Research Article

Received: 18 June 2012 Revised: 5 October 2012 Accepted article published: 29 October 2012 Published online in Wiley Online Library: 6 December 2012

(wileyonlinelibrary.com) DOI 10.1002/ps.3450

Target-site mutation associated with cross-resistance to ALS-inhibiting herbicides in late watergrass (*Echinochloa oryzicola* Vasing.)

Nikolaos S Kaloumenos,[a] Sofia L Chatzilazaridou,[b] Photini V Mylona,[c] Alexios N Polidoros[d] and Ilias G Eleftherohorinos[b]*

Abstract

BACKGROUND: Studies were carried out to elucidate the mechanism of resistance to ALS-inhibiting herbicides in 29 *Echinochloa* accessions from water-seeded rice fields of northern Greece and to discriminate the *Echinochloa* species.

RESULTS: Two *E. oryzicola* accessions were found to be cross-resistant to penoxsulam, bispyribac-sodium, imazamox, foramsulfuron, nicosulfuron and rimsulfuron, whereas all accessions were susceptible (S) to profoxydim. Sequencing of the *ALS* gene revealed that resistant (R) accessions had a Trp574Leu mutation, which was also confirmed by *Tsp*RI endonuclease digestion. Use of cpDNA sequence comparison analysis of *Echinochloa* species discriminated successfully *E. crus-galli* and *E. oryzicola* accessions.

CONCLUSION: This is the first report of *Echinochloa oryzicola* cross-resistance to ALS-inhibiting herbicides as a result of Trp574Leu mutation. The cpDNA sequence comparison analysis is a reliable tool for discrimination of conventionally classified *E. crus-galli* and *E. oryzicola* accessions.

Keywords: *ALS* mutation; target-site resistance; restriction digestion; *Echinochloa* species discrimination; penoxsulam resistance

1 INTRODUCTION

The genus *Echinochloa* comprises ~50 species that are among the most important weed species occurring in both tropical and temperate regions of the world.[1,2] Barnyardgrass [*Echinochloa crus-galli* (L.) Beauv.] is the most common weed species in a wide range of summer crops such as rice (*Oryza sativa* L.), maize (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), soybean (*Glycine max* L.), tobacco (*Nicotiana tabacum* L.) and sugar beet (*Beta vulgaris* L.), whereas late watergrass (*E. oryzicola* Vasing.) and early watergrass [*E. oryzoides* (Ard.) Fritsch.] are also considered to be serious weeds of water-seeded rice in many rice production areas.[3,4] As regards *E. oryzicola*, it has recently become a serious weed of rice fields in northern Greece.[5]

The taxonomy of the *Echinochloa* species is still poorly understood owing to their morphological similarity at the interspecific and intraspecific levels. Thus, *E. oryzicola* has been referred to as *E. phyllopogon* (Stapf) Stapf, *E. crus-galli* var. *oryzicola* (Vasinger) T. Koyama, tetraploid var. *oryzicola* and *E. oryzoides* (Ard.) Fritsch.[6] Although some of these taxa can be conventionally identified on the basis of their spikelet morphological traits and ecological habits, a significant amount of skill is required for their accurate identification, as the morphological features of the spikelet frequently overlap within and between species/taxa.[7]

Molecular techniques have offered many new tools for studying the evolutionary history and species divergence of plants, as well as for solving taxonomic confusion between species. More specifically, a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) method has proven very useful for discriminating *E. oryzicola* from *E. crus-galli* and *E. oryzoides* in Turkish accessions.[8] Also, chloroplast DNA (cpDNA) with a slow evolution rate and no recombination owing to the maternal inheritance of the organelle genome has been useful in examining relationships among different plant species.[9,10] Molecular analysis of specific regions of the cpDNA provided useful tools for species discrimination, analysis of phylogenetic relationships and divergence among different *Echinochloa* species.[7,11] Nucleotide sequencing of three non-coding regions (*trn*T-L, *trn*L-F intergenic spacers and *trn*L intron) of cpDNA in 30 accessions belonging to nine species of the genus *Echinochloa* provided information on phylogenetic relationships and new tools for species identification.[10] More specifically, sequence comparison between the different species revealed

* *Correspondence to: Ilias G Eleftherohorinos, Laboratory of Agronomy, School of Agriculture, Aristotle University of Thessaloniki, 541 24 Thessaloniki, Greece. E-mail: eleftero@agro.auth.gr*

a *Syngenta, Jealott's Hill International Research Centre, Bracknell, Berks, UK*

b *Laboratory of Agronomy, School of Agriculture, Aristotle University of Thessaloniki, Thessaloniki, Greece*

c *Agricultural Research Centre of Northern Greece, NAGREF, Greece*

d *Department of Genetics and Plant Breeding, School of Agriculture, Aristotle University of Thessaloniki, Thessaloniki, Greece*

that *E. oryzicola* and *E. phyllopogon* accessions displayed a single nucleotide repeat $(A)_{12}$ in the *trn*L intron between bases 464 and 484, while all other *Echinochloa* species had 5–6 A-repeats at the same position. Therefore, the cpDNA sequence comparison could be used for identification of morphologically classified *E. crus-galli* and *E. oryzicola* or *E. phyllopogon* accessions.

The herbicides propanil, pretilachlor, molinate, thiobencarb, quinclorac, clomazone, mefenacet, fentrazamide, oxadiazon, pendimethalin, bispyribac-sodium, penoxsulam, fenoxaprop-ethyl, cyhalofop-butyl and profoxydim control *Echinochloa* in rice effectively.[12–15] Additionally, the introduction of imidazolinone-resistant Clearfield™ rice cultivars makes selective control of *Echinochloa* and red rice (*Oryza sativa* L.) possible with the use of imazamox and imazethapyr.[16]

The repeated use of herbicides in rice grown globally has resulted in resistant (R) *Echinochloa* accessions to herbicides with different mechanisms of action.[17] For example, an *E. oryzicola* accession from rice grown in the Sacramento Valley of California has developed multiple resistance to the herbicides molinate, thiobencarb, clomazone, fenoxaprop-ethyl, bispyribac-sodium and penoxsulam.[18–20] In addition, *E. oryzoides* in Turkey has also developed multiple resistance to bensulfuron-methyl, bispyribac-sodium, cyhalofop-butyl and penoxsulam.[17,21]

The herbicides bispyribac-sodium, penoxsulam and imazamox are acetolactate synthase (ALS; EC 2.2.1.6)-inhibiting herbicides and have a propensity rapidly to select for R weed accessions.[22] This is confirmed by the fact that 126 weed species have already developed R accessions to ALS-inhibiting herbicides.[17] In most cases, resistance is due to point mutations in one of the following codons: Ala122, Pro197, Ala205, Asp376, Arg377, Trp574, Ser653 and Gly654, numbered on the basis of the sequence of *Arabidopsis thaliana* (L.) Heyhn.[23] However, in some cases, such as that of *E. oryzicola*, cross-resistance to bispyribac-sodium and penoxsulam was due to herbicide metabolism via cytochrome P450 monooxygenases (EC 1.14.14.1, P450s).[19,20,24–26]

During the 2008 and 2009 growing periods, rice growers from northern Greece (counties of Thessaloniki and Serres) noticed unsatisfactory *Echinochloa* control after the application of the recommended rates of penoxsulam. Although herbicide failure can be attributed to weed growth stage, herbicide application or environmental conditions, when these factors are eliminated, resistance could explain the lack of satisfactory weed control. Therefore, whole-plant response studies were conducted to determine whether the reduced *Echinochloa* control in the water-seeded rice fields of northern Greece was due to the evolution of resistance to ALS-inhibiting herbicides. Additionally, molecular studies were carried out to elucidate the mechanism of resistance to ALS-inhibiting herbicides and to classify the evaluated *Echinochloa* accessions in species.

