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Characterization of *acetolactate synthase gene* (*ALS*) in *Echinochloa colona* (L.) Link., a hexaploid weed species

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Abstract: Junglerice (*Echinochloa colona*) is one of the most problematic weed species in rice fields in Colombia. Herbicides inhibitors of the enzyme acetolactate synthase (ALS) have been widely used to control junglerice and other grass species. ALS inhibitors have the highest reports of resistance worldwide, and Colombia has recent reports of ALS resistance in *E. colona*. The timely and accurate detection of resistance sources is imperative for mitigating and managing herbicide resistance. However, for *E. colona* there are no published sequences of the *ALS gene*. In this research, primer design, RNA extraction, cloning, miniprep, and PCR were used to obtain the first partial sequence of the *ALS gene* on susceptible and resistant accessions of *E. colona*. The sequences did not present nucleotide differences that could be associated with target-site resistance to ALS inhibitors.

Keywords: Herbicide resistance; Metabolic resistance; Mode of action; Sequencing; Target-site resistance; Weed management

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1. Introduction

Echinochloa spp. is a weed considered one of the most difficult to control in more than 60 countries and the most invasive in rice fields worldwide (Massod et al., 2016; Amaro-Blanco et al., 2021). The most troublesome species infesting rice are the hexaploids *E. crus-galli* and *E. colona* (Wu et al., 2022; Panozzo et al., 2013; Yabuno, 1962; Brown, 1950).

In Colombia, *E. colona* (junglerice) is the major problem, causing yield reductions of up to 92% in association with other grasses (Castro, Almario, 1990). Diverse ecotypes, high seed production, short dormancy, rapid growth, competitive potential, allelopathic interaction, and resistance against various herbicides, make junglerice a big challenge for weed management (Masood et al., 2016).

Herbicides have been the most important tool for weed control worldwide since the late 1960s (Perotti et al., 2020). Historically, those targeting the synthesis of amino acids (e.g. acetolactate synthase inhibitors, ALS; glyphosate) have covered most of the market (Sparks, Brayan, 2021). By *Echinochloa* spp., ALS inhibitors are most commonly used because of their effective control (Song et al., 2017). These herbicides exert their activity by inhibiting acetohydroxy acid synthase (AHAS, E.C. 2.2.1.6), also known as ALS, which is the first enzyme in the branched-chain amino acid biosynthesis pathway (valine, leucine, and isoleucine) (Garcia et al., 2017).

The widespread and persistent use of ALS inhibitors has resulted in the rapid evolution of many weed populations resistant to these herbicides (Panozzo et al., 2013). *Echinochloa* spp. has developed resistance to these herbicides; in Argentina, Australia, Bolivia, Costa Rica, Egypt, Salvador, Guatemala, Honduras, Iran, Nicaragua, Panama, the United States, and Venezuela (Heap, 2021). In Colombia in 2015, resistant accessions to penoxsulam, bispyribac-sodium, and imazamox were reported (Carranza, Plaza, 2015), later in Tolima State, the most important rice production area of this country, 91% of the surveyed populations were resistant to bispyribac-sodium (Zabala et al., 2019).

Resistance mechanisms can be on the target site (TSR) or non-target site (NTSR). The TSR is caused by an alteration in the target enzyme or an increased number of TSR, thus diluting or losing the herbicide effect (Duke, Heap, 2017). NTSR mechanisms include reduced absorption or translocation and increased sequestration or metabolic degradation of the herbicidal molecule (Gaines et al., 2020). During the second half of the 20th century, most of the reported cases of resistance were associated with TSR (Leon et al., 2021), especially with ALS inhibitors, however, today there are reports of both TSR and NTSR for the main chemical families of

ALS-inhibiting herbicides (Gaines et al., 2020). In the past, most studies were focused on detecting target-site mutations, and rarely on examining the non-target resistance site resistance (Yu, Powles, 2014; Leon et al., 2021).

