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Mechanisms of resistance in populations of *Echinochloa* sp. from Portugal and Spain

Mecanismos de resistencia en poblaciones de *Echinochloa* sp. en Portugal y España

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ABSTRACT

Weed resistance to herbicides affects the main rice producing countries in the Iberian Peninsula. Over-reliance on acetolactate synthase (ALS) and acetyl-CoA carboxylase (ACCase) inhibitor herbicides, as well as low diversity of cultural practices have increased selection pressure towards resistant populations. Recently, the emergence of weeds with multiple resistance has also been observed. In the Iberian Peninsula, different populations of *Echinochloa* spp. have shown resistance to penoxsulam and profoxydim. The main objective of this work is to study the mechanisms involved in the resistance present in these populations through the sequencing of *ALS* and *ACCase* genes and gene expression studies by means of qRT-PCR. The study encompasses three populations of *Echinochloa* sp. from Portugal and seven from Spain, all of which had previously confirmed herbicide resistance through dose-response assays. None of the populations exhibited mutations already described as involved in resistance to ALS and ACCase inhibitors. However, in three resistant Portuguese accessions, mutations in the *ACCase* gene causing amino acid substitutions in the protein were found, but have not yet been confirmed to confer herbicide resistance. The relative expression of *ALS1* and *ACCase* genes was also assessed. Our study reveals the existence of different levels of overexpression among accessions and differences between roots and leaves within the same accession. These results are indicative of target site type resistance mechanisms, specifically involving gene overexpression between roots and leaves within the same accession. These results are indicative of target site type resistance mechanisms based on gene overexpression.

Keywords: multiple resistance, ALS, ACCase, overexpression

RESUMEN

La resistencia de las malas hierbas a los herbicidas afecta a los principales países productores de arroz de la Península Ibérica. La excesiva dependencia de herbicidas inhibidores de la acetolactato sintasa (ALS) y de la acetil-CoA carboxilasa (ACCasa), así como la escasa diversidad de prácticas culturales han aumentado la presión de selección hacia poblaciones resistentes. Últimamente, también se ha observado la aparición de malas hierbas con resistencia múltiple. En la Península Ibérica, diferentes poblaciones de *Echinochloa* spp. han mostrado resistencia a penoxsulam y profoxidim. El objetivo principal de este trabajo es el estudio de los mecanismos implicados en la resistencia presente en estas poblaciones, mediante secuenciación de los genes *ALS* y *ACCasa* y estudios de expresión génica mediante qRT-PCR. Se incluyeron 3 poblaciones de *Echinochloa* sp. de Portugal y 7 de España. La resistencia a los herbicidas se confirmó previamente mediante ensayos dosis-respuesta. No se encontró en ninguna población las mutaciones ya descritas como implicadas en la resistencia a los inhibidores de ALS y ACCasa. Sin embargo, en tres accesiones portuguesas resistentes, se encontraron mutaciones en el gen ACCasa que causan sustituciones de aminoácidos en la proteína, pero aún no se ha confirmado que confieran resistencia a herbicidas. También se evaluó la expresión relativa de los genes *ALS1* y *ACCasa*. Nuestro estudio revela la existencia de diferentes niveles de sobreexpresión entre accesiones y diferencias entre raíces y hojas dentro de la misma accesión. Estos resultados son indicativos de mecanismos de resistencia tipo *target site*, basados en la sobreexpresión génica.

