had a target site mutation. [45] Besides, the inheritance studies in many glyphosate resistant weed species (e.g., *E. indica* and *L. rigidum*) showed genetic control of a single, semi dominant nuclear gene in all cases where resistance was due to target site mutation. [20, 46] Furthermore, Wakelin and Preston [18] showed that the restricted glyphosate translocation resistance is inherited as a single, nuclear, dominant inheritance pattern in *L. rigidum*. While we have been unable to identify one of the commonly reported mechanisms of resistance to glyphosate in this population, this suggests there may be other potential resistance mechanisms.

The populations of *P. annua* tested had relatively low levels of glyphosate resistance; however, these low levels of resistance are sufficient to allow the weeds to survive the low rates used by greenkeepers. Employing higher rates is not an option for greenkeepers, as damage to the desired turf grasses will occur.[24] Use of low rates means that plants with weak mechanisms of resistance are able to survive the herbicide application. [47] This is reflected in the *P. annua* populations here where at least two resistance mechanisms are present. Only one of these mechanisms, gene amplification, was identified and other common mechanisms of glyphosate resistance were excluded. It is likely that *P. annua* has glyphosate resistance mechanisms that have not been reported in other species.

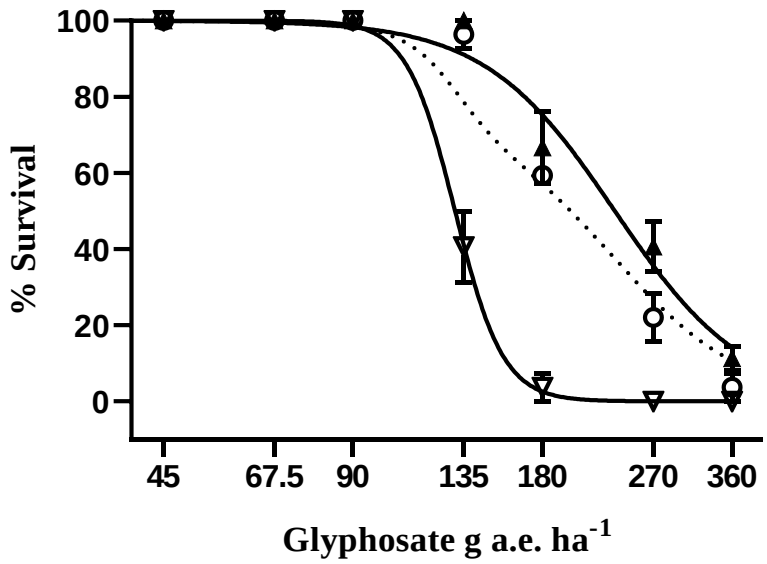



Figure 6. Glyphosate dose-response of susceptible (▽), P262-16 (▲) and F2 (○) populations of *P. annua*. The dotted line is the predicted response (3:1) of $F_2$ for resistance conferred by a single dominant allele. Data points are the means ± 95% CI for three-replicate × two repeats.

Table 4. Survival of parental and $F_2$ (P262-16 $\times$ S) populations at 180 and 270 g $ha^{-1}$ glyphosate with probability from exact binomial goodness of fit to the expected ratio for two different inheritance models.

| | S | P262-16 | $F_2$ (P262-16 $\times$ S) |
|---|---|---|---|
| | | 180 g $ha^{-1}$ | |
| Treated | 54 | 54 | 54 |
| Alive | 3 | 36 | 32 |
| Dead | 51 | 18 | 22 |
| Survival % | 5 | 67 | 59 |
| P (3:1) | | | 0.277 |
| P (1:3) | | | $8.7\times10^{-10}$ |
| | | 270 g $ha^{-1}$ | |
| Treated | 54 | 54 | 54 |
| Alive | 0 | 22 | 12 |
| Dead | 54 | 32 | 42 |
| Survival % | 0 | 41 | 22 |
| P (3:1) | | | 0.237 |
| P (1:3) | | | 0.006 |

## 4. CONCLUSION

Our research demonstrated the evolution of glyphosate resistance in five *P. annua* populations collected from the southeastern part of Australia. We identified the resistance mechanism for one *P. annua* population (HT) as due to *EPSPS* gene amplification and increased *EPSPS* expression. We were unsuccessful to identify the mechanism of glyphosate resistance in populations (P18, P27, P262-16 and P413-17) that were previously confirmed as multiple and cross-resistant to *ALS* inhibitors, *ACC*ase inhibitors, PSII inhibitors, inhibitors of microtubule assembly and inhibition of serine-threonine protein phosphatase. The inheritance of glyphosate resistance in P262-16 appeared to be controlled by a single, nuclear, dominant gene. This research demonstrates that glyphosate resistance has evolved more than once in *P. annua* in Australia and that more than one glyphosate resistance mechanism may be present.

## 5 ACKNOWLEDGEMENTS

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## DISCLOSURE STATEMENT

The author declared no conflict of interest.

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# CHAPTER 4: INHERITANCE AND MECHANISM OF PROPYZAMIDE RESISTANCE IN MULTIPLE-RESISTANT ANNUAL BLUEGRASS (*POA ANNUA* L.)

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**Pest Management Science: Submitted paper**

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| Contribution to the Paper | Planned the study, conducted all experiments, analyzed and interpreted data, wrote manuscript and acted as corresponding author. |
| Overall percentage (%) | 85% |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. |
| Signature | Date: 17.8.2021 |

## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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| Signature | Date: 17/08/2021 |

# Inheritance and mechanism of propyzamide resistance in multiple-resistant annual bluegrass (*Poa annua* L.)

**Short running title:** Propyzamide resistance in *Poa annua*

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**ABSTRACT**

**BACKGROUND:** Two populations of *Poa annua* from Victoria, Australia with multiple resistance to herbicides, including propyzamide, were investigated.

**RESULTS:** Crosses were made between two resistant populations and a susceptible population, with the resistant populations used as the pollen donor and the susceptible as the pollen receptor. Six $F_1$ plants from P18×S and four $F_1$ plants from P413-17×S were obtained. As propyzamide resistant plants were also resistant to ALS inhibiting herbicides, confirmation of true $F_1$ hybrids was obtained by sequencing the *ALS* gene to show the presence of a known mutation in the resistant parent plant. A detailed dose-response performed with $F_2$ populations indicated that a single dominant allele gives resistance in the P18 population, while more than one allele appeared to be involved in resistance in P413-17. Sequencing analysis identified no previously reported mutation in either *α-tubulin* or *β-tubulin* genes known to be associated with resistance. Studies using organophosphate insecticides (PBO and Malathion) found an antagonistic effect on propyzamide activity, suggesting enhanced degradation by cytochrome P450s is unlikely to contribute to propyzamide resistance.

**CONCLUSION:** While the specific mechanism of propyzamide was not identified, different inheritance patterns in two resistant populations suggest that there is likely more than one mechanism of resistance to propyzamide and that this mechanism may be complex and involve more than one gene.

**Keywords:** *Poa annua*, Annual Bluegrass, Propyzamide, Inheritance, *α-tubulin*, *β-tubulin*

## 1. INTRODUCTION

Annual bluegrass (*Poa annua* L.) is considered one of the most problematic annual weeds in temperate managed turf across the world. [1] According to a survey by the Weed Science Society of America (WSSA) conducted in North America in 2017, *P. annua* is ranked as the 4$^{th}$ most problematic weed in turf. [2] Features that make *P. annua* a problematic weed include prolific seed set of up to 200,000 seeds m$^{-2}$ [4] and year round germination under favourable conditions. [5] Historically *P. annua* has been effectively controlled by both PRE and early POST-emergent herbicides in managed turf. [6] However, in recent years, the repeated use of herbicides has led to herbicide resistance evolution in *P. annua*. Herbicide resistance in *P. annua* was first reported in 1970s, although those populations were not selected in turf. [7] More recently, increased reports of herbicide resistance in *P. annua* have been documented with 48 cases globally. [8]

Microtubules are protein dimers that consist of alpha (α) and beta (β) *tubulin*, [9] and are the major proteins necessary for cell division and expansion. [10] They are also the target of microtubule assembly inhibitor (MTI) herbicides (HRAC group K1), which are classified into four families: benzamide, benzoic acid, dinitroaniline, and pyridine. Herbicides in the benzamide family produce shortened tufts of microtubules specifically around the kinetochores. [11] Other MTI herbicides produce different effects e.g. dinitroanilines herbicides bind to the tubulin protein preventing the polymerization of the tubulin protein dimer [12] and, pyridine herbicides target the microtubule-associated proteins that function to stabilize microtubules. [13]

Propyzamide (pronamide), a MTI herbicide in the benzamide family, is commonly used for pre-emergence control of *P. annua* in turf, however, repeated use of propyzamide has selected for resistant populations, often with cross-resistance to different MTI families. [14] To date, 12 weed species, including *P. annua,* have developed resistance to MTI herbicides. [8] Of

five published reports of MTI herbicide resistant *P. annua*, three cases were resistant to the dinitroaniline herbicide prodiamine. These reports were in *P. annua* populations from Georgia and South Carolina, [15] North Carolina, [16] and Alabama. [17] Resistance was found to be attributed to either *α* or *β tubulin* gene mutations. Only two reports have been published so far on propyzamide herbicide resistance in *P. annua*. A population from Georgia was found to have a reduced absorption and translocation mechanism of resistance, conferring >10 times higher resistance compared to the S biotype. [18] In the second report from Australia, seven populations of *P. annua* collected from golf courses across south-eastern Australia were found to be resistant to propyzamide. [19] The genetic basis of resistance in these populations is still unknown.