TSR for ALS is nuclear controlled and presents different levels of dominance according to the species (Yu, Powles, 2014). It occurs primarily in five conserved protein domains (A, B, C, D, and E) (Amaro-Blanco et al., 2021), caused by amino acid substitutions in up eight sites: Ala_{122}, Pro_{197}, Ala_{205}, Asp_{376}, Arg_{377}, Trp_{574}, Ser_{653}, and Gly_{654} (Yu, Powles, 2014; Heap, 2021). This exceeds mutations number in other important TSR of herbicides and is expected to increase although at a reduced rate, indicating that ALS is one of the most susceptible targets to resistance (Yu, Powles, 2014).

Worldwide, there are at least twice as many weed species resistant to ALS inhibitor herbicides than to any other mode of action (MoA) (Moss et al., 2019). Colombia is high dependence on ALS inhibitors and applications are frequent because it is possible to have almost rice 2,3 rice harvests per year (Zabala et al., 2019). Then, few resistance cases and the gap in resistance mechanisms research could be correlated to a lack of diagnostic methods for Colombian ecotypes. In fact, of the *ALS gene* sequences reported in the NCBI (National Library of Medicine, 2004) for the genus *Echinochloa* spp., none of them correspond specifically to *E. colona*.

Identification of resistance mechanisms (TSR and/or NTSR) in a given weed population is essential for weed management decisions (Perotti et al., 2020). For target-resistance research, molecular markers based on PCR or target gene sequencing are used. However, most tools have been developed for diploid species, which limits TSR genotyping in polyploid species (Yu, Powles, 2014). Specific gene primers or markers for herbicide target genes in polyploids should be developed.

Major advances in whole-genome sequencing technologies and the development of bioinformatics tools and new statistical methods have provided new methods for studying the evolution of resistance and fitness of weed populations (Leon et al., 2021). However, PCR-based diagnostic techniques are inexpensive and allow rapid confirmation of herbicide resistance-mutations. Diagnostic techniques for ALS resistance, have predominantly been developed for grass weed species and despite that, there are many *ALS gene* sequences and specific primers for the genus *Echinochloa* (Iwakami et al., 2012; Kaloumenos et al., 2013; Riar et al., 2013; Panozzo et al., 2013; Matzenbacher et al., 2014), currently, there are no available *ALS* sequences to *E. colona*, necessary to diagnosis TSR. Therefore, the objective of this work was to sequence the *ALS gene* from resistant and susceptible accessions of *E. colona*, providing a molecular tool to facilitate a high-performing and accurate diagnosis of TSR in this species.

2. Material and Methods

2.1 Vegetal material

The *E. colona* accessions used were characterized by Carranza and Plaza (2015) as susceptible (CP_1) and resistant (TV_1) to penoxsulam. CP_1 came from Puerto Salgar (Cundinamarca State), an area without exposition to the herbicide. TV_1 came from a field located in Saldaña, Tolima a region intensively cultivated with rice. The populations were multiplied by two generations in separate locations and self-pollination was allowed. The mean effective dose (ED_{50}) of TV_1, in previous dose-response curve experiments, could not be calculated because it is well above the label dose (40 g ha^{-1}) and the maximum dose evaluated (40,960 g ha^{-1}).

Seeds were immersed in a 0.2% KNO_3 solution for 48 hours, later they were placed in Petri dishes with wet filter paper in a growth chamber at 35°C. Once the first leaf emerged, they were transplanted into pots with a mixture of soil and sterile peat.

2.2 RNA extraction and cDNA synthesis

Leaf tissue was used for extraction by independently macerating from five individuals of each accession. A modified method for extraction using CTAB and 4M LiCl was performed (Chang et al., 1993). RNA was diluted in 50 µL of RNase-free water. The integrity was verified by electrophoresis in agarose 1% and purity and quantification were done with NanoDrop™Spectrophotometer.

