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First report of target-site resistance to glyphosate in *Amaranthus hybridus* L. in the Republic of South Africa

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Abstract

Amaranthus hybridus L. is a major weed for summer crops. Although *A. hybridus* has been a known crop weed in the Republic of South Africa (RSA) for a long time, herbicide resistance has not been a problem. Nevertheless, *A. hybridus* populations from KwaZulu Natal (KZN) Province have caught the attention of farmers since glyphosate has been progressively becoming less effective in controlling the species. This study aimed to evaluate herbicide resistance in *A. hybridus* and the underlying mechanisms of resistance. Seeds from 50 glyphosate-resistant plants were collected from fields at Bergville and Winterton in KZN and compared with a susceptible population from Hendrina (Mpumalanga Province). Glasshouse screening was conducted where glyphosate (Roundup® PowerMax) was applied at 6-leaf stage, at doses 0, 540, 1080 (recommended dose), 2160 and 4320 g ae ha^{-1}. Surviving plants were sampled for molecular analysis to establish any target site mutations in the *EPSPS* gene that confer glyphosate resistance. Dose–response assay indicated 100% control in the Hendrina population, variation in the control of the Winterton population and 100% survival in the Bergville population. Molecular analysis indicated a rare triple mutation (TAP-IVS) in the KZN populations. This kind of mutation endows a high level of glyphosate resistance, which explains why these populations survived even the 4× dose. These findings confirmed the first cases of glyphosate-resistant *A. hybridus* and established the mechanism of resistance as target site mutations in the *EPSPS* gene reported in the RSA. These findings will serve as a base for other herbicide resistance cases and the development of initiatives to control and minimize the spread of this weed in the RSA.

KEYWORDS
Amaranthus hybridus L., *EPSPS* gene, glyphosate resistance, triple mutation

1 | INTRODUCTION

Amaranthus genera belong to the Amaranthaceae family originating from South America (Janovská et al., 2012). These genera contain about 70 species of which most are cultivated and used as leafy vegetables (Ebert et al., 2011). Species such as *Amaranthus palmeri* S. Watson (Palmer amaranth), *Amaranthus hybridus* L. (smooth pigweed), *Amaranthus deflexus* L. (Argentina amaranth), *Amaranthus viridis*

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L. (slender amaranth), *Amaranthus retroflexus* L. (redroot pigweed) and *Amaranthus spinosus* L. (spiny amaranth) are a threat to crops. These species are very competitive, which could be attributed to their C_4 photosynthetic mechanism (Aguyoh & Masiunas, 2003), vigorous growth, prolific seed production and discontinuous seed germination (Aguyoh & Masiunas, 2003; Netto et al., 2016). Among these species, *A. hybridus* is one of the most aggressive weeds in the world and can reduce crop yields by up to 90% (Costea et al., 2004). Due to the competitive nature of weeds against cultivated crops for growth resources, farmers have adopted the use of chemicals as a control measure (Craine & Dybzinski, 2013; Pieterse, 2010). However, herbicide resistance evolution has become a major concern in crop fields due to the selection pressure exerted by farmers through chemical control (Perotti et al., 2019). Since glyphosate's introduction, this herbicide has been widely used due to the introduction and use of glyphosate-resistant (GR) crops. Glyphosate, a non-selective, broad-spectrum and systemic herbicide has been extensively used on GR crops and under reduced tillage systems. The high popularity of glyphosate and its recurring use in GR crops has resulted in the evolution of GR weeds (Heap, 2023; Powles, 2008).

There are two herbicide resistance mechanisms by which weeds develop resistance to herbicides: target-site resistance (TSR) and non-target-site resistance (NTSR) (Dekker & Duke, 1995). TSR occurs when the enzyme targeted by the herbicide acquires a mutation rendering it to be functional but less sensitive to the herbicide (Hanson et al., 2013; Yu et al., 2015), or overexpression of the target gene through gene amplification (Gaines et al., 2010; Heap, 2023; Reinhardt et al., 2022; Sammons & Gaines, 2014). On the other hand, NTSR is linked with morphological and physiological mechanisms that result in less herbicide reaching the intended site of action (SOA). It includes reduced absorption and translocation (Fernandez-Moreno et al., 2017), or increased vacuolar sequestration (Ge et al., 2012) as well as enhanced metabolism (Rojano-Delgado et al., 2012).

Glyphosate acts on enolpyruvyl-5-shikimate-3-phosphate-synthase (EPSPS), an enzyme, that is, found in the shikimic acid (SA) pathway of plants and microorganisms (McCue & Conn, 1990). The mechanism of action of glyphosate is to inhibit the EPSPS enzyme in plant chloroplasts, resulting in the accumulation of shikimate acid, the natural substrate of EPSPS. Downstream products of the SA pathway include the three aromatic amino acids phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Typ) (Chen et al., 2015; Ge et al., 2012). Biosynthetic inhibition of these amino acids results in cell death (Koger et al., 2005). The most common form of TSR to glyphosate involves mutation or amplification of the *EPSPS* gene (Adhikary & Pratt, 2015; Gaines et al., 2010; Nandula et al., 2014; Perotti et al., 2019; Yu et al., 2015).

Mutation at position 106 of the nucleotide in the conserved domain of the *EPSPS* gene has been widely reported in different weed species. This mutation can include a change from proline to other amino acids such as serine (Pro106Ser) in *Eluesine indica* (Ng et al., 2004; Sammons & Gaines, 2014), alanine (Pro106Ala) in *Lolium multiflorum* (Jasieniuk et al., 2008) and threonine (Pro106Thr) in *Lolium rigidum* (Bostamam et al., 2012). However, single point mutations at position 106 endow low levels of glyphosate resistance (Ng et al., 2004). Double mutation has also been reported in a GR *E. indica* population that had a threonine (Thr) to isoleucine (Ile) at position 102 (Thr102Ile) together with Pro106Ser mutation. This kind of gene mutation is also known as TIPS double mutation, endowing high levels of glyphosate resistance (Yu et al., 2015). In 2019, the first report of a triple mutation in the *EPSPS* gene conferring resistance to glyphosate was published (García et al., 2019). The authors observed amino acid substitution in the conserved domain of the *EPSPS* gene involving Thr102Ile, Ala103Val and Pro106Ser (TAP-IVS mutation) in two *A. hybridus* populations from Argentina. Perotti et al. (2019) contended that this kind of mutation, also known as TAP-IVS mutation, endows high-level glyphosate resistance and weeds with this kind of mutation can tolerate repeated applications of glyphosate. Since then, this kind of mutation in *A. hybridus* has been widely reported. García et al. (2020) reported another *A. hybridus* population from Argentina, and the most recent *A. hybridus* population and other unresolved *Amaranthus* spp. were reported by Sulzbach et al. (2024) in Brazil.