Resistance to MTI herbicides can be conferred by target-site (TS) and non-target-site (NTS) mechanisms. Six different mutations; Leu-125-Met, Leu-136-Phe, Val-202-Phe, Thr-239-Ile, Arg-243, Met-268-Thr in the *α-tubulin* gene have been reported to confer resistance to dintroaniline herbicides in other weed species. [20-23] However, only one of these target-site mutations in *α-tubulin* (Thr239Ile) has been reported in *P. annua* to date. [17] Only two *β-tubulin* target-site mutations have been reported, one in *P. annua* (Arg241Lys) conferring dinitroaniline resistance [15] and the other in *Chlamydomonas reinhardtii* (Lys350Glu/Met) conferring resistance to colchicine, propyzamide and dinitroaniline herbicides. [24] Non-target site resistance mechanisms, namely a reduced absorption and translocation mechanism have been reported in *P. annua* [18], with enhanced metabolism been reported in *L. rigidum*, although the genetic basis of the resistance has not been identified. [25, 26]

In many weed species, herbicide resistance is the result of a single gene. [27] For example, trifluralin resistance was caused by a single nuclear recessive gene in two self-pollinated weed species: *E. indica* [28] and *S. viridis*. [29] In both cases, resistance was due to target-site resistance, which was later identified as *α-tubulin* mutations, Thr-239-Ile and Met-268-Thr in *E. indica* [30,

[31] and, Leu-136-Phe and Thr-239-Ile in *S. viridis.* [20] Similarly, trifluralin resistance in *L. rigidum* was reported as a single, recessive, nuclear trait. [32] The inheritance of propyzamide resistance has not been examined in any weed species. The focus of this study was to identify the mode of inheritance and the mechanism of the resistance to propyzamide in two resistant populations of *P. annua*.

## 2. MATERIALS AND METHODS

### 2.1 Plant materials

Two populations of *P. annua,* P18 and P413-17, collected from two golf courses in Victoria were used in this study. The two populations were previously confirmed as resistant (R) to five mode of action herbicides including *ALS*-inhibitors and propyzamide. [19] A population collected from a residential lawn in South Australia was used as a susceptible (S) control. The study was conducted at the University of Adelaide, Waite Campus, Urrbrae, Australia (34.9670°S, 138.6360°E).

### 2.2 Inheritance to resistance to propyzamide

#### *2.2.1 Formation of initial cross*

*P. annua* has very small flowers and is predominantly self-pollinated with low outcrossing. [33, 34] Due to the difficulty of emasculation, crosses were performed using the alternative approach of Preston and Malone [35]. Briefly, R plants that survived 1000 g a.i. $ha^{-1}$ propyzamide (Kerb® 500 g $L^{-1}$ propyzamide, Corteva Agriscience Australia Limited, Chatswood, Australia) were allowed to set seed, and this seed was used as the parent seed in the crosses. Both R and S seeds were sown in seedling trays (330 × 200 × 50 mm). Germinated seedlings were transplanted into 17 cm diameter pots (one plant per pot) containing standard potting mixture. [36] The pots

were transferred into a glasshouse and watered daily as required. Once plants had flowered, crossing was conducted every morning by dusting the pollen of individual resistant plants onto individual susceptible plants, with susceptible flowers bagged immediately afterwards. Individual flowers for both R and S parent were also bagged and allowed to self. At maturity, seeds from both selfed panicles and crosses were harvested separately, dried and stored at 4°C.

To identify true $F_1$ hybrids, advantage was taken of the fact that both resistant populations were also resistant to the ALS-inhibiting herbicide rimsulfuron. [19] Seeds from each attempted cross, as well as selfed seed from the resistant and susceptible parents were sown in 40 x 30 x 5 cm trays containing standard potting mix. [36] At the 1-leaf stage, seedlings were treated with rimsulfuron (Coliseum, Turf Culture Pty Ltd, Sunbury, Australia) at 25 g a.i. $ha^{-1}$ with a laboratory moving boom sprayer equipped with a twin nozzle (TeeJet® 110° flat fan, Spraying Systems, Wheaton, IL) delivering an output of 118 L $ha^{-1}$ at a pressure of 250 kPa and speed of 1 $ms^{-1}$. Putative $F_1$ plants that survived were rescued and transplanted into the 8 L pots to produce $F_2$ seeds. Since only a small number of seeds were produce by the $F_1$ plants, $F_2$ seeds were pooled within crosses from the same parents to generate one $F_2$ family for each cross.

### *2.2.2 Confirmation of $F_1$ through ALS gene sequencing*

The two resistant parent populations contained two different *ALS* mutations; Pro-197-Ser in P18 and Trp-574-Leu in P413-17. [19] DNA was extracted from leaf tissue of $F_1$ plants that survived the rimsulfuron treatment above, and the *ALS* gene amplified using *ALS* gene specific primers (Table 1) as previously described [19] to confirm $F_1$ status. The Australian Genome Research Facility (AGRF) of the University of Adelaide sequenced the amplified fragment. The sequences were assembled and analysed using the ContigExpress and AlignX programmes in the VectorNTi software suite (11.5) (Invitrogen Waverley, Victoria, Australia).

### *2.2.3 Segregation and dose-response of the $F_2$ populations*

Seeds from confirmed $F_1$ plants (the $F_2$ population), along with the resistant and susceptible parents, were germinated and at the 1-leaf stage, the seedling were transplanted, nine seedlings per 9.5 x 8.5 x 9.5 cm pot (Masrac Plastics, Adelaide, SA) with three replications. At the two-leaf stage, propyzamide was applied at rates of 0, 67.5, 125, 250, 375, 500, 750, 1000, 1250, 1500 and 2000 g $ha^{-1}$ as described earlier. Plants were returned outside and the herbicide response was evaluated 28 DAT (days after treatment) and plants with new tillers were considered to be survivors. [37] The experiment was repeated on three separate occasions.

The dose-response data were pooled after two-way ANOVA revealed no differences between the three experimental runs. The dose response data were analysed using a log-logistic equation using GraphPad software v. 8.0 (GraphPad Inc., San Diego, CA, USA). [38] A model with a single dominant gene was created by combining 0.75 (equivalent to 75%) the survival of the resistant parent with 0.25 (equivalent to 25%) the survival of the sensitive parent. A model with a single recessive allele was created by combining 0.25 the survival of resistant parents with 0.75 the survival of sensitive parents. The models were compared to the response of the $F_2$ populations. [39] Inheritance was tested using an exact binomial test that was two-sided and used the method of small P values [40] with expected survival values corrected for mortality of the resistant population and survival of the susceptible population at the dose chosen. For the $F_2$-P413-17 $\times$ S, six different two gene models were also tested: two alleles dominant and independent (15:1), one dominant and one recessive allele that are independent (13:3), two dominant alleles that are additive (9:7), two recessive alleles that are independent (7:9), one dominant and one recessive allele that are additive (3:13), and two recessive alleles that are additive (1:15).

### *2.2.4 Confirmation of $F_2$ through ALS gene sequencing*

To verify the previously confirmed *ALS* target-site mutation was also present among $F_2$ survivors, individual leaf tissues were collected from 15 individual plants of each $F_2$. Genomic DNA was extracted and the *ALS* gene sequenced by PCR as described above.

### 2.2.5. Mechanism of resistance to propyzamide

#### *2.2.5.1 Sequencing of α- and β-tubulin genes*

A 841-bp region of the *β-tubulin* gene containing known mutation sites Arg241Lys in *P. annua* [15] and Lys350Met in *C. reinhardtii*. [24], and *a* 746-bp fragment of *α-tubulin*[19], were sequenced from 15 individual plants of each population (resistant, susceptible and two $F_2$ populations). Total RNA was isolated from 0.1 g tissue from fully expanded leaves using Isolate II Plant RNA kit (Bioline, Alexandrima, NSW, Australia). The $F_2$ and R population leaf tissue was collected from individuals that survived 500 g $ha^{-1}$ propyzamide and the susceptible population from the control treatment. Tetro cDNA synthesis kit (Bioline, Alexandrima, NSW, Australia) was used to synthesise cDNA according to the manufacturer's instructions. An additional DNase treatment was performed using the RNAse free DNAse set (Qiagen) to ensure no DNA contamination in the RNA to ensure no DNA contamination in the RNA. PCR for *α-tubulin* was conducted using primers previously described [22] and the primers used for *β-tubulin* (Table 1) were designed using sequence from the *P. annua* transcriptome shotgun assembly, NCBI accession number GCZY00000000. [41] For both *α-tubulin* and *β-tubulin* the same PCR method was used as described by Barua *et. al* [19] but with primers specific for each gene (Table 1). The amplified fragments were sequenced by AGRF, using the same primers as used for amplification for *α-tubulin* and two internal primers (Table 1) for *β-tubulin.* Sequence data wasanalysed using Geneious Prime 2020 (Biomatters, New Zealand).

