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SPECIAL ISSUE

Amaranthus spp.: a genus of troublesome weeds

RESEARCH ARTICLE

PCR-based assay to detect the EPSPS TAP-IVS substitution in *Amaranthus hybridus*

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Abstract: Background: *Amaranthus* spp. are problematic weeds and competitors for nutrients in several crops, especially in soybean and corn. Resistance to glyphosate has been detected in several weed species, and a triple mutation in its EPSPS target gene was detected recently in *Amaranthus hybridus*.

Objective: The aim of this work was to develop a simple polymerase chain reaction (PCR) method to detect the EPSPS triple mutation in *A. hybridus*.

Methods: Two pairs of primers were designed for PCR-based detection of the EPSPS TAP-IVS triple mutation, which confers resistance to glyphosate, in *A. hybridus*.

Results: These sets of allele-specific primers were tested on five *Amaranthus* species and in 65 different field accessions. The PCR reaction using one set of the primers amplifies the wildtype (TAP) allele while the PCR reaction using the other pair of primers amplifies the triple mutation (IVS) allele. The presence of PCR products in both sets of primers identifies the heterozygous resistant individuals, and PCR product amplified only with the triple mutation set of primers identifies the homozygous resistant individuals. A DNA concentration test was performed and the recommend DNA amount to be used is 100 ng.

Conclusions: We developed and tested two sets of primers to detect the EPSPS TAP-IVS triple mutation and the results showed a 100% genotypic to phenotypic association. The triple mutation detection assay is easy to use and can be applied in a molecular laboratory with basic equipments. Early detection of resistance helps to better manage and control its spreading.

Keywords: smooth pigweed; glyphosate; herbicide; resistance; weed control; 5-enolpyruvylshikimate-3-phosphate synthase; Amaranthaceae

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1. Introduction

Weed resistance to the herbicide glyphosate is currently a problem in several crop producing regions worldwide and 53 weed species (26 dicots and 27 monocots) were identified with resistance (Heap, 2021). Glyphosate resistance in plants can be a result of either target-site (TSR) or non-target-site resistance (NTSR), or even the occurrence of both TSR and NTSR mechanisms in the same individual. The NTSR mechanisms reported for glyphosate are reduced uptake, reduced translocation, vacuolar sequestration, rapid necrosis followed by regeneration (the Phoenix phenomenon), and enhanced degradation of aminomethylphosphonic acid (AMPA) and glyoxylate by elevated aldo-keto reductase (AKR) activity (reviewed by Duke, 2019). A combination of both, TSR and NTSR, was observed in several species, for instance, *Amaranthus tuberculatus* and *Lolium rigidum*, in which there was one-codon change in the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene and reduced translocation of glyphosate (Duke, 2011; Bostamam et al., 2012; Nandula et al., 2013).

The TSR mechanisms observed are point mutations in the active site of the EPSPS gene or EPSPS gene duplication (on an extrachromosomal circular DNA (eccDNA) or in a tandem duplication at a single locus or in multiple loci throughout the genome (Gaines et al., 2010; Jugulam et al., 2014; Lorentz et al., 2014; Nandula et al., 2014; Koo et al., 2018; Patterson et al., 2018). The mutations can result either in one-, two- or three-codon changes and were reported in various weed species (Sammons, Gaines, 2014; Yu et al., 2015; Perotti et al., 2019). Species were observed with a single mutation at position 106 leading to substitutions from a proline to serine for *Eleusine indica* (Baerson et al., 2002), a proline to leucine on *Lolium rigidum* (Kaundun et al., 2011), *L. multiflorum* (Gonzalez-Torralva et al., 2012), and *Digitaria insularis* (Carvalho et al., 2012). A complete list of publications with species and their identified mutations in the EPSPS gene is available in weedscience.org (Gaines, Heap, 2021). The *Amaranthus* species with reported resistance to glyphosate are *A. palmeri*, *A. tuberculatus* (syn. *A. rudis*), *A. spinosus*, and *A. hybridus* (syn. *A. quitensis*) (Heap, 2021). In *A. tuberculatus* and *A. palmeri* a single codon change at the protein position 106 was reported and the resistance levels were usually low (≤ 10 fold) (reviewed by Sammons, Gaines, 2014). The double amino acid substitution in the EPSPS gene, known as TIPS, was reported for instance in *E. indica* (Yu et al., 2015) and *Bidens pilosa* (Alcántara-de la Cruz et al., 2016), and the double mutation known as TIPT

