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Johnsongrass resistance to nicosulfuron

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Abstract: **Background:** Johnsongrass (*Sorghum halepense* (L.) Pers.) is one of the most problematic weeds worldwide, and in recent years, control failures with herbicides inhibiting the enzyme acetolactate synthase (*ALS*) have been reported. **Objective:** Determine the resistance in different biotypes of Johnsongrass to nicosulfuron, as well as elucidate its possible resistance mechanism. **Methods:** Dose-response bioassays were conducted with four biotypes, three of them from corn fields with consecutive history of nicosulfuron applications (Veracruz-Mexico) and a susceptible biotype (Guanajuato-Mexico). A partial sequence of the *ALS* gene was obtained for each biotype to identify mutations conferring target-site resistance to this herbicide. **Results:** The dose-response curves showed high rates of resistance in the biotypes from Veracruz (IR: 30, 63, and 77 times more resistant than the susceptible biotype); at the molecular level, a mutation was found, which resulted in the substitution of amino acid (Asp376Glu). **Conclusions:** This mutation could be involved in resistance in the Oro Verde, El Marcial, and Francisco Villa populations.

Keywords: Herbicide Resistance; *ALS*-Inhibitors; Bioassays; Dose-Response Curve

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1. Introduction

Sorghum halepense (Pers) L. (Johnsongrass) has been categorized as one of the ten most troublesome weeds in the world (Holm, 1969). This species reproduces through rhizomes and seeds and has been reported as a weed in over 30 crops, mainly maize, cotton, and sugar cane (Oyer et al., 1959; Warwick, Black, 1983). Its interference reduces maize grain yields by 57% to 88% (Mitskas et al., 2003). Nicosulfuron is an herbicide that has effectively controlled Johnsongrass (Gubbiga et al., 1995).

Nicosulfuron (DPX-V9360/SL-950, Du-Pont/lSK) is an herbicide of the sulfonylurea family, recommended to control Johnsongrass in the early post-emergence of maize (Foy, Witt, 1990; Obrigawitch et al., 1990; Saari et al., 1994). Sulfonylurea herbicides inhibit the acetohydroxyacid synthase enzyme (*ALS*, EC 2.2.1.6), which is responsible for synthesizing essential branched-chain amino acids (valine, leucine, and isoleucine) in plants and microorganisms (Duggleby et al., 2008). *ALS*-inhibiting herbicides have broad weed control, low mammalian toxicity, selectivity in several crops, and low application rates (Brown, 1990). However, they can rapidly lead to resistant weed populations (Tranel, Wright, 2002).

ALS inhibitor herbicides were introduced in 1982 (Saari et al., 1994). However, only five years later, the first case of resistance in *Lactuca serriola* was reported in the United States (Mallory-Smith et al., 1990). To date, a total of 170 weed species are reported to be resistant to *ALS* herbicides, including Johnsongrass. In recent years, Johnsongrass resistant to *ALS* herbicides has been reported in several countries including the United States (2000), Italy (2007), Chile (2009), Mexico (2009), Venezuela (2010), Serbia (2014), Hungary (2015), Spain (2015), and Israel (2018), in crops such as maize, soybeans, and cotton (Heap, 2022).

Target-site resistance is the most frequent mechanism of weed resistance to herbicides (Heap, 2014) and for *ALS* herbicides, it is mainly attributed to a mutation in the *ALS* enzyme conferred by a single dominant gene (Tranel, Wright, 2002). Its mechanism is based on non-synonymous substitutions generating amino acid changes, causing point mutations limiting the binding of the herbicide to the enzyme (Powles, Yu, 2010). Molecular studies in Johnsongrass have confirmed substitutions in the positions Trp574Leu and Asp376Glu confer resistance to *ALS* inhibitors (Hernández et al., 2015; Panozzo et al., 2017). Our objective was to determine, in the treated Mexican Johnsongrass biotypes, the resistance to nicosulfuron and elucidate its possible mechanism of resistance at the target-site level.

