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**Rapid increase of herbicide resistance in *Echinochloa* spp. consequent to repeated applications of the same herbicides over time**

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(Article begins on next page)

1 **Rapid increase of herbicide resistance in *Echinochloa* spp. consequent to repeated**  
2 **applications of the same herbicides over time**

3

4 Francesco Vidotto<sup>a</sup>, Natalino Dalla Valle<sup>b</sup>, Silvia Fogliatto<sup>a</sup>, Marco Milana<sup>a</sup>, Fernando De  
5 Palo<sup>a</sup>, Maurizio Tabacchi<sup>c</sup> and Aldo Ferrero<sup>a</sup>

6

7 <sup>a</sup>Dipartimento di Scienze Agrarie, Forestali e Alimentari, Università degli Studi di Torino,

8 Grugliasco, Italy; <sup>b</sup> CORTEVA Agrisciences, Cremona, Italy; <sup>c</sup> ISIDRO s.r.l., Vercelli, Italy

9

10 **CONTACT:** Silvia Fogliatto; Dipartimento di Scienze Agrarie, Forestali e Alimentari,

11 Università degli Studi di Torino, Grugliasco, Italy; Email:silvia.fogliatto@unito.it

12

13 **Abstract**

14 Weed control in rice fields has become particularly difficult because of the increased

15 occurrence of herbicide resistance. The objective of the study was to assess in field

16 conditions, in Italy, if repeated applications of the same herbicides on *Echinochloa* spp.

17 populations already showing an initial level of resistance would increase their level of

18 resistance over a short time. Repeated applications of penoxsulam and cyhalofop-butyl were

19 performed at different rates, either alone, in combination, or in sequence for one, two or three

20 consecutive years (2010-2012) in the same plots. Confirmation of resistance was performed in

21 greenhouse on plants derived from untreated plots and from those that survived the field

22 treatments. Penoxsulam efficacy was lower than that obtained with cyhalofop-butyl. The

23 average efficacy across herbicide treatments declined over time as it was 52%, 32%, and 13%

24 at 28 days after treatments in plots treated for one, two and three years, respectively. The

25 treatments performed in greenhouse confirmed the low herbicide efficacy observed in the

26 field. Study results highlighted that repeated applications of ALS and ACCase inhibitor

27 herbicides can accelerate resistance selection in *Echinochloa*, and even herbicide rotation,  
28 sequencing, or mixing might not be effective if applied at time intervals of insufficient length.

29

30 **Keywords:** barnyardgrass, herbicide resistance, ALS-inhibitor herbicides, ACCase inhibitor  
31 herbicides

## 32 **Introduction**

33 Weeds continue to be the major problem in rice production worldwide, and play a key role in  
34 reducing crop quality and yield (Shaner 2014). Widespread use of herbicides in rice  
35 cultivation since the 1960s and improvements in the stability of weed control efficacy have  
36 led to three dramatic changes in rice cultivation: a reduction in manpower requirements, a  
37 shift from transplanted to direct-seeded cultivation in some areas (e.g. Europe), and a boost in  
38 profitability (Rao et al. 2007; Kraehmer et al. 2014). Weed control strategies also changed  
39 during these years and across areas of cultivation with a tendency to move to fewer widely  
40 used herbicides, monoculture cultivation, and continuous use of the same herbicides with  
41 limited modes of action. This situation has led to a reduction in herbicide efficacy due to the  
42 emergence of weed resistance biotypes (Shaner 2014). In particular, a rapid rise in cases of  
43 rice weeds resistant to ALS and ACCase inhibitor herbicides, currently the most commonly  
44 used herbicide classes in rice cultivation worldwide, was recorded between 1980 and 1995  
45 (Tranel and Wright 2002; Burgos et al. 2013). ALS-inhibiting herbicides have been widely  
46 used in rice cultivation due mainly to their high efficacy against several weeds, residual  
47 activity, crop safety, and relatively low toxicity (Tranel and Wright 2002). They act on  
48 acetolactate synthase, an enzyme that catalyzes the production of branched-chain amino acids  
49 valine, leucine, and isoleucine, but that also exhibits a strong tendency to develop gene point  
50 mutations (target site resistance) through amino acid substitution at a specific position (Riar et  
51 al. 2013; Heap 2014).

