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Occurrence of Resistance to ALS Inhibitors in European *Cyperus esculentus* L.: Characterisation and Implications for Management

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Abstract: Yellow nutsedge (*C. esculentus*) is a perennial geophyte and invasive weed which is very difficult to control in rice and other irrigated row crops. Acetolactate synthase (ALS) inhibitors are the most commonly used herbicides to control sedges in rice. Failure to control *C. esculentus* was recently reported in a rice field in north-western Italy. The resistance status of this *C. esculentus* population was determined through a whole-plant bioassay. The mechanism underlying the resistance was elucidated, and the available chemical and non-chemical control options were discussed. The population proved to be resistant to halosulfuron and azimsulfuron at the recommended field rate. The *ALS* trancripts amplified from resistant and susceptible plants revealed the presence of a Pro_{197}-to-Arg amino acid substitution in resistant plants, indicating that the resistance mechanism is target-site mediated. This is the first confirmation of herbicide resistance in *C. esculentus* in Europe. Resistance management should be based on an integrated approach, through the combination of diversified cultural and agronomic practices that can limit its spread and propagation through tubers.

Keywords: yellow nutsedge; invasive weed; tubers; ALS resistance; control strategies; herbicide; integrated weed management; target site resistance

1. Introduction

Cyperus esculentus L. (yellow nutsedge) is an emerging weed of increasing importance in spring-sown crops. It is now listed as an invasive plant species in Europe with a high potential threat to the environment and biodiversity (EPPO list of invasive plant species) [1]. Yellow nutsedge has a C_4 photosynthetic pathway [2], it is an obligate outcrosser and polyploid, although the number of chromosomes has not yet been defined for European accessions [3]. It is a perennial geophyte that produces tubers, rhizomes and stolons. These latter degenerate after formation of the tubers, while rhizomes are persistent organs with storage and vegetative reproduction functions [4,5]. The tubers are ovoid, subglobose, whitish when they are young and reddish-brown later. They can be dormant for several years and a single plant can produce thousands of tubers [6–8]. These underground organs can be affected by low temperatures and periods of frost, which reduce the number able to sprout in the following growing season [3].

Yellow nutsedge can also produce a large number of seeds, but the fate of seedlings in cultivated areas varies depending on the conditions that influence their viability, germination and growth [3,9]. It is generally accepted that seeds are less important than tubers for the propagation of this species [3].

Yellow nutsedge is distributed worldwide in areas with humid tropical to warm temperate climates. It is currently widespread in southern, western and parts of central Europe [3]. In Italy, it is present in several regions along the Po river, as well as in some central and southern areas [10,11].

Until recent years it was confined to crop field margins but it is now expanding in cultivated areas, mainly rice and irrigated row crops (e.g., maize) of north-western Italy and in some cases it has rapidly become the dominant weed species [12]. In Switzerland, yellow nutsedge appeared in regions with intensive vegetable production and then expanded rapidly outside of those regions, likely because *C. esculentus* was able to overcome the techniques used to control it [13].

Traditional rice cropping systems in Italy are characterised by widespread monoculture and water-seeding. However, in recent years there has been an increased adoption of dry-seeding in areas where water supply is not continuous. The reduced availability of water has led to the colonization and spread in rice fields of some weed species such as *C. esculentus* that have spread rapidly in areas where dry seeding has been adopted. Crop rotation with maize and soybean is sometime practiced to better control complex and very competitive weed communities, often including herbicide resistant populations [14].

Complexity of weed control in rice is exacerbated by the decreased number of available herbicides due to the strict EU legislation. The available pre-emergence and post-emergence herbicides provide various options but the registered products frequently only provide transient suppression [15,16]. In the last decade the most used herbicides to control *C. esculentus*, as well as other sedges and weeds in rice, are two acetolactate synthase (ALS) inhibitors, azimsulfuron and halosulfuron, as they guarantee a high level of control. However, the high reliance on herbicides with this site of action has led to the evolution of several ALS-resistant weed species in Italian rice: *Alisma plantago-aquatica* L. (common water-plantain), *Schoenoplectus mucronatus* (L.) Palla (ricefield bulrush), *Cyperus difformis* L. (smallflower umbrella sedge), *Echinochloa* spp., *Oryza sativa* L. var. *sylvatica* (weedy rice) and recently *Ammania coccinea* Rottb.(valley redstem) [17–19].

