Research Article

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Distribution, frequency and molecular basis of clethodim and quizalofop resistance in brome grass (*Bromus diandrus*)

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Abstract

BACKGROUND: Brome grass (*Bromus diandrus* Roth) is prevalent in the southern and western cropping regions of Australia, where it causes significant economic damage. A targeted herbicide resistance survey was conducted in 2020 by collecting brome grass populations from 40 farms in Western Australia and subjecting these samples to comprehensive herbicide screening. One sample (population 172-20), from a field that had received 12 applications of clethodim over 20 years of continuous cropping, was found to be highly resistant to the acetyl-CoA carboxylase (ACCase)-inhibiting herbicides clethodim and quizalofop, and so the molecular basis of resistance was investigated.

RESULTS: All 31 individuals examined from population 172-20 carried the same resistance-endowing point mutation causing an aspartate-to-glycine substitution at position 2078 in the translated ACCase protein sequence. A wild-type susceptible population and the resistant population had similar expression levels of plastidic ACCase genes. The level of resistance to quizalofop, either standalone or in mixture with clethodim, in population 172-20 was lower under cooler growing conditions.

CONCLUSION: Target-site resistance to ACCase-inhibiting herbicides, conferred by one ACCase mutation, was selected in all tested brome plants infesting a field with a history of repeated clethodim use. This mutation appears to have been fixed in the infesting population. Notably, clethodim resistance in this population was not detected by the farmer, and a high future incidence of quizalofop resistance is anticipated. Herbicide resistance testing is essential for the detection of evolving weed resistance issues and to inform effective management strategies.
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Supporting information may be found in the online version of this article.

Keywords: ACCase-inhibiting herbicides; brome grass; polyploidy; target-site mutation

1 INTRODUCTION

Although annual ryegrass (*Lolium rigidum* Gaud.) is the most troublesome grass weed of southern Australian cropping systems, brome grass (mainly *Bromus diandrus* Roth) infestations cost Australian farmers >$20 million annually in yield loss and control measures.[1] There are relatively few herbicide options for selective control of brome grass in cereal crops, and delayed sowing can be necessary in no-till systems because brome grass seeds are dormant at maturity and require cold-stratification for optimal germination, which is more likely to occur in late autumn or early winter.[2] Brome grass is also highly competitive with the crop, reducing wheat yield by more than 30%,[3] and field observations combined with simulation modelling demonstrated that brome seed production is so high that the soil seed bank can increase steadily even when herbicides are applied to control the weed population.[4]

Herbicides, however, are still the most effective option for controlling brome grass. Pre-emergence herbicides such as prosulfocarb

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and pyroxasulfone are successfully used on this genus, but the most common chemical control measures are the acetolactate synthase (ALS) and acetyl-CoA carboxylase (ACCase) inhibitors, applied post-emergence. As clearly illustrated by the widespread and high-level resistance of annual ryegrass to these modes of action,[5–8] repeated use of ALS and ACCase inhibitors can lead to the rapid evolution of resistance in weed populations. Resistance to these herbicide modes of action has been reported in *Bromus* spp. from across Australia, Europe, and the USA.[9,10] The molecular mechanisms conferring resistance to ALS inhibitors in brome grass have been relatively well-studied and are similar to those in other weed species, namely: point mutations of the ALS enzyme which decrease its affinity for the herbicide[11–13]; overexpression of the *ALS* gene, providing higher levels of enzyme to maintain flux through its metabolic pathway[14]; and enhanced detoxification of the herbicide, decreasing the concentration of ALS inhibitor in the plant cell.[11,14,15] By contrast, mechanisms of resistance to ACCase inhibitors have been elucidated only in one study with *Bromus tectorum* L.[16] Extensive research in other resistant grass weeds, however, has revealed that metabolic detoxification of ACCase inhibitors, likely mediated by cytochrome P450 mono-oxygenase(s) (in concert with glutathione transferase in some cases), occurs in a number of species.[17–20] There have also been 16 different point mutations, at seven positions, observed in the plastidic ACCase gene across the various resistant grass weeds (conferring different levels of resistance and cross-resistance to the three chemical classes of ACCase inhibitors, sometimes in a species-specific manner) and higher levels of ACCase expression in some resistant populations (reviewed in Takano *et al.*[21]).

In the *B. diandrus-rigidus* complex, both hexaploid ($2n = 6x = 42$) and octoploid ($2n = 8x = 56$) races have been identified.[22] The dynamics of evolution of target-site resistance in polyploid weed species such as brome grass, wild oat and Johnsongrass is more complex than in diploids such as annual ryegrass, because the effect of target-site mutations in one genome could be diluted by wild-type susceptible genes in the other genome(s); alternatively, the speed of adaptation and fixation of beneficial alleles is known to increase with increasing ploidy level.[23] Therefore, empirical observations of the response of polyploid weed populations to herbicide selection are necessary.

A baseline resistance screening of 91 brome grass populations collected randomly from across the Western Australian cropping region in 2010 yielded only one population with resistance to the two ACCase-inhibiting herbicides tested (fluazifop, a member of the aryloxyphenoxypropionate or FOP class of ACCase inhibitors, and clethodim, from the cyclohexandione or DIM class),[24] and the resistance mechanism was not investigated. No populations resistant to ACCase inhibitors were detected in a subsequent random survey of 97 populations collected from the same region in 2015.[25] In 2020, Western Australian growers who had perceived problems with a number of hard-to-kill weed species in their cropping fields submitted 40 brome grass populations (together with seeds from other weed species) to the University of Western Australia for resistance testing. The aims of the work reported here were to identify any potential cases of pre- or post-emergence herbicide resistance in these brome grass populations, and to fill a knowledge gap in *B. diandrus* by characterising the mechanism of ACCase inhibitor resistance in any populations surviving treatment with this group of herbicides.