2. Material and Methods

2.1 Plant Material

Seeds were collected from four Johnsongrass populations using a random sampling method in each population. Three of the population from the municipality of Ciudad Isla, Veracruz, Mexico, denoted as Oro Verde, El Marcial, and Francisco Villa, which, according to the farmers, had nicosulfuron applied for more than 10 years in the maize crop in the direct sowing system. In addition, a population with no history of nicosulfuron application, Comonfort, from the State of Guanajuato, Mexico, was included as a control (Figure 1).

2.2 Dose-response bioassay

Studies were conducted at the Agricultural Parasitology Department of the Autonomous University Chapingo. The dormancy of the seeds was broken through manual scarification. They were placed in a growth chamber (APT.line® KBWF E5.2, Binder, Tuttlingen, Germany). At 30 °C and 70% constant relative humidity for 48 hours and transplanted into 0.5-liter pots. Seedlings with 3 to 4 leaves were sprayed with nicosulfuron (Sanson® 6 OD, 60 g.a.i. L^{-1}, France). Doses of 0, 0, 3.75, 7.5, 15, 30, 60, 120, 240, 480, 960 g ha^{-1} ai. were used for the Oro Verde, El Marcial, and Francisco Villa biotypes, and for control Comonfort, the doses were 0, 0, 0.93, 1.87,