ALS inhibitors have proved to be most prone to evolve resistance [20,21] with resistance cases reported in 165 weed species to date [22]. Resistance to ALS inhibitors is often due to mutations in the ALS gene that decrease the affinity of the ALS enzyme for herbicides (target-site resistance) [23]. The ALS resistance cases reported to date are due to amino acid substitution at 8 different codons of the ALS sequence Ala_{122}, Pro_{197}, Ala_{205} Asp_{376}, Arg_{377}, Trp_{574}, Ser_{653} or Gly_{654} (the position is referred to the *Arabidopsis thaliana* L. (Heynh.) sequence) [22,24].So far, resistance to ALS inhibitors in *C. esculentus* has been reported only once in the USA in Arkansas [25]. This biotype was resistant to halosulfuron and the resistance was attributed to a Trp_{574}-to-Leu substitution in the ALS gene, pointing out that the main resistance mechanism is target-site mediated.

In 2017, a population of yellow nutsedge from Vercelli (north-western Italy) was not adequately controlled by halosulfuron at the recommended field dose. The objectives of this study were (1) to confirm the resistance of the *C. esculentus* population to halosulfuron and investigate the resistance pattern to azimsulfuron; (2) to determine the resistance mechanism involved, in particular to verify the possible target-site resistant mechanism involved and (3) to discuss the management of ALS-resistant *C. esculentus*.

2. Materials and Methods

2.1. Plant Material

Tubers from *C. esculentus* plants that had survived a treatment with halosulfuron were collected from rice fields in the Vercelli province during September 2017 (population 17-2). The field presented a patchy infestation and had been treated with ALS inhibitors for at least 3 years. A susceptible population (reference) was also collected from an area never treated with herbicides on a farm near Vercelli (population 17-3). Tubers were put in pots (30 cm diameter × 27 cm height) containing a mix composed of 50% silty loam soil and 50% peat and placed in a greenhouse at Legnaro, north-eastern Italy (45°21′ N, 11°58′ E). Experiments were conducted during the September-October period and plants were light supplemented using 400 W metal-halide lamps, which provided a Photosynthetic Photon Flux Density (PPFD) of about 400 μmol m^{-2} s^{-1} and a 16 h photoperiod. Average minimum and

maximum temperatures were 17/29 °C and 17/28 °C for the first and second experiment, respectively. Young sprouts at 2–3 leaf stage were then transplanted into plastic trays (32.5 × 26.5 × 9.5 cm) containing a non–sterile potting mix (60% silty loam soil, 15% sand, 15% perlite and 10% peat).

2.2. Whole-Plant Bioassay

A whole-plant bioassay was performed to confirm the resistant and susceptible status of both populations. Experimental layout was a complete randomized design with two trays (replicates) with 15 plants for each herbicide treatment. When the young sprouts reached the 5–6 leaf stage, herbicides were applied using a precision bench sprayer delivering 300 L ha^{-1} at a pressure of 215 kPa and speed of about 0.75 ms^{-1}, with a boom equipped with three flat-fan (extended range) hydraulic nozzles (Teejet®®, 11002). Plants of both populations were sprayed with halosulfuron methyl at the recommended field rate, 30 g a.i. ha^{-1} (Permit®®, 75%, WG, Nissan Chemical Europe, Auvergne-Rhône-Alpes, France) and with azimsulfuron at 22.5 g a.i. ha^{-1} (Gulliver®®, 50%, WG, Cheminova Agro, Bergamo, Italia) in addition to the recommended surfactant.

Four weeks after herbicide application, the number of surviving plants in each tray was counted along with a visual estimation of the biomass (VEB). The VEB scores were assessed using a scale of 0 to 10, where 0 was given to trays where plants were all killed and 10 when plants were not affected by the herbicide, i.e., they looked similar to the untreated control trays. Standard error (SE) was calculated for each data mean and the experiment was repeated. A *t*-test ($p < 0.05$) was performed to identify significant differences between the two experiments.