was detected in *B. subalternans* (Takano et al. 2020). Thus far, double mutation in the EPSPS gene was not observed in *Amaranthus* spp. The only known triple mutation in the EPSPS gene conferring high resistance levels to glyphosate was reported for *A. hybridus* in Cordoba, Argentina (Perotti et al., 2019; Garcia et al., 2019). The triple amino acid substitution occurs at position 102 (ACA to ATA, Thr to Ile), at position 103 (GCC to GTG, Ala to Val), and at position 106 (CCA to TCA, Pro to Ser). The TAP-IVS triple amino acid substitution (T102I, A130V, and P106S), based on *in silico* conformational studies, is reported to generate an EPSPS enzyme with a functional active site and with increased restriction to glyphosate binding, due to a likely smaller active site (Perotti et al., 2019).

The process to confirm whether a population is resistant or not is composed of bioassays and molecular assays. In the bioassays, seeds from the resistant population are sowed and the herbicide of interest is applied to the seedlings at the recommended stage, and this whole process can take several weeks to a few months to be executed and the results to be available (Burgos, 2015). In the other hand, molecular assays aiming to detect the resistance at the genetic level, which means finding changes in the DNA that are associated with resistance, can be faster performed from several days to a few weeks (Délye et al., 2015). However, molecular assays can be more expensive when costly equipments are required, such as Sanger sequencing platforms. It is very important to highlight the complementarity of bioassay and molecular assay results, and the information each one brings to the understanding of weed resistance.

Thus, this study was performed with the aim of developing a simple molecular method to detect the EPSPS triple mutation in *A. hybridus*. We took advantage of the polymerase chain reaction (PCR) and the PCR amplification of specific alleles (PASA) (also called allele-specific PCR, ASPCR) (Corbett, Tardif, 2006) strategies to design allele-specific primers for the mutations leading to the EPSPS triple amino acid substitution which confers high levels of resistance to glyphosate.

2. Material and Methods

2.1 Sample preparation and DNA extraction

Leaf samples were collected and used for DNA isolation. Samples were either dried leaves collected in the field and brought to the laboratory or fresh leaf tissue from germinated seeds collected in the greenhouse. Genomic DNA was extracted from approximately 100 mg of leaf tissue using the Wizard Genomic DNA Purification Kit (Promega) and following the manufacturer's instructions. DNA quality was verified on agarose gels and quantity was measured using the DeNovix instrument (Wilmington, Delaware). DNA samples were stored at -20°C until further use.

2.2 Primer design and PCR assays for EPSPS triple mutation detection

The EPSPS DNA sequences from *A. hybridus* available in Genbank under accession numbers MG595170.1 (201 bp; resistant) and MG595171.1 (201 bp; sensitive) along with internally available sequenced samples of *A. hybridus*, *A. viridis*, *A. deflexus*, and *A. spinosus*, were used for designing the primers. The sequences were aligned, and a forward primer was designed upstream of the EPSPS triple mutation region corresponding to 102, 103, and 106 amino acid positions. This forward primer is common for wildtype and mutant alleles and was named 'Wildtype Forward' (WT-F; Table 1). Two reverse primers were designed: the 'Wildtype Reverse' (WT-R) primer amplifies the wildtype (TAP) allele, and the 'Triple Mutation Resistant Reverse' (TMR-R) primer amplifies the triple mutation (IVS) allele. Primers designed in this study are listed in Table 1. Primers EPSF1 and EPSR8 are available in the literature (Gaines et al., 2010) and were used to amplify a 195 bp long fragment of EPSPS gene which contains the 102-106 positions, and this amplification was used as a PCR positive control to confirm that the DNA of all samples are of good quality for PCR purposes. All primers were synthesized by and purchased from the company Exxtend (Paulínia, São Paulo).