A. hybridus has not only been confirmed to be GR. This species has been reported to have evolved resistance to herbicides with different modes of action (MOA). In a soyabean [*Glycine max* (L.) Merr.] crop in Argentina, an *A. hybridus* population initially found to be GR in 2013 was later reported to have evolved resistance to imazethapyr (García et al., 2019). In 2018, multiple resistance to 2,4-D, dicamba and glyphosate was reported in an *A. hybridus* population in Argentina (Dellaferrera et al., 2018). To date, *A. hybridus* has become an extremely troublesome weed for summer crops globally because it has evolved herbicide resistance to different MOAs such as the EPSPS, acetolactate synthase (ALS), photosystem II (PSII), protoporphyrinogen oxidase (PPO) and synthetic auxins (Busi et al., 2018; Larran et al., 2018).

Weed species from *Amaranthus* genera have not been of great concern in the Republic of South Africa (RSA) until a non-native *A. palmeri* population was detected in the 2017–2018 growing season in Douglas district of the Northern Cape Province. This species was growing in association with crops such as alfalfa (*Medicago sativa* L.), cotton (*Gossypium herbaceum* L) and maize (*Zea mays* L.). It was also growing with another weed from the *Amaranthus* genera, *A. hybridus L.* ssp. *hybridus* var. *hybridus* (Cape pigweed) (Sukhorukov et al., 2020). The *A. palmeri* population from Douglas gained attention because it was difficult to control with glyphosate in Roundup Ready® (RR®) cotton fields (Sukhorukov et al., 2020). Reinhardt et al. (2022) later demonstrated that this *A. palmeri* population was resistant to glyphosate because of elevated *EPSPS* gene copy number. In a study by Gaines et al. (2012), the frequency of hybridization between *A. palmeri* (pollen source) and *A. hybridus* was calculated to be 0.01% when they coexist in the field. The findings of Gaines et al. (2012) probably explain why there were no reported cases of apparent glyphosate resistance in *A. hybridus* populations occurring together with *A. palmeri* at the Douglas location in the RSA. Nevertheless, *A. hybridus* populations from KZN Province in the RSA have caught the attention of farmers since glyphosate is progressively becoming less effective in the control of the species. This research is aimed at confirming glyphosate

resistance for the *A. hybridus* populations from field-collected seeds, and the heritability of resistance into the next generation for the KZN populations, as well as performing molecular analysis to determine the mechanism of resistance.

2 | MATERIALS AND METHODS

2.1 | Plant material sources

A. hybridus seeds were collected from two different farms in the KZN Province of the RSA in 2021. These farms are located in Bergville (28°43′23″ S 29°20′37″ E) and Winterton (28°49′42″ S 29°32′5″ E) districts and are within a 20 km radius. At the Bergville location, the farmer was rotating RR® maize and soyabeans, and fully relied on the use of glyphosate every season. *A. hybridus* population from Bergville was classified as highly resistant to glyphosate based on the anecdotal farmer's report because all plants were surviving field doses. In contrast, the Winterton farmer was rotating herbicides with different MOA, and based on the field report, there were intermittent cases of glyphosate resistance. *A. hybridus* seeds were collected from 50 randomly selected matured plants that survived glyphosate treatment on both farms. For a glyphosate-susceptible population, 50 plants were randomly collected from a farm in Hendrina [Mpumalanga Province (26°09′20″ S 29°44′02″ E)], located about 500 km away from the populations in KZN Province. Seeds collected from the plants were separately bulked and stored at 4°C for later use.

2.2 | Plant growth under glasshouse conditions

The study was carried out under glasshouse conditions at the Experimental Farm of the University of Pretoria (25°44′59″ S 28°15′19″ E). Seeds were planted on 50 × 30 cm seedling trays filled with potting mix consisting of pure sand [source: Silica Quartz (Pty) Ltd] and coconut peat [source: Pelemix (Pty) Ltd] in a 6:1 sand: coconut peat ratio (w/w). Seedling trays were maintained under glasshouse conditions, with an average day/night temperature of 25°C and a photoperiod of 16/8 h day and night. Seeds were watered three times a week with tap water, and after emergence, seedlings were fertilized with Hygroponic NPK 1.6:1:5 fertilizer [source: Hygrotech, RSA] at 1 g fertilizer to 1 L water two times a week. As recommended by Martínez-Núñez et al. (2019), plants were transplanted at the 4-leaf stage into 12.5 cm diameter plastic pots with a 6:1 ratio (w/w) sand: coconut peat mixture. Two plants were transplanted in each pot and were kept under glasshouse conditions. Pots were watered four times a week and fertilizer was applied as mentioned above. It is difficult to categorize *Amaranthus* populations at the species level through morphological characteristics alone. This is due to limited differences in plant morphology among these closely related species, and the presence of considerable inter-population variability within species (Costea & DeMason, 2001). Therefore, before spraying, molecular characterization of the internal transcribed spacer (ITS1 and ITS2) region was done to confirm the identity of *Amaranthus* species following the procedure explained in Section 2.4.

2.3 | Evaluation of glyphosate resistance in *A. hybridus*

Glyphosate (Roundup® PowerMax) from Bayer (Pty) Ltd, RSA was applied when weeds were at the 6-leaf development stage, BBCH Code-12, 33 days post-seeding. The design of the experiment was a completely randomized factorial. The first factor was population with three levels (Bergville, Winterton and Hendrina); the second factor was glyphosate dose with five levels (0, 0.5×, 1×, 2× and 4×) where × was equal to the recommended dose of 1080 g ae ha^{-1}. These doses, respectively, correspond to 0, 540, 1080, 2160 and 4320 g ae ha^{-1}. Each herbicide dose was replicated five times (with two plants per pot representing an experimental unit). Herbicide was applied using an Oxford Small-Plot Precision Sprayer calibrated to deliver a spray volume of 300 L ha^{-1} at 180 kPa through a TeeJet E80015 EVS nozzle. Roundup® PowerMax solution was prepared with 1% ammonium sulphate [source: ATP AMSUL-50, Villa Crop Protection, RSA]. Spraying was carried out in an enclosed spraying room where pots were placed in a 1 × 1 m quadrant. The enclosed environment was used to prevent contamination of glyphosate to non-target plants.