2 MATERIALS AND METHODS

2.1 Plant material and herbicide application

Brome grass (*Bromus diandrus* Roth) seed was collected at harvest in 2020 (October–November) from 40 sites across the Western Australian grain belt (Fig. 1) in the same way as described by Busi *et al.*,[8] and transported to the University of Western Australia for threshing. Cleaned seeds were allowed to after-ripen under warm, dry conditions for at least 2 months to permit release of primary dormancy. Herbicide screening was performed on plants grown outdoors from May to July in 2021. Seeds were sown in 12-cell trays (20 seeds per cell) filled with commercial potting mix (50% river sand, 25% pine bark, 25% peat moss),[8] and watered as required to maintain >80% field capacity. Pre-emergence herbicides (Table 1) were applied directly to seeds lying on the soil surface, which were then covered with a layer of fresh potting mix and watered well. Seedlings for post-emergence herbicide application were fertilised weekly with commercial soluble fertiliser (Diamond Red: N 27%, P 5.7%, K 10.9%, trace elements: Campbells Fertilisers, Laverton North, Australia) and treated with herbicide

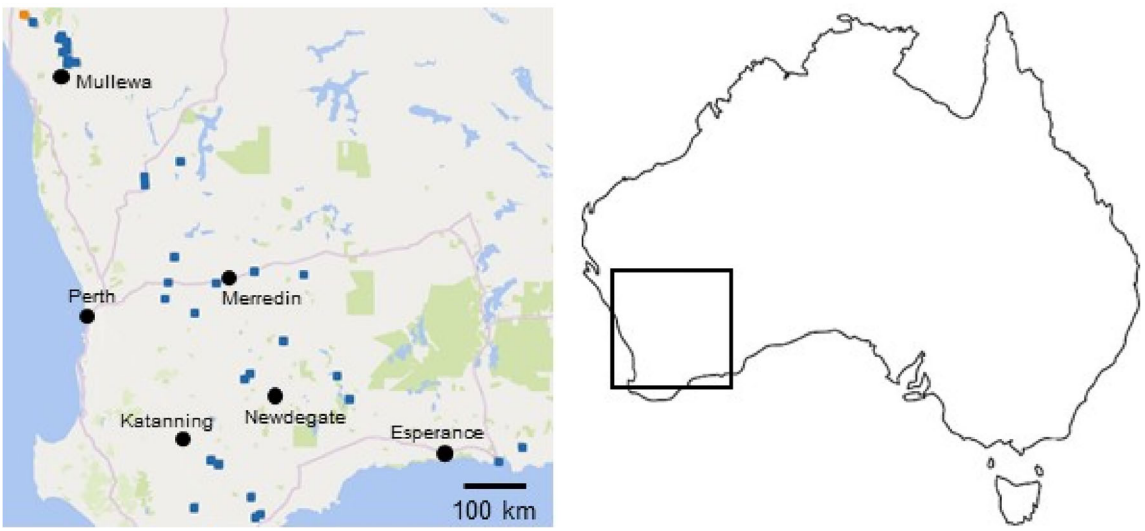


Figure 1. Collection sites of brome grass populations sampled from the Western Australian grain belt in late 2020. The orange square denotes the population (172-20) with resistance to acetyl-CoA carboxylase (ACCase) inhibitors; blue squares represent the other populations.

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Table 1. Herbicide treatments used in the resistance screening of brome grass populations from Western Australia. In each case, the dose applied represents the recommended label rate

Herbicide	Mode of action	Use	Formulation	Dose (g a.i. ha^{-1})
Clethodim	ACCase inhibitor	Post (standalone)	Clethodim 240[†] (240 g L^{-1})	60
Quizalofop	ACCase inhibitor	Post (standalone)	Targa[‡] (99.5 g L^{-1})	12.5
Clethodim + quizalofop	ACCase inhibitor + ACCase inhibitor	Post (tank mixture)		60 + 12.5
Imazamox + imazapyr	ALS inhibitor + ALS inhibitor	Post (formulated mixture)	Intervix[§] (33 + 15 g L^{-1})	25 + 11
Sulfometuron	ALS inhibitor	Post (standalone)	Oust[¶] (750 g kg^{-1})	25
Glyphosate	EPSPS inhibitor	Post (standalone)	Crucial[††] (600 g L^{-1})	480
Propyzamide	Microtubule assembly inhibitor	Pre (standalone)	Dargo 500 WG[‡‡] (500 g kg^{-1})	500
Carbetamide	Microtubule organisation inhibitor	Pre (standalone)	Ultro 900 WG[§§] (900 g kg^{-1})	990
Triallate	Lipid synthesis inhibitor	Pre (standalone)	Avadex Xtra[††] (500 g L^{-1})	1500

ACCase, acetyl-CoA carboxylase; ALS, acetolactate synthase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase.
[†] FMC Australia, Sydney, Australia.
[‡] Sipcam Pacific Australia, Geelong, Australia.
[§] BASF, Melbourne, Australia.
[¶] DuPont Australia, Sydney, Australia.
[††] Nufarm Australia, Laverton North, Australia.
[‡‡] Conquest Crop Protection, Perth, Australia.
[§§] ADAMA Australia, Sydney, Australia.