2 MATERIALS AND METHODS

2.1 Seed source and plant material

A roadside survey was conducted during the summer of 2009 in rice monoculture fields located in northern Greece.[27] The purpose of the survey was to locate and mark fields with poor control of *Echinochloa* spp. after the application of penoxsulam. Therefore, a total of 112 fields (92 in Thessaloniki and 20 in Serres) with poor control of *Echinochloa* spp. were surveyed, and mature seeds, before rice harvest, were collected from 23 fields located in Thessaloniki and from five fields located in Serres. In particular, 17 *E. oryzicola* and 11 Pollacci barnyardgrass [*E. erecta* (Pollacci) Pign.] samples were collected from the rice fields. Additionally, one *E. crus-galli* sample (accession A06) was collected from a rice field margin located in Thessaloniki, as surviving plants were not found in the rice fields surveyed. During seed collection, care was taken to obtain a representative sample from each field, which was labelled as separate accession. The collected *Echinochloa* seeds were initially placed in big plastic bags. Afterwards, the seeds were transferred to the laboratory, where they were air dried, threshed, placed in plastic bags and stored at 3–5 °C for further use in the following experiments. Seeds from an *E. crus-galli* accession susceptible to ALS- and ACCase-inhibiting herbicides were purchased from Herbiseed (Twyford, Berks, UK) and used as S-control (accession A05). During the 2011 summer period, plant material (leaf blades) was collected from *E. oryzicola* plants that had survived in two rice fields (not sampled before) after penoxsulam or imazamox treatment respectively. The collected plant material during the 2011 summer survey was subjected only to genomic DNA extraction, which was used for *ALS* gene sequencing and restriction digestion analysis.

2.2 Whole-plant response

In three separate experiments, 29 *Echinochloa* accessions were screened to identify possible R accessions to penoxsulam, bispyribac-sodium and profoxydim, and five selected accessions were further evaluated for cross-resistance to imazamox and the sulfonylurea herbicides foramsulfuron, nicosulfuron and rimsulfuron. All whole-plant trials were carried out in plastic pots (9 by 9 by 10 cm) filled with soil:pit mixture 4:1 (v/v). In each pot, 15 seeds were placed on the surface and then covered with 1 cm of the same mixture. Fertilisation and irrigation were performed as needed to achieve vigorous plant growth. All pots were randomly placed in a net protected area at the University farm (Thessaloniki, northern Greece) and allowed to grow during the 2010 summer period. Herbicides were applied POST at the 3–5-leaf stage with a portable field plot sprayer (AZO-SPRAYERS, The Netherlands) using a 2.4 m wide boom fitted with six 8002 flat-fan nozzles (Teejet Spray System Co., Wheaton, IL) and calibrated to deliver 300 L ha^{-1} of water at 250 kPa pressure. Prior to herbicide treatment, seedlings were thinned to four per pot. The efficacy of each herbicide treatment on the *Echinochloa* accessions was evaluated 4 weeks after treatment (WAT) by recording the number of surviving plants and determining their above-ground fresh weight from each pot. Fresh weight data were then transformed to percentage of untreated control and used without further transformation in analysis of variance (ANOVA).

For each experiment conducted to evaluate resistance to penoxsulam, cross-resistance to imazamox or cross-resistance to sulfonylurea herbicides, a completely randomised experimental design was used with four replications (pots) for each herbicide treatment. All experiments included an untreated control treatment for each accession evaluated. Each experiment was performed twice, and ANOVA combined over experiment runs was performed because Bartlett's test indicated that the error variances of the data obtained from the two experiments (time replicated) were homogeneous. In addition, because the ANOVAs indicated no significant treatment by experiment run interaction, data were averaged over the experiment repetitions (experimental runs). Differences among treatment means were compared at the 5% level of significance using the LSD test.

2.3 Penoxsulam, bispyribac-sodium and profoxydim resistance

Twenty-nine *Echinochloa* accessions along with the S-control accession (A05) were tested for resistance to penoxsulam (Viper® 20.4 g L^{-1} OD; K+N Efthimiadis), cross-resistance to bispyribac-sodium (Adora® 400 g L^{-1} SC; Bayer Cropscience Hellas) and multiple resistance to profoxydim (Aura® 200 g L^{-1} EC; BASF Agro Hellas). The experiment was performed twice during the 2010 summer period. Penoxsulam was applied at 40 (recommended rate according to Greek herbicide label), 80 and 160 g AI ha^{-1}; bispyribac-sodium rates were 30 (recommended rate according to Greek herbicide label), 60 and 120 g AI ha^{-1}, and the respective rates of profoxydim were 200 (recommended rate according to Greek herbicide label), 400 and 800 g AI ha^{-1}. The data obtained from these experiments, as described previously, were analysed using a 3 by 3 by 30 (three herbicides by three herbicide rates by 30 *Echinochloa* accessions) factorial design combined over experimental runs. It is worth noting that the 3 by 3 by 30 factorial experiment was used for the ANOVA, although the 30 accessions were screened in two separate experiments conducted under similar conditions. More specifically, the first screening experiment was conducted from 5 May to 20 June 2010 and included 14 accessions originating from the sampled rice area and the purchased S-control A05 accession, whereas the second screening experiment was conducted from 8 May to 23 June 2010 and included 15 accessions originating from the rice area and the S-control A05 accession (for comparison). The second run of these two separate experiments was carried out in a similar way as described previously from 22 June to 6 August 2010. The accessions that were effectively controlled (>80% of the treated plants killed) by the recommended rate in Greece were labelled 'S' (A05, A06), whereas the accessions that were not controlled (<80% of the treated plants killed) by 4 times the recommended rate of either herbicide were labelled 'R' (A01, A02). Finally, the accessions that were not controlled (<80% of the treated plants killed) by the recommended rate but that were controlled (>80% of the treated plants killed) by 4 times the recommended rate were labelled 'less S' (less susceptible) (A03, A04).

2.4 Imazamox cross-resistance

The efficacy of imazamox against two *E. oryzicola* accessions resistant to penoxsulam (A01, A02), two less susceptible *E. oryzicola* accessions (A03, A04) and one susceptible *E. crus-galli* accession (A05) was evaluated in a separate time-replicated pot experiment. Treatments included imazamox (Pulsar® 40 g L^{-1} SL; BASF Agro Hellas) applied at 80 (recommended rate according to Greek herbicide label) and 160 g AI ha^{-1}, as well as penoxsulam and profoxydim applied at the recommended rate. The herbicide rates and adjuvants used are described in Table 1. The data obtained (expressed as percentage of untreated control) from the two experimental runs (repetition in time) were analysed (ANOVA) using a 4 by 5 (four herbicide treatments by five *Echinochloa* accessions) factorial design combined over experimental runs.