Glyphosate efficacy was assessed 16 days after treatment (DAT) based on leaf chlorosis, necrosis, meristem turgidity and complete plant death on a scale of 0%–100%, with zero indicating no herbicide injury and 100% indicating complete plant death (necrotic). At 35 DAT, plant survival was assessed based on visual appearance and meristematic turgidity, which are indicators of the plant's potential to recover, grow and produce seeds. Survived and dead plants were clipped at soil level and oven-dried at 80°C for 48 h to calculate the percentage biomass (biomass of treated plants expressed as a percentage of untreated control biomass). To confirm the heritability of any glyphosate resistance, F1 seed lines were created from the Bergville and Winterton field populations and also screened with glyphosate. To do this, a further 50 plants from the Bergville and Winterton populations were grown and treated with glyphosate at 1080 g ae ha^{-1}. Surviving plants from both the Bergville and Winterton populations were grown in different glasshouses to avoid cross-pollination between the two populations. For the Bergville and Winterton populations, 50 and 26 plants survived field doses of glyphosate treatment, respectively. The seeds collected from these surviving plants represented the F1 generation seed lines, hereafter referred to as F1 Bergville and F1 Winterton. These F1 seed lines were then screened for glyphosate resistance by following the same procedure explained on the field populations. The glyphosate susceptible population collected from Hendrina was also screened again for glyphosate sensitivity along with these F1 seed lines, for comparison of resistance. The first and second screening on the Hendrina field population are hereafter named Hendrina-A and Hendrina-B, respectively.

2.4 | Molecular characterization of ITS region and partial *EPSPS* gene sequence

Leaf tissues from each surviving plant and controls were collected, frozen in liquid nitrogen and ground into a fine powder using a sterile mortar and pestle for the DNA extraction process. The Quick DNA™ Plant/Seed Miniprep Kit (Zymo Research, Inqaba, RSA) was used to extract genomic DNA. To confirm the identity of *A. hybridus*, the ITS region was amplified and sequenced for each population. Primers used for the ITS region included ITS-F (5′-TCCTCCGCTTATTGATATGC-3′) and ITS-R (5′-GGAAGTAAAGTCGTAACAAGG-3′). Partial sequencing of the *EPSPS* gene was also done to identify possible target site mutations. Primers EPSPS-F (5′-ATGTTGGACGCTCTCAGAACTCTTGGT-3′) and EPSPS-R (5′-TGAATTTCCTCCAGCAACGGCAA-3′) from Gaines et al. (2012) were used. Each PCR reaction (*EPSPS* gene and ITS region) contained 12.5 μL DreamTag Master Mix (Thermo Fisher, RSA), 1 μL of each primer for both forward and reverse, 9.5 μL dH_2O and 1 μL DNA sample (from extraction) to a total volume of 25 μL. Thermoprofile conditions consisted of initial denaturing at 95°C for 5 min; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 52 and 59°C for 30 s, for the ITS and *EPSPS* gene, respectively. Extension was done at 72°C for 2 min, and final extension at 72°C for 10 min. The specificity of the PCR products was verified in 1% agarose gel electrophoresis and subsequently purified by ethanol precipitation.

For Sanger sequencing, each reaction contained about 40–200 ng purified PCR product, 0.5 μL BigDye (Promega, RSA), sequencing buffer (5×) and dH_2O to a total volume of 10 μL. The same PCR primers were used for sequencing and samples were sequenced in both directions. A general sequencing protocol was used with primer specific annealing temperatures. Purified reactions were submitted to the ACGT Sequencing Facility at the University of Pretoria, Hatfield Campus, RSA. Sequence analyses were carried out using CLC Genomic Workbench 23.0.3 (CLC Bio, a QIAGEN company, Aarhus, Denmark). Generated EPSPS and ITS consensuses were subjected to BLASTn in the National Centre of Biotechnology Information (NCBI) database for closely related species. Multiple sequence alignments were carried out with MAFFT version 7 (Katoh & Standley, 2013), and phylogenetic analysis adopted the maximum likelihood statistical model using MEGAX software (Kumar et al., 2018).

2.5 | Statistical analysis

For Bergville (highly resistant population) and Hendrina (highly susceptible population), percentage visual control (PVC) and percentage biomass were regressed over glyphosate doses using a four-parameter log-logistic non-linear regression model (Equation 1) in R (version 4.3.0.) using the 'drc' package described by Ritz and Streibig (2005) to generate dose–response curves. The dose–response curves were used to determine the amount of herbicide dose required to cause injury or biomass reduction of 50% (GR_{50}). Resistance ratio (R/S), also called resistance factor (RF), was calculated to determine the relative level of glyphosate resistance between the Bergville and Hendrina populations. The data on PVC, survival percentage and percentage biomass were analysed using the analysis of variance (ANOVA) with glyphosate doses and populations as fixed effects in R. Significant treatment means were separated using the Fisher's protected Least Significant Difference (LSD) test at $p \leq 0.05$.

$$Y = c + \frac{d - c}{1 + \exp(b(\log(x) - \log(e)))} \quad (1)$$

y = above-ground dry weight expressed as a percentage of the mean untreated control, c = lower limit, d = upper limit, e = herbicide dose required to reduce growth by 50% (GR_{50}), b = slope of the curve around GR_{50} and x = the herbicide dose.

$$\text{Resistance level}(R/S) = \frac{GR_{50}\text{ of population}}{GR_{50}\text{ of the average susceptible population (Hendrina)}} \quad (2)$$

3 | RESULTS

3.1 | Confirmation of *A. hybridus* identity

The phylogenetic tree for the ITS sequences was constructed to assess their phylogenetic relatedness with reference species (Figure 1). Populations from Bergville and Winterton formed a distinct cluster (KwaZulu Natal Cluster) associated with references *A. hybridus* ([KY968931], [KY968853], [MT811921]), *A. hypochodriacus* (KU310615) and *A. cruentus* (KY968887) with 70 bootstrap supports. The second cluster consisted of the Hendrina population and reference *A. hybridus* (MT811924) with 73% bootstrap support. The KZN populations clustered with *A. hybridus* species complex, as demonstrated by the phylogenetic tree (Figure 1). *Amaranthus palmeri* and *A. spinosus* formed distinct clusters from the KZN and Hendrina populations and were supported by 99% bootstrap.