Palabras-clave: resistencia múltiple, ALS, ACCasa, sobreexpresión

INTRODUCTION

One of the major weeds encountered by rice growers across the globe is *Echinochloa* spp. These Poaceae species are polyploid and C4 plants what makes them very competitive against rice. *Ehinochloa crus-galli* (L.) P. Beauv. can remove 60 to 80% of the available nitrogen in the soil, especially in the first phase of the vegetative cycle. The presence of 9 plants per m^2 is enough to cause a reduction in crop density of around 57 % (Maun and Barret, 1986). It also has a high seed production capacity (Norris, 1992), which results in a significant seed bank. Despite the success of herbicides in managing these weeds, the rice fields in Portugal and Spain are still dominated by *Echinochloa phyllopogon* (Stapf) Stapf ex Kossenko (Carretero, 1981; Vasconcelos *et al*., 2021). Moreover, the constant use of the same herbicides has turned the control of these species increasingly difficult due to the evolving of herbicide resistance mainly to the ACCase- and ALS- inhibitor herbicides, the most widely used (Heap, 2023). Current occurrences observed in Portugal and Spain (Torra *et al.,* 2022; Calha *et al*., 2023) suggested potential cross-resistance between herbicides characterized by distinct modes of action. These instances indicate the possible involvement of diverse resistance mechanisms. Knowledge of the resistance mechanism is a fundamental tool for defining management strategies for *Echinochloa* spp. populations and reducing the impact of herbicides on the environment. In metabolic resistance, the cytochrome P450 monooxygenase complex (CitP450) plays a major role in the detoxification of both mode of actions (HRAC 1 and 2). Multiple resistance, where more than one mechanism of resistance is present in the same individual could be due to mutations in several gene(s), causing insensitivity in both ALS and ACCase enzymes, for instance (Riar *et al*., 2013). A very common resistance mechanism is substitution of a nucleotide in the genes encoding herbicide target sites leading to an amino acid substitution in the resulting protein. The conformational alteration in the binding site leads to the insensitivity of the protein for this herbicide (Powles & Yu, 2010). Other resistance mechanism is associated with gene copy number. For instance, some occurrences in *Echinochloa* spp. possess duplicated copies of *ACCase* genes (Iwakami *et al*., 2012, 2015). Gene expression studies have played a significant role in clarifying the relative level of expression of target genes linked to herbicide resistance. qRT-PCR is one of the most used strategies to compare gene expression. The $2^{-\Delta\Delta Ct}$ method has been extensively used as a relative quantification strategy between different samples (Schmittgen & Livak, 2008).

In the present study, we explored target site resistance mechanisms including gene expression for *ALS* and *ACCse* genes in populations of *Echinochloa* spp. from Portugal and Spain.

MATERIAL AND METHODS

Plant Material

Mature seeds from 10 populations of *Echinochloa* spp., were selected from three rice fields in Alcácer-do-Sal (South Portugal) and seven from Extremadura (Spain) that had been confirmed as highly resistant (RRR) by dose-response assays (Ibañez *et al*., 2019; Calha *et al*., 2023). Table 1 shows the origin of each population, the applied treatment and the resistance/susceptibility profile.

Table 1 - Resistance profiles to ALS and ACCase-inhibitor herbicides of *Echinochloa* sp.populations, from Portugal (PT) and Spain (SP), and exposure to herbicide solutions in bioassay

Species	Populations (R/S)	Treatment	
		Penoxsulam (5mg.L^{-1})	Profoxydim (5mg.L^{-1})
Echinochloa phyllopogon (PT)	A01-18 (S)	x	x
	A06-18 (R); A07-18 (R)	x	x
Echinochloa spp. (SP)	16-21 (R); Ech 5 (S) Ech 3 (S)	x	x
	1-16 (R); 19-18 (R)	x	-
	Ech 7 (R); Ech 9 (R)	-	x

Resistant (R) and Susceptible (S) populations

Incubation

Seeds were stored at 3 ºC in a dry atmosphere, in the dark until they were used. Twenty pre-treated seeds (chemical scarification with H_2SO_4) from each population were prepared in Falcon tubes for incubation in an herbicide solution (5 mg L^{-1} of penoxsulam or profoxydim) in a growth chamber (22± 1 ºC / 18 ± 1 ºC, 16 h light, 250- 300 µE m^{-2} s^{-1} PAR) for 24 h. A control without herbicide was used per population (Table 1). Susceptible populations and those with cross-resistance were exposed to both herbicides; Populations resistant to only one herbicide were exclusively exposed to the corresponding herbicide.