RNA samples were treated with DNase I. Subsequently, cDNA synthesis was performed with a First Strand cDNA Synthesis Kit (Thermo Scientific), using oligodT primers and incubating first at 65 °C for 5 min, after chilling on ice was put MMLV enzyme, 5X buffer, RNAse inhibitor, and dNTPs, then the reaction was incubated by 60 min at 37 °C. The enzyme deactivation was done at 70 °C for 10 min.

2.3 Primer design

For the amplification of the *ALS gene*, primers were designed. cDNA sequences, available in the NCBI database of different species of Poaceae and *Echinochloa* spp. were aligned in the ClustalX2 program (Larkin et al., 2007). Conserved and similar regions were identified and from this, sequences from *E. crus-galli* (LC006063.1, LC006062.1, LC006061.1, LC006060.1, LC006058.1 LC006059.1), and *E. phyllopogon* (AB636581.1, AB636580.1) were selected. Again, selected sequences were aligned as previously described and a consensus sequence was obtained by Genedoc (Nicholas et al., 1997). Subsequently, using Primer3 (Koressaar et al., 2018), five primer pairs (Table 1) were designed, overlapping each other to obtain a complete sequence of the target region (Figure 1). Thermodynamic properties were verified with mFold.

Table 1 - Primers to amplify *ALS* and β-*tubulin* genes in susceptible and resistant accessions of *E. colona*. Base pair, bp

ID	Primer sequence (5′–3′)	°T annealing	Expected size (bp)
ECHCO-1F	CTTGCCACCCTCCCCAAA	58 °C	674
ECHCO-1R	TGTCGAGGACGAGGTAGTTGT		
ECHCO-2F	GTCATMGCCAACCACCTC	55 °C	490
ECHCO-2R	CACCAACAAGACGCAGCAC		
ECHCO-3F	STTCTTCCTCGCCTCCTCT	56 °C	682
ECHCO-3R	CTGTGGCTGAATCTCCTCATC		
ECHCO-4F	CCTGTTCTTTATGTTGGTGGTG	56 °C	775
ECHCO-4R	CCTTCACTGGGAGGTTCTCRA		
ECHCO-5F	GATGAGGAGATTCAGCCACAG	57 °C	636
ECHCO-5R	ATACACGGTCCTGCCATCA		
TUB_ECO_F	ACGAYTGCATGGTKCTTGAC	55 °C	389
TUB_ECO_R	ACCTCCTTKGTGCTCATCTT		

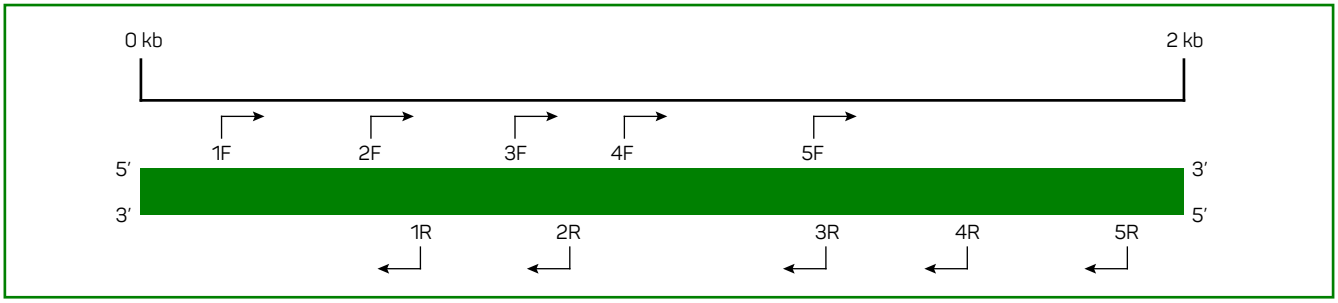

Figure 1 - Primer location used to amplify the *ALS gene* in susceptible and resistant accessions of *E. colona*

Additionally, a set of primers was designed to amplify a partial sequence of the β-*tubulin* gene (Tab. 1) used as a reference gene in primers validation for *ALS* and future works in *E. colona*. For this, RNA sequences from, *E. phyllopogon* (AB775463.1; AB775464.1; AB775465.1), *Hordeum vulgare* (Y09741.1; AM502849.1; AM502850.1), *Triticum aestivum* (U76745.1; U76744.1), *Oryza sativa* (L33263.1; X79367.1), and *Zea mays* (NM_001111988.1; NM_001111986.1) were aligned, the design was performed as described above.