2.3. ALS Gene Assembly and Primer Design

To identify potential target-site mutations in resistant *C. esculentus* (R, isolated from 17-2 population) compared to susceptible (S, isolated from 17-3 population) plants, the ALS gene sequence accessions of five *Cyperus* sp. were downloaded from National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) database. Extracted ALS genomic and expressed partial sequences were retrieved and subjected by alignment to generate a consensus sequence of 1730 bp using Geneious and Clustal Omega tools (http://www.ebi.ac.uk/Tools/msa/clustalo/). The accession numbers and relative properties of all the sequences considered in this study are reported in Table 1. After in silico sequence translation and following alignment of obtained protein sequences, different pairs of primers were designed to amplify portions of the ALS gene sequence, including the main conserved domains among different specific species sequences. The sequences and relative coordinates of designed primers on consensus sequence are reported in Table 2.

Table 1. Accession numbers of five ALS sequences used for the alignment, downloaded from NCBI website on March 2019 and the following obtaining of consensus sequence.

NCBI Accession Number.	Weed Species	Sequence Length (bp)	Position Referred to Consensus Sequence (from 1 to 1730 bp)	% Similarity With Consensus Sequence	Identical Sites (%)
KM624613.1	*Cyperus esculentus*	716	from 958 to 1673	94.4%	640 bp (94%)
EF061294.2	*Cyperus difformis*	1709	from 22 to 1730	94.7%	1606 bp (94%)
KM235318.1	*Cyperus difformis*	1706	from 1 to 1706	94.7%	1603 bp (94%)
KT150718.1	*Cyperus compressus*	668	from 995 to 1662	94.4%	595 bp (89.1%)
KT150720.1	*Cyperus compressus*	668	from 995 to 1662	94.4%	595 bp (89.1%)

Table 2. Primers sequences, and relative coordinates in consensus sequence, used for amplification and sequencing of ALS target fragments.

Primer	Sequence 5′ –> 3′	Position (bp) in Consensus Sequence (From-To)
1_Fw	CAGCACCAAATGTGGGC	1180–1196
1_Rev	TCATTGGCAAGACGTGCTC	1654–1675
2_Fw	CCTCGTCAGTGGCTATCCAG	1222–1241
2_Rev	TGCGGTACGATCACATCC	1632–1649
3_Fw	GCCACTTCAGGCCGTC	445–460
3_Rev	GAGCGACTAGCAAATGCCTC	868–887
4_Fw	CCTCGCATCATCAAAGAGGC	415–434
4_Rev	CAGTATTTGGTTCATGCCTTGC	978–992
5_Fw	GATGTTCTCGTTGAGGTTCTCGAGAG	22–47
6_Fw	ATGTCTTCGCCTATCCAGGCGGAGC	62–86
5_Rev	GAC GGC CTG AAG TGG C	445–460

2.4. ALS Gene Expression Analyses and Sequencing

The RNA extraction procedure for ALS gene expression determination, and its subsequent transcript sequencing, was performed considering two susceptible and seven resistant plants collected separately from 17-3 and 17-2 populations, defined S1 and S2 and R1, R2, R3, R4, R5, R6 and R7, respectively. Total RNA was extracted from 100 mg of young leaf tissue (one leaf/per plant) using an InviTrap plant RNA Mini Kit (Stratec Biomedical AG, Birkenfeld, Germany) and subjected to an additional step on column DNase I (RNase free) treatment (Takara Bio Group, Saint-Germain-en-Laye, France) introduced before the last washing step and elution. Nucleic acid concentration was measured at 260 nm using a NanoDrop 2000c Spectrophotometer (NanoDrop Products, Waltham, MA, USA).

Complementary DNA (cDNA) synthesis was performed with an ImProm II reverse transcriptase kit (Promega, Madison, WI, USA), according to the manufacturer's instructions but with some step modifications: 500 ng of target RNA and 0.5 µg of oligo dT15 were mixed with nuclease free water to a final volume of 20.5 µL. Samples were incubated for 5 min at 70 °C and then quick-chilled for 5 min on ice. After this denaturation step, the reaction mix (2.25 mM MgCl2, 0.5 mM dNTP mix, 1 µL of Improm-II Reverse Transcriptase, 1× concentration of supplied buffer, and nuclease free water to a final volume of 19.5 µL) was added to each sample and they were placed in a T1 plus Thermocycler 96 (Biometra, Göttingen, Germany) for 5 min at 25 °C, 1 h at 42 °C, and 15 min at 70 °C.