2.3 EPSPS gene amplification and Sanger sequencing

An EPSPS fragment of 195 bp, which contains the 102-106 positions, was amplified from all samples using primers EPSF1 and EPSR8 (Table 1) as previously described (Gaines et al., 2010). The PCR reaction was performed in a final volume of 25 µL containing 5.0 µL of 5X GoTaq Buffer, 0.5 µL of 10 mM dNTPs (Sinapse), 1.5 µL of 25 mM $MgCl_2$, 0.5 µL of each primer at 10 µM, 0.2 µL of GoTaq G2 Hot Start Polymerase (Promega), and 14.8 µL of ultrapure nuclease-free water (Sigma). The PCR conditions were as follow: 95°C for 3 min, 32 cycles of 95°C for 30 s, X°C (see Table 1 for specific annealing temperatures) for 30 s, 72°C for 1 min, and a final extension step of 72°C for 5 min. PCR amplification was verified on 1% agarose gels and using the 100 bp molecular weight (ladder, Sinapse). PCR products were purified using the ExoSAP-IT™ Express reagent

Table 1 - List of primers used in this study

Primer ID	Primer Sequence 5' → 3'	Fragment	Tm
WT-F	GGACGCTCTCAGAACTCTTGG	152 bp	50°C[1]
WT-R	GGCGCATYGCTGTTC		
WT-F	GGACGCTCTCAGAACTCTTGG	152 bp	50°C[1]
TMR-R	ATCGCATGACTATTC		
EPSF1	ATGTTGGACGCTCTCAGAACTCTTGGT	195 bp	57°C[2]
EPSR8	TGAATTTCCTCCAGCAACGGCAA		

[1] This study; [2] Gaines et al., 2010.

(Thermo Fisher) following manufacturer instructions. Purified PCR products were 10X diluted and 1 µL was used in the sequencing reaction. Sequencing reactions were performed with the BigDye cycle sequencing terminator kit (Thermo Fisher) and sequencing was performed in an Applied Biosystems 3500 Genetic Analyzer instrument (Thermo Fisher). All sequence electropherograms were visually checked for quality and consistency before sequences were assembled and aligned using UGENE (Unipro, 2012; Okonechnikov et al., 2012).

2.4 Testing the EPSPS triple mutation detection assay

The primer pairs WT-F + WT-R and WT-F + TMR-R were tested in *Amaranthus* samples from the following five species: *A. palmeri*, *A. hybridus*, *A. viridis*, *A. deflexus*, and *A. spinosus*. The EPSF1 and EPSR8 primers (Gaines et al., 2010) were used to amplify a 195 bp long fragment of EPSPS gene, which contains the 102-106 positions, and the fragment was sequenced for all the samples tested for the five species listed above. Another test was performed with samples from *A. hybridus* known to be glyphosate resistant. All the tests were performed three times in three different PCR reactions and runs. The *Amaranthus* species determination was performed using the PCR assay with intron 1 sequence from the EPSPS gene as previously published (Wright et al., 2016; data not shown).

2.5 DNA concentration and sensitivity of EPSPS triple mutation detection assay

This sensitivity assay was performed to test three amounts of DNA to detect the EPSPS triple mutation. The DNA samples of a wildtype TAP-allele (sensitive) and two resistant (a heterozygous TAP/IVS and a homozygous IVS) *A. hybridus* samples were diluted to three concentrations: 100, 10, and 1.0 ng μL^{-1}, and PCR reactions were performed as described above. PCR amplification was verified on 1% agarose gels and used the 100 bp molecular weight (ladder, Sinapse).