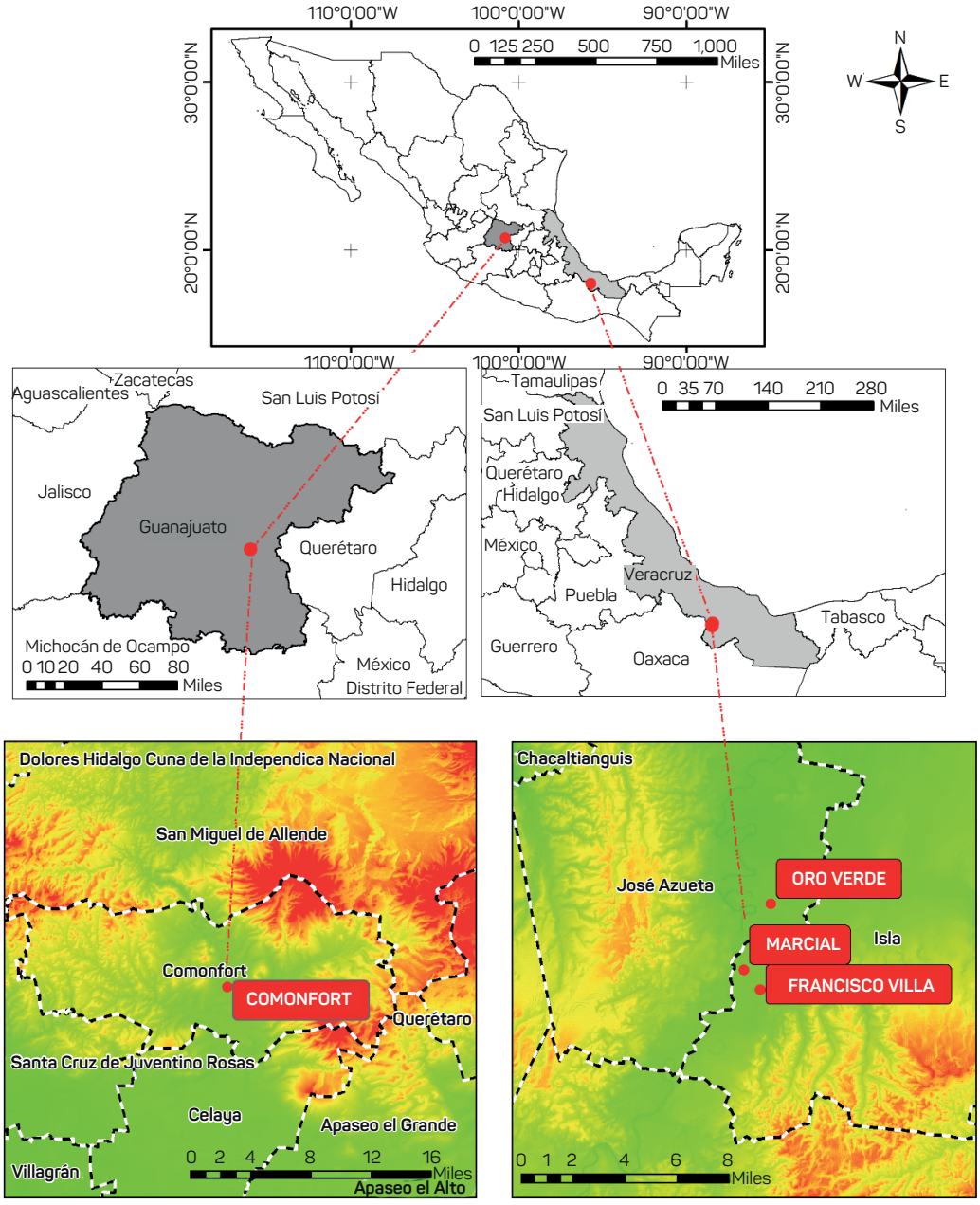


Figure 1 - Geographic location of sampling area of the Francisco Villa, Oro Verde, El Marcial, and Comonfort populations (Mexico)

3.75, 7.5, 15, 30, 60, 120, 240 g ha^{-1}. The commercial dose of this product farmers use is 30 to 60 g ha^{-1}. Spraying was performed with a CO_2-based sprayer calibrated to spray 200 L ha^{-1}. Four replicates were used for each dose, and two seedlings were used per pot. At 21 days after application, the seedlings were cut at ground level, weighed and GR50 was estimated (required dose of nicosulfuron to inhibit the weight of fresh by 50%) and the resistance index (RI) was calculated by dividing the GR_{50} of the susceptible by the GR_{50} of each of the suspected resistant populations (Oro Verde, El Marcial, and Francisco Villa). The experiment was carried out twice.

2.3 *ALS* gene sequencing

Leaves were collected using three replicates of each biotype. Genomic DNA was extracted following the methodology of (Doyle, Doyle 1987) and quantified in a Nanodrop (NanoDrop 2000, Spectrophotometer, Thermo Scientific, EE. UU.). The primers used for the PCR reaction were as described by Hernández et al. (2015) (Forward: 5´ CTGTTCTTTATGTTGGTGGT 3´; Reverse: 5´ TATCTGTAGCAAAAGGCACT 3´) that amplify a partial sequence of the *ALS* gene including the mutation sites Asp-376, Arg-377, Trp-574, Ser-653, and Gly-654. To 50 µL PCR reaction were added 36.6 µL of H2O, 5 µL of the mixture Buffer + Mg Cl2, 2 µL of dNTPs (SIGMA-ALDRICH®), 2 µL (10 uM) of forward primer (10 uM), 2 µL of reverse primer (10 uM), 0.4 µL (5 units /µL) of Taq DNA Polymerase (SIGMA-ALDRICH®), and 2 µL of DNA (100 ng/µL). The PCR conditions were one cycle of denaturation at 94 °C for 1 minute, 35 cycles at 94 °C for 30 seconds, 57 °C for 30 seconds, and 72 °C for 90 seconds, plus a final cycle of 72 °C for 10 minutes. The PCR products were observed on a 0.8% agarose gel (SIGMA-ALDRICH®) stained with Ethidium Bromide where they were observed in a photodocumenter. (UVP MultiDoc-It™, EE. UU). Sequencing was performed by the Sanger method with Applied Biosystems™ HITACHI, 3,500 xL. The sequences were cleaned and deposited at NCBI (https://www.ncbi.nlm.nih.gov/nuccore/OP615945) (one sequence per population) with the accession numbers OP615945, OP615946, OP615947, and OP615948.

The results were cleaned and subjected to a BLAST (Basic Local Alignment Search Tool) at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Finally, the *Arabidopsis thaliana* template sequence of the *ALS* gene (NM_114714.3) (Dr. Patrick J. Tranel, personal communication) was employed to establish the universal numbering of points mutations in the *ALS* gene. Sequence alignment was performed using Unipro UGENE software (Okonechnikov et al., 2012).