Genbank ALS coding sequences from other Cyperaceae species were aligned and primers targeting conserved regions in the alignment were designed manually. The sequence of forward and reverse primers used in following PCR reactions is reported in Table 2.

PCR amplifications were performed using a GoTaq DNA Polymerase kit (Promega, USA) in a 25 µL mixture including 5 µL of 5× Colorless GoTaq Flexi Buffer, 5% DMSO, dNTPs mix (200 µM each), MgCl2 (1 mM), forward and reverse primers (0.4 µM each), 0.2 µL GoTaq DNA Polymerase, and 1ul of cDNA t.q. The thermocycler programme was as follows: 95 °C for 6 min; 35 cycles of 95 °C for 60 s, 58 °C for 30 s, 72 °C for 40 s; 72 °C for 10 min. PCR products were purified with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co., Düren, Germany) following the manufacturer's instructions. Once purified, PCR products obtained from each plant were sequenced by BMR Genomics (Padova, Italy). The sequences were retrieved and a multi-alignment was done using Geneious tool.

3. Results and Discussion

3.1. Herbicide Cross-Resistance

No significant differences were recorded in survival rate and VEB values between the first and second experiment (*t*-test with $\alpha = 0.05$), therefore the data were pooled and analysed. The susceptible reference population (17-3) was totally controlled by both ALS-inhibitors, halosulfuron and azimsulfuron, at the recommended field rate. Contrary, all plants of population 17-2 survived the treatment with either halosulfuron and azimsulfuron, and their biomass was similar to the untreated control plants (Figure 1). This result confirms that population 17-2 is highly resistant to halosulfuron and azimsulfuron.

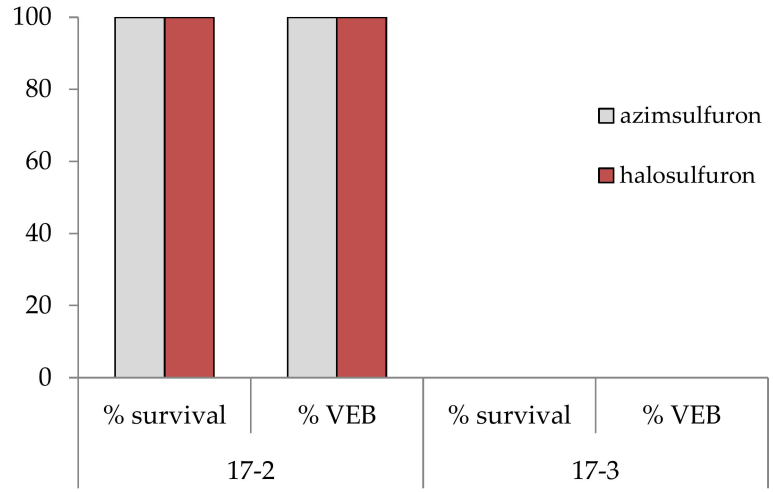

Figure 1. Whole plant bioassay conducted on *Cyperus esculentus* populations (17-2 and 17-3 at Legnaro (North Italy) in autumn 2017. Percentage of plant survival and visual estimation biomass (VEB) four weeks after treatment with two ALS-inhibiting herbicides applied in post-emergence.

This is the first documented case of *C. esculentus* resistant to ALS inhibitors in Europe. Previously, one case of yellow nutsedge resistant to ALS inhibitors was reported in rice fields in the USA (Arkansas) and that biotype similarly resulted highly cross-resistant to halosulfuron and other ALS inhibitors [25]. Halosulfuron represents the key herbicide to control yellow nutsedge in rice because of its great efficacy to control and limit tuber production [26]. Other chemical options are less effective and are often used in mixture to exploit the additive effect of multiple active ingredients [15,16].