ALS and ACCase gene sequencing

DNA isolation - Genomic DNA was extracted from leaves from both resistant and susceptible populations using the DNeasy Plant Mini Kit (Qiagen, Germany). Three plants from each population were analysed independently. For each plant, a pool of three leaves was grinded with N_2 (liquid) and three subsamples of 100 mg were taken for DNA extraction; **Sequencing** - To assess the nucleotide composition of the *ALS* and *ACCase* genes, PCR was conducted with primers flanking the regions previously identified as harbouring mutations linked to herbicide resistance (Table 2) and a proofreading Taq polymerase (Supreme NZYTaq II, NZYTech, Portugal). The amplicons were treated with ExoSAP-IT™ (Applied Biosystems, NL) to remove the excess of PCR reagents and sent for sequencing to the Molecular Biology laboratory of INIAV (Portugal).

Gene expression analysis by qRT-PCR

RNA isolation - Total RNA was extracted from leaf sheaths and oots of the same plants used in the incubation assay, using the RNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer recommendations. Purification was achieved through the digestion with PerfeCta DNase I (Quanta, Beverly, MA), to eliminate completely any potential genomic DNA contamination. The resulting RNA was immediately stored at -80 °C until further use. **cDNA synthesis and amplification** - Total RNAs (230 ng) underwent reverse transcription into cDNAs and these cDNAs were subsequently amplified in triplicates using FastGene ICGreen One Step Mix (Nippon Genetics, Europe GmbH) following the manufacturer's instructions. The transcription and amplification reactions were conducted in a final volume of 20 µL, containing 1 µL of template RNA, 10 µL of 2x Fast Gene IC Green One Step Mix (Nippon Genetics, Europe), 1 µL of 20x FastGene Scriptase, and 0.8 µL of each primer (10 µM). The reactions were carried out in a qTower® Real-Time Thermal Cycler (Analytik Jena, Jena, Germany) with the following conditions: cycle included an initial transcription step at 45°C for 10 minutes, followed by a general denaturation step at 95 °C for 2 minutes, and 40 cycles of 95 °C for 5 seconds and annealing/polymerisation at 57 °C for 20 seconds with the measurement of fluorescence after the polymerisation. Melting curves of

Table 2 - Primers used for the ALS1 and ACCase and genes and their known mutations

Gene	Primer	Sequence (5'-3')	Position/Known mutation (*Alopecurus myosuroides*)	Referência
ALS1	ALS1-F	CACCCCCACGCACAAATG		Iwakami *et al.* (2011)
	ALS1-R	AAGCTACTTAAGATTACCATACCAGAGT		
ACCase	ACCP1	CAACTCTGGTGCTCGGATTGGCA	1781/Ile-309-Leu	
	ACCP1R	GAACATAGCTGAGCCACCTCAATATATT		
	CNACCaseF	AGAGCTGGATCATTTGGCCC		This study
	ACCP4	CAGCTTGATTCCCATGAGCGATC	2027/Trp-109-Cys 2041/Ile-151-Asn 2078/Asp-262-Gly 2088/Cys-292-Arg 2096/Gly-316-Ala	
	ACCP2R	CCATGCAGTCTTGGAGTTCCTCTGA		
β-actin	β-actin-F1	CGGAGAATAGCATGAGGAAGTG		Laforest *et al.* (2017)
	β-actin-R1	AGTGGTCGAACAACTGGTATTG		

the amplicons were obtained with temperatures ranging from 55 °C to 95 °C with a 0.1 ºC increase in temperature every second. A positive amplification was obtained when a sigmoidal curve was obtained and the derivative of the melting curve had a pick at the expected melting temperature of the amplicon. **Relative gene expression** - Estimation of *ALS1* and *ACCase* gene expression was achieved through relative quantitation by means of real-time PCR. The amplification fold change was estimated by double delta Ct analysis (Schmittgen & Livak, 2008) using the β-actine gene as a reference assay as this is a single copy gene in the *Echinochloa* genome (Iwakami *et al.,* 2015). Amplification efficiencies for both genes in all populations (albeit herbicide exposure in the bioassay) were determined to ensure identical cycling performance during real-time PCR: Fold change = $2^{-\Delta\Delta Ct}$