Table 1. Primer name with fragment size used for gene sequencing

| Target gene | Primer name | Primer sequence 5′ –3′ | Fragment size | Reference |
|---|---|---|---|---|
| *ALS* | Poa_*ALS* F574 | TGGGCGGCTCAGTATTACAC | 479-bp | McElroy et al.[45] |
| | Poa_*ALS* R574 | ATAGGCAGCACATGCTCCTG | | |
| | Poa *ALS*_1F | ACCCGCATCAGGTGCTCCACGGT | 454-bp | GeneBank acc no. KT346395 |
| | Poa *ALS*_1R | AGGAGGCGAGGAAGAAGGCTTCCT | | |
| *α-tubulin* | AW08F | GGAGATTGTTGACCTGTGCCT | 746-bp | GenBank acc no. *E. indica* AJ005599 [22] |
| | AW05R | TGGGTGGCTGGTAGTTGATAC | | |
| *β-tubulin* | Poa b-tub seq F | TCACCACCCCTAGCTTTGGTGAT | | |
| | Poa b-tub seq R | TCATCCTCGTACTCACCCTCCT | | |
| | Poa b-tub 241 F1 | AACAACTGGGCCAAGGGNCACT | 841-bp | |
| | Poa b-tub 241 R1 | CGGAACATCTCCTGGATGGANGT | | |

### *2.2.5.3 Effects of Cytochrome P450 inhibitors*

A detailed dose-response experiment using rates of 0, 125, 250, 375, 500, 750, 1000, 1250, 1500 and 2000 g $ha^{-1}$ propyzamide in the presence or absence of the cytochrome P450 inhibitors piperonyl butoxide (PBO) (PBO 800, 800 g $L^{-1}$ PBO, Adama, St Leonards, Australia) and malathion (Fyfanon® 440 EW, FMC, North Ryde, Australia) was conducted. The experiment was designed with four-replications. Seeds from the R and S populations were germinated and transplanted at the 1-leaf-stage into a 500 ml black plastic tub (26 cm × 19 cm × 9 cm) containing 400 ml of polypropylene black beads to support the seedlings. The base of a 500 ml black plastic tub was perforated and placed into a 750 ml black plastic tub containing modified Hoagland nutrient solution. [42] After transplanting, plants were transferred to a growth room with the following conditions; 20 °C day/15 °C night, 12 hr light/dark photoperiod, light intensity 300 µmol $m^{-2}$ $s^{-1}$., Milli-Q-water with Hoagland nutrition solution was added every third day to replace evapotranspiration losses. At the 2-leaf stage, the Hoagland solution was replaced 24 hr prior to herbicide treatment with a new solution with or without 70 µM PBO or 70 µM malathion. Herbicide was applied 24 hr after application of cytochrome P450 inhibitors using a laboratory moving boom sprayer as mentioned above. In both trials, survivors were counted 28 days after herbicide application. Both experiments were repeated twice. The dose-response data were pooled after two-way ANOVA revealed no differences between the three experimental runs. The dose response data were analysed using a log-logistic equation using GraphPad software v. 8.0 (GraphPad Inc., San Diego, CA, USA). [38]

## 3. RESULTS

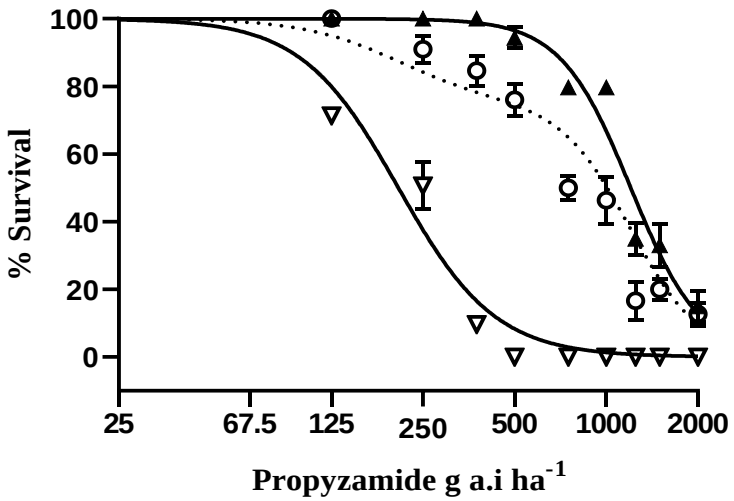
### 3.1 Inheritance of resistance to propyzamide

All seedlings from the selfed S plants died when treated with 25 g $ha^{-1}$ rimsulfuron, whereas no mortality occurred in selfed seeds of the resistant parents. Only 6 putative $F_1$ plants (<1%)

from the P18♂ × S♀ cross and 4 plants (<1%) from the P413-17♂× S♀ cross survived after rimsulfuron application. Confirmation through *ALS* sequencing revealed that all the six surviving $F_1$ plants from the P18 × S cross contained the Pro-197-Ser *ALS* mutation of the resistant parent P18. Likewise, the four surviving $F_1$ plants from the P413-17 × S cross contained the Trp-574-Leu mutation as in the P413-17 parent. Similarly, *ALS* sequence analysis of the $F_2$ populations confirmed that these mutations were present in 80% of $F_2$ plants from P18 × S, and 60% of $F_2$ plants from P413-17 × S.

A detailed dose response experiment with 0 to 2000 g $ha^{-1}$ propyzamide showed both $F_2$ populations exhibited an intermediate response compared to the parents (Fig. 1 and 2). For $F_2$-P18×S, the dose response curve showed a single step, with survival declining to about 75% at 500 g $ha^{-1}$ propyzamide and then declining further at higher rates. The dose response curve mostly fitted a model for a single dominant allele (dotted line) (Fig. 1). This type of response is an indication of segregation for resistance in the $F_2$ population for a single dominant allele. [35] In contrast, the $F_2$-P413-17×S population had a sharper decline with survival of about 25% at 500 g $ha^{-1}$ propyzamide application and a further decline in survival at higher rates. The dose response curve did not fit a model for a single recessive allele (dotted line) (Fig. 2).

**a)**




**b)**

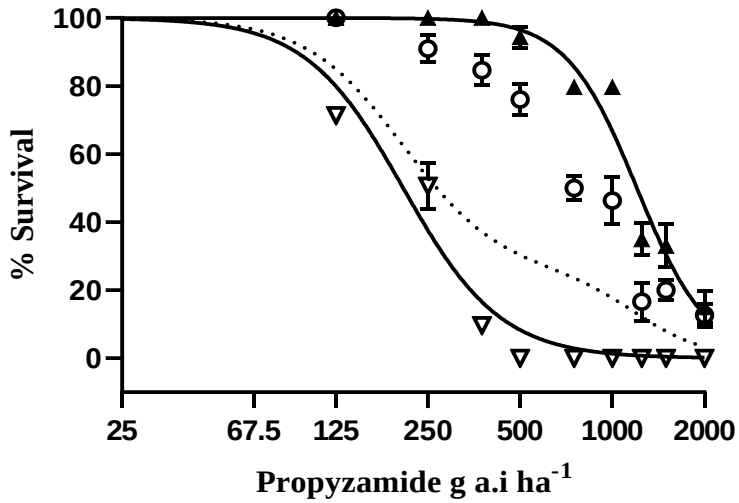
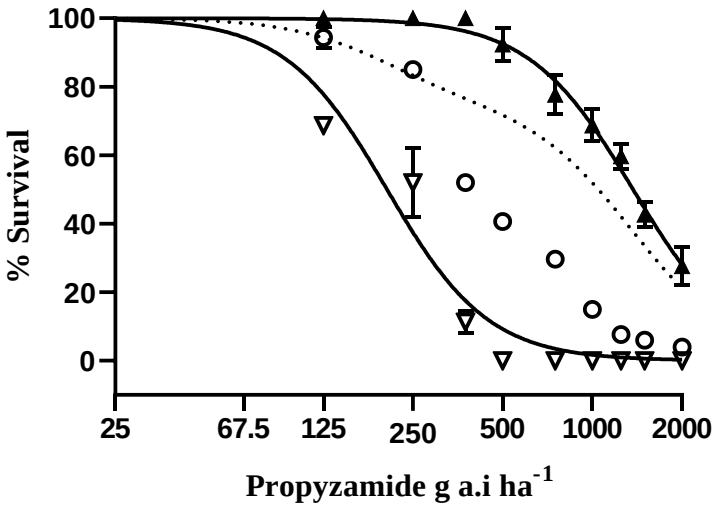



Fig. 1 Dose-response of susceptible (▽), resistant (P18) (▲) and, $F_2$ (P18×S) (○) populations of *P. annua* to propyzamide. The dotted line represents the expected response for resistance caused by a single allele dominant 3:1 (a) and a single recessive 1:3 (b) at low doses. Data points are means±95% confidence intervals (CI) for three replicates × three repeats.

**a)**




**b)**

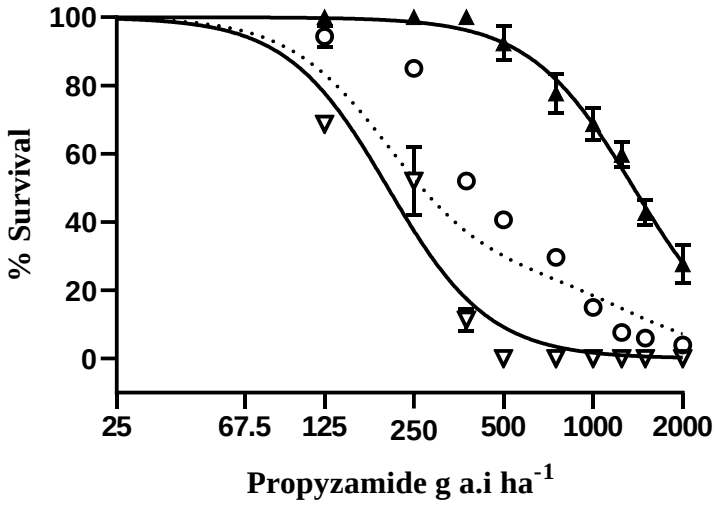



Fig. 2 Dose-response of susceptible (▽), resistant (P413-17) (▲) and, $F_2$ (413-17×S) (○) populations of *P. annua* to propyzamide. The dotted line represents the expected response for resistance caused by a single allele dominant 3:1 (a) and a single recessive 1:3 (b) at low doses. Data points are means±95% confidence intervals (CI) for three replicates × three repeats.

The data were further analysed using an exact binomial test for a single-gene-model with either a dominant allele or a recessive allele using the survival values for 500 g $ha^{-1}$, where, 3:1 represents a single dominant, and 1:3 is a single recessive model. The survival data fitted the 3:1 ratio for $F_2$-P18×S, indicating a single dominant allele in this population (Table 2) and failed to fit the 1:3 ratio for a single recessive allele. In contrast, the response of $F_2$-P413-17×S did not fit either single gene model; suggesting more than one gene may be involved for propyzamide resistance. Of the two gene models (9:7, 7:9; 15:1, 1:15; and 13:3, 3:13), the data for $F_2$-P413-17 × S fitted both the 9:7 ratio and a 7:9 ratio (Table 3), but not any of the other two gene models (13:3, 3:13 and 15:1, 1:15) (Table 3).