3.2. ALS Gene Mutation

Partial coding sequences of ALS transcripts were obtained and assembled for both susceptible and resistant plants. The amplified fragments obtained with primers 6_Fw and 5_Rev encompassed the codons 122 and 197; with primers 3_Fw and 4_Rev they encompassed the codons 376, and finally with primers 1_Fw and 1_Rev they encompassed the codons 574.

Regarding the investigation of polymorphic allele variations, alignments of the nucleotide sequence, before, and in silico translated amino acid sequence, after, revealed no differences between R and S biotypes in the putative mutation point and along the entire length of the fragment. The only allele variation exception observed was detected at 242 nt position of the 321 sequenced nucleotides of the first fragment obtained with 6_Fw and 5_Rev, where a double peak was determined in all tested R biotypes: based on this evidence we can state that in R plants two different ALS transcript isoforms were present, responsible for a Pro_{197}-to-Arg amino acid substitution allelic variation due to a CGT change in the CCT codon. On the contrary, in S biotype, the sequencing at the same nt position revealed one unique peak, corresponding with Pro_{197} (corresponding to CCT codon) (Figure 2). The double

peaks observed at codon 197 can indicate the presence of an ALS isoform resulting from heterozygosity or homologous differences in a polyploid weed, either of which confer resistance to ALS inhibitors. In *Schoenoplectus mucronatus*, a polyploid weed species infesting Italian rice fields, mutations detected in one ALS transcript have been shown to determine ALS resistance [27].

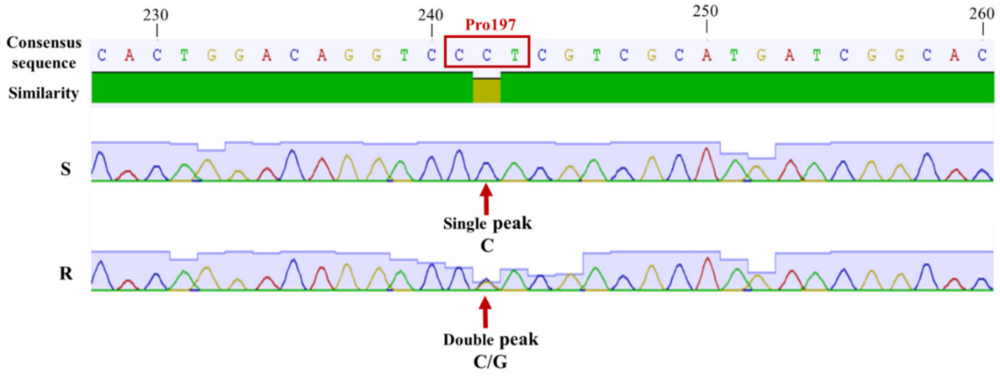

Figure 2. Schematic magnification of nucleotide sequence alignment of *C. esculentus* ALS sequenced fragments obtained from S and R biotype sequences around the Pro_{197} codon position in the red box. The chromatogram reveals the single peak corresponding to C in S biotype (at 242 nt position), while the double peak for C and G is evident for R biotype. The % level of similarity between the aligned sequence is indicated in light green.

The Pro_{197}-to-Arg substitution in the ALS gene of the R biotype confirmed the hypothesis that the target-site resistant amino acid substitution is the possible mechanism involved in ALS-inhibiting herbicide resistance in yellow nutsedge. Our result is different from the previous study considering *C. esculentus* where a Trp_{574}-to–Leu substitution was detected in an American biotype conferring resistance to ALS inhibitors [25]. The *ALS* mutated allele observed in our study has been reported in other weed species and in all cases it confers resistance to the sulfonylureas i.e., the herbicides tested in this work [22].