2.6 Testing the EPSPS triple mutation detection assay in multiple field accessions

This EPSPS triple mutation detection assay was used in our large-scale resistance monitoring program for *Amaranthus* spp. with samples collected in 2020. For this study, *Amaranthus* spp. seeds were collected from soybean growing regions throughout Brazil. These seeds were sown in plastic containers containing substrate and plants were kept in a greenhouse under 32°C/25°C (day/night) in a 16 h photoperiod. The samples were collected from Rio Grande do Sul (RS, 33 samples), Paraná (PR, 15 samples), Minas Gerais (MG, 1 samples), and Mato Grosso (MT, 16 samples) states in Brazil. Leaf samples were harvested from individual plants and were used for DNA extraction as described above. A total of 65 samples were analyzed using this method and sequenced using the Sanger Sequencing method (described above) for confirmation purpose.

3. Results and Discussion

Two sets of allele-specific primers were developed for detection of the triple mutation in the EPSPS gene, which confers resistance to glyphosate and was first identified in *A. hybridus*. The primer pair WT-F + WT-R was designed to amplify the wildtype (TAP) allele and the primer pair WT-F + TMR-R was designed to amplify the triple mutation (IVS) allele. For a better understanding and helping the interpretation of results, a schematic diagram is presented in Figure 1. Both primer pairs were tested and amplified one band only (Figure 2). All the results were confirmed through Sanger sequencing the amplified fragments (Figure 3). Using this detection assay, two PCR reactions are performed to determine whether the samples are wildtype or are carrying the triple mutation, as shown in Figure 1. Thus, the presence of a PCR band when using the WT-F + WT-R primers, and absence of PCR band amplified with the WT-F + TMR-R primers, showed that the sample has the wildtype (sensitive) allele. When there was a PCR band amplified with the WT-F + WT-R primers and a PCR band amplified with the WT-F + TMR-R primers, for the same sample, it showed that the sample has the TAP-IVS (resistant) allele in a heterozygous state. When there was no PCR band amplified with the WT-F + WT-R primers, and only a PCR band amplified with the WT-F + TMR-R primers, it showed that the sample has the homozygous IVS (resistant) allele.

These sets of allele-specific primers were tested on five *Amaranthus* species and they were able to amplify the EPSPS fragments in all the tested species (Figure 2). There is a high conservation in the EPSPS gene region corresponding to the active target site (Padgette et al., 1991; Sammons, Gaines, 2014), where the triple mutation is located. The

Primer Pair	Sample 1	Sample 2	Sample 3	
EPSF1 + EPSR8 (EPSPS Fragment)	+	+	+	Presence = good quality DNA
WT-F + WT-R (TAP-Allele)	+	+	-	
WT-F + TMR-R (IVS-Allele)	-	+	+	
The assay result is:	Wildtype sensitive sample	Heterozygous resistant sample	Homozygous resistant sample	

Figure 1 - Schematic diagram of expected results using the PCR-based detection of EPSPS triple mutation assay. The plus sign (+) denotes presence of PCR band and the minus sign (-) denotes absence of PCR band in the agarose gel. Presence of PCR band when using EPSF1and EPSR8 primer pair is required to verify the quality of the DNA and as a PCR positive control

195 bp EPSPS gene fragment amplified (as described in the methods) was sequenced for all five species and confirmed the results obtained with the allele-specific primers. Due to this high EPSPS sequence conservation the allele-specific primers can be used to genotype other *Amaranthus* species, although the EPSPS triple mutation was found only in *A. hybridus* until now (Garcia et al., 2019; Perotti et al., 2019). Another important information is that double mutations as the TI-PS and TI-PT which are reported in *E. indica* (Yu et al., 2015) and *B. subalternans* (Takano et al., 2020), respectively, were not observed in any of the *Amaranthus* species studied so far. This information about the double mutation is important due to the fact that the reverse primer (TMR-R) might be able to amplify the double mutant allele depending on its sequence if it happens to arise in nature.