2.5 Sulfonylurea cross-resistance

Additionally, the efficacy of foramsulfuron (Equip® 22.5 g L^{-1} OD; Bayer Cropscience Hellas), nicosulfuron (Milagro® 40 g L^{-1} SC; Syngenta Hellas) and rimsulfuron (Rush® 250 g kg^{-1} WG; DuPont Agro Hellas) was evaluated against the five previously described *Echinochloa* accessions (A01, A02, A03, A04, A05). Foramsulfuron was applied at 52 (recommended rate according to Greek herbicide label), 104, 208 and 416 g AI ha^{-1}; nicosulfuron rates were 40 (recommended rate according to Greek herbicide label), 80, 160 and 320 g AI ha^{-1}, and the respective rates of rimsulfuron were 10 (recommended rate according to Greek herbicide label), 20, 40 and 80 g AI ha^{-1}. Also, the recommended rate of penoxsulam along with the untreated control treatment were included. The data obtained (expressed as percentage of the untreated control) from the experimental runs (repetition in time) were analysed (ANOVA) using a 4 by 4 by 5 (four herbicides by four herbicide rates by five *Echinochloa* accessions) factorial design combined over experimental runs.

2.6 Molecular identification of *Echinochloa* species

Leaves of individual plants from two *E. oryzicola* accessions resistant to penoxsulam (A01, A02), two less susceptible *E. oryzicola* accessions (A03, A04) and two susceptible *E. crus-galli* accessions (A05, A06) were used for DNA extraction. DNA was isolated, according to Doyle and Doyle,[28] by using CTAB. For each accession, the isolation was performed 3 times from tissue taken from three different plants. A quantity of 5 μL of DNA (about 250 ng) was used as template in PCR with primers amplifying the cpDNA *trn*L intron (*trn*L-F: CGA AAT CGG TAG ACG CTA CG, *trn*L-R: GGG GAT AGA GGG ACT TGA AC). The KAPA Taq PCR kit was used to perform the PCR reactions (KAPA BIOSYSTEMS). The PCR mixture of 25 μL total volume contained 1× PCR buffer A, 0.2 mM of each dNTP (New England Biolabs), 0.8 μM of each primer and 1.2 units of KAPA Taq polymerase. The amount of genomic DNA added to the PCR mixture was 250 ng diluted in 5 µL. PCR amplification was carried out in a Veriti 96-well thermal cycler (Applied Biosystems). The following touchdown PCR programme was employed. An initial denaturation at 94 °C for 2 min was followed by ten amplification cycles of denaturation at 94 °C for 30 s, annealing at 58 °C (−0.5 °C per cycle) for 30 s and elongation at 72 °C for 50 s. The touchdown cycles were followed by 30 standard amplification cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and elongation at 72 °C for 50 s. The final elongation step was performed at 72 °C for 10 min. Afterwards, the amplification products were resolved on a 1.2% agarose gel, and single bands of the expected size were observed.[28] The bands of the *trn*L intron were excised from the gel, and DNA was recovered using a modified freeze-squeeze method.[11] Briefly, the band slice in a 1.5 mL reaction tube was frozen in liquid nitrogen and centrifuged for 5 min at maximum speed, and the liquid phase was recovered in a new DNA collection tube. The agarose was crushed and mixed with a small amount of sterile distilled water which was added to the original tube with a pipette tip. The freezing–centrifugation cycle was repeated, and the recovered liquid phase was added to the DNA collection tube.[29] The DNA solution was sent for sequencing with no further purification to the Department of Immunology and Histocompatibility (Medical School, University of Thessaly, Larissa, Greece). DNA was directly sequenced using the *trn*L PCR primers. The DNA sequences were aligned and compared using ClustalX 2.[30]

2.7 Amplification and sequencing of the *ALS* gene fragment

A pair of degenerate primers was designed to amplify a 336 bp *ALS* gene fragment containing the Trp574 codon (TGG) (numbering standardised to *Arabidopsis thaliana*). The primers were the up primer (D-Up) 5′-CTGGyGCykCTGTGGCyAAC-3′ and the down primer (D-Down) 5′-CwGGrGTbTCrAGCATCTTC-3′. Because no information regarding the *ALS* gene sequence of any *Echinochloa*

Table 1. Fresh weight reduction (% of untreated) of six *Echinochloa* accessions as a result of the application of penoxsulam, bispyribac-sodium, profoxydim and imazamox treatments. Mean values are averaged over the two experiments

	Herbicide (g AI ha^{-1})										
	Penoxsulam			Bispyribac-sodium			Profoxydim			Imazamox	
Echinochloa accession	40[a]	80	160	30[a]	60	120	200[a]	400	800	80[a]	160
	Control (% of untreated)										
A01 (*E. oryzicola*)	1	8	16	3	3	12	100	100	100	0	6
A02 (*E. oryzicola*)	4	19	33	5	17	31	100	100	100	0	0
A03 (*E. oryzicola*)	85	91	100	76	87	98	100	100	100	100	99
A04 (*E. oryzicola*)	87	97	99	76	95	99	100	100	100	100	100
A05 (*E. crus-galli*)	100	100	100	100	100	100	100	100	100	100	100
A06 (*E. crus-galli*)	100	100	100	95	100	100	100	100	100		
$LSD_{0.05}$						2					1

[a] The recommended rate on the Greek herbicide label.

species was available, primer design was based on the nucleotide sequences of *ALS* genes from the following Poaceae species: *Bromus tectorum* L. (AF488771), *Lolium rigidum* Gaud. (EF411171), *O. sativa* L. (AY885674), *Hordeum vulgare* L. (AF059600), *H. murinum* L. (EF540587), *Avena fatua* L. (FJ997632) and *Z. mays* L. (X63553).