(Table 1) when they had reached the two-leaf stage. Herbicides were delivered by a twin-nozzle (TeeJet XR11001 flat fan) laboratory sprayer calibrated to deliver 110 L water ha^{-1} at 210 kPa, and applied at the recommended label rate (Table 1). Each population was sprayed with each herbicide and the experiment was repeated.

Plant survival, defined as seedling emergence and establishment following pre-emergence herbicide treatment, or as the production of healthy new growth following post-emergence treatment, was assessed 4 weeks after herbicide application by counts of dead or alive plants.[8] Herbicide-susceptible brome grass populations, used as controls, always showed 100% mortality at the recommended label rate of each herbicide.

2.2 Dose–response experiments with ACCase inhibitors

A population (designated 172-20) surviving the recommended field rates of the ACCase-inhibiting herbicides clethodim and quizalofop was further investigated in a dose–response study with butroxydim, clethodim, fluazifop and quizalofop applied as a standalone and in two- and three-way mixtures, as listed in Table 2 (standalone doses ranged from 12.5 to 400 g ha^{-1} for butroxydim, clethodim and quizalofop, and from 25 to 800 g ha^{-1} for fluazifop). One of the populations showing 100% mortality in the initial herbicide screening (designated 76-20) was used as a susceptible control. Plants were grown, sprayed and assessed as above. There were three replicates of each herbicide treatment and dose. The original experiment with all herbicides was performed in November 2021 (mean day and night temperatures: 23 and 15 °C, respectively). Because of the decreased ability of grass weeds to metabolise quizalofop at lower temperatures,[26] the treatments involving quizalofop and/or clethodim were repeated outdoors in June 2022 (mean day and night temperatures: 17 and 8 °C, respectively).

2.3 ACCase gene sequencing

Leaf samples were taken from individuals of populations 76-20 and 172-20 for sequencing of known mutation sites in the carboxyltransferase domain of the ACCase genes. Leaves from

Table 2. Herbicide treatments used in the dose–response study on the putative acetyl-CoA carboxylase (ACCase) inhibitor-resistant brome grass population 172-20

Herbicide	Formulation	Dose (g a.i. ha^{-1})
Butroxydim	Factor WG[†] (500 g kg^{-1})	12.5, 25, 50, 100, 200, 400
Clethodim	Clethodim 240[‡] (240 g L^{-1})	12.5, 25, 50, 100, 200, 400
Clethodim (winter)		1.6, 3.1, 6.3, 12.5, 25, 50, 100, 200
Fluazifop	Fusilade Forte[§] (128 g L^{-1})	25, 50, 100, 200, 400, 800
Quizalofop	Targa[¶] (99.5 g L^{-1})	12.5, 25, 50, 100, 200, 400
Quizalofop (winter)		1.6, 3.1, 6.3, 12.5, 25, 50, 100, 200
Butroxydim + clethodim		50, 100, 200, 400
Butroxydim + quizalofop		50, 100, 200, 400
Clethodim + quizalofop		50, 100, 200, 400
Clethodim + quizalofop (winter)		3.2, 6.3, 12.5, 25, 50, 100, 200, 400
Butroxydim + clethodim + quizalofop		75, 150, 300, 600

The doses listed for the tank mixes represent the total amount of active ingredient applied, with each component making up an equal part of the whole. The susceptible control population 76-20 was treated with the standalone herbicides at each dose, but not with the mixtures. All treatments were performed in summer, with additional treatments of clethodim and quizalofop in winter, as indicated.
[†] Nufarm Australia, Laverton North, Australia.
[‡] FMC Australia, Sydney, Australia.
[§] Syngenta Australia, Sydney, Australia.
[¶] Sipcam Pacific Australia, Geelong, Australia.

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susceptible population 76-20 were samples from untreated plants, or following plant death after herbicide treatment. Leaves from individuals of resistant population 172-20 were collected as independent samples, with at least three replicates sampled from each of the following groups: (i) untreated plants; (ii) survivors of 100 g ha^{-1} clethodim standalone, 25 or 100 g ha^{-1} quizalofop standalone, or a 100 + 25 g ha^{-1} clethodim + quizalofop mixture; (iii) plants killed by 200 g ha^{-1} clethodim or quizalofop standalone, or a 100 + 100 g ha^{-1} clethodim + quizalofop mixture. In addition, seeds were harvested from 172-20 plants surviving clethodim, quizalofop or a mixture, germinated in the absence of herbicide, and coleoptile tissue was sampled from these progeny of the original surviving plants.