The sensitivity of the PCR for EPSPS triple mutation detection assay was tested using three DNA concentrations in the PCR, 100, 10 and 1 ng, and the assay showed consistent results for the three amounts (Figure 4). However, the 1 ng concentration resulted in a faint band and could lead to dubious interpretation of the results. Thus, the recommended DNA amount to be used in this triple mutation detection assay is 100 ng in a 25 µL reaction. PCR reactions with DNA amounts below 1 ng were not tested and thus, are not recommended for use.

A set of 65 field accessions of *Amaranthus* spp., collected from various agricultural regions in Brazil in a resistance monitoring program, was genotyped using the

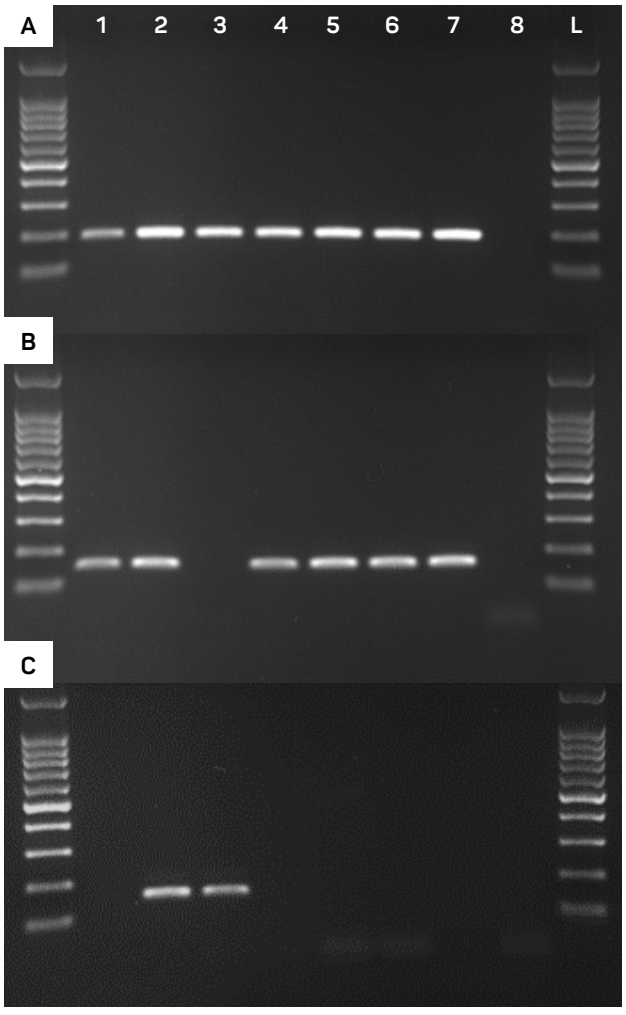

Figure 2 - Electrophoresis of PCR products amplified with primers for detection of EPSPS triple mutation in five *Amaranthus* spp.: 1) *A. hybridus*, 2) *A. hybridus* heterozygous with triple mutation, 3) *A. hybridus* homozygous with triple mutation, 4) *A. viridis*, 5) *A. spinosus*, 6) *A. deflexus*, 7) *A. palmeri*, 8) PCR negative control. Primers for amplifying an EPSPS gene fragment and confirm DNA quality (EPSF1 and EPSR8; Gaines et al., 2010) (A); primers (WT-F + WT-R) for amplifying the wildtype allele (no triple mutation. B); primers (WT-F + TMR-R) for amplifying the allele with triple mutation (resistant IVS-allele; C). L is the ladder with 100 bp molecular weight [sizes from bottom to top: 100, 200, 300, 400, 500 (brightest), 600, 700, 800, 900, 1,000 bp]