Leaves of individual *Echinochloa* plants from the previously described six accessions (A01, A02, A03, A04, A05, A06) were used for DNA extraction. In particular, leaf blade tissue was collected from the two resistant *E. oryzicola* accession plants (A01 and A02) that survived with no visible adverse symptoms after treatment with the penoxsulam recommended rate. Also, plant material was collected from the two less sensitive *E. oryzicola* accessions (A03, A04) and from the two barnyardgrass S accessions (A05, A06) prior to herbicide treatments. In addition, DNA was extracted from leaf tissue that was collected during the 2011 field survey from *E. oryzicola* plants (identified on the basis of the morphological traits on remaining *Echinochloa* mature plants before rice harvest) that had survived in each of the two rice fields treated with the penoxsulam (A07 putative R accession) or imazamox (A08 putative R accession) recommended rate. DNA was extracted from leaf samples taken from three individual plants of each *Echinochloa* accession. Extraction was performed from 100 mg ground leaf tissue using the cetyl trimethylammonium bromide (CTAB) method according to the protocol outlined in the NucleoSpin® Plant II kit (MACHEREY NAGEL GmbH & Co. KG, Düren, Germany). The polymerase chain reaction (PCR) mixture consisted of 0.8 μM of each primer, 200 μM of each deoxyribonucleotide triphosphate (dNTP), 1.5 mM of $MgCl_2$, 5% dimethyl sulfoxide (DMSO), 5 μL of the supplied 10× thermophilic buffer, 5 μL of DNA sample and 1.2 enzyme units (U) of *Thermus aquaticus* (Taq) polymerase (DNA polymerase, recombinant; HyTest, Finland) in 50 μL. Amplifications were carried out in a heated-lid PCR machine (MJ Research, model PTC-200) with the following cycle: denaturing at 95 °C for 3 min, denaturing at 95 °C for 30 s, annealing at 59–52 °C with a ramp of 0.2 °C s^{-1}, elongation at 72 °C for 45 s and cycling to the second denaturing step 34 more times. A final elongation step was performed at 72 °C for 5 min. Afterwards, each PCR product was separated on 1.4% agarose gels and purified according to the protocol outlined in the NucleoSpin® Extract II kit (MACHEREY NAGEL GmbH & Co. KG, Düren, Germany). The purified DNA was sent immediately for sequencing to the Department of Immunology and Histocompatibility (Medical School, University of Thessaly, Larissa, Greece). Each PCR product was sequenced twice, once with the forward primer and once with the reverse primer. The sequencing chromatograms were visualised and edited using BioEdit 7 (Ibis Biosciences, Carlsbad, CA). The same software was used to translate the DNA sequences to the respective peptide sequences. The DNA and peptide sequences were aligned and compared using ClustalX 2.[30]

2.8 Restriction analysis

The same PCR product (336 bp) was subjected to digestion by *Tsp*RI (restriction endonuclease; New England Biolabs, Ipswich, UK) for 4 h at 65 °C. The restriction point of this endonuclease, NNCASTGNN↓ (S refers to C or G), overlaps the codon Trp574 and appears twice in the specific *ALS* fragment from the wild-type *Echinochloa ALS* allele. Each digestion reaction contained 3 μg of DNA of the PCR product, 1 U (the amount of enzyme capable of digesting 1 μg of λ DNA h^{-1} at 65 °C in 50 μL total reaction volume) and 0.1 μg μL^{-1} of BSA in 30 μL total reaction volume. The digested DNA was separated by agarose (2%) electrophoresis in TAE buffer at 95 V for 40 min. The resulting bands were visualised under ultraviolet (UV) light after being stained with ethidium bromide solution (1 μg mL^{-1}). The digestion profile for each accession was compared with the respective digestion profile of the S-control (A05). A completely randomised design was used for these experiments, with three repetitions. Gel images were captured with a Kodak DS DC40 camera (Eastman Kodak Company, Rochester, NY), while the Quantity-One® software (Bio-Rad Laboratories, Greece) was used for gel image analysis and band comparisons.

3 RESULTS AND DISCUSSION

3.1 Penoxsulam, bispyribac-sodium and profoxydim resistance

Penoxsulam applied at the recommended rate reduced the fresh weight of 15 *E. oryzicola* accessions by 88–100% by comparison with the untreated control (data not shown). Similarly, bispyribac-sodium applied at the recommended rate reduced the fresh weight of nine *E. oryzicola* accessions by 83–100%. These accessions were labelled 'S'. However, bispyribac-sodium applied at the recommended rate reduced the fresh weight of six *E. oryzicola* accessions by 63–77%. These accessions (e.g. A03 and A04 in Table 2) were labelled 'less S'. Penoxsulam and bispyribac-sodium applied at the recommended rate reduced the fresh weight of the 11 *E. erecta* accessions by 95–100% and 90–99%

Table 2. Fresh weight reduction (% of untreated) of five *Echinochloa* accessions as a result of the application of foramsulfuron, nicosulfuron, rimsulfuron and penoxsulam treatments. Mean values are averaged over the two experiments.

		Control (% of untreated)			
Herbicide	Accession	Rate (g AI ha^{-1})			
Foramsulfuron		**52**[a]	**104**	**208**	**416**
	A01 (*E. oryzicola*)	29	37	42	51
	A02 (*E. oryzicola*)	17	22	34	39
	A03 (*E. oryzicola*)	99	100	100	100
	A04 (*E. oryzicola*)	100	100	100	100
	A05 (*E. crus-galli*)	100	100	100	100
Nicosulfuron		**40**[a]	**80**	**160**	**320**
	A01 (*E. oryzicola*)	3	17	44	90
	A02 (*E. oryzicola*)	7	30	46	83
	A03 (*E. oryzicola*)	98	100	100	100
	A04 (*E. oryzicola*)	90	100	100	100
	A05 (*E. crus-galli*)	100	100	100	100
Rimsulfuron		**10**[a]	**20**	**40**	**80**
	A01 (*E. oryzicola*)	13	27	41	60
	A02 (*E. oryzicola*)	14	31	43	47
	A03 (*E. oryzicola*)	97	98	100	100
	A04 (*E. oryzicola*)	86	100	100	100
	A05 (*E. crus-galli*)	100	100	100	100
Penoxsulam		**40**[a]	**80**	**160**	**320**
	A01 (*E. oryzicola*)	12	35	39	44
	A02 (*E. oryzicola*)	14	18	37	38
	A03 (*E. oryzicola*)	91	97	93	100
	A04 (*E. oryzicola*)	88	90	99	100
	A05 (*E. crus-galli*)	100	100	100	100
$LSD_{0.05}$				4.4	

[a] The recommended rate on the Greek herbicide label.

respectively compared with the untreated control (data not shown). Nevertheless, both penoxsulam and bispyribac-sodium applied at 4 times the recommended rate reduced the fresh weight of two *E. oryzicola* accessions (A01, A02) only by 16–33% and 12–31% respectively (Table 2). These accessions were labelled 'R'. Finally, profoxydim applied at the recommended rate reduced the fresh weight of all evaluated accessions by 95–100% (e.g. A01, A02, A03 and A04) (Table 1), and all herbicide treatments resulted in a high level of control of the two *E. crus-galli* accessions (A05 and A06) (Table 1).

3.2 Imazamox and sulfonylurea cross-resistance

The recommended rate of imazamox reduced the fresh weight of the A05 *E. crus-galli* and A03 and A04 *E. oryzicola* accessions by 99–100% by comparison with the untreated control (Table 2). However, the same herbicide rate did not have any effect on the fresh weight of the A01 and A02 *E. oryzicola* accession plants, whereas 2 times the recommended rate reduced their fresh weight by 0 and 6% respectively.

Foramsulfuron, nicosulfuron and rimsulfuron applied at the recommended rate reduced the fresh weight of the A05 *E. crus-galli* accession by 100% compared with the untreated control (Table 2). Similarly, the same rate of the three herbicides reduced the fresh weight of the *E. oryzicola* A03 and A04 accessions by 97–99% and 86–100% respectively. However, their recommended rate applied on the *E. oryzicola* A01 and A02 accession plants reduced their fresh weight by 3–29%, whereas the respective fresh weight reduction due to 4 times their recommended rate was 41–46%. Finally, penoxsulam applied at the recommended rate reduced the fresh weight of the *E. oryzicola* A01 and A02 accessions by 12 and 14% respectively, whereas 4 times the recommended rate reduced their fresh weight by 39 and 37% respectively.