IDENTXX GmbH (Stuttgart, Germany: www.identxx.com) performed the DNA extraction, amplification and sequencing. Air-dried leaf samples (~0.5 cm^2) were placed in tubes containing two steel beads of 4.5-mm diameter and homogenised in a shaker mill (TissueLyser II, Qiagen, Hilden, Germany), and DNA was extracted in the KingFisher™ Flex Magnetic Particle Processor (Thermo Fisher Scientific, Schwerte, Germany) using the Chemagic Plant 400 kit (PerkinElmer, Rodgau, Germany) with slight modifications (no pre-extraction step and lower buffer volumes) to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification was performed using a MangoTaq DNA polymerase (Bioline, Luckenwalde, Germany) and specific proprietary primers (IDENTXX; Supporting Information, Fig. S1) for the commonly mutated positions 1781, 2027, 2041, 2078 and 2096 of the ACCase gene (codon positions are relative to the *Alopecurus myosuroides* ACCase protein sequence). Target DNA was amplified in a thermal cycler (T100 PCR thermal cycler, Bio-Rad Laboratories GmbH, Feldkirchen, Germany) using standard conditions of 3 min initial denaturation at 95 °C, 45 cycles of 10 s denaturation at 95 °C/35 s primer annealing at the appropriate temperature for each primer/30 s elongation at 72 °C, 5 min final elongation at 72 °C. Successful amplification was checked using agarose gel electrophoresis. The PCR products were analysed for single nucleotide polymorphisms via pyrosequencing on a PyroMark Q24 (Qiagen)[27] using specific, proprietary pyrosequencing primers (IDENTXX). During the sequencing reaction, all incorporated nucleotides of a short region that encompass the position of interest were detected and reported by creating a pyrogram in a pyrorun file. Subsequently, the file was read by the PyroMark Q24 software (version 2.0.7) and visually evaluated for mutations. PCRs were repeated for randomly selected samples and sequenced via Sanger sequencing (SeqLab-Microsynth, Göttingen, Germany) to validate the results using an alternative sequencing method. Chromatograms of the sequences were analysed using Geneious software (version 9.1.8, Biomatters, Auckland, New Zealand).

Table 3. Herbicide resistance in 40 brome grass populations collected from the Western Australian grain belt in 2020

	No. populations in each resistance category		
Herbicide	Susceptible (≤5% survival)	Developing resistance (6–19% survival)	Resistant (≥20% survival)
Clethodim	39	0	1
Quizalofop	39	0	1
Clethodim + quizalofop	39	1	0
Imazamox + imazapyr	39	1	0
Sulfometuron	29	8	3
Glyphosate	40	0	0
Propyzamide	40	0	0
Carbetamide	40	0	0
Triallate	40	0	0

All herbicides were applied at the recommended rate as listed in Table 1.

2.4 Determination of chromosome number

Seeds of the resistant population 172-20 were germinated at 25 °C in the dark on moistened filter paper for 6–7 days. Actively growing root tips of ~10–20 mm were excised, pretreated with 2 mM 8-hydroxyquinoline for 4 h to accumulate metaphase cells, fixed in freshly prepared ethanol/glacial acetic acid (3:1) for 30 h, and finally stored at −20 °C in the same solution until use.[28] Chromosome preparations from root tip cells were performed according to Mehravi *et al.*[29] Briefly, root tips were washed twice in ice-cold water and then twice in citrate buffer (0.01 M

Table 4. Dose–response analysis of susceptible (76-20) and resistant (172-20) brome grass populations treated with clethodim, quizalofop or a 50:50 mixture under summer and winter conditions

	LD_{50} (g ha^{-1})		172-20 *versus* 76-20		Summer *versus* winter (172-20)	
Herbicide	76-20	172-20	RI	*P*-value	RI	*P*-value
Summer						
Clethodim	23.5	107.8	4.6	<0.01	3.9	0.65
Quizalofop	4.1	179.9	43.9	<0.01	2.1	<0.01
Mixture	4.1	132.6	32.3	<0.01	6.1	0.02
Winter						
Clethodim	<3.1[†]	30.9	>10.0	Nd[‡]		
Quizalofop	<3.1[†]	100.7	>32.5	Nd		
Mixture	<3.1[†]	19.9	>6.4	Nd		

The herbicide dose causing 50% mortality (LD_{50}) was estimated from dose–response curves, and the resistance index (RI) was calculated as the ratio of LD_{50} values of population 172-20 and susceptible population 76-20, or of summer *versus* winter for population 172-20 only.
[†] Survival of susceptible population 76-20 was lower than lowest tested rate of 3.1 g ai ha^{-1}.
[‡] Not determined by non-linear regression analysis as lower than lowest tested dose.

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citric acid, 0.01 M sodium citrate, pH 4.8), and the meristematic portion of the root tips (1–1.5 mm from the apical end) were digested with 30 mL of enzyme solution (1% pectolyase, 0.7% cellulase, 0.7% cellulase R10, 1% cytohelicase in citrate buffer) for 1 h at 37 °C. Meristems were then washed twice in citrate buffer and once in ethanol, and placed in 70 mL glacial acetic acid/methanol (9:1). The tissue was carefully broken using a dissecting needle until a cell suspension had formed, and 7 µL of this was transferred to a glass slide and placed inside a box controlled at 50% relative humidity. After gradual drying, slides were stored in 70% ethanol. Cells were stained with a droplet of 1 µg mL^{-1} 4′,6-diamidino-2-phenylindole, and high-resolution images of the chromosomes were obtained with a Nikon A1Si laser scanning confocal microscope (Nikon Instruments Inc., Tokyo, Japan).