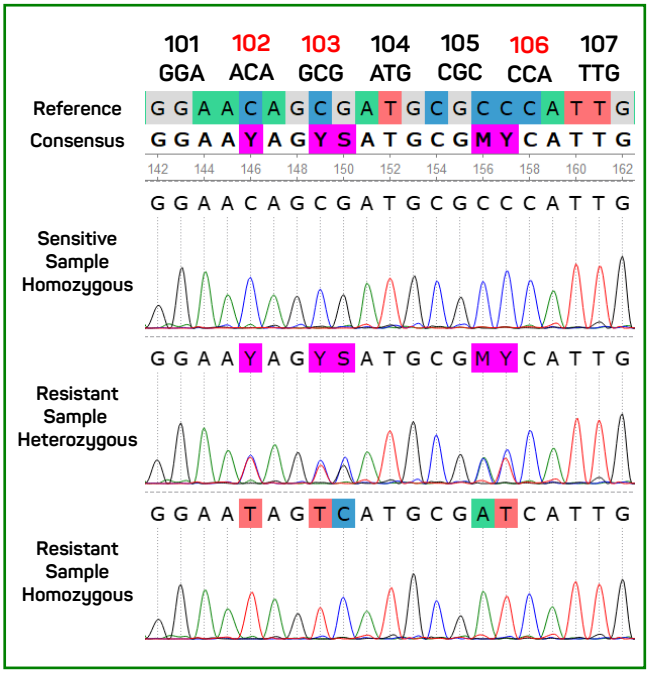

Figure 3 - Electropherogram alignment of EPSPS gene fragments. Alignment showing the codons for 101 to 107 amino acid. The triple amino acid substitution TAP-IVS corresponding to positions 102 (ACA to ATA, Threonine to Isoleucine), 103 (GCG to GTC, Alanine to Valine), and 106 (CCA to TCA, Proline to Serine) are highlighted in red. In position 105 there is a mutation in the resistant homozygous sample leading to a synonymous substitution (CGC to CGA, arginine). The *A. hybridus* EPSPS sequence (MG595171.1) was used as "Reference"

PCR detection assay for the triple mutation in the EPSPS target gene. The distribution of the number of samples per state and the results are detailed in Table 2. Sensitive and resistant (IVS-allele) samples were observed in the Paraná and Rio Grande do Sul states, whereas in the Minas Gerais and Mato Grosso states all the sampled plants were sensitive (Table 2). For this study, only one sample from Minas Gerais was tested and interestingly it was identified as *A. viridis* and showed the wildtype genotype.

A few things can influence the PCR detection assay and can affect the results, giving false negatives or even false positives, and thus are important to be mentioned here. False negative results can be due to the presence of PCR inhibitors, for instance, substances originated from the sample or were introduced during sample processing or even remaining from the DNA extraction process (Schrader et al., 2012). Such organic or inorganic substances, among them calcium ions, urea, phenol, polysaccharides, and proteinases, can be present in the DNA solution and interfere with different steps of a PCR and ultimately inhibit the amplification of the targeted amplicon. Troubleshooting the DNA extraction process will only be required if the PCR with primers EPSF1 and EPSR8 result in no amplification at all. Thus, there should be a minimum DNA quality where the PCR reaction can occur without interference, and most DNA purification/extraction kits commercially available result in high quality DNA.