3.2 | Dose–response assay

Based on the three glyphosate efficacy parameters evaluated; PVC, survival percentage and percentage biomass, populations had differential responses to glyphosate doses. This experiment confirmed high glyphosate resistance in the Bergville population and high glyphosate susceptibility in the Hendrina population. In contrast, there was variation in the response of the Winterton population to different doses. Due to the significant variation (highly resistant and highly susceptible plants within a population), a dose–response curve could not be plotted for this population. Phytotoxic effects were observed on leaves of

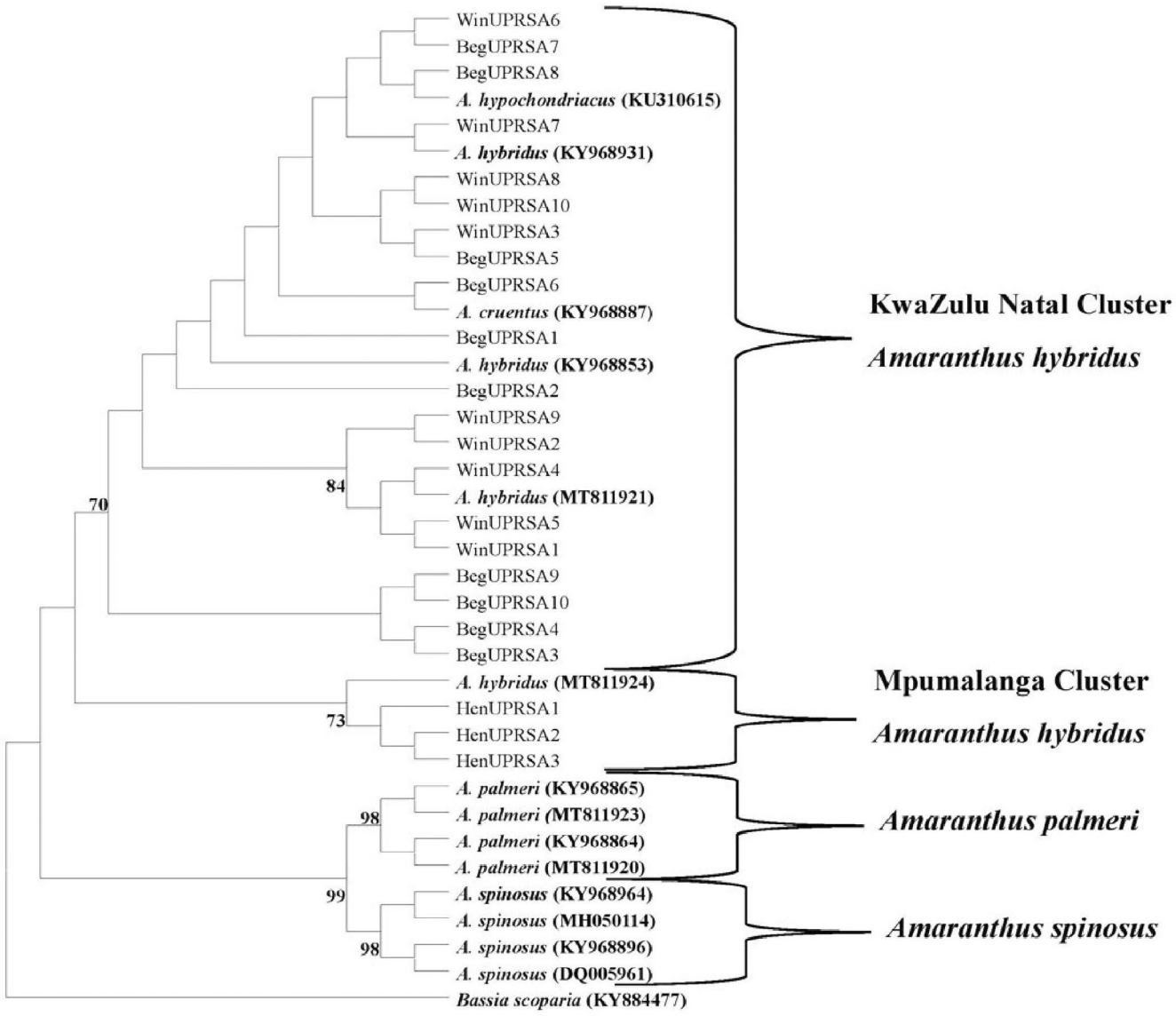

FIGURE 1 The phylogenetic tree constructed by ITS region sequences. Molecular analysis for Bergville (Beg), Winterton (Win) and Hendrina (Hen) was done at the University of Pretoria (UP) in RSA. The bootstrap values (1000 replicates) are indicated at each node of the tree which shows the percentage of trees in which the associated taxa clustered together. All bootstrap values less than 70 were excluded (since they are not significant). *Bassia scoparia* (KY884477) was used as an outgroup.

all populations, with young developing leaves becoming chlorotic. For susceptible plants, there was wilting in the first 6 days followed by chlorosis and necrosis by 28 DAT. For resistant plants, mild chlorotic leaves turned green, and plants continued to grow until seeding (Figure 2).

Log-logistic non-linear regression model predicted that the dose that will cause 50% herbicide injury for Hendrina-A was 305.30 g ae ha^{-1} compared to Bergville (38 964 g ae ha^{-1}). The Bergville population showed an RF of 127.6 folds compared to the susceptible population. A similar trend was observed when the F1 generation was compared to Hendrina-B. The GR_{50} for herbicide injury was 90.48 g ae ha^{-1} for Hendrina-B compared to 33 451 g ae ha^{-1} for F1 Bergville (Table 1 and Figure 3A). The F1 generation of Bergville showed an RF of 367.7 compared to Hendrina-B. For percentage biomass, the GR_{50} for Hendrina-A was 582.61 g ae ha^{-1} compared to Bergville, with 39 317 g ae ha^{-1}. The RF for Bergville was 67.48 fold compared to Hendrina-A. For F1 Bergville and Hendrina-B, the GR_{50} of percentage biomass was 10 831 and 418.47 g ae ha^{-1}, respectively, with an RF of 25.88 fold (Table 2 and Figure 3B).