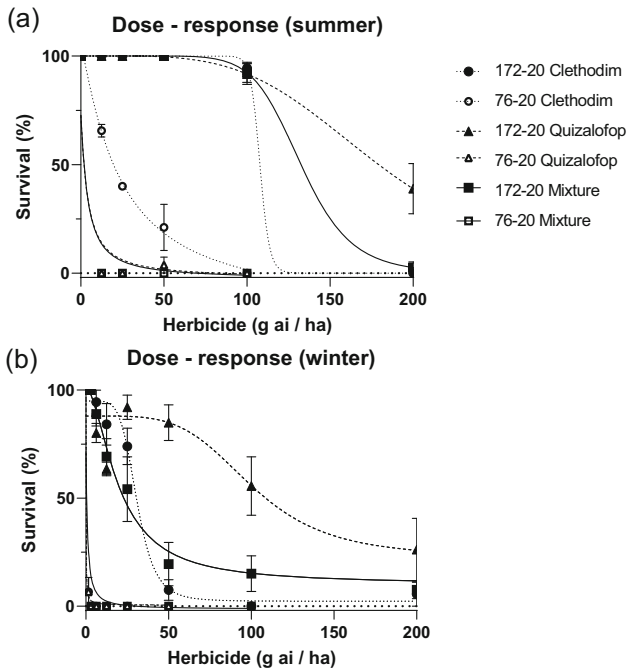


Figure 2. Dose–response curves for resistant brome grass population 172-20 and susceptible population 76-20 following treatment with clethodim, quizalofop and a 50:50 mixture of these herbicides under summer (a) and winter (b) conditions. Values are means ± SE (n = 3).

2.5 Relative expression of ACCase

Seeds of both populations were germinated on 0.6% (w/v) agar in the dark at 4 °C for 7 days and then transferred to 24 °C for a further 3 days. Germinated seedlings were transplanted into 9.5 × 8.5 × 8.5 cm pots containing commercial potting mix and grown in a naturally lit glasshouse maintained at an average diurnal temperature range of 13–23 °C and with a photoperiod of ∼10 h. When seedlings had reached the two-leaf stage, four herbicide treatments were applied: (i) untreated control, (ii) 50 g ha^{-1} clethodim, (iii) 25 g ha^{-1} quizalofop, and (iv) 50 + 25 g ha^{-1} clethodim + quizalofop. There were nine seedlings per population and treatment, of which three seedlings were sampled at 6 h post-treatment and their leaves immediately flash-frozen in liquid nitrogen. This sampling time-point was chosen as ACCase gene expression was significantly upregulated in ACCase inhibitor-resistant barnyard grass (*Echinochloa crus-galli*) populations compared with susceptible populations, 6 h after treatment with three different ACCase-inhibiting herbicides.[30]

RNA was extracted from 100 mg of finely ground leaf tissue per biological replicate using an Isolate II RNA Plant Kit (Meridian Bioscience) according to the manufacturer's recommendations. A second DNase treatment of 10 µg of RNA per sample was conducted using a TURBO DNA-free Kit (Invitrogen). RNA quality and successful removal of genomic DNA was assessed via agarose gel electrophoresis. Tetro cDNA Synthesis Kits (Meridian Bioscience) were used to synthesise 1 µg of complementary DNA (cDNA) per sample, using 60 µM each of oligo(dT) and random hexamer primers in 20-µL reactions.

Table 5. Sequencing of the acetyl-CoA carboxylase (ACCase) gene at amino acid position 2078 in susceptible brome grass population 76-20 and resistant population 172-20

Population	Treatment	Survivors	No. individuals sequenced	Nucleotide sequence at position 2078	Amino acid at position 2078
76-20	None	Yes	3	GAT	Asp
76-20	Fluazifop (25 g ha^{-1})	Yes	3	GAT	Asp
76-20	Clethodim (100 g ha^{-1})	No	3	GAT	Asp
172-20	None	Yes	3	G(A/G)T	Asp/Gly
172-20	Clethodim (100 g ha^{-1})	Yes	6	G(A/G)T	Asp/Gly
172-20	Quizalofop (25 g ha^{-1})	Yes	3	G(A/G)T	Asp/Gly
172-20	Quizalofop (100 g ha^{-1})	Yes	3	G(A/G)T	Asp/Gly
172-20	Clethodim + quizalofop (100 + 25 g ha^{-1})	Yes	4	G(A/G)T	Asp/Gly
172-20	Clethodim + quizalofop (100 + 100 g ha^{-1})	No	3	G(A/G)T	Asp/Gly
172-20	Clethodim (200 g ha^{-1})	No	3	G(A/G)T	Asp/Gly
172-20	Quizalofop (200 g ha^{-1})	No	3	G(A/G)T	Asp/Gly
172-20 progeny seed	None	Yes	3	G(A/G)T	Asp/Gly

The wild-type codon is GAT. Leaf samples were taken from individuals that had not received herbicide treatment, as well as from individuals that had either survived or died after treatment with clethodim and/or quizalofop. Seed from 172-20 plants that had survived herbicide treatment was also germinated and the young coleoptiles sequenced. In all cases, identical results were obtained from each individual sequenced in each treatment.

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Relative chloroplastic ACCase expression was determined via quantitative PCR using the ACcp4 and ACcp2R primers designed to selectively discriminate against cytosolic ACCase genes.[31] Each 10-µL reaction contained 25 ng of cDNA template, 3 pmol each of forward and reverse primers and 5 µL of SensiFast SYBR Lo-ROX (Meridian Bioscience), and was assayed using a 7500 Fast Real-