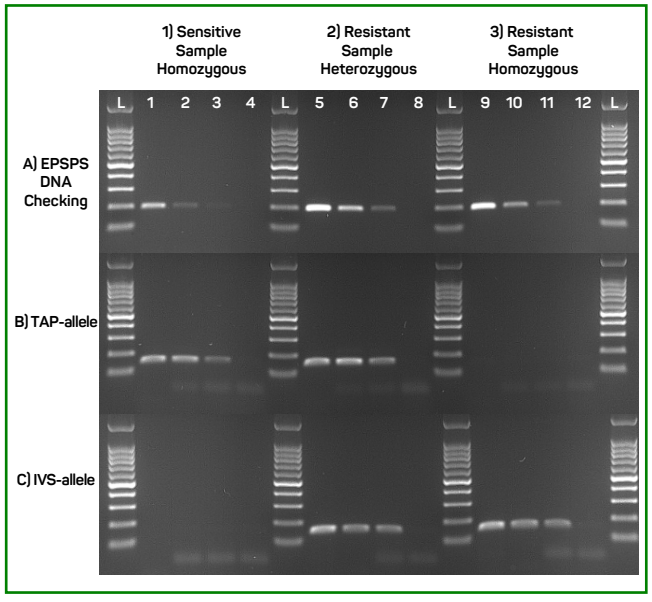

Figure 4 - Electrophoresis of PCR products amplified with primers for detection of EPSPS triple mutation and three DNA concentrations for testing the sensitivity of the assay. Primers (EPSF1 and EPSR8; Gaines et al., 2010) for amplifying an EPSPS gene fragment and confirm DNA quality as a PCR positive control (A); primer pair (WT-F + WT-R) for amplifying the wildtype (TAP) allele (B); primers (WT-F + TMR-R) for amplifying the IVS allele (C). Presence of PCR band in B only denotes a wildtype (TAP) allele; presence of PCR bands in B and C denotes the heterozygous resistant (TAP/IVS) allele; presence of PCR band in C only denotes the homozygous resistant (IVS) allele. DNA dilutions: 100 ng (well 1, 5, 9), 10 ng (well 2, 6, 10), 1 ng (well 3, 7, 11), negative control (well 4, 8, 12), L is the ladder with 100 bp molecular weight [sizes from bottom to top: 100, 200, 300, 400, 500 (brightest), 600, 700, 800, 900, 1,000 bp]

Table 2 - Genotyping results using the PCR detection assay for the triple mutation of the EPSPS target gene. A total of 65 *Amaranthus* accessions collected from four Brazilian states (Paraná, Rio Grande do Sul, Minas Gerais, and Mato Grosso). The species column shows the *Amaranthus* spp. found in each state

State	Total	Wildtype	IVS-Allele (Resistant)		Species
			Heterozygous	Homozygous	
Paraná	15	2	5	8	*A. hybridus*
Rio Grande do Sul	33	16	9	8	*A. hybridus*
Minas Gerais	1	1	-	-	*A. viridis*
Mato Grosso	16	16	-	-	*A. hybridus*
Total	**65**	**35**	**14**	**16**	

Overall, this PCR-based assay for detecting the EPSPS triple mutation conferring resistance to glyphosate in *A. hybridus* will fulfil the gap of lacking a simple method for detecting glyphosate resistance, which is spreading in Brazil and South America. This assay will enable laboratories with basic equipments, such as PCR thermocycler and electrophoresis apparatus, to identify *Amaranthus* plants harboring the IVS triple mutation. By having the results in a few days on whether the *Amaranthus* plants in a crop field are resistant or not, the growers and weed management technical teams can make faster and better decisions on approaches for controlling the weed.

4. Conclusions

We developed and tested two sets of primers to detect the EPSPS triple mutation in *A. hybridus* and the results showed a 100% genotypic to phenotypic association. The triple mutation detection assay is easy to use and can be applied in a molecular laboratory with basic equipments and help in the assessment of *Amaranthus* with resistance to glyphosate. The identification and confirmation of glyphosate resistance in *Amaranthus* plants can improve the decision on the management strategy to be applied in the problematic fields.

Author's contributions

SMM, and DDR: Conceptualization of the manuscript and development of the methodology. SMM: Data

collection and curation, data analysis, data interpretation, supervision, writing the original draft of the manuscript. SMM, CO, LNL, and DDR: Funding acquisition and resources, writing, review and editing. SMM, and DDR: project administration. All authors read and agreed to the published version of the manuscript.

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