2.4 cDNA amplification and gene sequencing

The amplification conditions were performed for 3 min at 94 °C, 30 cycles for 30 s at 94 °C, 30 s of annealing (temperature according to the pair of primers used, Tab. 1), and 1 min at 72 °C, and a final extension of 3 min at 72 °C. Amplification was of 50 µL containing cDNA 2 µl, Buffer 10X 5 µL, $MgCl_2$ 2.5 µL (2.5 mM), dNTPs 1.5 µL (0.3 mM), 1.5 µL of each primer (0.4 µM), Taq Platinum 5 units (Invitrogen) and water 35 µL. The amplicons were evaluated by electrophoresis (1% agarose, 0.5X TBE buffer), 10 µL of PCR product were evaluated for 40 min at 70 volts, stained with EZ-vision, and visualization was done with ChemiDoc MP System (BioRad) image documentary.

The amplicons were sequenced and the analysis was done with the Geneious program, an alignment was carried out to obtain consensus sequences of the *ALS gene*. The identity of sequences was confirmed through the Blastn and BlastX algorithms of the NCBI portal (Johnson et al., 2008).

2.5 Cloning and transformation

The amplicons obtained with ECHCO 3F-3R, 4F-4R, and 5F-5R primers were eluted from electrophoresis gel and individually cloned in the PCR™ 2.1-TOPO® vector and then transformed into competent *E. coli* DH5α cells, using the TOPO-TA Cloning® kit (Invitrogen), following the methodology recommended by the supplier. Subsequently, the plasmid DNA extraction of 12 recombinat colonies of each amplicon by each accession, was carried out using a protocol modified from that described by (Sambrook, Russel, 2000). Positive cloning was verified by PCR amplification using the ECHCO 3F-3R, 4F-4R, and 5F-5R primers, then amplification was carried out with the M13 forward (–20) and M13 reverse primers included in the

cloning kit, the conditions were as described above and the annealing temperature was 50 °C. The fragment size was verified by electrophoresis and the samples were sequenced by the Sanger method.

2.6 Obtaining the sequence

The cleaning and alignment were carried out in the Geneious program, using as a reference map a consensus sequence obtained from the alignment of the partial sequences of the *ALS gene* in *Echinochloa crus-galli*, and *Echinochloa phyllopogon*, obtained from cDNA, available in NCBI (Matzenbacher et al., 2014; Iwakami et al., 2012; Panozzo et al., 2013; Riar et al., 2013; Iwakami et al., 2015). The susceptible and resistant accession sequences were aligned separately, generating a consensus sequence of the susceptible accession and a consensus sequence of the resistant accession.

3. Results and Discussion

3.1 *ALS gene* amplification in *Echinochloa colona*

Three (3F/3R, 4F/4R, and 5F/5R) of five primers (Table 1) resulted in positive amplification, which in electrophoresis presented the expected size (Figure 2). Likewise, primers designed to β-*tubulin* produced a fragment close to 389 bp as contemplated in the design (Figure 2). Amplicons obtained for both accessions after eluted were sequenced. However, the results obtained were not conclusive.

E. colona is a hexaploid species (Wu et al., 2022). Polyploids can have multiple *ALS gene* copies, some even with introns. All copies may be expressed at different levels, being silenced, or even being pseudogenes (Yu, Powles, 2014). Therefore, the cloning strategy looked to capture the diversity of *ALS gene* copies in *E. colona*.