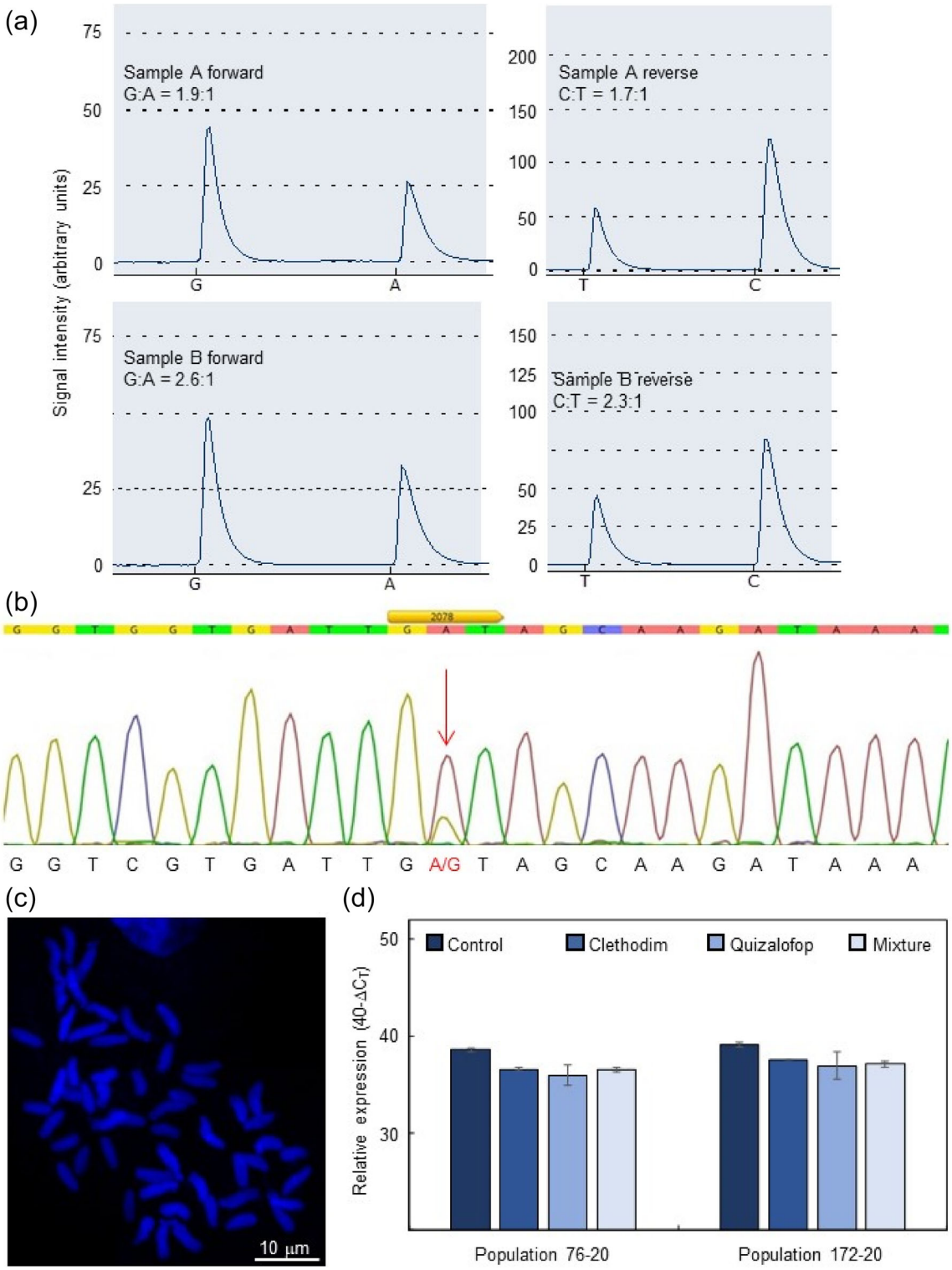


Figure 3. Molecular analysis of acetyl-CoA carboxylase (ACCase) in brome grass populations. (a) Representative pyrograms in the forward and reverse directions from two individuals from resistant population 172-20 showing the ~2:1–2.5:1 signal ratio of wild-type (adenine forward, thymine reverse) and mutant (guanine forward, cytosine reverse) nucleotides within the codon at position 2078 of the ACCase sequence. (b) Representative Sanger sequencing chromatogram from a resistant individual in population 172-20 showing the ~3.5:1 peak ratio of adenine and guanine nucleotides at position 2078. (c) Representative chromosome squash from root tips of population 172-20 showing 56 chromosomes. (d) Relative expression of plastidic ACCase in susceptible population 76-20 and resistant population 172-20 under no herbicide treatment (control) and at 6 h after treatment with clethodim, quizalofop or a mixture of the two herbicides. Values are means ± SE (n = 3).

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time PCR system (Applied Biosystems) with the following cycling protocol: (i) 30 s initial denaturation at 95 °C, and (ii) 40 cycles of denaturation at 95 °C for 3 s followed by primer annealing and extension at 60 °C for 30 s. ACCase expression was normalised against *ACTIN*, which was identified by NormFinder[32] as the most stably expressed of five reference genes among the populations and herbicide treatments examined in this study (see Supporting Information, Table S1 for primer sequences). The *ACTIN* reference gene and ACCase gene-of-interest primer pairs had acceptable amplification efficiencies of 2.016 (100.8%) and 1.876 (93.8%), respectively, which were calculated using LinRegPCR (version 2018.0).[33]

2.6 Data analysis

After the initial herbicide-screening experiment, brome grass populations were placed in three arbitrary resistance categories as previously described[8]: populations were considered susceptible if survival (percentage of total herbicide-treated individuals remaining alive) after treatment with the recommended rate of a herbicide was ≤5%; 'developing resistance' if survival was 6–19%; and 'resistant' if survival was ≥20%. The resistance category of each population was the same and non-significantly different by the chi-squared heterogeneity test in both independent screening experiments, so the data presented in Table 3 are representative of both experiments.

