CROSS-RESISTANCE TO ALL ALS-INHIBITING HERBICIDE GROUPS AND PHYLOGENETIC RELATIONSHIP OF ALS GENE IN *Cyperus difformis* L.

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Resistance (R) of *Cyperus difformis* L. (CYPDI, smallflower umbrella sedge) to ALS (acetolactate synthase)- inhibiting herbicides is a classical example of rapid evolution of herbicide-R. Only after four consecutive seasons of bensulfuron-methyl (ALS inhibitor) use in California (CA) rice areas, resistant biotypes of CYPDI were found in two rice fields of the Sacramento valley. After that, ALS-R in CYPDI had evolved in several fields all over CA rice area. Mechanism of resistance to ALS-inhibitors is described as target site for one biotype (Osuna et al. 2002). However, the mechanism of R in biotypes where additional patterns of cross-R occurs in not known. In addition, the specific mutation resulting in the target site R in CYPDI is not understood. There is no molecular information available regarding genes sequences for CYPDY or other *Cyperaceae* and the ploidy level for CIPDY is not known either. Molecular and crossing studies using the ALS–R as a marker indicate that this species is highly selfing (Merotto Jr., in press). Development of curative and preventive strategies for R management require knowledge of the cross-resistance patterns of ALS-resistant CYPDI, the mechanism of resistance, and the molecular basis of the resistance. Based on this knowledge, correct diagnostic of resistance evolution can be developed, and mainly, adequate herbicide usage programs can be projected for current or news ALS-inhibitors.

The objectives of this study were to: i) assess the cross-R patterns for all five ALS-inhibiting herbicide classes in CYPDI; ii) investigate the mechanism of R using biochemical and molecular approaches; iii) analyze the phylogenetic relationship of the ALS gene from CYPDI with that of other plant species reported in the literature.

Four CYPDI biotypes were used for the determination of cross-R patterns among ALS -inhibiting herbicides. Seeds from the IR biotype were collected in Northern Italy (Po Valley) and seeds from WA, BI and AS biotypes were collected in California's Northern Sacramento Valley. Cross-R to the ALS-inhibiting herbicides was performed at whole-plant level and through an "in vitro" ALS enzyme activity assay. The four-parameter logistic model was fitted to the data through the "drc" package from "R" program.

Leaf samples from the same plants used for the ALS activity assay were individually collected for total DNA extraction through the CTAB protocol and RNA extraction using the RNeasy Plant mini kit (Qiagen). Eighteen regular and degenerated oligonucleotide primers for polymerase chain reaction (PCR) amplification were designed based on the nucleotide sequences from ALS genes available in the GenBank database. Standard PCR amplifications were carried out in a total volume of 15 µl. A degenerated oligonucleotide primer produced a clear 551 bp correspondent to the expected fragment size. Sequencing of this fragment was done by the BigDye Terminator v3.1 Cycle sequencing in an ABI PRISM 3100 DNA Analyser. Two chromosome walking and a RACE (Rapid Amplification of cDNA Ends) procedures were conducted in order to obtain the remaining 5' sequence of the ALS gene. The partial sequences obtained with these fragments were arranged in an unified contig of 1709 bp (Figure 1) that has been deposited in the GenBank database under the accession number EF061294. A phylogenetic analysis was performed among the found CYPDI ALS gene sequence and the sequences used for primer designing based on a 350 bp using the PHYLIP software.

The herbicide cross-R evaluated at enzymatic level through the "in vivo" ALS assay indicated that the WA biotype was resistant to all herbicides tested. The IR biotype was susceptible to halosulfuron-methyl and moderately resistant to bispyribac-sodium and penoxsulam (Table 1). These results are in agreement with the whole-plant assay (Table 1). Resistance to ALS-inhibitors in biotypes IR and WA is due to a target site related

mechanism. The reduced sensitivity of their ALS enzyme may be due to a mutation on the ALS gene or to an over expression of this gene. The susceptibility to herbicide halosulfuron-methyl exhibited by the IR biotype suggests that ALS overexpression is not the R-mechanism.

Because the ALS "in vitro" assay indicated that a target site modification could be the cause of R in the WA and IR biotypes we attempted to isolate and sequence the ALS gene. Since no information was available regarding the DNA sequence of the ALS gene for CYPDI nor for any other *Cyperaceae*, a set of degenerated primers were designed. An analysis of the obtained sequence (Figure 1) corresponding to the "A" region of ALS gene shows a GC content of 71%, which could have caused of difficulties encountered in the amplification of this region. The ALS gene sequence obtained for CYPD in this study (Figure 1) corresponds to a 1709 bp coding region and is equivalent to the 609 to 2317 nucleotide position in the standard *Arabidopsis* ALS gene sequence X51514.