Table 2 Survival of parental and $F_2$ populations at 500 g $ha^{-1}$ propyzamide with probability from exact binomial goodness of fit to the expected ratio for two different single gene inheritance models.

| | S | P18 | $F_2$ (P18 × S) |
|---|---|---|---|
| | | 500 g $ha^{-1}$ | |
| Treated | 81 | 81 | 81 |
| Alive | 0 | 77 | 62 |
| Dead | 81 | 4 | 19 |
| % Survival | 0 | 95 | 75 |
| P (3:1) | | | 0.33 |
| P (1:3) | | | $1.7\times10^{-23}$ |
| | S | P413-17 | $F_2$ (P413-17 × S) |
| | | 500 g $ha^{-1}$ | |
| Treated | 81 | 81 | 81 |
| Alive | 0 | 76 | 35 |
| Dead | 81 | 5 | 46 |
| % Survival | 0 | 95 | 43 |
| P (3:1) | | | $5.03\times10^{-7}$ |
| P (1:3) | | | 0.000104 |

Table 3 Survival of parental and $F_2$ (P413-17 × S) populations at 500 g $ha^{-1}$ with probability from exact binomial goodness of fit to the expected ratio for six different multiple gene inheritance models.

| | S | P413-17 | $F_2$ (P413-17 × S) |
|---|---|---|---|
| | | 500 g $ha^{-1}$ | |
| Treated | 81 | 81 | 81 |
| Alive | 0 | 76 | 35 |
| Dead | 81 | 5 | 46 |
| % Survival | 0 | 95 | 43 |
| P (9:7) | | | 0.095 |
| P (7:9) | | | 0.74 |
| P (13:3) | | | $2.7\times10^{-10}$ |
| P (3:13) | | | $1.3\times10^{-7}$ |
| P (15:1) | | | $5.7\times10^{-22}$ |
| P (1:15) | | | $1.1\times10^{-22}$ |

### 3.2 Mechanism of resistance to propyzamide

#### *3.2.1 Target site mutation*

Target-site resistance to MTI herbicides has been shown to result from missense mutations causing amino acid substitutions at any of six sites in *α-tubulin* gene in higher plants: Leu-125-Met, Leu-136-Phe, Val-202-Phe, Thr-239-Ile, Arg-243, Met-268-Thr. [17, 20-23] A conserved region of 746-bp for *α-tubulin* was sequenced that covered all previously reported mutations in this gene found in higher plants. However, none of these mutations were present in the resistant or $F_2$ individuals tested. Likewise, sequencing 841-bp of the *β-tubulin* gene in these plants did not identify either of the two amino acid substitutions, Arg241Lys in *P. annua* [15] and Lys350Met in *C. reinhardtii*, [24] reported to provide MTI resistance.

#### *3.2.2 Effect of PBO and Malathion on propyzamide herbicide*

In the absence of herbicide, the cytochrome P450 inhibitors PBO and malathion at 70 $\mu M$ did not affect plant growth. The susceptible population was fully controlled at the recommended field rate (500 g $ha^{-1}$) of propyzamide in the absence of inhibitors, but the $LD_{50}$ for this population increased with the addition of either inhibitor. The same occurred for the two resistant populations. The $LD_{50}$ value for population P18 was 1008 g $ha^{-1}$ when treated with herbicide only, and increased to 2123 g $ha^{-1}$ with the addition of PBO and 2295 g $ha^{-1}$ with malathion (Table 4). Similarly, for population P413-17, there was an increase in $LD_{50}$ from 1264 g $ha^{-1}$ with propyzamide alone to 1980 g $ha^{-1}$ with PBO, and to 4060 g $ha^{-1}$ with malathion (Table 4).

Table 4 Pooled dose-response data of propyzamide herbicide with or without inhibitor (PBO/Malathion) required to kill 50% of R and S population of *P. annua* with 95% confidence intervals (CI) in parentheses and resistant index (RI)

| Propyzamide +/- PBO (Inhibitor) | | | | |
|---|---|---|---|---|
| Population | Inhibitor | LD50 g a.i $ha^{-1}$ | RI | P |
| 18 | - | 1008.1 (813.2, 1265.4) | 12 | <0.01 |
| | + | 2123.4 (1655.6, 2875.4) | 7 | <0.01 |
| 413 | - | 1264.2 (1002.5, 1630.1) | 15 | <0.01 |
| | + | 1980.2 (1558.4, 2636.6) | 6 | <0.01 |
| S | - | 83.1 (55.7, 114.5) | - | <0.01 |
| | + | 312.1 (251.5, 384.9) | - | <0.01 |
| Propyzamide +/- Malathion (Inhibitor) | | | | |
| 18 | - | 1206.6 (1009.3, 1456.7) | 8 | <0.01 |
| | + | 2295.1 (1679.2, 3397.2) | 9 | <0.01 |
| 413-17 | - | 1453 (1099.9, 2041.6) | 10 | <0.01 |
| | + | 4060 (2221.7, 8917) | 16 | <0.01 |
| S | - | 144.5 (119.3, 174.6) | - | <0.01 |
| | + | 246.9 (193.3, 315.5) | - | <0.01 |

## 4. DISCUSSION

This study has identified that resistance to propyzamide in two *P. annua* populations is the result of two different inheritance patterns. For P18, a single dominant allele (3:1) fitted the data best, whereas for P413-17, resistance is likely due to more than one gene and both 9:7 and 7:9 models fitted the data. The variable patterns of inheritance in the two populations of *P. annua* suggest that more than one mechanism likely confers resistance to propyzamide. Genetic inheritance for target site resistance to MTI herbicides appears to be as a single recessive allele, similar to the inheritance of trifluralin resistance in *E. indica* [28] and *S. viridis*. [29] A single recessive allele was not identified in the present study; however, a two gene model of 2 recessive alleles (7:9) did fit the data for P413-17. The lack of recessive inheritance was confirmed by the lack of target site mutations identified. Previously, six different mutations (Leu-125-Met, Leu-136-Phe, Val-202-Phe, Thr-239-Ile, Arg-243, Met-268-Thr) in the *α-tubulin* gene, [20, 21, 23] and one *β-tubulin* target-site mutation (Arg241Lys) [24] have been reported in higher plants. However, in our study none of these mutations was observed. The parts of the genes sequenced (746-bp for *α-tubulin* and 841-bp for *β-tubulin*) covered all the previously reported mutation sites. However, there remains the possibility of other unknown target site mutations may contribute to resistance in P413-17.

Non-target site mechanisms are also known to contribute to MTI herbicide resistance. [17, 18] The inheritance data of F2-P18×S showed the inheritance of propyzamide resistance was controlled by a single, nuclear dominant allele, which would suggest a non-target site resistance mechanism, rather than a target site mechanism. To explore this, we examined the effects of cytochrome P450 inhibitors on propyzamide response in these populations. Previous studies reported synergistic interactions between a cytochrome P450 inhibitor phorate and MTI herbicides trifluralin [43] and propyzamide [44] in *L. rigidum*. However, in our study, neither PBO nor malathion showed a synergistic effect on propyzamide. This suggests that cytochrome

P450-mediated metabolic resistance is unlikely to be the mechanism of resistance; although, cytochrome P450s not affected by these inhibitors could be involved.

In addition to cytochrome P450-based resistance mechanisms, other non-target site mechanisms, such as reduced absorption and translocation of propyzamide [18] are possible and need to be examined.

## 5 CONCLUSION

In conclusion, this study identified two inheritance patterns for propyzamide resistance in *P. annua* populations. P18 exhibits a single-nuclear, dominant gene inheritance, whereas in P413-17 more than one gene contributes to resistance. No previously reported mutation in either *α-tubulin* or *β-tubulin* was identified in either resistant population. Additionally, two cytochrome P450 inhibitors failed to synergise the effect of propyzamide. The fact that two different inheritance patterns are present suggests that at least two resistance mechanisms could provide resistance to propyzamide.

## 6. ACKNOWLEDGEMENTS

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## DISCLOSURE STATEMENT

The author has declared no conflict of interest.

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# CHAPTER 5: ALTERNATIVE HERBICIDES FOR CONTROLLING HERBICIDE-RESISTANT ANNUAL BLUEGRASS (*POA ANNUA* L.) IN TURF

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*Article*

# Alternative Herbicides for Controlling Herbicide-Resistant Annual Bluegrass (*Poa annua* L.) in Turf

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**Abstract:** *Poa annua* is a cosmopolitan, cool-season grass species regarded as one of the most significant weeds of turfgrass. It is mainly controlled by herbicides; however, repeated use of herbicides in golf turf has resulted in the evolution of multiple-herbicide resistant *P. annua*. Four field experiments were performed in autumn and spring in golf turf to identify effective herbicide options to control multiple herbicide-resistant *P. annua*. In herbicide resistance screening, the trial site population (SA1) was found to be susceptible to amicarbazone and terbuthylazine, but resistant to simazine and metribuzin at the field rate of each herbicide. Consistent with the results of the pot study, the PSII-inhibiting herbicides amicarbazone and terbuthylazine provided the best control (80–100%) of *P. annua* in both autumn and spring trials with minimal damage to the turf. In contrast, the other two PSII-inhibiting herbicides, metribuzin and simazine, were relatively ineffective in controlling *P. annua* in the field. Indaziflam also performed well in both autumn trials and reduced *P. annua* occurrence by >75%. Pyroxasulfone and s-metolachlor only provided moderate weed control in both the autumn and spring trials, reducing *P. annua* occurrence by 50%. Among the nine different herbicides, amicarbazone and terbuthylazine were found to be most effective for spring and autumn application in turf. As resistance to some PSII-inhibiting herbicides has already evolved in this field population, the use of amicarbazone and terbuthylazine needs to be integrated with other herbicide modes of action and non-chemical tactics to delay the onset of resistance to them.