RESULTS AND DISCUSSION

ALS and ACCase gene sequencing

The mutations already described as being involved in resistance to ALS and ACCase inhibitors were not found in any population. However, in three resistant Portuguese accessions, mutations were found in the *ACCase* gene that causes amino acid substitutions in the protein, but have not yet been confirmed as conferring herbicide resistance: Arg-498-Trp, Tir-507-Ser, Asp-580-Asn, Leu-68-Ser, Tir-170-His, Ile-173-Leu, Val-273-Leu, Gli-297-Pro, Ser-270-Fen, Val-276-Ala, Tre-366-Pro, Tir-408-His, Asn-483-Tre, Ser-513-Pro, Met-519-Leu, Ile-540-Leu, Asp-136-Asn, Fen-142-Cis, Glu-157-Arg and Arg-358-Lis. It was concluded that none of the already known mutations are governing the confirmed resistance in the *Echinochloa* populations. This finding has already been reported in other studies with *Echinochloa phyllopogon* (Iwakami *et al*., 2012).

Gene expression by qRT-PCR

To evaluate the effect of the treatments on the expression of the internal control gene, the mean Ct from replicate runs of β-actine gene of the treated samples was compared with the mean Ct value of the control samples. The fold change in the internal control in the treated samples compared to the untreated was 1.08. The herbicide treatment did not have an effect on the expression of the internal control, β-actine gene. The relative expression of *ALS1* and *ACCase* genes were also assessed in the populations at each herbicide treatment, penoxsulam and profoxydim, respectively (Table 3).

The expected results were observed in the susceptible populations (A1_18, Ech 5 and Ech3) with no overexpression detected in either gene, except for Ech3, which exhibited a 60-fold increase in the *ALS1* gene when exposed to penoxsulam. Overexpression of the *ALS1* gene (fold change > 1) was observed in populations 16_21; A7_18; ECh3 and

Table 3 - Fold change ($2^{-\Delta\Delta Ct}$) variation in the expression of both *ALS* and *ACCase* genes in the roots of populations treated with penoxsulam and profoxydim, respectively

Treatment	penoxsulam		profoxydim	
gene	*ALS1*	*ACCase*	*ALS*	*ACCase*
A1-18	0.74	0.03	0.45	0.103
A6_18	0.42	0.01	0.20	0.0003
A7_18	26.20	487.8	7.89	0.257
Ech3	61.82	0.001	2.05	0.301
Ech5	0.015	0.06	0.21	0.340
Ech7	-	-	13.93	3.14
Ech9	-	-	1.8	1.2
	-	-	85.04	15.15*
16_21	4.07	551	4.6	1156
01_16	6.18	168.31	-	-
19_18	0.06	0.008	-	-

* shoots

01_16 following exposure to penoxsulam. However, this mechanism was also present in populations exposed to profoxydim, particularly Ech7 and 16_21. Overexpression of *ACCase* gene was present in all R populations; Ech 7; 16_21; 01_16; A7_18; and Ech9 after 24 h exposure to profoxidym. Interestingly, higher values of overexpression were even observed in populations exposed to penoxsulam, 01_16; A7_18 and 16_21, which was not expected. Our study unveils varying degrees of overexpression among accessions resistant to ALS-inhibiting. Populations showing resistance to the ALS-inhibiting herbicide penoxsulam (A07-18, 16-21 and 1-16) also exhibit high overexpression of the *ACCase* gene although varying considerably (fold change from 168 to 551). However, these populations did not show resistance to profoxydim when applied independently, but population 16-21 showed a high overexpression of *ACCase* gene when profoxydim was used. These results are indicative of populations with cross-resistance between penoxsulam and profoxydim, mainly after the application of penoxsulam, what can result from the herbicide selection pressure imposed by the repeated application of both herbicides in rice fields.Our study confirmed target site type resistance mechanisms based on gene overexpression.

CONCLUSION

Our study reveals the existence of different levels of overexpression between accessions of *Echinochloa* spp. with different resistant profile, resistant to penoxsulam or to profoxydim only or to both herbicides. These results are indicative of target site type resistance mechanisms based on gene overexpression. The resistant plants contain 4-758 fold more copies of transcripts of the *ALS1* gene and 2-103 of *ACCAse* gene than the susceptible plants. However, the overexpression of *ALS1* gene after exposure to profoxydim, was unexpected and requires further studies to explain these results at the physiological and genetic levels. The occurrence of gene amplification as an herbicide resistance mechanism in naturally occurring weed populations was reported for the first time in the Portuguese and Spanish populations of *Echinochloa* spp.

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