Dose–response data were analysed using the drc package in the statistical software R (version 4.1.0)[34] and fitted to a three-parameter non-linear logistic model:

$$y = \frac{d}{1 + \exp[b(\log x - \log e)]} \quad (1)$$

where y is plant survival (expressed as a percentage), d is the upper asymptotic value of y (100%), b is the slope of the curve, e is the herbicide dose required to kill 50% of the population (LD_{50}) and x is the herbicide dose (g ha^{-1}). Significant differences between LD_{50} values were identified using t-tests in the drc package.

Final relative ACCase gene expression was presented on a $\log_2$ scale of 40-ΔC_T values,[35] and analysed by analysis of variance and Tukey post-hoc statistical tests in R.

3 RESULTS

3.1 Response of brome grass populations to single-dose herbicide application

None of the 40 populations tested survived the recommended rates of glyphosate, propyzamide, carbetamide or triallate (Table 3). There was one population resistant (≥20% survival) to clethodim and quizalofop applied standalone, and this population also showed lower levels of survival (12%) to the mixture of these two ACCase inhibitors (Table 3). Resistance to sulfometuron was comparatively high, with three populations showing survival ranging from 21% to 39%, whereas one population showed low (16%) survival to the formulated mixture of imazamox + imazapyr and was therefore classified in the 'developing resistance' category (Table 3).

3.2 Characterisation of ACCase inhibitor response in a putative resistant population

The population surviving clethodim and quizalofop application (population 172-20) was highly resistant to butroxydim and fluazifop applied standalone, with even the highest doses resulting in >80% survival (Supporting Information, Fig. S2). Therefore, LD_{50} values could not be calculated and no further experiments were performed using butroxydim and fluazifop.

Under the warmer conditions in November 2021 (summer), the resistance indices of population 172-20 compared with susceptible population 76-20 were 5 and 44 for standalone clethodim and quizalofop, respectively, and 32 for the binary mixture (Table 4; Fig. 2). In June 2022 (winter), survival of population 76-20 was so low that an LD_{50} could not be calculated for any herbicide treatment, whereas quizalofop either standalone or in a mixture with clethodim was more effective on population 172-20 than it had been in summer (Table 4; Fig. 2).

3.3 ACCase gene sequencing

Susceptible population 76-20 had codons corresponding to the wild-type amino acids at all positions sequenced in the ACCase gene. Resistant population 172-20 also had the wild-type sequences at positions 1781, 2027, 2041 and 2096. However, in all resistant individuals sequenced, there was an A to G point mutation at position 2078, which causes an aspartate-to-glycine amino acid substitution (Table 5). The pyrograms in the forward and reverse directions gave an average ratio of A to G signals of 2.1:1 (Fig. 3(a)), whereas the Sanger sequencing chromatograms gave a ~3.5:1 ratio (Fig. 3(b)), indicating the presence of both wild-type and mutated alleles within individuals. To clarify this result, given that members of the *B. diandrus-rigidus* complex can be tetraploid, hexaploid or octoploid,[20] the number of chromosomes in population 172-20 was determined, and the relative expression levels of plastidic ACCase were quantified in both populations 76-20 and 172-20.

3.4 Chromosome number and ACCase gene expression

Fifty-six chromosomes were counted in population 172-20, indicating octoploidy ($2n = 8x = 56$) (Fig. 3(c)).

Relative plastidic ACCase gene expression was similar between populations 76-20 and 172-20 in the absence of herbicide treatment, with an average difference of 1.6-fold in relative expression between populations ($P > 0.05$; Fig. 3(d)). Application of standalone or mixed ACCase-inhibiting herbicides did not change gene expression at 6 h after treatment in either population (Fig. 3(d)).

4 DISCUSSION

The herbicide resistance problem in Western Australian populations of brome grass is not yet as serious as that in annual ryegrass or wild radish (*Raphanus raphanistrum* L.), but the lack of selective herbicide options for brome grass control makes preservation of the existing herbicides even more important. Six populations of brome grass from Western Australia were previously identified as having resistance (probably via metabolic detoxification) to sulfonylurea-class ALS inhibitors.[36] Now, a population with high-level resistance to both the FOP and DIM classes of ACCase inhibitors, conferred by an Asp-2078-Gly mutation in the ACCase gene, has also been identified, in a field with a history of repeated clethodim use. Interestingly, resistance to ACCase-inhibiting herbicides was not suspected by the grower who submitted the samples. This is probably because the grower would have used clethodim at the registered label rate of 120 g ha^{-1} for control of annual ryegrass, and as the dose–response analysis in the current study shows, no plants from brome grass population 172-20 survived clethodim applications of more than 100 g ha^{-1}.

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The grower would therefore currently be achieving satisfactory control of brome grass in the field.