**Keywords:** *Poa annua* L.; annual bluegrass; amicarbazone; terbuthylazine; indaziflam; weed management



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## 1. Introduction

Annual bluegrass (*Poa annua* L.) is one of the most problematic weeds in sports turf, particularly in temperate climates [1,2]. It reduces the turf quality for sport and creates an uneven surface that affects ball roll [3,4]. This weed also competes for water and nutrients with the desired turfgrass species and reduces turf growth. It often produces panicles below the turf cutting height, which reduces the effectiveness of mowing for weed control [3]. *P. annua* is a genetically diverse weed species that typically germinates in autumn, grows in winter, and produces seed in spring; however, some germination can occur in spring as well [5,6]. During summer *P. annua* senesces, resulting in dead, bare, and unsightly patches that reduce the aesthetic value of turf [7]. It is considered the most problematic weed of golf courses in Australia and other countries, such as the USA. One feature that makes it difficult to control *P. annua* is its large seed bank of up to 200,000 seed $m^{-2}$ [6] and potential for year-round germination [8].

Several alternative management strategies including manual, cultural, biological and chemical control are available for controlling *P. annua* [9,10]. However, chemical control tends to be most commonly used, because of the ease of application and reliability of weed control. Both PRE and POST herbicides are used to control *P. annua* in turf [11]. However, repeated use of herbicides has resulted in the evolution of herbicide resistant *P. annua*

populations [12–14]. The loss of herbicides to resistance requires new weed management practices including additional herbicides to control *P. annua*.

Greenkeepers of the golf course used for this study had reported difficulty in controlling *P. annua* with several herbicides. Seeds were collected from the golf course trial site in October 2017 to screen for herbicide resistance. The population was confirmed resistant to four different herbicide modes of action HRAC (Herbicide Resistance Action Committee) group, 1, 2, 5 and 31 in initial research, with >9-fold resistance to HRAC group 1, 2 and 31 and >2-fold resistance to HRAC group 5. Therefore, alternate herbicides with different mode of action could be an option for controlling this population. Hence, four field trials were conducted at a golf course in spring and autumn seasons between 2018 and 2020. The treatments were selected based on some commonly used herbicides currently registered for controlling *P. annua* in golf turf in Australia and additional herbicides that are not currently registered for turf, but may be suitable for controlling *P. annua*. The objective of the study was to identify suitable herbicide options to control multiple herbicide-resistant *P. annua* in a bermuda grass (*Cynodon dactylon*) turf during autumn and spring.

## 2. Materials and Methods

### 2.1. Experimental Site and Field Trial Design

Field trials were conducted on a Bermuda grass (*Cynodon dactylon*) turf at a golf course (34.896°S 138.51°E) in spring and autumn between 2018 and 2020. The chosen site was a practice green, which received the same weed control measures as the remainder of the golf greens. The turf was well managed with regular mowing (twice a week) at a height of 9 mm and watered every third night depending on the weather conditions. The soil type of the trial site is sandy in texture with 1.48% organic matter (Table 1). Liquid fertilizer MP Brilliance (20-0-0 + 6Fe, 1Mg) was applied at 37 L $ha^{-1}$ every 3-4 weeks to maintain the fertility of the golf course. Meteorological and soil properties of the experimental sites are presented in Tables 1 and 2.

**Table 1.** Calendar dates and environmental conditions during herbicide application in field trials in 2018–2020.

| Application Timing | Trial | Date | Air Temperature at Spraying (°C) | Average Maximum Temperature during Trial (°C) | Average Minimum Temperature during Trial (°C) | Total Rainfall during Trial (mm) |
|---|---|---|---|---|---|---|
| Spring | Trial 1 | 24 October 2018 | 17.7 | 23.1 | 13.3 | 35.5 |
| | Trial 2 | 30 September 2019 | 21.7 | 23.4 | 11.8 | 15.6 |
| Autumn | Trial 1 | 8 March 2019 | 23.7 | 25.6 | 15.5 | 9.0 |
| | Trial 2 | 24 March 2020 | 20.5 | 24.9 | 15.0 | 4.2 |

**Table 2.** Soil analysis data of the trial site.

| Soil pH | Organic Matter (%) | Salinity EC 1:5 (dS $m^{-1}$) | Texture | Physical Properties (%) | | |
|---|---|---|---|---|---|---|
| | | | | Sand | Silt | Clay |
| 7.6 | 1.48 | 0.073 | Sand | 92 | 6.6 | 1.7 |

Nine different herbicide treatments were applied in the spring trial established in October 2018 and repeated in September 2019 (Table 3). Similarly, a field trial with nine herbicide treatments was undertaken in the autumn at the same location in March 2019 and repeated in March 2020 (Table 3). Dates of herbicide application and weather conditions are presented in Table 1. The timing of herbicide application in these trials is consistent with the weed management programs used by local greenkeepers. A range of both PRE and POST herbicides were used in both spring and autumn trials as emergence of *P. annua* occurs over a long period. Therefore, some herbicides e.g., s-metolachlor, indaziflam, which are mainly recommended for PRE application, were applied in both autumn and spring trials to test for both PRE and POST herbicide activity. Additionally, herbicides not currently registered for *P. annua* control were tested in these field trials. The trial was established in a

randomised complete block design with four replications. The plot size was 5 m × 2 m with a 0.25 m gap between plots. A non-treated check treatment was included in each trial. Herbicides were applied using a $CO_2$-pressurized boom sprayer with medium droplet size flat-fan nozzles (TT01 and LD110-015) delivering 100 L ha$^{-1}$ volume at a pressure of 200 kPa. The trials were assessed 28 DAT (days after treatment) and again 42 DAT.

**Table 3.** List of herbicides used in spring and autumn treatment.

| Herbicide | Trade Name | HRAC Group [15] | Registered for Turf in Australia | Recommended Application Time | Used in Which Trial | Rate (g a.i. ha$^{-1}$) Used in Experiment | Company |
|---|---|---|---|---|---|---|---|
| Pendimethalin | Rifle® 440 | 3 | Yes | PRE | Autumn | 1496 | Nufarm |
| Propyzamide (Pronamide) | Kerb® 500 SC | 3 | Yes | PRE, EPOST | Spring Autumn | 600 | Corteva Agrisci. |
| Metribuzin | Mentor® WG | 5 | No | PRE, EPOST | Spring Autumn | 210 | Adama |
| Simazine | Gesatop® 600 SC | 5 | Yes | PRE, POST | Spring Autumn | 1200 | Syngenta |
| Terbuthylazine | Terbyne® Xtreme® 875 WG | 5 | No | PRE, POST | Spring Autumn | 875 | Sipcam Pacific |
| Amicarbazone | Amitron® 700 WG | 5 | Yes | PRE, POST | Spring Autumn | 700 | Arysta LifeScience Australia Pty Ltd. |
| Pyroxasulfone | Sakura® 850 WG | 15 | No | PRE | Spring Autumn | 100.3 | Bayer |
| S-Metolachlor | Pennmag® | 15 | Yes | PRE | Spring Autumn | 1920 | Syngenta |
| Indaziflam | Specticle® | 29 | Yes | PRE, EPOST | Spring Autumn | 50 | Bayer |
| Endothall | Poachek® | 31 | Yes | EPOST, POST | Spring | 262.5 | Campbell Chemicals |

### 2.2. Pot Trial Methodology

As most of the PRE herbicides applied in the trial were ineffective, a follow-up pot study was undertaken with the PRE herbicides (Table 4) to determine the resistance status of the *P. annua* population from the trial site (hereafter referred to as SA1). The *P. annua* population (SA1) used in this study was collected from the trial site during October 2018. One susceptible population collected from a non-golf course area was used as the susceptible control. Seed bulking was performed in 2019 and the pot trial conducted during July 2019 and repeated in June 2020 at the University of Adelaide following the method used by Barua, et al. [16]. Approximately 100 seeds were measured by volume (0.2 mL), placed onto the surface of standard potting mix [17] and herbicide applied directly onto the seed. Herbicides (Table 4) were applied using a laboratory moving boom sprayer equipped with a twin nozzle (Tee-jet 110° flat fan Spraying Systems, Wheaton, IL, USA) delivering an output of 118 L ha$^{-1}$ at a pressure of 250 kPa and speed of 1 m s$^{-1}$. Immediately after herbicide application, the seeds were covered with 5 mm of potting mix. The pots were placed outside and watered as required. The experiment was assessed for seedling emergence 28 DAT (days after herbicide treatment). Plants that emerged and grew to the two-leaf stage were considered resistant to the herbicide treatment. The experiment was repeated.

In order to further investigate resistance in *P. annua* to PSII inhibiting herbicides, an additional pot trial was undertaken. In this dose-response pot trial herbicide rates used were the following: amicarbazone (0, 26.3, 52.5, 105, 210, 420, 840 and 1680 g a.i ha$^{-1}$), terbuthylazine (0, 32.8, 65.6, 131.3, 262.5, 525, 1050 and 2100 g a.i ha$^{-1}$), simazine (0, 26.3, 52.5, 105, 210, 420, 840 and 1680 g a.i ha$^{-1}$) and metribuzin (0, 13.1, 26.3, 52.5, 105, 210 and 420 g a.i ha$^{-1}$). The methodology used was the same as Barua et al. [16]. The susceptible population mentioned above was used as the susceptible control. Seeds were sown in trays

(330 by 200 by 50 mm) located outdoors. One to two leaf seedlings were transplanted into punnet pots (95 by 85 by 95 mm) containing the already described standard potting mix, with 5 plants per pot and replicated three times. At the 2-3 leaf stage, plants were treated with the herbicides using the laboratory moving boom sprayer mentioned above. Plants actively growing with new leaves after 28 days were classified as survivors, while plants with severe stunting or dead were considered susceptible [19]. The experiment was repeated.