The study on penoxsulam and bispyribac-sodium efficacy against the *Echinochloa* accessions indicates that the reported unsatisfactory control by the farmers in most sampled fields could be attributed to weed growth stage, improper herbicide application or unsuitable environmental conditions. However, the lack of acceptable penoxsulam and bispyribac-sodium efficacy against the *E. oryzicola* A01 and A02 accessions suggests evolution of cross-resistance to these herbicides. This could be attributed to the herbicide selection pressure imposed by the rice monoculture applied as main practice in the sampling area, along with the repeated use of these two herbicides for a 6 year period. Similarly, an *E. oryzicola* accession was found to be cross-resistant to bispyribac-sodium and penoxsulam by other researchers.[18–20]

The fact that imazamox applied at 2 times the recommended rate did not have any effect on the *E. oryzicola* A01 and A02 accession plants indicates clearly that these accessions, in addition to their previously confirmed cross-resistance to penoxsulam and bispyribac-sodium, have also developed cross-resistance to imazamox.

The poor efficacy of foramsulfuron, nicosulfuron and rimsulfuron against the *E. oryzicola* A01 and A02 accessions strongly supports the evidence that these accessions, in addition to their previously confirmed cross-resistance to penoxsulam, bispyribac-sodium and imazamox, have also developed cross-resistance to these sulfonylurea herbicides registered for use in corn. This pattern of cross-resistance has been associated with mutation in position Trp574, which usually confers cross-resistance to different ALS-inhibiting herbicides, whereas the respective mutation in position Pro197 usually causes resistance mainly to sulfonylurea herbicides, as shown for other dicot and grass species.[23] It is worth noting that the *E. oryzicola* cross-resistance to ALS-inhibiting herbicides penoxsulam (triazolopyrimidines), bispyribac-sodium (pyrimidinylthiobenzoates), imazamox (imidazolinones) and formsulfuron, nicosulfuron and rimsulfuron (sulfonylureas) is reported for the first time worldwide. Similar results concerning cross-resistance only to bispyribac-sodium and penoxsulam were also reported for *E. oryzoides* in Turkey and for *E. oryzicola* in the Sacramento Valley of California.[17–21]

The high level of profoxydim efficacy against the 30 *Echinochloa* accessions studied suggests that none of these accessions has developed resistance to this ACCase-inhibiting herbicide. The lack of *Echinochloa* R accessions to profoxydim in Greece could be attributed to the lower selection pressure imposed as a result of its very limited use in the rice area. On the other hand, an *E. oryzicola* (syn. *E. phyllopogon*) accession from the Sacramento Valley in California was found R to the ACCase-inhibiting herbicide fenoxaprop-p-ethyl, whereas another *E. oryzicola* (syn. *E. phyllopogon*) accession was reported R to the ACCase-inhibiting herbicide cyhalofop-butyl.[31,32]

3.3 Molecular identification of *Echinochloa* species

A search at the NCBI nucleotide database (performed in December 2011) with the query "trnL AND intron AND '*Echinochloa*'[porgn:__txid45618]" retrieved 63 sequences that

Table 3. Alignment of the A-repeat region of the *trn* L intron of cpDNA of 13 species of the genus *Echinochloa* along with the six *Echinochloa* samples examined. The alignment is representative of the one performed with 63 accessions of *Echinochloa trn* L intron sequences retrieved from the NCBI nucleotide database and shows all the observed variation in the A-repeat region of this sequence

Echinochloa species	GenBank Acc. No.	Sequence Alignment
E. crus-galli	AB353425	TACTTTAAAAAA-----GTGGA
E. crus-galli var *formosensis*	AB223107	TACTTTTAAAAA-----GTGGA
E. crus-galli var *praticola*	AB353427	TACTTTTAAAAA-----GTGGA
E. crus-pavonis	AB353419	TACTTTAAAAAA-----GTGGA
E. colona	AB223115	TACTTTAAAAAA-----GTGGA
E. esculenta	AB223111	TACTTTTAAAAA-----GTGGA
E. frumentacea	AB353421	TACTTTAAAAAA-----GTGGA
E. oryzoides	AB353428	TACTTTTAAAAA-----GTGGA
E. picta	AB353429	TACTTTAAAAAA-----GTGGA
E. stagnina	AB223117	TACTTTTAAAAA-----GTGGA
E. turneriana	AB353420	TACTTTAAAAAA-----GTGGA
E. walteri	AB353430	TACTTTAAAAAA-----GTGGA
A05 (*E. crus-galli*)		**TACTTTTAAAAA-----GTGGA**
A06 (*E. crus-galli*)		**TACTTTTAAAAA-----GTGGA**
E. phyllopogon	AB353422	TACTTAAAAAAAAAAAAGTGGA
E. oryzicola	AB223091	TACTTAAAAAAAAAAAAGTGGA
A01 (*E. oryzicola*)		**TACTTAAAAAAAAAAAAGTGGA**
A02 (*E. oryzicola*)		**TACTTAAAAAAAAAAAAGTGGA**
A03 (*E. oryzicola*)		**TACTTAAAAAAAAAAAAGTGGA**
A04 (*E. oryzicola*)		**TACTTAAAAAAAAAAAAGTGGA**
E. oryzicola	AB223098	TACTTAAAAAAAAAA-GTGGA
E-obtusiflora	AB223120	TACTTTAAAAAAAAA-GTGGA
E. stagnina	AB223118	TACTTAAAAAAAAAA-GTGGA

were classified according to the source annotation as different *Echinochloa* species and accessions: *E. crus-galli* (33 accessions), *E. oryzicola* (10), *E. esculenta* (4), *E. crus-galli* var. crus-galli (3), *E. crus-galli* var. praticola (3), *E. colona* (3), *E. crus-galli* var. formosensis (2), *E. oryzoides* (2), *E. frumentacea* (2), *E. stagnina* (2), *E. crus-pavonis* (2), *E. obtusiflora* (1), *E. phyllopogon* (1), *E. turneriana* (1), *E. picta* (1) and *E. walteri* (1). The retrieved sequences, aligned by using MEGA5 software, revealed that nine *E. oryzicola* and one *E. phyllopogon* accession contained 12 A-repeats, one *E. stagnina* and one *E. oryzicola* accession had 11 A-repeats, one *E. obtusiflora* had ten A-repeats, while the remaining sequences contained 5–6 A-repeats at the respective positions.[33] The same alignment performed in the A-repeat region, including representative accessions for *Echinochloa* species and the sequenced six samples of this study, indicated that the samples taken from A01, A02, A03 and A04 accession plants contain 12 A-repeats matching the pattern of *E. oryzicola* and *E. phyllopogon*, whereas the samples taken from A05 and A06 accession plants have five A-repeats matching the pattern of the *E. crus-galli* motif (and also the remaining species with 5–6 A-repeats) (Table 3).