Then was made individual cloning of each fragment and recombinant vectors were transformed into *E. coli*. Plasmid DNA was used to PCR with the ALS primers in Table 1 (3F/3R, 4F/4R, and 5F/5R). By electrophoresis was visualized the expected band sizes again (Figure 3). After, sequencing was performed by Sanger, using universal primers M13 forward (–20) and M13 reverse (Figure 4).

3.2 *ALS gene* sequencing in *Echinochloa* colona

The alignment was made, joining each clone sequence with its three amplicons (3F/3R, 4F/4R, and 5F/5R). A partial consensus sequence *ALS gene* was obtained. However, because of the richness of guanine and cytosine in the first part of the gene, which is difficult to primer design, it was not possible to obtain the complete coding sequence of the *ALS gene*.

The CP_1 and TV_1 sequences contain 1,298 bp (base pairs). These were deposited in NCBI with the accession numbers: OL604241 (CP_1) and OL604242 (TV_1). The nucleotide sequences showed 99% and 98% similarity with the *ALS* sequences gene reported for accessions of *E. cruss-galli* and *E. phyllopogon*, respectively. The coverage percentage was 99%.

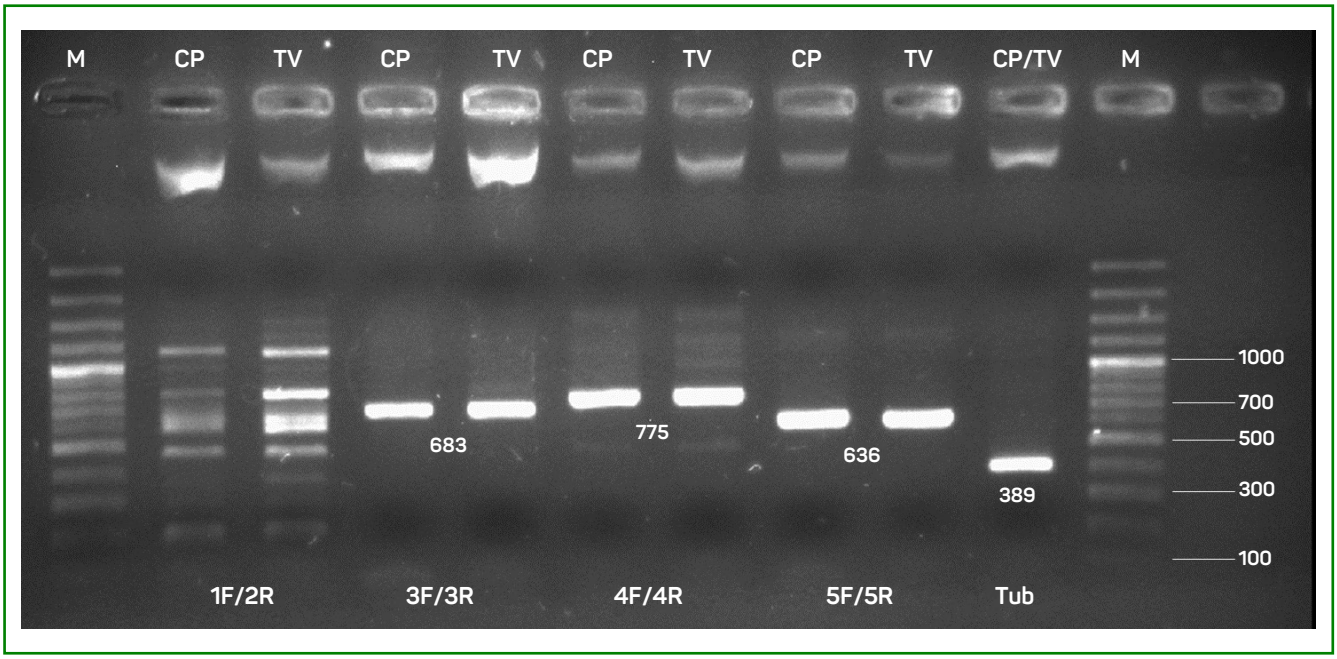

Figure 2 - Amplicons from cDNA of susceptible (CP_1) and resistant (TV_1) accessions of *E. colona*. The expected fragments sizes are 3F/3R=683 bp; 4F/4R = 775 bp; 5F/5R = 636 bp; and β-tubulin, Tub = 389 bp. The 1F/2R combination should have at least an 858 bp band. M = Marker, 100 bp DNA ladder

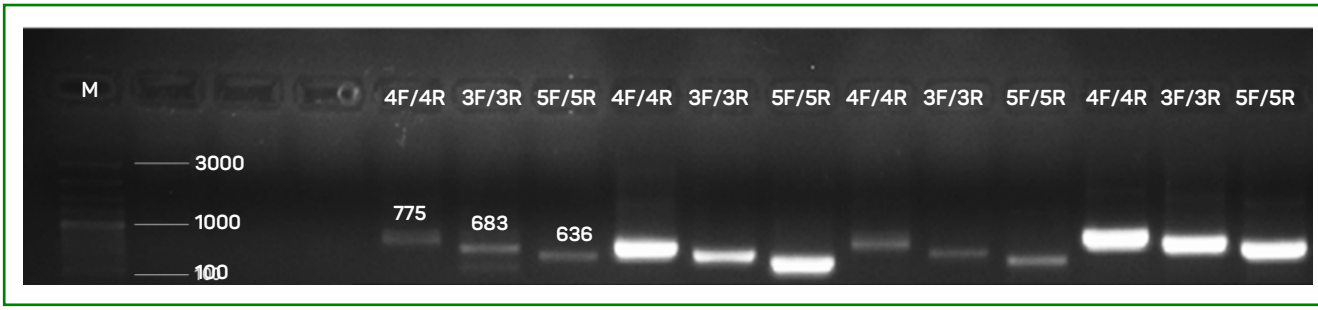

Figure 3 - Amplicons from plasmid DNA of *E. coli* colonies, transformed with recombinant vectors. The expected fragments sizes are 3F/3R=683 bp, 4F/4R = 775 bp; and 5F/5R = 636 bp. M = Marker, 100 bp DNA ladder