**Table 4.** Rate of herbicides used and survival (%) of the field population SA1 to herbicides at PRE application in a pot study.

| Chemical Name | HRAC Group [15] | Rate (g a.i. ha$^{-1}$) Used in Study | % Survival | Resistance Status * |
|---|---|---|---|---|
| Pendimethalin | 3 | 1496 | 10 | S |
| Propyzamide (Pronamide) | 3 | 600 | 15 | S |
| Metribuzin | 5 | 210 | 30 | R |
| Simazine | 5 | 1200 | 34 | R |
| Amicabazone | 5 | 700 | 0 | S |
| Terbuthylazine | 5 | 875 | 19 | S |
| Pyroxasulfone | 15 | 100.3 | 0 | S |
| S-Metolachlor | 15 | 1920 | 14 | S |
| Indaziflam | 29 | 50 | 0 | S |

* R—Resistant: More than 20% survival compared to the untreated control is considered as resistant [18], S—Susceptible.

### 2.3. Data Collection and Analysis

Prior to herbicide treatment, a low density of *P. annua* was present on the site in the autumn trials, but a much larger number of *P. annua* plants were uniformly distributed at the trial site in spring trials. The occurrence of *P. annua* in the trials after treatment was determined with the use of a 1 by 1 m grid divided into four hundred 50 by 50 mm squares. The grid was placed in the centre of each plot at three random locations and the number of squares containing *P. annua* plants counted to determine the % occurrence. The treatments were visually assessed for turf phytotoxicity at 7 DAT, 28 DAT and 42 DAT using a scale of 0—100 where 0 = no visual injury and 100 = no green tissue [20].

The data of percent occurrence of *P. annua* were subjected to two way analysis of variance (ANOVA) with GenStat version 19 (VSN International Ltd. Hemel Hempstead, UK) with herbicide and experiment run treated as variables. As there was a significant variation between experimental runs ($p < 0.05$), the data of each run is presented separately. The data were square-root transformed before statistical analysis to normalize the distribution of the residuals. Where treatment differences were significant, the means of the transformed data were compared using Fisher's Protected LSD at $p = 0.05$. As there were no significant differences in turf quality between the two runs, the data were pooled for statistical analysis.

Dose response trials were set up as a completely randomized design and repeated. Data was subjected to three way ANOVA for each herbicide with population, rate and run as variables. For every herbicide there was a significant effect of rate ($p < 0.0001$) and population ($p < 0.0001$), but not for run. Therefore, data for each herbicide were pooled across experiments. Survival at each rate was converted to mortality and the data analyzed using PriProbit (1.63) [21] with the $LD_{50}$ with 95% confidence intervals (CI) determined and resistance ratios calculated as $LD_{50}$ Resistant/$LD_{50}$ Susceptible. Population responses to the herbicides were considered different if confidence intervals of the $LD_{50}$ did not overlap.

## 3. Results

### 3.1. Spring Trial Assessment

The occurrence of *P. annua* in the non-treated control for the two spring field trials varied between 86 and 100% of grids (Table 5), which indicates a relatively uniform spatial distribution at the site. In both spring trials, amicarbazone (2% occurrence) and terbuthy-

lazine (15–20% occurrence) provided the greatest control. The remaining treatments were less effective, particularly endothall, which was ineffective in both spring trials due to herbicide resistance confirmed previously at this site Barua, et al. [16]. These less effective treatments showed inconsistency in weed control over the two years.

**Table 5.** Effect of spring herbicide treatments on % occurrence of *P. annua* in golf turf in 2018 and 2019 after 28 DAT.

| Treatment | *P. annua* (% Occurrence) [a] | |
|---|---|---|
| | 2018 | 2019 |
| Propyzamide (Pronamide) | 62.4 d | 64.3 ef |
| Metribuzin | 60.4 d | 49.8 d |
| Simazine | 66.3 d | 57.7 e |
| Terbuthylazine | 14.6 b | 19.6 b |
| Amicarbazone | 1.9 a | 1.6 a |
| Pyroxasulfone | 50.4 c | 39.4 c |
| S-Metolachlor | 55.5 cd | 41.1 c |
| Indaziflam | 59.6 d | 39.9 c |
| Endothall | 81.4 e | 67.2 f |
| Non-treated | 99.5 f | 86.3 g |

[a] values with different letters within each column are significantly different.

In 2018, amicarbazone provided the highest level of control by reducing *P. annua* occurrence to 2% followed by terbuthylazine that reduced *P. annua* occurrence to 14% (Table 5). However, the other two PSII-inhibiting herbicides (simazine and metribuzin) provided moderate control and reduced the occurrence of *P. annua* between 60 to 66% (Table 5). Other mode of action herbicides, such as indaziflam and propyzamide, also reduced occurrence to 60 to 62% (Table 5).

Amicarbazone and terbuthylazine were also the most effective weed control treatments in the 2019 spring trial, followed by pyroxasulfone, indaziflam and s-metolachlor. Amicarbazone reduced the occurrence of *P. annua* to 2% followed by terbuthylazine to 20% (Table 5). Simazine and metribuzin provided moderate control of *P. annua* similar to the results obtained in 2018. The next most effective treatments were pyroxasulfone and indaziflam that reduced *P. annua* occurrence to 39% followed by s-metolachlor to 41% (Table 5).

### 3.2. Autumn Trial Assessment

The occurrence of *P. annua* was high (>96%) in untreated plots for both 2019 and 2020 autumn trials (Table 6). Amicarbazone was once again the most effective herbicide in autumn 2019, followed by terbuthylazine, indaziflam, pyroxasulfone, and s-metolachlor. In contrast, pendimethalin, propyzamide and simazine were less effective treatments in both autumn trials. The occurrence of *P. annua* was reduced to 0% by amicarbazone, 6% by terbuthylazine, 22% by indaziflam, 43% by pyroxasulfone, and 45% by s-metolachlor (Table 6).

**Table 6.** Effect of autumn herbicide treatments on % occurrence of *P. annua* in golf turf in 2019 and 2020 after 28 DAT.

| Treatment | *P. annua* (% Occurrence) [a] | |
|---|---|---|
| | 2019 | 2020 |
| Pendimethalin | 83.5 f | 80.5 g |
| Propyzamide (Pronamide) | 84.9 f | 56.1 e |
| Metribuzin | 55.8 e | 59.2 ef |
| Simazine | 89.8 fg | 68.4 f |
| Terbuthylazine | 6.2 b | 8.0 b |
| Amicarbazone | 0.0 a | 1.4 a |
| Pyroxasulfone | 43.2 d | 40.6 d |
| S-Metolachlor | 44.9 de | 55.0 e |
| Indaziflam | 22.6 c | 26.3 c |
| Non-treated | 99.9 g | 96.4 h |

[a] values with different letters within each column are significantly different.

In 2020, amicarbazone was again the best treatment and reduced *P. annua* occurrence to below 2% (Table 6). Terbuthylazine was the second best treatment and reduced *P. annua* occurrence to 8%, followed by indaziflam to 26%, pyroxasulfone to 40%, and s-metolachlor to 55% (Table 6). Consistent with the spring trials, simazine and metribuzin were less effective than amicarbazone or terbuthylazine.

A difference in the performance of two herbicides (propyzamide and simazine) was observed in the two autumn trials (Table 6). As the soil type at the study site is sandy in texture (Table 2), higher rainfall in 2019 (Table 1) may have moved these herbicides through the soil profile reducing their performance in that year.

In all four trials, amicarbazone was the most effective treatment; however, it produced mild turf grass injury (data were not shown). This persisted for four weeks after herbicide application, followed by complete recovery of the turf at six weeks. No other treatments were phytotoxic to the turf. Perry, et al. [22] reported that amicarbazone provided superior control of *P. annua* through foliar and root uptake at 371 g $ha^{-1}$ as compared to atrazine at 2025 g $ha^{-1}$. Another study suggested that amicarbazone is more active than other PSII inhibitors such as atrazine Dayan, et al. [23]. Amicarbazone has been recently registered for turf in Australia at 210 g $ha^{-1}$ [24], a much lower rate than 700 g $ha^{-1}$ used in our field trials that were initiated prior to the registration of amicarbazone. It is possible the lower rate on the label will provide less effective control of *P. annua* than observed in this trial and in other studies [22,23]. The efficacy of amicarbazone at lower rates on *P. annua* needs to be validated. Of all the herbicide treatments investigated, terbuthylazine consistently resulted in good turf quality followed by indaziflam, propyzamide and amicarbazone at six weeks after herbicide application. In both the autumn and the spring trials there was rapid recovery of turf grass and no difference in turfgrass density (data not shown here) at 6 weeks after herbicide application. However, terbuthylazine, which was the second most effective herbicide in these trials, is not registered for use in turf in Australia. Indaziflam was the next most effective herbicide for PRE application in autumn and is registered for use in Australia [25]. Two other herbicides, pyroxasulfone and s-metolachlor, provided similar levels of control (40−55%) in all the trials. S-metolachlor is registered in turf for *P. annua* control during the autumn season. Pyroxasulfone should be considered as an option for use in turf and would increase the range of herbicide options for *P. annua*. The use of herbicide rotations can help to slow the evolution of resistance in weeds.

### 3.3. Pot Trial Screening

To investigate why some PRE herbicides were not effective in the autumn field trial, a pot study was conducted with the trial site population (SA1) using these herbicides. Moderate levels of resistance (30 to 34% survival) were detected to metribuzin and simazine (Table 3), which could explain the poor efficacy of these two herbicides in the field. However, SA1 was susceptible to the other herbicides used in the field trials (Table 3). Therefore, other factors, such as weed growth stage, weed competition and environmental conditions appear to be responsible for the poor control of *P. annua* by these herbicides.