Factorial ANOVA for PVC between Bergville, F1 Bergville, Hendrina-A and Hendrina-B demonstrated that glyphosate doses, populations and their interactions were highly significant (p-value <0.001). For the Bergville population, only the highest glyphosate dose (4320 g ae ha^{-1}) elicited significant glyphosate damage (Figure 4A), with a 6% (±4.59) increase in visual symptoms (PVC) on

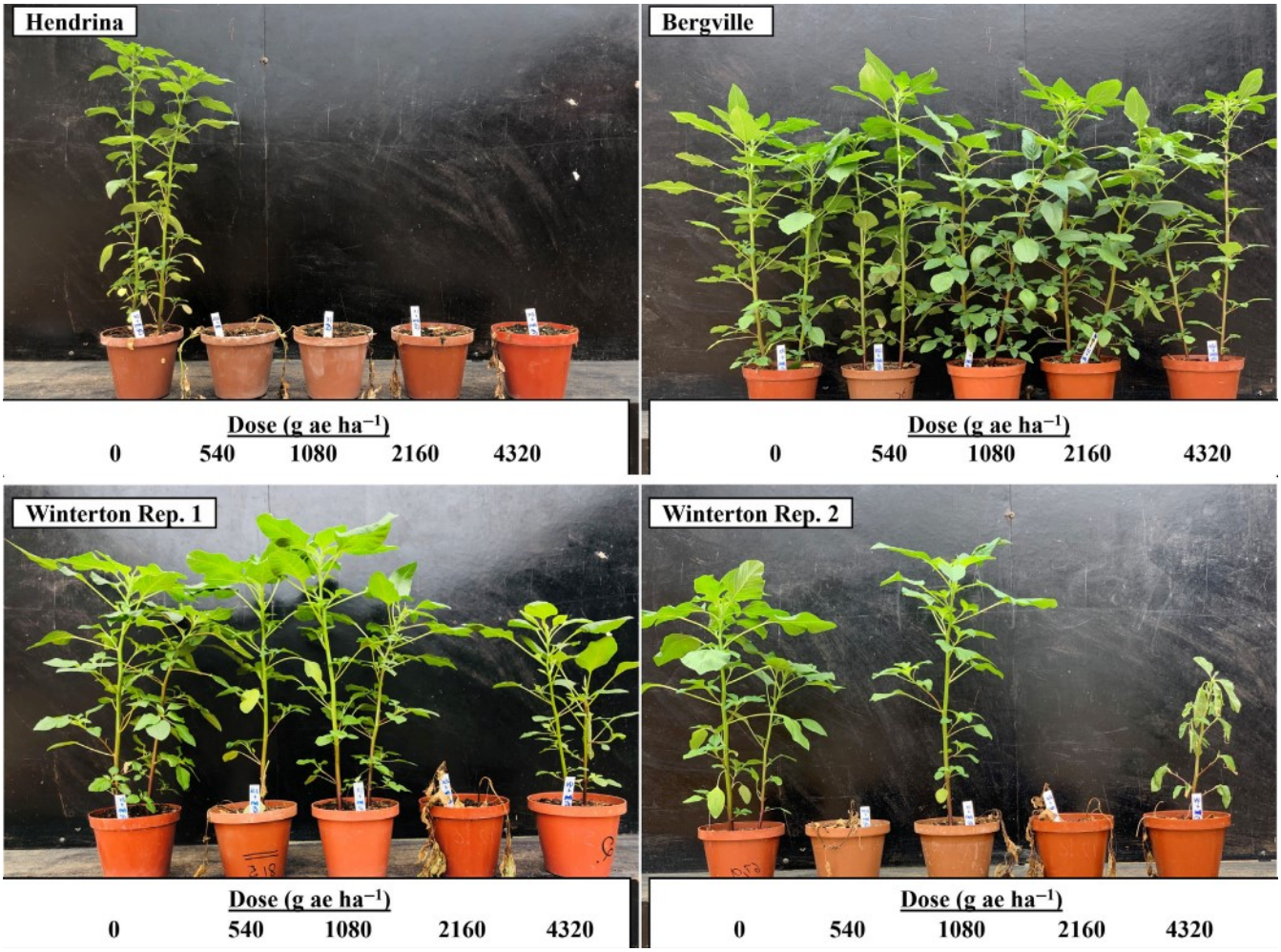

FIGURE 2 Response of three *Amaranthus hybridus* populations to different doses of glyphosate at 21 DAT. The Winterton population shows a variation in glyphosate response (Rep. 1 and 2 are replicates).

TABLE 1 Estimates calculated from a four-parameter log-logistic non-linear regression model of glyphosate dose–response resulting in 50% injury of highly resistant and highly susceptible *A. hybridus* populations.

Population	*d* ± SE	*b* ± SE	GR_{50}[a] (g ae ha^{-1}) ± SE	R/S ratio
Hendrina	101.25 ± 2.35	−1.75 ± 0.44	305.30 ± 42.15	—
Bergville	11.36 ± 72.05	−0.49 ± 0.96	38 964 ± 739 370	127.6
Hendrina	103.43 ± 5.55	−0.92 ± 0.60	90.48 ± 81.51	—
F1 Bergville	58.16 ± 763.40	−1.07 ± 1.27	33 451 ± 511 490	369.7

Note: Estimates represent GR_{50} calculated from PVC expressed as percentage of untreated control ± standard error in g ae ha^{-1}.
[a]GR_{50}—glyphosate dose resulting in 50% injury and resistance factor at GR_{50} value, respectively.

the F1 generation compared with untreated controls. The Hendrina population was significantly damaged by all glyphosate doses compared to untreated controls and the Bergville population. For the recommended dose, PVC was 91 (±9.66), and 93% (±9.19) for Hendrina-A and Hendrina-B, respectively, while Bergville and F1 Bergville had a PVC of 1.5% (±2.42) each. The percentage biomass of the Hendrina population was significantly reduced compared with the Bergville population. The lowest percentage biomass was 2.32% from Hendrina-B at 4320 g ae ha^{-1} compared with 85.1% (±15.7) from Bergville. At the recommended dose, the percentage biomass of Hendrina-A (20.85% ± 15.14) and -B (16.60% ± 7.46) was significantly reduced (Figure 4B).