2.4 Statistical Analysis

Johnsongrass aboveground fresh weight data were transformed into percentages of the untreated control, using the nonlinear 4-parameter regression model with an adjustment for standard errors to make it more robust for the heterogeneity of variances (Knezevic et al., 2007; Ritz, Streibig, 2005; Ritz et al., 2015).

equation [1]:

$$Y = c + \frac{d\text{-}c}{1 + exp[b(logx - log\ GR_{50})]} \quad [1]$$

where (Y) is the plant response to herbicide doses, (*d*) is the upper limit, (*c*) the lower limit, (*b*) the slope of the curve, (*x*) nicosulfuron doses, and (GR_{50}) is the required dose of nicosulfuron to inhibit the fresh weight of Johnsongrass plants by 50% (Seefeldt et al., 1995). The data were analyzed in the R programming environment using the drc package (Ritz et al., 2015).

3. Results and Discussion

3.1 Dose-response bioassay

The dose-response curve (Figure 2) showed that the collected biotypes were resistant to nicosulfuron. Francisco Villa, Oro Verde, and El Marcial biotypes required 133, 109, and 52 g ha^{-1} of nicosulfuron to inhibit 50% of the fresh weight. The Comonfort biotype required 1.72 g ha^{-1} to reduce 50% of its fresh weight, 2.86% of the maximum commercial dose (60 g ha^{-1}). The resistance levels were 30 and 77 times higher than for the susceptible biotype (Table 1).

3.2 *ALS* gene sequencing

The amplicons obtained were above 1300 bp. The cleaned sequences had 99% similarity to accession MF462186.1, corresponding to the *ALS* gene of *Sorghum halepense* (Panozzo et al., 2017). We examined 11 of the 12 samples sequenced and compared sites Asp376, Arg377, and Trp574, sites between the *Arabidopsis thaliana* template sequence (NM_114714.3), the sequences showing variation in position 376 for the Oro Verde, El Marcial, and Francisco Villa biotypes (Figure 3). This variation consisted of substituting the amino acid Asp (aspartic acid) for the

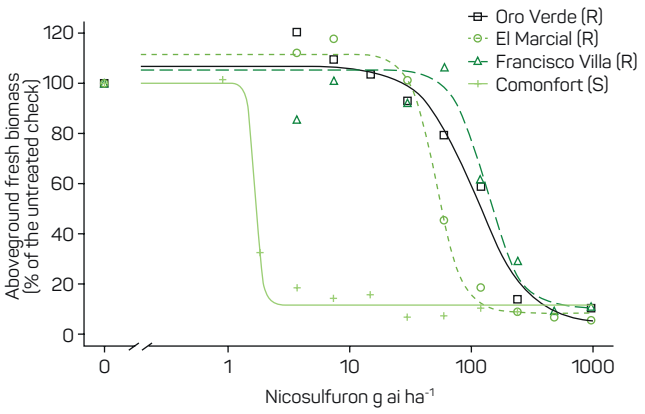


Figure 2 - Response of Johnsongrass biotypes to nicosulfuron doses aboveground fresh biomass

Table 1 - Log-logistic equation parameter estimates the dose-response curves for Johnsongrass biotypes to nicosulfuron by the aboveground fresh biomass

Biotypes	Log-logistic model parameters				
	b (±SE)[1]	c (±SE)	d (±SE)	GR_{50} g ha^{-1} (±SE)	RI
Francisco Villa	3.07 (±1.87)	10.37 (±6.04)	105.77 (±6.6)	133.13 (±22.51) ***	77.4
Oro Verde	1.96 (±0.99)	3.96 (±4.61)	106.24 (±5.46)	109.35 (±24.04) ***	63.5
El Marcial	3.98 (±3.32)	8.72 (±2.71)	111.99 (±6.52)	52.19 (±5.88) ***	30.3
Comonfort	14.29 (±27.03)	11.63 (±1.28)	100.58 (±17.62)	1.72 (±0.23) ***	

[1] Standard Error *** Represent the significance of the parameter estimate

amino acid Glu (glutamic acid) and showed the heterozygous nature of the biotypes examined.