These findings of molecular differentiation of the six *Echinochloa* accessions studied strongly support the evidence that the *Echinochloa* A01 and A02 accessions with cross-resistance to ALS-inhibiting herbicides belong to *E. oryzicola* species. Also, the less S A03 and A04 accessions belong to *E. oryzicola* species, whereas the S A05 (purchased as *E. crus-galli*) and A06 accessions (collected from the field margin and classified conventionally as *E. crus-galli*) were found to belong to *E. crus-galli* species. Therefore, these results indicate that the cpDNA sequence comparison could be used for identification of conventionally classified *E. crus-galli* and *E. oryzicola* accessions. Similar results were reported by Yamaguchi *et al.*[11] who found that the nucleotide sequencing of cpDNA in 30 accessions belonging to nine species of the genus *Echinochloa* provided information on phylogenetic relationships and new tools for species identification.

3.4 Amplification and sequencing of the *ALS* gene fragment

The use of degenerate primers led to successful amplification of the targeted *ALS* gene fragment obtained from three individuals of each of the eight *Echinochloa* accessions studied. The sequences of the PCR product correspond to a 320 bp coding region (Fig. 1), equivalent to nucleotide positions 1879 to 2198 of the standard *Arabidopsis ALS* gene sequence X51514. As nucleotide and amino acid sequence variation was not detected among the individuals of each accession, the consensus sequence is presented for each accession. Nevertheless, nucleotide polymorphisms were observed among the *Echinochloa* accessions studied. In particular, the sequence alignment comparison between the S *E. crus-galli* (A05 and A06) and R *E. oryzicola* (A01 and A02) and putative R *E. oryzicola* (A07 and A08) accessions revealed a heterozygous mutation in R and putative R accessions at the second nucleotide of codon 574. In particular, the codon's second guanine (G) was substituted by thymine (T) and thus resulted in the Trp574 (TGG) amino acid substitution by Leu (TTG) (Trp574Leu). However, the less S *E. oryzicola* (A03 and A04) accessions did not show any mutation at the 574 codon when compared with the S *E. crus-galli* (A05 and A06) accessions. Nevertheless, nucleotide polymorphisms were observed in other regions of the *ALS* fragment, which resulted in amino acid substitutions not related to herbicide resistance (Fig. 1). It is worth noting that the S *E. crus-galli* (A05 and A06) accession revealed more nucleotide polymorphisms irrelevant to herbicide resistance than the *E. oryzicola* accessions.

The lack of point mutations at codon 574 of the sequenced *ALS* gene from the S *E. crus-galli* (A05, A06) accessions is in agreement with present findings of the whole-plant response experiments. Also, the absence of point mutations at the same codon of the sequenced *ALS* gene from the less S *E. oryzicola* (A03, A04) accessions is in agreement with the findings of the whole-plant response experiment and suggests that their less sensitivity recorded as regrowth might be due to an unknown penoxsulam and bispyribac-sodium resistance mechanism and not due to an

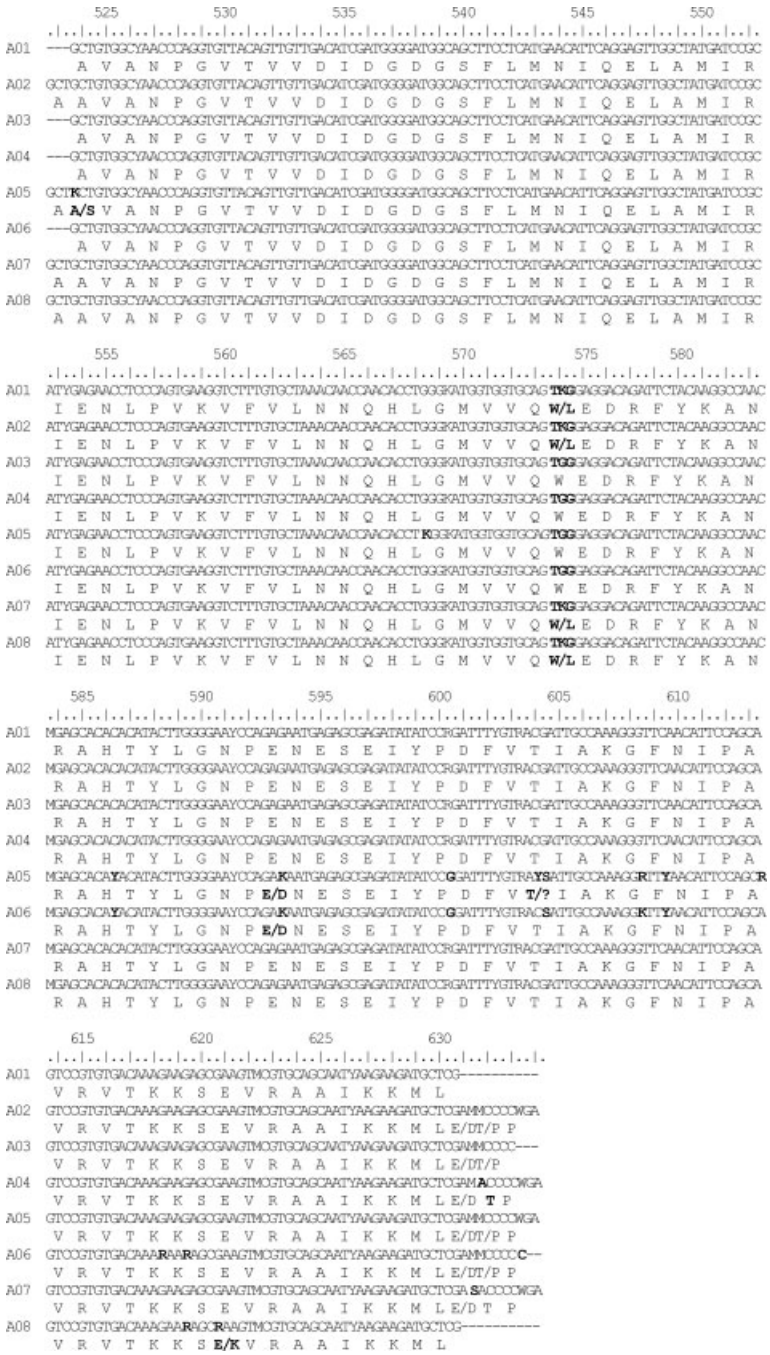

Figure 1. Nucleotide and deduced amino acid sequence alignment of *ALS* gene fragments, originating from two resistant (R) *E. oryzicola* (A01, A02), two putative R *E. oryzicola* (A07, A08), two less susceptible (S) *E. oryzicola* (A03, A04) and two S *E. crus-galli* (A05, A06) accessions. Observed polymorphisms are marked in bold. The codon positions refer to the wild-type mouse-ear cress ALS gene (GenBank: X51514).

altered target-site mechanism of action. However, the presence of Trp574Leu mutation in the 12 analysed *E. oryzicola ALS* sequences from the R *E. oryzicola* (A01, A02, A07, A08) accessions supports the evidence that their (A01, A02) previously detected cross-resistance to ALS-inhibiting herbicides was due to mutant *ALS* alleles encoding an amino-acid replacement at codon 574. The detection of the Trp574 mutation in one of the two alleles suggests that the 12 R sequenced individual plants were heterozygous (RS) for the resistant Trp574Leu allele.