52 Numerous cases of resistance have also been reported for ACCase inhibitor herbicides, which  
53 have had a 30-year history of use primarily for grass weed management in rice (Kukorelli et  
54 al. 2013; Altop et al. 2014). They inhibit the enzyme acetyl-CoA carboxylase (ACCase) that  
55 catalyzes the first step in fatty acid synthesis (Focke and Lichtenthaler 1987; Burton et al.  
56 1989), and reduces production of phospholipids required for cell growth. Today, both ALS  
57 and ACCase inhibitor herbicides continue to be used against most of the main weed  
58 complexes that infest rice fields worldwide, such as weedy rice (*Oryza sativa* L.),  
59 barnyardgrass (*Echinochloa* spp. complex), the *Cyperus* complex, club rushes  
60 (*Schoenoplectus* spp. and *Scirpus* spp.), and broadleaved monocots (e.g. *Alisma* spp.,  
61 *Heteranthera* spp.) (Panozzo et al. 2013; Kraehmer et al. 2014). Among these, *Echinochloa*  
62 spp. are polyploid species with a C4 photosynthetic pathway, able to infest several crops  
63 worldwide, including rice. They are also among the few plants that can adapt to both dry and  
64 flooded conditions (Vidotto et al. 2016; Serra et al. 2018). Morphology is known to vary  
65 widely both across *Echinochloa* species and among populations within the same species.  
66 Sensitivity to herbicides can be equally variable, such as reported in a previous paper in which  
67 *E. crus-galli* (L.) P. Beauv. generally exhibits more sensitivity compared to *E. phyllopogon*  
68 (Stapf) Koso-Pol. and *E. erecta* (Pollacci) Pignatti (Vidotto et al. 2007).

69 In Europe, rice weed control strategies evolved in much the same way, relying most on  
70 herbicide use. In addition, European strategies developed to solve specific, and often  
71 changing, weed species threats, such as those found across cultivation areas that varied in soil  
72 and climate conditions (Rao et al. 2007). From the 90s, weed control problems were further  
73 complicated by EU regulatory actions. Particularly restrictive regulations, such as Directive  
74 91/414/EEC and Regulation (EC) No 1107/2009, that have established the process of  
75 periodical assessment for the renewal of approval of the pesticide molecules, have further  
76 reduced the number and modes of action of usable herbicides (Prather et al. 2000).

77 Management of *Echinochloa* spp. in European direct-seeded rice is primarily performed with

78 ALS and ACCase inhibitor herbicides, and their continuous use has resulted in resistant  
79 populations (Vidotto et al. 2007; Altop et al. 2014; Peterson et al. 2018).  
80 In Italy, rice weed control has mirrored global and EU history, with a few additional  
81 considerations. Italian rice is often cultivated in monoculture across a large area located  
82 mostly in the northern regions of Piemonte, Lombardia, and Veneto (Bordiga et al. 2014). The  
83 territory represents about 90% of the total Italian rice area (about 219,300 ha in 2018) (Ente  
84 Nazionale Risi 2019) and is characterized by common environmental conditions, and by  
85 farms with specialized labor organizations and equipment (Ferrero and Vidotto 2010). The  
86 common environment, lack of crop rotation, and adoption of few, widely used herbicides in  
87 Italy results in its rice fields having similar herbicide-based weed control programs.  
88 Knowledge of the response of *Echinochloa* populations to repeated field application of  
89 different herbicides is of primary importance to understand resistance evolution and  
90 consequently to plan appropriate weed management strategies (Vidotto et al. 2007). In  
91 particular, we tested the hypothesis that *Echinochloa* populations, already showing some  
92 individuals escaping herbicide treatments with ALS and ACCase inhibitors, would increase  
93 their level of resistance over a short time if herbicides with same SOA (site of action) were  
94 continuously applied over three years. We also tested the opposite hypothesis, in which  
95 alternating SOAs would maintain or lower the resistance level of the populations. To this end,  
96 we conducted a field study over three years (2010, 2011, and 2012) by repeating the  
97 application of the same herbicides in naturally occurring infestations of *Echinochloa* spp.