The results showed a high level of resistance the Francisco Villa, Oro Verde, and El Marcial biotypes to nicosulfuron, which could be due to several factors described in the literature (Gressel, 1978; Jasieniuk et al., 1996). This resistance to *ALS* inhibitors (sulfonylureas) can be attributed to a high frequency of *ALS* mutations. 2.2×10^{-5} to 1.2×10^{-4} in weeds such as *Lolium rigidum* not previously treated (Preston, Powles, 2002).

The mutation in our biotypes, has been reported more frequently in dicotyledonous species such as *Amaranthus hybridus*, *A. powellii*, *Conyza canadensis*, *Schoenoplectus juncoides*, and *Descurainia sophia*, withresistance indexes to sulfonylureas from 14 to 3261 (Whaley et al., 2007; Ashigh et al., 2009; Zheng et al., 2011; Sada et al., 2013; Xu et al., 2015). Another study showed that homozygous *Raphanus raphanistrum* plants with the Asp376Glu mutation were resistant to sulfonylurea, triazolopyrimidine, and imidazolinone herbicides with GR_{50} R/S ratios of > 6.9, 3.8, and 2.4 respectively (Yu et al., 2012).

In grasses, the mutation was described in *Lolium perenne* (Menegat et al., 2016) and *S. halepense*, the latter with an average RI of 14.85 (Panozzo et al., 2017). This mutation may cause cross-resistance to *ALS* inhibitors (Wei et al., 2016), even if another herbicide of a different chemical group, but with the same mechanism of action, has never been applied before. In addition, the number of mutations reported in the *ALS* gene so far is 180 (100%) of which 51% represent the Pro197 mutation followed by Trp574 (23%), Ser653 (8%), Asp376 (7%), Ala122 (7%), Ala205 (3%), Gly654 (1%), Arg377 (1%), Gly654 (1%), and Arg377 (1%) (Heap 2022). However, target site resistance (TSR) is not the only resistance mechanism; there are often other important resistance mechanisms such as non-target-site resistance (NTSR), which is sometimes more complex than TSR due to different processes and the involvement of large gene families such as Cytochrome P450 and Glutathione S- transferases (GS) (Gaines et al., 2020).

In our studied populations, extending the sequencing of the complete *ALS* gene would be advisable to determine if other mutations influence resistance to nicosulfuron. In addition, focusing on studies of non-target-site resistance (NTSR), a more complex and still poorly understood mechanism, therefore deserve our focus (Delye et al., 2011; Jugulam, Shyam, 2019).

4. Conclusions

The three Johnsongrass populations were highly resistance to nicosulfuron. The Francisco Villa, Oro Verde, and El Marcial populations exhibited resistance factors to nicosulfuron of 77.4, 63.5, and 30.3 respectively. The Sangers' DNA sequencing analysis of the *ALS* gene showed an SNP mutation in the position 376, leading to an amino acid substitution from Asp (aspartic acid) to Glu (glutamic acid) in the enzyme.

These results suggest that mutations in the *ALS* gene are one of the key mechanisms for the observed phenotypic resistance. However, it is important to note that point mutations are not the only documented resistance mechanism. Therefore, further research in other areas, such as NTSR, is essential due to its greater complexity of study, often involving multiple genes.

This study contributes to understanding the molecular basis of resistance in Mexican biotypes of *S. halepense*, to consider implementing integrated weed management strategies that include use of herbicides with different modes of action, crop rotation, and cover crops.

Author's contributions

All authors read and agreed to the published version of the manuscript. JISA, and AB: Conceptualization of the manuscript and development of the methodology. JISA, MV, and AB: data collection and curation. JISA, LAA, and MV: data analysis. JISA, AB, MV, LAA, and AAM: data interpretation. AB, AAM, and LAA: funding acquisition and resources. AB, AAM, and LAA: project administration. AB: supervision. JISA: writing the original draft of the manuscript. JISA, AB, LAA, MV, and AAM: writing, review, and editing.