### 3.4. Dose-Response Trial

To further investigate the extent of resistance to the PSII inhibiting herbicides used in the trial, dose response experiments were conducted on SA1. Compared to the S population, SA1 had 2.6-fold resistance to simazine and 2.2-fold to terbuthylazine. The $LD_{50}$ of SA1 population to metribuzin was 1.5-fold higher than the S population and for amicarbazone was 1.2-fold higher (Table 7). Low-level resistance does not always result in a failure of the herbicide in the field. Terbuthylazine controlled the S population well below the field rate, so this herbicide was effective despite the low-level resistance present. In contrast, metribuzin at the field rate only controlled the S population, so the increase in tolerance of SA1 is the likely cause of poor performance in the field. The dose response study confirmed the SA1 population exhibited greater resistance to simazine and lower resistance to terbuthylazine, but little resistance to amicarbazone.

**Table 7.** The dose required for 50% mortality ($LD_{50}$) of susceptible (S) and trial site population (SA1) to various PSII inhibiting herbicides with 95% confidence intervals (CI) in parentheses. R/S is the ratio of $LD_{50}$ of resistant and susceptible populations.

| Herbicides | | Population | |
|---|---|---|---|
| | | S | SA1 |
| | | $LD_{50}$ g a.i. ha$^{-1}$ | |
| Simazine | | 249.1 (193.9, 319.6) | 638.4 (500.6, 818.6) |
| | R/S | - | 2.6 |
| Amicarbazone | | 284.0 (227.8, 353.9) | 354.5 (284.7, 442.2) |
| | R/S | - | 1.2 |
| Metribuzin | | 72.8 (57.6, 91.9) | 109.0 (86.3, 137.1) |
| | R/S | - | 1.5 |
| Terbuthylazine | | 258.2 (194.3, 342.9) | 575.2 (440.3, 751.8) |
| | R/S | - | 2.2 |

## 4. Discussion

Resistance to 10 herbicide modes of action has been reported in *P. annua* worldwide [12], which restricts the number of herbicide options available for its control in turf. Most of the herbicides used in the autumn trial exhibited poor control of *P. annua* in the field (Table 6), despite resistance not being confirmed in pot studies (Table 3). This indicates that factors other than herbicide resistance were responsible for the poor performance, such as high seed bank, herbicide application timing relative to weed emergence, soil type, thatch layer, and environmental conditions [10,26].

The timing of a herbicide application is important to maximise PRE herbicide effectiveness [11]. In the current study, herbicide timings were similar to those used by local greenkeepers. In our field study, the performance of indaziflam was greater in the autumn trial than the spring trial. Brosnan, et al. [27] reported that soil application of indaziflam was more effective than foliar application to control *D. ischaemum* and *P. annua*. They concluded that indaziflam needs to be absorbed by the roots to maximise POST control. Therefore, time of application is important for the success of indaziflam. It is likely that when this herbicide was applied in autumn some *P. annua* had already germinated, thus reducing the efficacy of the indaziflam. In previous research, indaziflam was shown to be more effective when used PRE and EPOST (early POST) [28], which could explain why it was less effective in our spring trials where weeds were established. Greenkeepers often reapply the same herbicide during the season to maximise activity on subsequent germinations, which was not done in these field trials. Multiple applications may be more effective than a single application. Thus, a follow-up application timing study is needed to determine the difference between a single and multiple applications of this herbicide.

Pronamide has been shown to provide good control *P. annua* with PRE and POST treatments [29]. However, in our study performance of pronamide only provided moderate efficacy (Tables 5 and 6). In a previous study, poor efficacy of pronamide used POST on *P. annua* in Georgia, USA was associated with reduced absorption and translocation [30]. Others have also claimed that unsuitable application timing (when plants are too large) can lead to the poor efficacy of pronamide [31].

Factors which influence the efficacy of PRE herbicides include solubility in soil solution, binding to organic matter and the half-life in soil [32]. Uptake of herbicides by the root occurs more readily when the herbicide is in soil solution. Out of the herbicides tested in the autumn trials, amicarbazone had the highest solubility in water [33] and was found to have the greatest efficacy. In contrast, terbuthylazine and indaziflam have lower water solubility that could account for their lower activity.

Soil organic matter content can also play an important role in PRE herbicide activity [34]. In our study, some the PRE herbicides were unsuccessful for controlling *P. annua*

in the autumn trial, even though no herbicide resistance was detected. The soil at the trial site was sandy with 1.48% soil organic matter content (Table 2). Soil low in organic matter (low cation exchange capacity) has a lower tendency to bind herbicides and allows greater herbicide availability for uptake by weeds. However, heavy rainfall or frequent irrigation can move the herbicides through the soil profile before the compound has a chance to bind to the soil colloids and organic matter [32]. Since most *P. annua* seeds germinate in the top layer of soil [10,35], leaching of herbicides to lower layers of soil could result in reduced weed control.

Another factor that may have reduced the performance of PRE herbicides is the thatch layer, which is primarily organic matter (stems, stolons, roots), in turf that develops between the turf and soil surface [36]. This limits the movement of air, water and nutrition into the soil [37]. The thatch layer can also bind PRE herbicides, thereby reducing their effectiveness [38,39].

In conclusion, amicarbazone was the most effective herbicide for the reduction of P. annua occurrence (98–100%) followed by terbuthlazine (>80%), indazaflam (>63%) and pyroxasulfone (>57%) in both autumn and spring trials. Indaziflam performance was greater in the autumn trial than the spring trial indicating that indazaflam could be a good option for autumn application. The availability of multiple herbicides with different modes of action allows rotating herbicides in an herbicide management program, which is important to reduce the risk of resistance. Pyroxasulfone, although not currently registered in turf, could be a viable option for *P. annua* control. Amicarbazone and terbuthylazine controlled the trial site population (SA1) in pots indicating it was susceptible to both herbicides (Table 3). In contrast, simazine and metribuzin were less effective (Table 3). Terbuthylazine could be a potential candidate for *P. annua* control in turf. Given the extensive presence of herbicide resistance, it would be invaluable for the greenkeepers to test their populations for resistance status, so they can make informed choices of herbicides for *P. annua* control. The currently effective herbicides need to be used with care otherwise, they could also lose their effectiveness due to resistance.

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# CHAPTER 6: GENERAL DISCUSSION AND CONCLUSION

## 6.1 General Discussion

Herbicides are used for controlling weeds, as they are the most effective and economically viable solution (Vargas Jr and Turgeon, 2003). However, the evolution of herbicide resistance has provided a major challenge to the effectiveness and sustainability of many herbicides (Norsworthy et al., 2012). Globally there are at least 502 unique cases of resistance to 21 herbicide mechanisms of action recorded (Heap, 2021). *Poa annua* is the most problematic weed in sports turf, particularly in temperate climates (Heide, 2001; Wódkiewicz et al., 2014). Herbicide resistance in *P. annua* was initially reported in the 1970s, but those populations were not from turfgrass systems (Darmency and Gasquez, 1983). Since then, herbicide resistance in *P. annua* has become increasingly common with 48 occurrences globally (Heap, 2021).

In 2017, the first case of herbicide resistance in *P. annua* was confirmed in Australia (Heap, 2021). Thirty-one populations of *P. annua* were screened for the resistance with five different herbicides (ALS inhibitors, ACCase inhibitors, PSII inhibitors, microtubule inhibitors, and endothall) and result showed that most of the populations were resistant to three or more herbicide modes of action, with half of them being resistant to all five modes of action tested (Chapter 2). The majority of populations were resistant to ALS inhibitors, PSII inhibitors, endothall, ACCase inhibitors, and micro tubulin inhibitors (MTI) at EPOST application, whereas, only 7% of populations were resistant to PRE-application of the MTI herbicide propyzamide (Chapter 2).

A detailed dose response study was conducted with four multiple resistant populations (P18, P27, P262-16 and P413-17) with five herbicide MOA at various rates (Table 1, Chapter 2). These populations had a high level of resistance to the ALS inhibitors rimsulfuron (>19

fold) and foramsulfuron (>56 fold), and moderate to low level of resistance to endothall (>7.9-fold), ACCase inhibitors (>4.3-fold), PSII inhibitors (>2-fold), and POST application of MTI herbicides (1.7-to 4.1-fold) (Chapter 2). Populations P18, P27, P262-16 and P413-17 also had low level of resistance to propyzamide used PRE (Chapter 2).

Sequencing of target genes revealed Trp574Leu substitution in the *ALS* gene giving a high level of resistance to both rimsulfuron (>19 fold) and foramsulfuron (>56 fold) in three *P. annua* populations (P27, P262-16 and P413-17). In contrast, Pro197Ser substitution in *the ALS* gene in P18 population provided a high level of resistance to rimsulfuron (>19 fold), but low level of resistance to foramsulfuron (>9 fold) (Chapter 2). Likewise, resistance to the ACCase inhibitor pinoxaden was due to a target-site mutation, Ile1781Leu, which gave >4.3 fold resistance (Chapter 2). Low to intermediate levels of resistance to propyzamide, simazine and endothall were confirmed in the four resistant populations (P18, P27, P262-16 and P413-17) but no target-site mutations were detected in *psbA* or *α-tubulin* genes in these populations. The resistance mechanism to these herbicides remains unknown (Chapter 2). There remains scope to further investigate resistance mechanisms for MTI, PSII inhibitors and endothall in *P. annua*.