For survival percentage, the number of surviving plants per pot (replicated five times) was determined. For the Hendrina population, no plants survived herbicide treatment, even at 540 g ae ha^{-1} (0.5×). For the highly resistant population, 100% of the plants survived glyphosate doses, even at 4× (4320 g ae ha^{-1}) for both field populations and F1 generation. In contrast, there was great variability in the

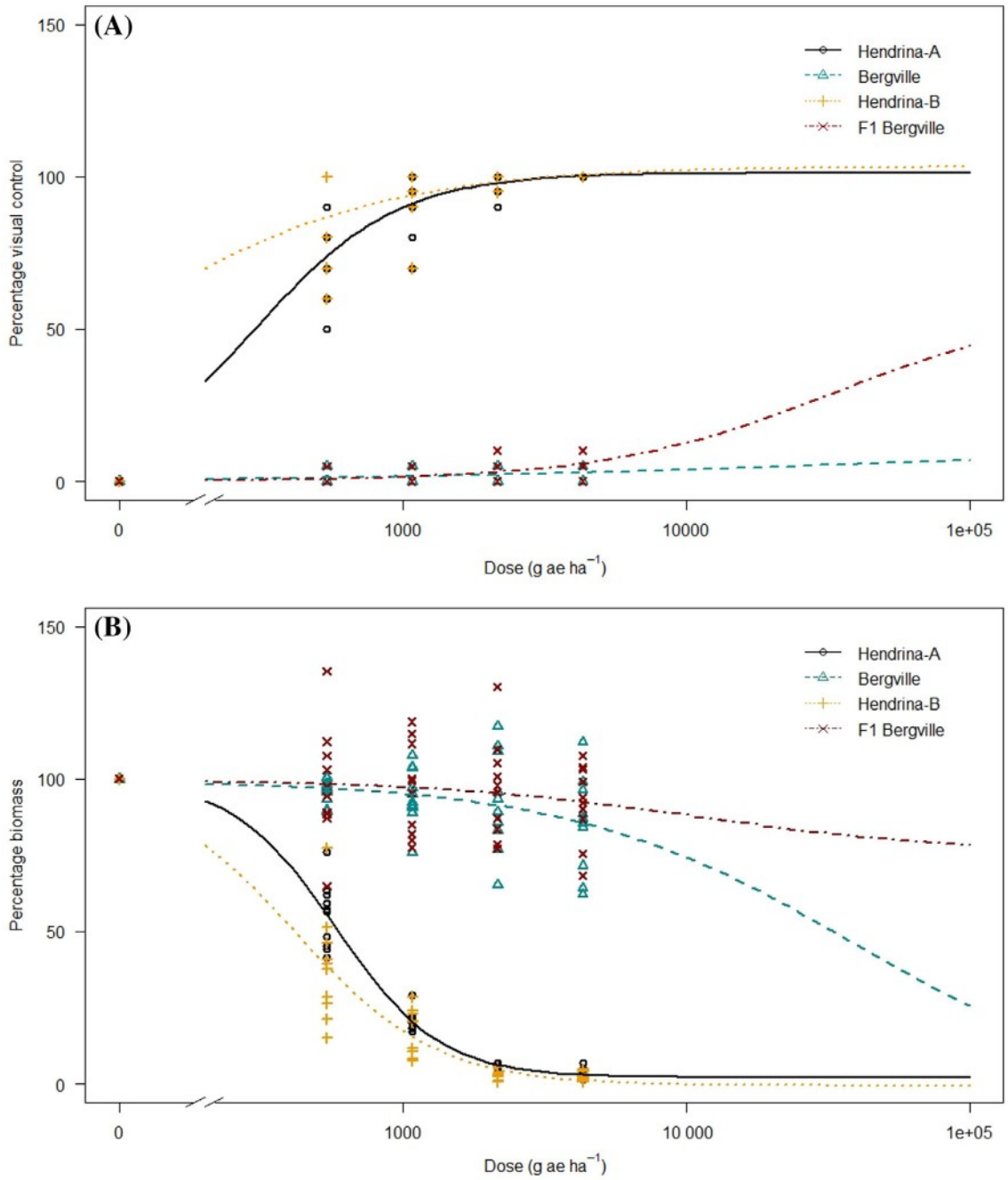

FIGURE 3 Dose–response curve for PVC (A) at 16 DAT and percentage biomass (B) at 35 DAT in glyphosate-resistant field populations and F1 seed lines compared with a susceptible population. Hendrina-A and -B are the same seeds treated with the field populations and F1 generation, respectively.

Population	*d* ± SE	*b* ± SE	GR_{50}[a] (g ae ha^{-1}) ± SE	R/S ratio
Hendrina	99.98 ± 3.22	2.38 ± 0.43	582.61 ± 37.71	_
Bergville	99.47 ± 3.34	0.86 ± 0.63	39 317 ± 235 150	67.48
Hendrina	99.98 ± 3.23	1.75 ± 0.49	418.47 ± 45.60	_
F1 Bergville	99.74 ± 3.42	0.93 ± 1.31	10 831 ± 65 738	25.88

Note: Estimates represent GR_{50} calculated from percentage biomass ± standard error in g ae ha^{-1}.
[a]GR_{50}—glyphosate dose resulting in 50% biomass reduction and resistance factor at GR_{50} value, respectively.

TABLE 2 Estimates calculated from a four-parameter log-logistic non-linear regression model of glyphosate dose–response resulting in 50% biomass reduction of highly resistant and highly susceptible *A. hybridus* populations.

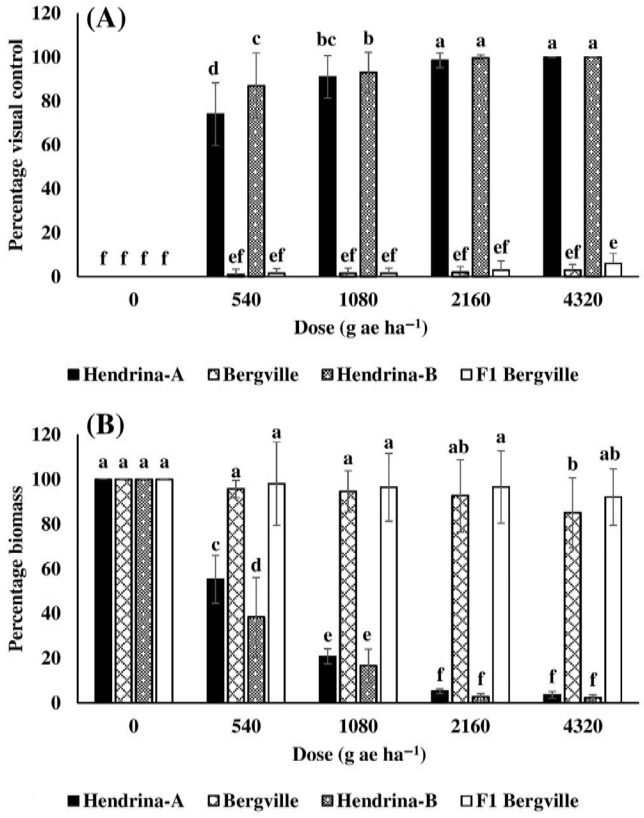

FIGURE 4 Percentage visual control (A) and percentage biomass (B) in *Amaranthus hybridus* populations measured 16 and 35 DAT with glyphosate, respectively. Bars with the same letters are not significantly different and bars with different letters are significantly different between populations and doses at $p < 0.001$ level of significance. Error bars = standard deviation.