However, the nucleotide and amino acids sequences did not vary within the 4 biotypes analyzed. Therefore, is expected that more than one ALS gene is present in CYPDI because at least three different nucleotide sequences should be obtained considering the different pattern of herbicide cross-R found in the biotypes IR and WA and the wild-type AS. Several studies had also found multiple copies of ALS gene in a complex organization of herbicide R in diploid and polyploidy plant species (White et al., 2003; Kolkman et al., 2004; Warwick et al. (2005). The phylogenetic analysis among the obtained CYPDI ALS gene and several mono and dicotiledoneae species revealed that there is no similarity among these species (Figure 2). There is also no similarity between the obtained CYPDI ALS gene and those from species where more than one gene is present (data do not shown).

Target-site herbicide-R exists in CYPDI to all groups of ALS –inhibiting herbicides. Cross-R to all ALS inhibiting herbicides is variable among two CYPDI biotypes studied. This variability is possibly caused by different mutations of the ALS gene or different mechanisms of R. The obtained ALS gene of CYPDI is not homologous to *Poaceae* or to other dicotiledoneous species evaluated.

Literature cited

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Table 1 - Resistance index (RI, ratio of I_{50} values) relative to the wild-type AS biotype of three CYPDI biotypes based on the log-logistic equation of dose-response curves of CYPDY aboveground fresh-weight and ALS enzyme activity as a function of ALS -inhibiting herbicides.

Herbicide/Biotype	IR		WA		BI	
	Fresh-weight	ALS activity	Fresh-weight	ALS activity	Fresh-weight	ALS activity
Bensulfuron-methyl	15*	526.3*	10.01 *	552.8*	1.7*	2.3^{ns}
Imazethapyr	7.2*	46,0**	6.7*	11.1**	1.76*	0.7^{ns}
Bispyribac-Na	6.1*	4.7*	16.4*	25.9*	0.9^{ns}	1.7^{ns}
Penoxsulam	2.3*	7.5^{ns}	11.1*	354.4*	1.0^{ns}	3.6^{ns}
Propoxycarbazone-Na	17.6*	32.0**	19.4*	167.4**	1.1^{ns}	1.0^{ns}
Halosulfuron-methyl	1.3ns	2.0**	16.7 *	148.6*	0.6^{ns}	0.6^{ns}
IR5878	17.1*	10655**	22.0*	21000*	0.97^{ns}	3.0*

* significant to P<0.05; ** significant to P<0.01; ^{ns} no significative (P<0.05)

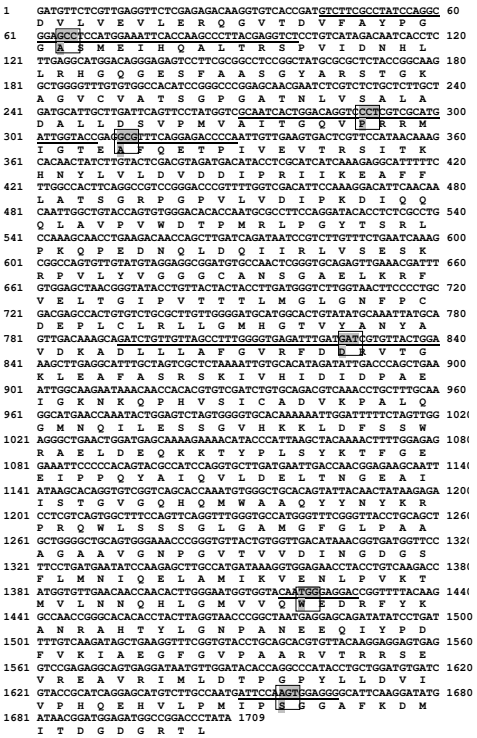

Figure 1 – Nucleotide and deduced amino acid of ALS gene form CYPDI. Underline sequence indicate highly conserved sequences of plants ALS gene. Bordered and shaded regions indicate the six point-mutation causing R to ALS inhibitors herbicides. GenBank accession number EF061294.

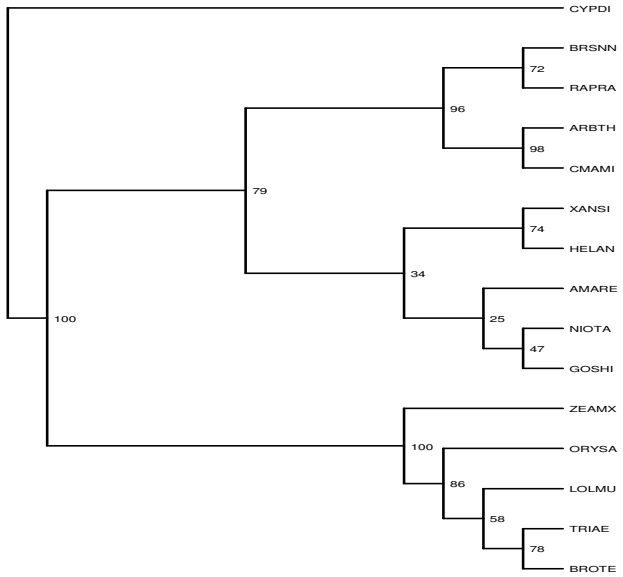

Figure 2 – Strict consensus phylogenetic tree of ALS gene flanking the region A where the domains C, A and D are located. Numbers refers to the percentage bootstrap support for the groups to the right.