Multiple herbicide resistance is a term often used when a weed species is resistant to at least two herbicides that belong to two different herbicide MOA family (Powles and Preston, 1995). Multiple herbicide resistance has been reported in many weed species (Heap, 2021). Despite the fact that, *P. annua* ranked second with resistance to ten herbicide MOA, just after *L. rigidum* (Heap, 2021), there are only a few reports available of multiple herbicide resistance in *P. annua* (Breeden et al., 2017; Brosnan et al., 2015; Singh et al., 2021). However, our study identified four populations (P18, P27, P262-16 and P413-17) of *P. annua* where multiple-resistance to six herbicide MOA was present (Chapter 2 and 3). In contrast, there a

number of published reports on multiple herbicide resistance in *L. rigidum* (Mahmood et al., 2016; Owen et al., 2014; Torra et al., 2021). *L. rigidum* is the most problematic weed in Australian cropping system (Gill, 1996; Yu et al., 2004) and in this cross-pollinated species, multiple-resistance occurs faster than in self-pollinated species, as resistance can be transferred from plant to plant through pollen (Busi, 2014; Owen et al., 2007) allowing the accumulation of different resistance alleles. *L. rigidum* has been shown to evolve resistance to herbicides to a greater extent than the self-pollinated species *A. fatua* (Busi et al., 2016). *P. annua* is a self-pollinated species with less than 15% out-crossing (Carroll et al., 2021). Therefore, it is expected that resistance will evolve in self-pollinated species through sequential selection pressure with herbicides, rather than populations sharing resistance genes dispersal via pollen (Busi et al., 2016). Therefore, resistance has likely arisen in *P. annua* through repeated applications of herbicides. The low tolerance for *P. annua* in golf turf means that the turf managers apply herbicides for control multiple times each year. The ability of *P. annua* to germinate through most of the year and rapidly produce seed means there may be more than one generation treated each year. This intense use of herbicides over multiple generations appears to have led to multiple resistance across numerous herbicide modes of action.

Managing multiple herbicide resistance is always challenging. Herbicides are the most common control method used to control *P. annua*, because of their easy application and reliability of weed control (Vargas Jr and Turgeon, 2003) and the fact that alternative practices are not effective in golf turf or are too labour intensive. Hence, field studies conducted in autumn and spring season during 2018 to 2020 on a multiple-resistant *P. annua* population in a bermudagrass (*Cynodon dactylon*) turf at a golf course in South Australia identified four different herbicide options. Amicarbazone was the most effective herbicide that reduced the occurrence of *P. annua* by 98-100% followed by tebuthylazine >80%, indaziflam >63%, and pyroxasulfone >57% (Chapter 5). The availability of multiple herbicides with different modes

of action allows rotating herbicides in a management program, which is important to reduce the risk of resistance.

Screening with glyphosate was conducted for 32 population (31 populations from the golf courses and a population collected from a residential area in South Australia where glyphosate was used to control weeds in the garden) (Chapter 3). Five populations (P18, P27, P262-16, P413-17 and HT) were found to be resistant to POST glyphosate application at 180 g $ha^{-1}$. A detailed dose response was performed at two different temperatures (16/12 °C and 27/20 °C) with various rates (0-360 g $ha^{-1}$) of glyphosate and confirmed presence of a low level of resistance (>2-fold) to glyphosate in five (P18, P27, P262-16, P413-17 and HT) populations of *P. annua* (Chapter 3). This is a relatively low level of resistance, but sufficient for *P. annua* to survive the low rate of glyphosate used by the greenkeepers.

Molecular, physiological and biochemical studies were conducted to explore the mechanisms of resistance (Chapter 3). No mutation was detected in the *EPSPS* gene in any of the resistant populations. However, an increased *EPSPS* gene copy number (1.7 to 9.3-fold) and *EPSPS* gene expression (3.5 to16.3-fold) compared to the susceptible was found in one of the resistant populations (HT). This resistance mechanism was not found in other four populations, suggesting other resistance mechanisms were operating. Reduced herbicide absorption or translocation, or increased activity of aldo-keto reductase were not detected in any resistant population. The resistance mechanism of four multiple resistance population (P18, P27, P262-16 and P413-17) remains unknown. Even though there are more known mechanisms of glyphosate resistance than for any other herbicide (Sammons and Gaines 2014), the mechanism of resistance in these *P. annua* populations was not identified. It is possible that these *P. annua* populations possess yet undocumented resistance mechanisms.

An inheritance study was conducted with a $F_2$ population obtained from the cross between a resistant (P262-16) and susceptible population and showed glyphosate resistance was due to a single, nuclear dominant gene in this population. This inheritance pattern for glyphosate has been reported in other weed species where the resistance mechanism was due to target site resistance (Ng et al., 2004; Nguyen et al., 2019; Preston et al., 2009), however, no target site resistance mechanism was identified in our resistant population.

This study showed a population with *EPSPS* gene amplification, but low level of resistance to glyphosate (>2-fold). Many previous studies found gene amplification resulted in a relatively high level of resistance (Gaines et al., 2010). This suggests that *EPSPS* gene amplification in *P. annua* may operate differently compared with other species. This is worthy of future research.

While the levels of resistance to glyphosate were low (Chapter 3), glyphosate is a non-selective herbicide and can damage turf species (Velsor et al., 1989). Therefore, glyphosate is used at low rates in turf when the turf grass is dormant. When used at low doses, plants with weak resistance mechanisms can survive and be selected (Busi et al., 2013). This may be why more than one mechanism of resistance to glyphosate was selected in the *P. annua* populations.

There is limited information available on resistance to propyzamide, as there are few examples of resistance to this herbicide (Brunton et al., 2020; McCullough et al., 2017). Hence, further molecular and physiological studies were conducted to explore the mechanism of resistance (Chapter 4). No mutation was detected in *α-tubulin* gene (Chapter 2) or in the *β-tubulin* gene (Chapter 4) in any of the resistant populations. Hence, a non-target site resistance mechanism is suspected. Studies with the cytochrome P450 inhibitors piperonyl butoxide (PBO) and organophosphate insecticide (malathion) were conducted to determine whether these inhibitors could synergise the herbicide. The results showed that PBO and malathion antagonized propyzamide activity resulting in an increase in the $LD_{50}$ in both resistant and

susceptible populations. Hence, the resistance mechanism is unlikely to be cytochrome P450-based resistance. Therefore, other mechanisms should be explored. A previous study by McCullough et al. (2017) has shown that reduced absorption and translocation can confer resistance (>10-fold) to POST propyzamide in a *P. annua* from Georgia.

Understanding the inheritance pattern can help in managing the evolution and spread of herbicide resistance (Maxwell et al., 1990). The inheritance of resistance in propyzamide has not been identified in any weed species. Inheritance of resistance to other MTI herbicides (e.g. trifluralin) was previously reported as a single, nuclear recessive gene in *E. indica* (Zeng and Baird, 1997) and *S. viridis* (Jasieniuk et al., 1994) and the resistance mechanism was due to a target site resistance in both cases (Anthony et al., 1998; Délye et al., 2004; Yamamoto et al., 1998). Inheritance studies were conducted with $F_2$ populations from crosses between two resistant (P18 and P413-17) and one susceptible parents (Chapter 4). A detailed dose-response analysis of $F_2$ conducted with propyzamide confirmed two different patterns of inheritance. $F_2$ progeny of P18 exhibited a single-nuclear, dominant gene inheritance (3:1), whereas in P413-17 more than one gene contributes to resistance. The pattern of inheritance indicated that the resistance mechanism to propyzamide could be complex and more than one resistance mechanism is involved in resistance in *P. annua*.

Propyzamide resistance in weeds is rare, but not impossible. Two reports of resistance in *P. annua* (McCullough et al., 2017) and *L. rigidum* (Brunton et al., 2020) have been reported. High selection pressure in golf turf over many years has selected a small number of *P. annua* populations with resistance to propyzamide. Only four of the 31 populations tested (Chapter 2) had resistance to this herbicide. However, continued use of propyzamide is likely to lead to more resistance. The low tolerance of *P. annua* in golf turf means that even low levels of resistance can create a management problem.

There are no reports on the fitness of propyzamide resistant populations and only a few reports are available on fitness studies with other MTI herbicides (Chu et al., 2018; Darmency et al., 2011). In *L. rigidum*, a severe fitness cost in plant growth due to target-site based dinitroaniline resistance was identified (Chu et al., 2018). Therefore, studies should be conducted on the fitness of propyzamide resistant *P. annua*.

## 6.2 Conclusion

This research provides a comprehensive exploration of resistance to some major herbicide groups in *P. annua* in Australia including ALS inhibitors, ACCase inhibitors, PS II inhibitors, microtubule inhibitors, *EPSPS* inhibitors, and endothall. Resistance to ALS inhibitors and ACCase inhibitors was confirmed due to target-site mutation. Glyphosate resistance in one of resistant populations was due to gene amplification. The mechanisms of resistance to the other herbicides remained unknown, but these multiple-resistant populations are likely to possess multiple resistance mechanisms. This was supported by the inheritance study of propyzamide resistance that found two different patterns suggesting at least two-resistance mechanisms are present in the populations. The variety of resistance mechanisms means *P. annua* is likely to evolve resistance to other herbicides and alternative practices will be required in the future. Herbicide rotation is not a permanent solution to stop resistance. Therefore, to identify a sustainable solution of this problem more research on an integrated management approach for *P. annua* control is required. The seed bank of *P. annua* is large and estimated at 200,000 seed $m^{-2}$ (Lush, 1989) and about 80% of the seed remains viable in the top layer (0-1 cm) of the soil (Branham et al., 2004). Therefore, seed bank reduction is critically important for managing herbicide-resistant *P. annua* (Norsworthy et al., 2012). Hence, fraise mowing (a new cultivation practice that physically removes large quantities of turfgrass sward) can be an alternative mechanical option for controlling a large *P. annua* seed bank (Brosnan et al., 2020).

Manual removal of *P*. *annua* could be one practice for controlling *P*. *annua* from turf; however, it is not practical at present due to the large labour requirement. Therefore, the use robotics for the control of *P*. *annua* in turf may be a good future option. For example, a fully-automated weed killing robot called AG-BOT has been developed, that can cut 90% of the weed control cost (Anonymous, 2016). A similar type of technology could be designed for controlling *P*. *annua* by uprooting it directly from turf, particularly as *P*. *annua* can be easily distinguished from most turf species. Finally, the findings reported here will provide guidance into understanding resistance to herbicides used to control *P*. *annua* in turf.

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