Winterton population for both the field populations and the F1 generation. Plants within this population were classified as highly resistant, tolerant or highly susceptible and therefore, the data were not suited for dose–response statistical analysis (Table 3).

3.3 | Target site mutations in *EPSPS* gene fragment

To further confirm the observed resistance to glyphosate and determine the mechanism of resistance, partial sequencing of the *EPSPS* gene was done. A PCR band of 195 bp was successfully amplified. Sanger sequencing of the *EPSPS* gene revealed amino acid substitutions in the Bergville (BegUPRSA1-10) and Winterton (WinUPRSA1-10) populations. The *EPSPS* gene from the KZN populations revealed a triple mutation (Figure 5). Amino acid substitutions were at positions 102 (Thr102Ile), 103 (Ala103Val) and 106 (Pro106Ser) of the *EPSPS* gene. This kind of triple amino acid substitution results in TAP-IVS mutation. All sequences from Bergville and Winterton were 100% similar with previously published *A. hybridus* species with GenBank reference: MG595170. However, sequences from the Hendrina population (HenUPRSA1-5) had no mutation in the *EPSPS* gene (Figure 5) and were 100% similar to reference *A. palmeri* (KC169785).

4 | DISCUSSION

4.1 | Confirmation of *Amaranthus* identity

The genus *Amaranthus* is often difficult to differentiate based on morphology due to a limited distinguishable trait between its species (Costea & DeMason, 2001). *A. hybridus* morphologically falls into two distinct groups (group 1 and 2) and are both geographically widespread throughout America. The *hybridus* group 1 plants are morphologically similar to *A. cruentus*, while *hybridus* group 2 individuals are more similar to *A. hypochondriacus* (Adhikary & Pratt, 2015). To confirm the identity of the collected populations we used molecular confirmation using the ITS marker region. The ITS region is an informative and universally used barcoding system to establish species identity on a molecular level in plants and fungi (Xu et al., 2018). Populations from KZN and Mpumalanga formed clusters associated with reference species *A. hybridus*, *A. hypochodriacus* and *A. cruentus* from the *A. hybridus* species complex (Adhikary & Pratt, 2015; Sauer, 1950). *A. hybridus* species complex is categorized into grain amaranth (*A. cruentus* and *A. hypochondriacus*) and weedy amaranths (*A. hybridus* and *A. quitensis* Kunth) as mentioned by Adhikary and Pratt (2015) and Sauer (1950).

4.2 | Response of *A. hybridus* populations to glyphosate

Our findings on the two KZN *A. hybridus* populations confirm that they are not controlled by the recommended dose ($1\times = 1080$ g ae ha^{-1}) and survived even $4\times$ the recommended dose (4320 g ae ha^{-1}). These findings support those reported by Bagavathiannan and Norsworthy (2016), where *A. palmeri* was highly resistant even to $16\times$ the recommended glyphosate field dose. Variation in glyphosate sensitivity was observed in the Winterton population compared with the Bergville population. In the Bergville population, plants survived all the tested doses, indicating a homogeneous population that has been under high selection pressure for glyphosate resistance. In the Winterton population, the number of plants that survived varied per treatment, but some plants survived all tested doses. These findings (survival approximately >50%) confirm those of Garetson et al. (2019) for *A. palmeri* that at the early stages of glyphosate resistance development, there could be great variation in herbicide response because resistant individuals are present at low frequencies. The mechanism of resistance in the KZN populations was, however, attributed to a triple TAP-IVS mutation in the EPSPS enzyme, although the present study did not investigate the roles of other mechanisms such as NTSR. It is most likely that resistance evolved at some stage and then spread since the two sites are located within a 20 km radius. Differences in the percentage of

Seed source	Location	Dose (g ae ha^{-1}) 0	540	1080	2160	4320	Effect L	D	L * D
Parent	Hendrina	100^{a}	0^{e}	0^{e}	0^{e}	0^{e}	***	***	***
	Bergville	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}			
	Winterton	100^{a}	20de	70^{b}	30^{cd}	30^{cd}			
F1 generation	Bergville	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}			
	Winterton	100^{a}	60^{b}	60^{b}	50^{bc}	50^{bc}			

TABLE 3 Survival percentage (35 DAT) of three field *Amaranthus hybridus* populations and F1 generations of resistant populations exposed to different glyphosate doses.

Note: L = location, D = dose, L * D = interaction between location and dose. Numbers with the same letters are not significantly different and numbers with different letters are significantly different between populations and doses at $p < 0.001$ level of significance.
***$p < 0.001$.