## 98 **Materials and Methods**

### 99 *Field trial*

#### 100 *Experimental site*

101 The study was carried out during 2010-2012 in a rice field located in Vercelli province, in  
102 northwestern Italy (45.37421° N, 8.27557° E; WGS84). The field had a long history of rice

103 cultivation in monoculture. At the start of the study, the abundance of *Echinochloa* spp. and  
104 other rice weeds was deemed representative of average infestation level found in the rice area.  
105 The majority of individuals (>90%) were classified as *E. crus-galli* (L.) Beauv. while the  
106 remaining as *E. erecta* (Pollacci) Pignatti. The *Echinochloa* complex present in the field was  
107 characterized by high morphological variability, even within a same species, as already  
108 observed previously in this and other weeds (Andres et al. 2015; Claerhout et al. 2016). The  
109 field was chosen as it was infested by *Echinochloa* populations already showing an initial  
110 level of resistance to both ALS- and ACCase inhibiting herbicides due to the use of these  
111 SOAs for many years, even though penoxsulam and cyhalofop-butyl, the two herbicides used  
112 in this study, have never been used in the field. In the year preceding the experiment, the  
113 efficacy of ALS-inhibitors was about 63%, while the efficacy of other ACCase herbicides was  
114 75% (data not shown). Every year, the field was chisel-ploughed twice (autumn and spring) to  
115 a depth of 25 cm and then harrowed about 10 days before seeding. The field was sown each  
116 year in the first ten days of May.

#### 117 *Experimental layout*

118 The experimental field was divided into three adjacent areas 22 m wide × 120 m long. Each  
119 area hosted a trial that commenced in a specific year and endured for different growing  
120 seasons. The following describes the three trials:

121 trial 1: started in 2010 and carried out during the 2010, 2011, and 2012 growing  
122 seasons;

123 trial 2: started in 2011 and carried out during the 2011 and 2012 growing seasons;

124 trial 3: started in 2012 and carried out during the 2012 growing season only.

125 Thus, among the three growing seasons, trials were performed for one, two, or three years.

126 This layout was chosen to distinguish the year effect from the herbicide efficacy effect. Each  
127 area hosting one of the three trials was further subdivided into 44 plots, 5.5 × 10 m in size.

128 During each growing season, the same set of treatments was applied to the same plots, and in  
129 accordance with the experimental layout of the previous year. All treatments were arranged in  
130 a randomized complete block design, in which each plot was an experimental unit. Four  
131 replicates were performed per treatment. Each year, in the part of the field surrounding the  
132 area occupied by plots, *Echinochloa* spp. was controlled by the use of profoxydim (“Aura”),  
133 applied at label rate.

#### 134 *Herbicide treatments*

135 The herbicides used were the ALS inhibitor herbicide penoxsulam (“Viper™”), and the  
136 ACCase inhibitor cyhalofop-butyl (“Clincher™”), applied at different timings and rates,  
137 either alone or in combination. Treatments were labelled 1–11, with 11 representing the  
138 untreated check (Table 1).

139 Some of the treatments were chosen to simulate repeated use of the same herbicide  
140 throughout different years. For such trials, repeated use of same herbicides with a high  
141 potential to induce resistance in weeds, such as ALS and ACCase inhibiting herbicides  
142 (treatments 1 to 7; Table 1), and treatments commonly recommended to maintain herbicide  
143 efficacy and prevent resistance development, as sequential application or mixed herbicides  
144 application within the same growing season (treatments 8 to 10), were chosen. Ethoxylated  
145 sorbitan monooleate (“Astrol nuovo” 12%) was always added to the mixture as the wetting  
146 agent at a rate of 2 l ha<sup>-1</sup> of formulated product when cyhalofop-butyl was used. For trials  
147 lasting for more than one year (trials 1 and 2), treatments were applied to the same plots each  
148 year. The only exceptions were when penoxsulam or cyhalofop-butyl (treatment 8) were  
149 applied every other year in the same plots.

#### 150 *Application methods and timing*

151 The herbicides were applied using an experimental backpack sprayer equipped with three flat  
152 fan nozzles, calibrated to deliver 300 L ha<sup>-1</sup> at a pressure of 203 kPa. Depending on the  
153 treatment, the herbicides were sprayed in one or two timings (timing A and timing B). First

154 applications (timing A) were performed between 23 (2012) and 25 (2010 and 2011) days after  
155 seeding, approximately at the beginning of June each year, while second applications (timing  
156 B) were done between 33 (2012) and 37 (2010) days after seeding, corresponding to 10-12  
157 days after timing A in mid-June. The growth stage of *Echinochloa* spp., according to the  
158 BBCH scale, ranged from 12 to 22 at timing A and from 13 to 24 at timing B (Lancashire et  
159 al. 1991).