The successful amplification and sequencing of the *ALS* gene of *Echinochloa* species using primers designed from sequence information of related grass weed taxa is reported for the first time worldwide. This method enabled the successful detection of an *ALS* mutation in four *E. oryzicola* accessions, which caused the amino acid substitution Trp574Leu conferring cross-resistance to most of the ALS-inhibiting herbicides. Tan *et al.*[34] also reported successful determination of the Trp574Leu mutation in the *ALS* gene of *L. rigidum* using primers designed from sequence information of related taxa. Additionally, Délye and Boucansaud[35] reported the development of a derived cleaved amplified polymorphic sequence (dCAPS) method that made it possible to detect Trp574Leu or Pro197Thr in most black-grass (*Alopecurus*

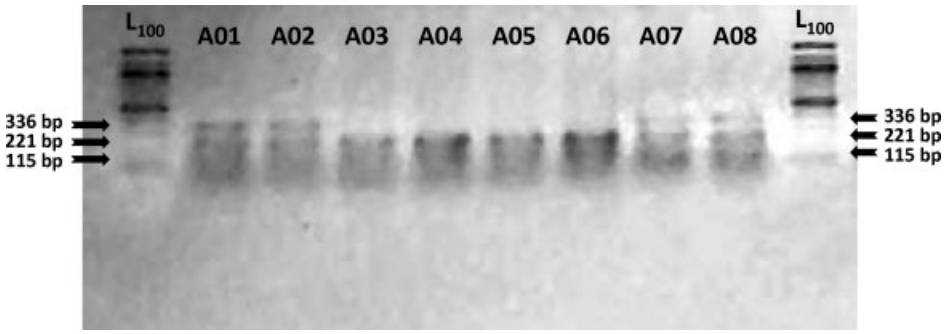

Figure 2. Digestion profile (*Tsp*RI) of the eight sequenced *Echinochloa* accessions. A01 and A02: resistant (R) *E. oryzicola*; A07 and A08: putative R *E. oryzicola*; A03 and A04: less susceptible (S) *E. oryzicola*; A05 and A06: S *E. crus-galli* accessions.

myosuroides Huds.) plants analysed from a field where the ALS-inhibiting herbicides failed after 3 years of use. Yu *et al*.[36] also developed cleaved amplified polymorphic sequence (CAPS) markers for detecting in *L. rigidum* plants the Pro-197-Ala, Pro-197-Arg, Pro-197-Gln, Pro-197-Leu, Pro-197-Ser and Trp-574-Leu mutations.

The high level of cross-resistance to ALS-inhibiting herbicides as a result of the Trp574Leu mutation present in the *E. oryzicola* accessions is consistent with other results reported.[17,23] However, this *E. oryzicola* target-site resistance is in contrast to results reported by other researchers who found an *E. oryzicola* accession with cross-resistance to bispyribac-sodium and penoxsulam as a result of enhanced herbicide metabolism via cytochrome P450 monooxygenases.[19,20,24]

3.5 Restriction analysis

The *Tsp*RI endonuclease (restriction enzyme) was chosen because it fully digests the S sequence at the Trp574 codon, but not the R sequence with a mutation within the restriction site. Thus, the digestion performed resulted in different profiles for R and S accessions. In particular, the digestion of the *ALS* fragment from the S *E. crus-galli* (A05 and A06) accessions resulted in the same restriction profile with two products (115 and 221 bp). Also, the restriction profiles of the less S *E. oryzicola* (A03 and A04) accessions were similar to those of the S accessions (A05 and A06). In contrast, the digestion of the *ALS* fragment from the R (A01 and A02) as well as from the putative R (A07 and A08) accessions revealed an intact *ALS* fragment (336 bp) and two digestion products with 115 and 226 bp (Fig. 2).

The same restriction profile (two products of 115 and 221 bp) of the digested *ALS* fragment from the S *E. crus-galli* (A05 and A06) accessions suggests the integrity of the restriction site and the absence of mutation in both *ALS* alleles. Also, the similar restriction profile of the less S *E. oryzicola* (A03 and A04) accessions to that of the S accessions supports the absence of any mutation at codon 574. In contrast, the presence of an intact *ALS* fragment (336 bp) and two digestion products (115 and 226 bp) in the digested *ALS* fragment from the R *E. oryzicola* (A01 and A02) and putative R *E. oryzicola* (A07 and A08) accessions suggests the coexistence of both R and S alleles in the R individuals (heterozygous plants, RS). These findings indicated that the use of *Tsp*RI endonuclease distinguished successfully the R and putative R accessions from the S and less S accessions, which confirmed the sequencing analysis and thus strongly supported the hypothesis that this method could be used to identify mutations at the Trp574 codon in *Echinochloa* species without the need for DNA sequencing.

4 CONCLUSIONS

This work clearly demonstrated that two *E. oryzicola* accessions out of the 30 *Echinochloa* accessions examined in whole-plant response experiments have developed cross-resistance to ALS-inhibiting herbicides penoxsulam, bispyribac-sodium, imazamox, foramsulfuron, nicosulfuron and rimsulfuron. The DNA sequencing revealed that all plants of the R *E. oryzicola* accessions had an *ALS* point mutation at codon 574 that resulted in the amino acid substitution Trp574Leu. The use of *Tsp*RI endonuclease distinguished the R from the S *Echinochloa* accessions and confirmed the sequencing analysis. The use of cpDNA sequence comparison analysis identified the conventionally classified *E. crus-galli* and *E. oryzicola* accessions. Therefore, taking into consideration the high level of cross-resistance to ALS-inhibiting herbicides as a result of the Trp574Leu mutation present in *E. oryzicola* accessions, measures should be taken for rotational use of herbicides with different modes of action to reduce selection pressure on a single mode of action and thus delay the evolution of R *Echinochloa* accessions in rice.