3.3. Implications for C. esculentus Management

The management of *C. esculentus*, especially resistant populations, should be based on an integrated control strategy aimed at significantly decreasing the selection pressure exerted by ALS herbicides, i.e., weed control tactics should be diversified in space and time [8]. The chemical options to control *C. esculentus* are very limited and include only two other herbicide modes of action for rice i.e., HRAC group G and C3 (Table 3). Glyphosate is the only product authorized to be used in pre-sowing (false seedbed preparation) and during the turnaround period. It can achieve a good control as it translocates into the tubers [16]. However, its efficacy can vary depending on the plant age, being more effective to control early sprouting of parent tubers [28]. S-metolachlor, a herbicide applied in pre-emergence and authorized in maize and soybean (Table 3) was reported to provide a good control of *C. esculentus* [29], especially when there is enough soil moisture [12]. Considering that in Italian rice growing areas the adoption of crop rotation with maize and soybean has increased, S-metolachlor can be considered a valid option. Post-emergence herbicides, other than ALS inhibitors, are generally less effective to control *C. esculentus*, however their use in combination with other herbicides mode of action and/or mechanical control is recommended to achieve a better control [30]. In USA, herbicides from different HRAC groups have been extensively studied to control *C. esculentus* and it was concluded that poor control is related to the limited absorption and translocation of the herbicide to the site of action, the dormancy and depth at which the tubers are located and environmental conditions [31]. It is worth mentioning that the active ingredients belonging to group O, i.e., MCPA and triclopyr, can have partial efficacy on *C. esculentus*.

Table 3. Active ingredients authorized in Italy in rice (R), maize (M) or soybean (S) effective to control ALS-resistant *C. esculentus*.

HRAC Group	Active Ingredient	Year of Registration	Application	Crop	References
G	glyphosate	1977	False seed bed, Pre-sowing, post-harvesting	R, M, S	[16]
C3	bentazon	1973	post-emergence	R, M, S	[29,31]
K3	S-metolachlor	2001	pre-emergence	M, S	[12,29,31]

HRAC stands for Herbicide Resistance Action Committee (https://hracglobal.com/tools/hrac-mode-of-action-classification-2020-map).

The chemical control of *C. esculentus* appears particularly difficult because only a few herbicides are able to devitalize its underground apparatus, therefore it is fundamental to treat when plants are at the early stage of growth and as soon as the first weed patches appear in the field.

To improve control of *C. esculentus*, non-chemical control options needs to be integrated with chemical treatments. Integrated weed management (IWM) allows to reduce the selection pressure imposed by herbicides, limiting the evolution of resistant weed populations as well as being more environmentally sustainable. Among the cultural options, the increase crop density can improve the crop competition. The shading caused by the crop and the rapid canopy closure can adversely affect the growth of *C. esculentus*, in particular maize and hemp can be good competitors for light [32,33]. Another important recommendation is to manage properly the turnaround period to prevent that *C. esculentus* plants continue to produce tubers until the end of the growth season. Being a perennial geophyte, it is believed that mechanical control can favor the propagation of tubers within the field and therefore would not be recommended. Instead, some studies have reported that frequent fallow tillage (monthly intervals), can bring the tubers closed to the soil surface where they can be devitalized [34]. It has been well established that low temperatures and periods of frosts can drastically reduce the sprouting of tubers during the following season [3,35].

To avoid the propagation of *C. esculentus* from other neighboring fields, it is recommended to frequently and carefully clean machinery and agricultural equipment. It has been suggested that yellow nutsedge seeds and propagules sticking to machines and wheels may have played an important role in the propagation of *C. esculentus* in Switzerland [13]. The increased spread of *C. esculentus* in Italian rice fields is associated to the increase in dry-seeding; therefore adopting the practice of water-seeding for a few years could limit its development as water submersion limits its establishment and spread.

In addition to cultural and mechanical control, physical option as solarization through the use of plastic mulches can limit the density of tubers. Johnson et al. 2007 reported a significant reduction in *C. esculentus* tuber density when a summer-long solarization was used [34]. However, solarization can affect only tubers that are located in the first 10 cm of soil depth [36]. This option may be a valuable tool especially in organic farming, where the control tactics against *C. esculentus* are even more limited in comparison to traditional farming.

This is the first confirmation of *C. esculentus* resistant to ALS inhibitors in Europe. The efficacy of chemical control in rice fields is at risk and needs to be integrated with cultural and agronomic practices to slow down the evolution and spread of herbicide resistance. The implementation of IWM should be a priority to manage *C. esculentus*, a weed that can potentially invade different cropping systems. Only the combination of diversified practices, in both space and time, can improve weed management.

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