Studies on numerous grass weed species such as barnyard grass,[30] goosegrass (*Eleusine indica*),[37] Italian ryegrass (*Lolium multiflorum*),[38] sterile wild oat (*Avena sterilis*)[39] and black grass (*Alopecurus myosuroides*),[40] as well as on recombinant wheat ACCase mutants expressed in yeast cells,[41] have demonstrated that, in contrast to certain other ACCase mutations, an amino acid substitution at position 2078 can confer high-level resistance to all three classes (FOP, DIM and DEN) of ACCase inhibitors. The Asp-2078-Gly mutation identified here in brome grass population 172-20 occurs in a population with resistance to clethodim, butroxydim, quizalofop and fluazifop, and the resistant phenotype persists when mixtures of FOP and DIM are applied. There is no evidence that any other form of target-site modification, apart from the Asp-2078-Gly mutation, is responsible for the FOP and DIM resistance observed in population 172-20, because there were no differences in ACCase expression between 172-20 and the susceptible 76-20 population either under control conditions or at 6 h after herbicide treatment. A previous study on goosegrass demonstrated that for those ACCase-inhibiting herbicides which stimulated ACCase expression in the resistant population (and not all did), the stimulation occurred within 6 h and then decreased back to control levels within 72 h.[30] Therefore, the observed similarity in relative ACCase expression levels in susceptible and resistant brome grass populations at 6 h is likely to be maintained at later time points.

Notwithstanding the inherent uncertainty of Sanger chromatograms in terms of the signal strength of individual bases,[42] the wild-type (A) to mutant (G) peak height ratio of ~3.5:1 is similar to that obtained with the more reliable[43] pyrosequencing method (~2:1). Because the chromosome count demonstrated octoploidy in population 172-20, a wild-type/mutant nucleotide ratio of 3:1 would indicate a homozygous mutation in one of the genomes, whereas a ratio of 2:1 would not be possible in an octoploid. Therefore, it is likely that one of the genomes of population 172-20 carries a homozygous ACCase mutation. Given that all 31 sequenced individuals gave identical results, it would appear that the Asp-2078-Gly mutation has become fixed in the population. In mutagenesis studies on wheat ACCase, it was observed that the level of resistance to ACCase-inhibiting herbicides depended on whether the mutation was located on the A, B or D genome; in hexaploid wild oat (*Avena fatua*), the resistance level conferred by single mutations in ACCase was lower than for the equivalent mutations in diploid annual ryegrass because of gene dilution (reviewed in Kaundun[44]). In the case of brome grass population 172-20, attempts to determine the relative expression level of the mutant *versus* wild-type copies of ACCase were unsuccessful (data not shown), so the extent of gene dilution remains unknown. Similarly, long-read sequencing is needed to determine whether biased fractionation[45] of the four genomes in brome grass has resulted in the loss of any wild-type ACCase, thus increasing the gene-dosage effect of the resistance-endowing Asp-2078-Gly mutation.

Although metabolism of ACCase-inhibiting herbicides was not measured directly in the current work, it is likely that quizalofop is metabolised to some extent in both brome grass populations studied. The resistant population 172-20 was significantly more sensitive to quizalofop under the cooler conditions of winter, whereas the susceptible population 76-20 was so sensitive in winter that an LD_{50} could not be calculated. This is in line with previous studies on quizalofop and other metabolisable herbicides showing slower metabolism, and thus greater herbicide efficacy, at lower temperatures (Bough *et al.*[26] and references therein). However, direct study of clethodim and quizalofop metabolism in these populations is required to determine whether: (i) quizalofop metabolism in 172-20 is enhanced relative to 76-20, thus contributing to resistance; and (ii) clethodim is metabolised (there are so far no published reports of metabolism-based clethodim resistance in weeds), because sensitivity to clethodim was also increased, although not statistically significantly, in both populations in winter.

Vila-Aiub *et al.*[46] found that the Asp-2078-Gly mutation in ACCase imposes a fitness cost in annual ryegrass, whereas the Ile-1781-Leu mutation does not; however, field studies on black grass and Italian ryegrass have shown that the frequency of individuals with resistance to ACCase inhibitors does not decrease under alternative methods of weed control (reviewed in Kaundun[44]). Therefore, avoidance of the use of ACCase inhibitors in fields with a resistance problem is required. Brome grass sheds a higher proportion of its seeds before harvest than does annual ryegrass, potentially making harvest weed seed control techniques less attractive. However, a modelling study concluded that removal of even 20% of the new brome grass seed population at harvest could lead to a 46% reduction in the seed bank.[4] This is important because brome grass was the most fecund, in terms of seed production, of 11 weeds (including annual ryegrass) studied over 3 years, and caused the largest wheat yield reduction.[3] These authors emphasised that the introduction of brome grass seeds into new cropping areas (for example, via contaminated crop seed) should be rigorously avoided, especially given the high probability that weed seeds collected with the grain originate from mother plants that have survived selective herbicide application.[47]

In conclusion, this study has identified the molecular basis of resistance to ACCase-inhibiting herbicides in a *B. diandrus* population collected from a field with a long history of clethodim use. The identification of target-site-based herbicide resistance in another polyploid weed species provides an opportunity for closer examination of how rapidly resistance evolves in these species under field conditions. This is especially relevant given the introduction of Co-Axium barley to Australia in 2022, which allows growers to selectively use quizalofop in barley crops. As demonstrated by this study, brome grass with target-site resistance to ACCase inhibitors will express a high level of phenotypic resistance at the rate of quizalofop (37 g ha^{-1}) used in barley. Therefore, it is anticipated that cases of quizalofop resistance in brome grass will increase. This, coupled with the fact that the existing case of clethodim resistance was not suspected by the grower, highlights the importance of closely monitoring the weed populations subjected to repeated herbicide selection so that problems can be identified before they become insurmountable, and alternative weed control strategies implemented.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Roberto Busi at https://research-repository.uwa.edu.au/en/persons/roberto-busi.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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