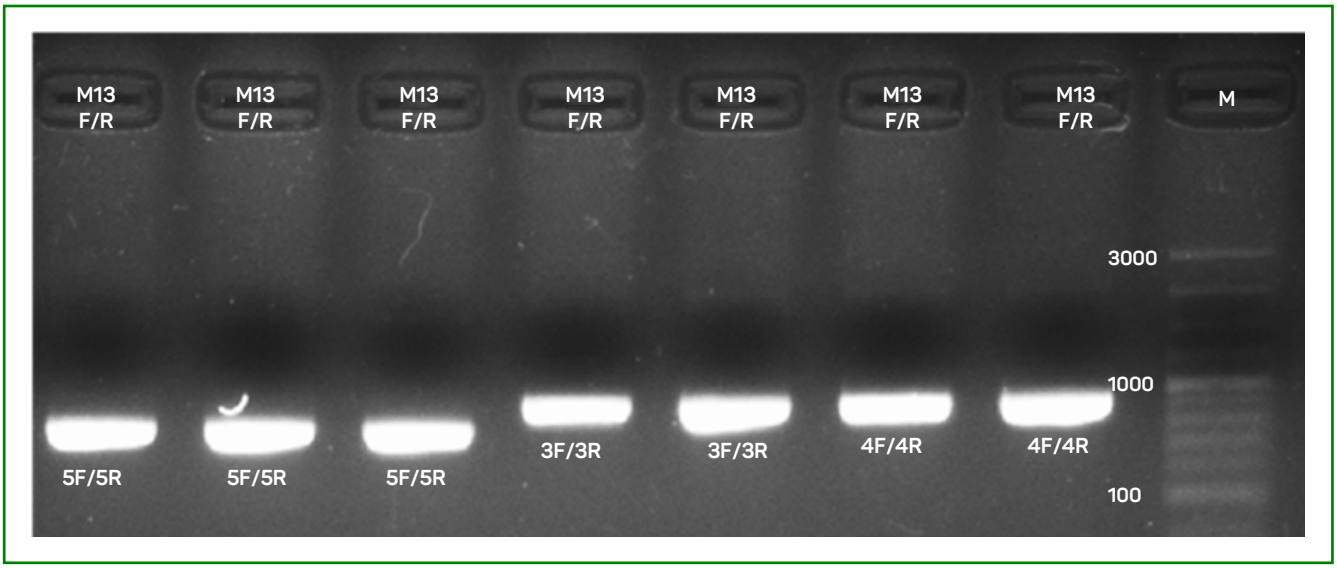

Figure 4 - Amplicons from plasmid DNA of *E. coli* colonies, transformed with recombinant vectors. PCR was performed with the universal primers, M13 forward (–20) and M13 reverse. The expected fragments sizes using M13 primers are 3F/3R=852 bp, 4F/4R=924 bp; and 5F/5R = 805 bp. M = Marker, 100 bp DNA ladder

The alignment of *E. colona* sequences with the *A. thaliana* NM_114714.2 sequence revealed no point mutations in the eight hotspots usually associated with ALS inhibitors resistance (Ala_{122}, Pro_{197}, Ala_{205}, Asp_{376}, Arg_{377}, Trp_{574}, Ser_{653}, and Gly_{654}) (Figure 5).

Of the eight hotspots in the *ALS gene*, for the *Echinochloa* spp. genus, three have been detected, Ala_{122}, Trp_{574}, and Ser_{653} (Riar et al., 2013; Matzenbacher et al., 2014; Panozzo et al., 2013; Kaloumenos et al., 2013). The codon corresponding to Trp_{574} in the susceptible and resistant accessions was identical to *A. thaliana*. Regarding Ser_{653}, although the codon for both accessions presented the substitution of thymine for cytosine in the terminal nucleotide (Figure 6), the resulting amino acid continued to be serine, corresponding to a silent mutation.

As for Ala_{122}, it was not possible to sequence this area of the *ALS gene* in *E. colona* due to the high GC content, typical of Poaceae (Iwakami et al., 2012), which makes it difficult to design primers in this area. Although several pairs of primers were designed, positive amplification of this area was not achieved, which corresponds, on average, to about 270 bp concerning the other sequences reported for *Echinochloa* sp. (Matzenbacher et al., 2014; Panozzo et al., 2013; Riar et al., 2013; Iwakami et al., 2015; 2012; Kaloumenos et al., 2013). However, there are several works on species of the genus *Echinochloa* sp., in which, despite obtaining the complete sequence of the *ALS gene*, they did not find mutations in the susceptible or resistant accessions (Iwakami et al., 2012; Iwakami et al., 2015). Likewise, additional works suggest that resistance to ALS inhibitor herbicides in some accessions of the genus *Echinochloa* spp. could also be conferred by the accelerated degradation of herbicides, mediated by the P450 enzyme (Fischer et al., 2000; Riar et al., 2013; Song et al., 2017; Yasuor et al., 2009; Yu, Powles, 2014; Wright et al., 2018). In fact, in studies done with accessions of *E. colona* resistant to ALS herbicides from the same area of the resistant accession evaluated here (TV_1), multiple resistance to three different modes of