REFERENCES

1 Holm LG, Plucknett DL, Pancho JV and Herberger JP, *The World's Worst Weeds: Distribution and Biology*, University Hawaii Press, Honolulu, Hawaii, 609 pp. (1977).
2 Michael PW, Taxonomy and distribution of Echinochloa species with a special reference to their occurrence as weeds of rice. *Proc Conf Weed Control in Rice*, Los Baños, Laguna, Philippines, pp. 291–306 (1983).
3 Carretero JL, El género *Echinochloa* en el suroeste de Europa. *An Jard Bot Madr* **38**:91–108 (1981).
4 Tabacchi M, Mantegazza R, Spada A and Ferrero A, Morphological traits and molecular markers for classification of *Echinochloa* species from Italian rice fields. *Weed Sci* **54**:1086–1093 (2006).
5 Damalas CA, Dhima KV and Eleftherohorinos IG, Morphological and physiological variation among species of the genus *Echinochloa* in northern Greece. *Weed Sci* **56**:416–423 (2008).
6 Aoki D and Yamaguchi H, Genetic relationship between *Echinochloa crus-galli* and *Echinochloa oryzicola* accessions inferred from internal transcribed spacer and chloroplast DNA sequences. *Weed Biol Manag* **8**:233–242 (2008).
7 Yasuda K, Yano A, Nakayama Y and Yamaguchi H, Molecular identification of *Echinochloa oryzicola* Vasing. and *E. crus-galli* (L.) Beauv. using a polymerase chain reaction–restriction fragment length polymorphism technique. *Weed Biol Manag* **2**:11–17 (2002).
8 Mennan H and Kaya-Altop E, Molecular techniques for discrimination of late watergrass (*Echinochloa oryzicola*) and early watergrass (*Echinochloa oryzoides*) species in Turkish rice production. *Weed Sci* **60**:525–530 (2012).
9 Clegg MT and Zurawski G, Chloroplast DNA and the study of plant phylogeny: present status and future prospects, in *Molecular Systematics of Plants*, ed. by Soltis DE and Doyle JJ. Chapman and Hall, New York, NY, pp. 1–13 (1992).

10 Wolfe KH, Li WH and Sharp PM, Rates of nucleotide substitution vary greatly among plant mitochondria, chloroplast, and nuclear DNAs. *Proc Natl Acad Sci USA* **84**:9054–9058 (1987).
11 Yamaguchi H, Utano AYA, Yasuda K, Yano A and Soejima A, A molecular phylogeny of wild and cultivated *Echinochloa* in East Asia inferred from non-coding region sequences of trnT-L-F. *Weed Biol Manag* **5**:210–218 (2005).
12 Damalas CA, Dhima KV and Eleftherohorinos IG, Control of *Echinochloa oryzoides* and *E. phyllopogon* with cyhalofop, clefoxydim, and penoxsulam applied alone and in mixtures with broadleaf herbicides. *Weed Technol* **20**:992–998 (2006).
13 Vidotto F, Tesio F, Tabacchi M and Ferrero A, Herbicide sensitivity of Echinochloa spp. accessions in Italian rice fields. Crop Prot **26**:285–293 (2007).
14 Damalas CA, Dhima KV and Eleftherohorinos IG, Bispyribac-sodium efficacy on early watergrass (*Echinochloa oryzoides*) and late watergrass (*Echinochloa phyllopogon*) as affected by coapplication of selected rice herbicides and insecticides. *Weed Technol* **22**:622–627 (2008).
15 Senseman SA, *Herbicide Handbook*, 9th edition. Weed Science Society of America, Lawrence, KS, 458 pp. (2007).
16 Webster EP and Masson JA, Acetolactate synthase-inhibiting herbicides on imidazolinone-tolerant rice. *Weed Sci* **49**:652–657 (2001).
17 Heap I, *The International Survey of Herbicide Resistant Weeds*. [Online]. Available: http:/www.weedscience.org/In.asp [25 August 2012].
18 Fischer AJ, Ateh CM, Bayer DE and Hill JE, Herbicide resistant Echinochloa oryzoides *and E. phyllopogon in California Oryza sativa fields. Weed Sci* **48**:225–230 (2000).
19 Yasuor H, Osuna MD, Ortiz A, Saldain NE, Eckert JW and Fischer AJ, Mechanism of resistance to penoxsulam in late watergrass [*Echinochloa phyllopogon* (Stapf) Koss.]. *J Agric Food Chem* **57**:3653–3660 (2009).
20 Yasuor H, TenBrook PL, Tjeerdema RS and Fischer AJ, Responses to clomazone and 5-ketoclomazone by *Echinochloa phyllopogon* resistant to multiple herbicides in Californian rice fields. *Pest Manag Sci* **64**:1031–1039 (2008).
21 Kaya-Altop E and Mennan H, *Multiple Resistant Early Watergrass (*Echinochloa oryzoides*) to Herbicides in Turkey*. [Online]. Available: http://www.weedscience.org/Case/Case.asp?ResistID=5530 [25 August 2012].
22 Tranel PJ and Wright TR, Resistance of weeds to ALS-inhibiting herbicides: what have we learned? *Weed Sci* **50**:700–712 (2002).
23 Tranel PJ, Wright TR and Heap IM, *ALS Mutations from Herbicide-resistant Weeds*. [Online]. Available: http:/www.weedscience.org/mutations/MutDisplay.aspx [25 August 2012].
24 Fischer AJ, Bayer DE, Carriere MD, Ateh CM and Yim KO, Mechanisms of resistance to bispyribac-sodium in an *Echinochloa phyllopogon* accession. *Pestic Biochem Physiol* **68**:156–165 (2000).
25 Osuna MD, Vidotto F, Fisher AJ, Bayer DE, De Prodo G and Ferrero A, Cross-resistance to bispyribac-sodium and bensulfuron-methyl in *Echinochloa phyllopogon* and *Cyperus difformis*. *Pestic Biochem Physiol* **73**:9–17 (2002).
26 Yun MS, Yogo Y, Miura R, Yamasue Y and Fischer AJ, Cytochrome P-450 monooxygenase activity in herbicide-resistant and -susceptible late watergrass (*Echinochloa phyllopogon*). *Pestic Biochem Physiol* **83**:107–114 (2005).
27 Beckie HJ, Heap IM, Smeda RJ and Hall LM, Screening for herbicide resistance in weeds. *Weed Technol* **14**:428–445 (2000).
28 Doyle JJ and Doyle JL, A rapid DNA isolation procedure from small quantities of fresh leaf tissue. *Phytochem Bull* **19**:11–15 (1987).
29 Koenen M, Recovery of DNA from agarose gels using liquid nitrogen. *Trends Genet* **5**:137 (1989).
30 Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F and Higgins DG, The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl Acids Res* **25**:4876–4882 (1997).
31 Bakkali Y, Ruiz-Santaella JP, Osuna MD, Wagner J, Fischer AJ and De Prado R, Late watergrass (*Echinochloa phyllopogon*): mechanisms involved in the resistance to fenoxaprop-p-ethyl. *J Agric Food Chem* **55**:4052–4058 (2007).
32 Ruiz-Santaella JP, De Prado R, Wagner J, Fischer AJ and Gerhards R, Resistance mechanisms to cyhalofop-butyl in a biotype of *Echinochloa phyllopogon* (Stapf) Koss. from California. *J Plant Dis Prot* **118**:95–100 (2006).
33 Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S, MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**:2731–2739 (2011).
34 Tan MK, Preston C and Wang GX, Molecular basis of multiple resistance to ACCase-inhibiting and ALS-inhibiting herbicides in *Lolium rigidum*. *Weed Res* **47**:534–541 (2007).
35 Délye C and Boucansaud K, A molecular assay for the proactive detection of target site-based resistance to herbicides inhibiting acetolactate synthase in *Alopecurus myosuroides*. *Weed Res* **48**:97–101 (2008).
36 Yu Q, Han H and Powles SB, Mutations of the ALS gene endowing resistance to ALS-inhibiting herbicides in *Lolium rigidum* populations. *Pest Manag Sci* **64**:1229–1236 (2008).