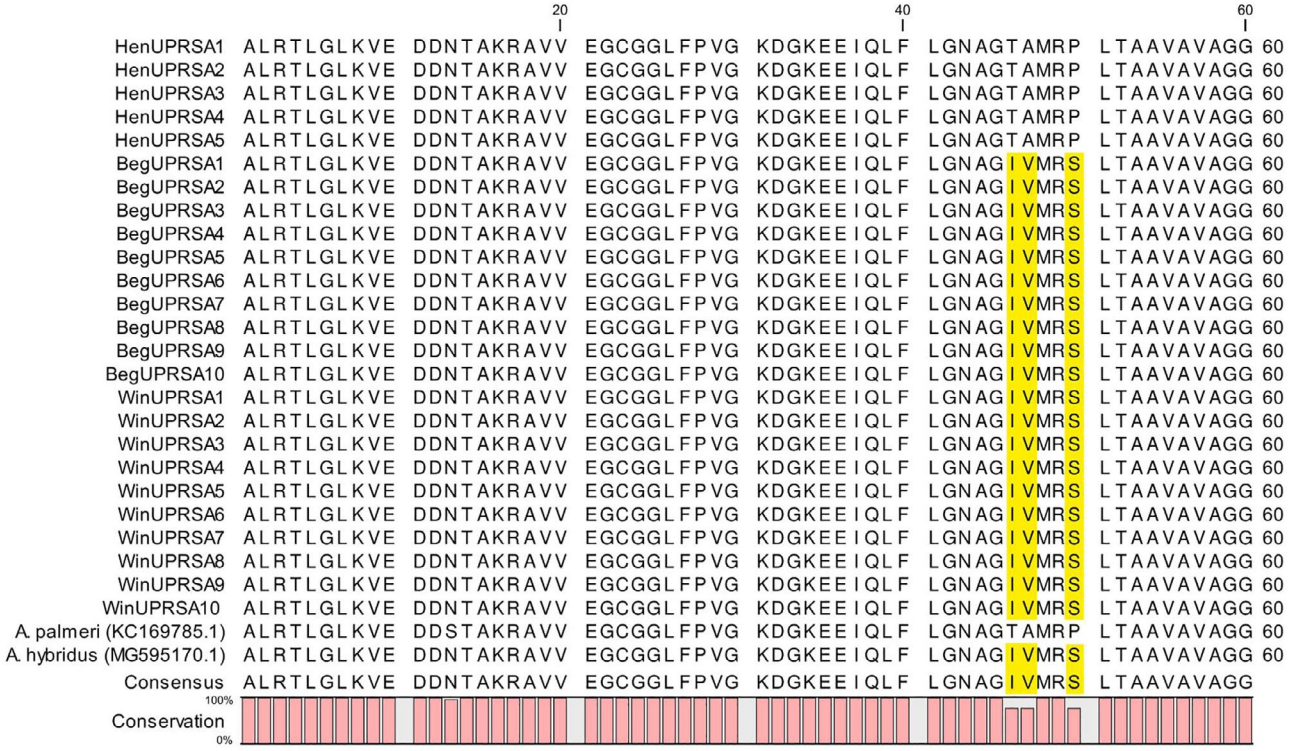

FIGURE 5 Translated protein sequence of the *Amaranthus hybridus EPSPS* gene sequence. Highlighted amino acids show target site mutations in the populations studied.

resistance in the two populations could be attributed to differences in the cropping practices and herbicide-use history. Glyphosate resistance in *A. hybridus* populations presents a serious threat to the agricultural industry of the RSA because these species can facilitate pollination with other *Amaranthus* species (Gaines et al., 2012). The agronomic implication of hybridization is that the *Amaranthus* species will not be manageable with glyphosate due to the transfer of adaptive traits among related species. In the event of continuous use of glyphosate on the Winterton population, inevitably, the whole population will sooner than later be highly resistant to glyphosate. That would be the result of continuous seed production from GR plants and/or hybridization between susceptible and resistant plants of different *Amaranthus* species.

4.3 | Target site mutation in the *EPSPS* gene

Populations from KZN (Bergville and Winterton) had a rare triple mutation (TAP-IVS) that has been reported in *A. hybridus* from Argentina (García et al., 2019, 2020; Perotti et al., 2019) and Brazil (Sulzbach et al., 2024). This kind of mutation confers higher levels of glyphosate resistance (García et al., 2019; Perotti et al., 2019;

Sulzbach et al., 2024), which explains why the plants from Bergville and Winterton survived even 4× (4320 g ae ha^{-1}) the recommended dose. However, alleles that endow high adaptive value in one environmental condition come at a cost to the plant's fitness in another environment. Fitness penalty under herbicide-free environments as a result of mutations conferring glyphosate resistance has been reported by Yanniccari et al. (2016). Fitness penalty associated with TSR has been documented to involve different physiological effects such as delayed flowering, reduced vegetative growth and decreased competitive ability compared with herbicide susceptible population/wild types (Frenkel et al., 2017). Fitness penalty associated with the TAP-IVS mutation has not been studied. However, speculations by other researchers infer that slow evolution of glyphosate resistance may reflect a high fitness penalty associated with mutations (Gaines et al., 2019). Therefore, there is a need for further studies to understand the potential evolutionary dynamics of the triple mutation (Figure 5).

A. hybridus is the first weed species recorded in South Africa (RSA) to have evolved a triple mutation in the EPSPS enzyme. Among South African *Amaranthus* species, *A. palmeri* from the Northern Cape in the RSA was found to have evolved glyphosate resistance due to elevated *EPSPS* gene copy number (Reinhardt et al., 2022). The high level of glyphosate resistance in *A. hybridus* and *A. palmeri* is becoming a big concern for the agricultural sector in the RSA because of the possibility of hybridization between them and other *Amaranthus* species (Gaines et al., 2012), which can result in species with two different modes of glyphosate resistance.

5 | CONCLUSION

This study aimed to confirm glyphosate resistance in *A. hybridus* collected from crop fields in KZN in the RSA. Using both glasshouse screening as well as molecular characterization of the *EPSPS* gene, we confirmed the presence of resistance and determined the mechanism of resistance. This study found the first case of a target site mutation involving a triple TAP-IVS mutation in the EPSPS enzyme in *A. hybridus* populations from the RSA. This mutation results in the inability of glyphosate to bind to the target enzyme and confers a high level of resistance in these populations. Previously, Reinhardt et al. (2022) reported glyphosate resistance in *A. palmeri* that is conferred by *EPSPS* gene amplification. The possibility of NTSR cannot be ruled out in these populations. Therefore, further studies are needed to verify the presence of NTSR mechanisms and the potential that both TSR and NTSR mechanisms could simultaneously contribute to the observed glyphosate resistance in the KZN populations. These cases are of considerable concern, especially with the high adoption rate of conservation agriculture and the over-reliance on chemical weed control in South African farming systems. Integrated weed management (IWM) practices such as the use of herbicides with different MOA, stricter sanitation practices, management of the weed seedbank and cultural practices such as crop rotation, should be adopted to slow down the evolution of GR weeds, as well as to prevent the evolution of weeds with new or stacked mechanisms of herbicide resistance. This study highlights the importance of resistance screening to detect and prevent the spread of resistant populations as early as possible.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available from the corresponding author upon request.

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