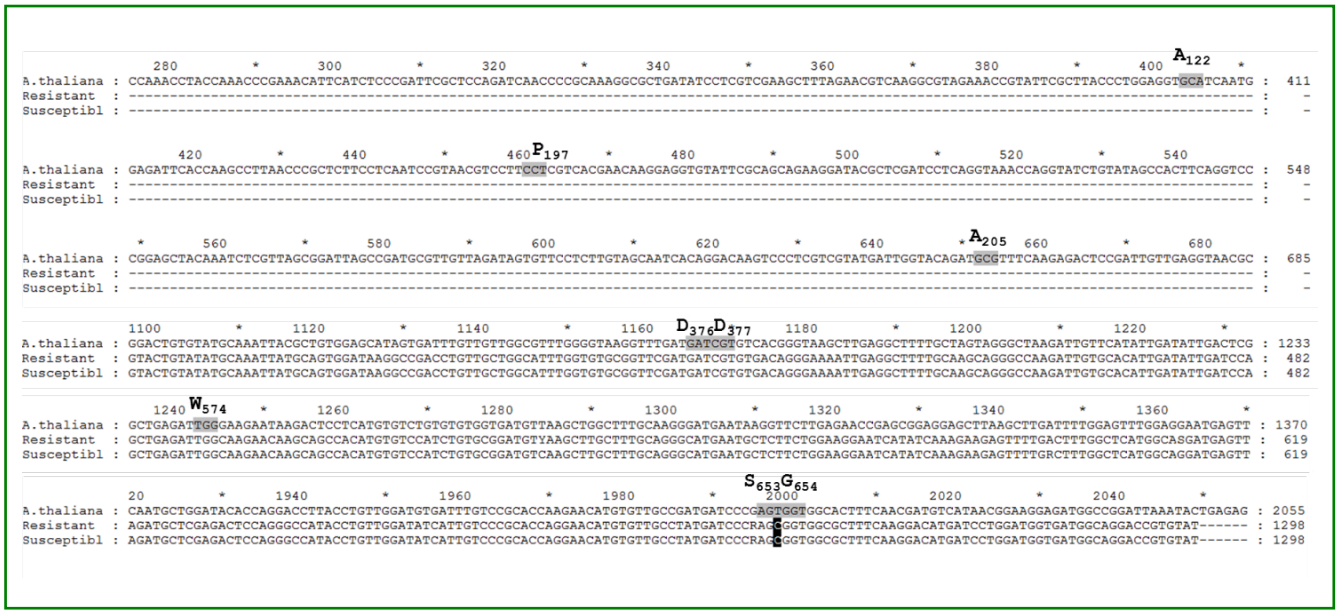

Figure 5 - Partial nucleotide sequence of the *ALS gene* of *E. colona*, in susceptible (CP_1) and resistant (TV_1) accessions, and the reference sequence NM_114714.2 of *Arabidopsis thaliana*. Codons in gray indicate the position of eight mutations usually associated with herbicide resistance. Bases that are different from the reference sequence are shown in black

action was reported (Zabala et al., 2019), which is usually associated with NTSR resistance. Additional studies must be done to determine the resistance mechanism in the resistant accession evaluated in this study.

4. Conclusions

In Colombia due to the lack of diagnostic-resistance tools, the official reports of ALS resistance do not coincide with the reported in the field. Using molecular techniques were designed specifical primers for *E. colona*, and was obtained the first partial coding sequence of the *ALS gene* for the species. Although were compared sequences from a susceptible accession and one with a high resistance index, could not be detected mutations associated with target-site resistance. Therefore, subsequent work should research, besides entire ALS-gene sequencing, gene copy numbers, expression levels, and mechanisms related to NTSR resistance.

Author's contributions

All authors read and agreed to the published version of the manuscript. NC and DZ: conceptualization of the manuscript and development of the methodology. DZ and NC: data collection and curation. DZ and NC: data analysis. DZ and NC: data interpretation. NC: funding acquisition and resources. GP, ET, and NC: project administration. GP and ET: supervision. DZ and NC: writing the original draft of the manuscript. DZ, NC, ET, and GP: writing, review, and editing.

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