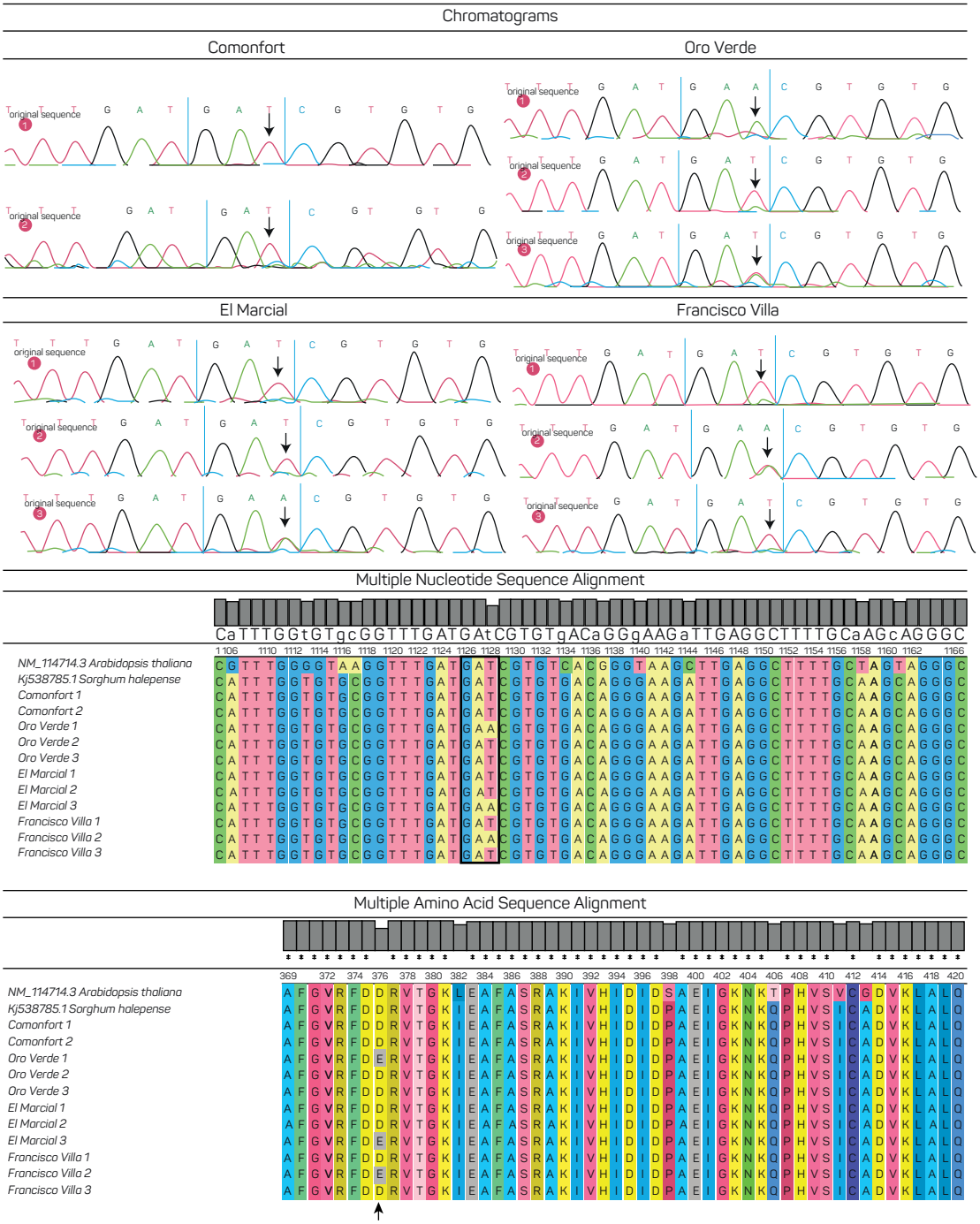


Figure 3 - Chromatograms obtained from the partial sequencing of the *ALS* gene from *Sorghum halepense*. Arrows show point mutation at position 376 in sequences of *S. halepense* biotypes. The mutation corresponds to substituting the amino acid D (aspartic acid) for E (glutamic acid) and indicates the heterozygous nature of the biotypes. The reference heterologous sequence corresponds to A. *thaliana* (NM_114714.3) and *S. halepense* (KJ538785.1)

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