## 160 *Assessments and data analyses*

### 161 *Efficacy of field treatments*

162 The efficacy of the various herbicidal combinations on *Echinochloa* was evaluated in all plots  
163 by counting *Echinochloa* plants present in three 0.5 x 0.5 m quadrats, randomly placed in  
164 each plot. Then, treatment efficacy was expressed as percentage reduction of plant density as  
165 compared to the control. This assessment was performed at 28 DATB (days after timing B) in  
166 2010, 2011, and 2012.

167 Efficacy data were subjected to an analysis of variance (ANOVA) using SPSS version 22 to  
168 test the effects of year, trial, and treatment. According to ANOVA, all these factors  
169 significantly affected herbicide efficacy. Despite an absence of significance of the second-  
170 order interaction (trial×treatment×year), the first-order interactions of trial×treatment and  
171 year×treatment were found to be significant ( $\alpha \leq 0.05$ ). For this reason, data from different  
172 years and trials were analyzed separately, and the REGWF test ( $\alpha \leq 0.05$ ) was employed to  
173 compare herbicide treatment efficacy within trial and within year.

174 The effect that repeated treatments over time within a trial (trials 1 and 2) had on herbicide  
175 efficacy was tested with GLM repeated measure ANOVA (SPSS Version 22), considering the  
176 growing season (year of study) as the repeated factor. Multiple comparisons for trials 1 and 2  
177 were performed to determine the significance of the different treatment efficacy among trial  
178 years using the Sidak method.



179 *Confirmation of herbicide resistance in greenhouse*

180 Seeds produced from *Echinochloa* plants that escaped the field treatments in trial 1 were  
181 collected at the end of the 2012 growing season. Seeds from plants present in the untreated  
182 plots were also collected at the same time. Thus, eleven seed bulks, one per treatment, were  
183 made from the plants present in each plot by collecting and then drying the seeds in the  
184 greenhouse of the Dipartimento di Scienze Agrarie, Forestali e Alimentari of Grugliasco,  
185 Torino (Italy). Each bulk was stored in the refrigerator for later use.

186 In the early spring of 2013, 36 seeds per bulk were randomly taken and sown (nine seeds per  
187 pot) in four pots (36 cm<sup>2</sup>) filled with commercial potting mix. The design resulted in four  
188 replicates per treatment, and the experiment was conducted twice. All pots were maintained in  
189 the same greenhouse and placed in trays containing a water layer about 5 cm deep to  
190 completely saturate the substrate. Each pot was placed in its own saucer to avoid pot-to-pot  
191 interference through water. The pots were arranged in bench in a completely randomized  
192 layout and allowed to grow to BBCH growth stage 13-14.

193 *Herbicide treatments*

194 Plants grown from seed collected from control and treatment plots were treated in the  
195 greenhouse with the same herbicides and rates used in the field; penoxsulam at 40 g ha<sup>-1</sup> and  
196 cyhalofop-butyl at 300 g ha<sup>-1</sup>. Plants coming from untreated plots were sprayed in greenhouse  
197 with both herbicides at the field rate. The herbicides were applied using a cabinet track  
198 sprayer equipped with a single flat fan nozzle (Teejeet DG8002-VS), calibrated to deliver 300  
199 L ha<sup>-1</sup> at a pressure of 203 kPa. After treatment, the plants were rearranged in the greenhouse  
200 bench until study end.

201 *Assessments and data analyses*

202 The effects of the applied products on the plants were assessed at 21 days after treatment by  
203 cutting them with scissors just above the soil level and measuring the fresh biomass. Fresh  
204 biomass data of the plants pertaining to each treatment were expressed as efficacy (percentage

205 reduction of plant biomass compared to the untreated control plants). Efficacy of penoxsulam  
206 and cyhalofop-butyl treatments on plants coming from different field treatments were tested  
207 separately by using ANOVA analysis and the REGWF test ( $p \leq 0.05$ ). As the test conducted in  
208 greenhouse was repeated twice, two separate analyses (ANOVA) were conducted on the data.  
209 The results of the ANOVA did not find any significant differences among the results of the  
210 two tests and thus the data were averaged. All the analyses were performed with the statistical  
211 package SPSS version 22.

## 212 **Results**

### 213 *Field trial*

#### 214 *Echinochloa* spp. density in untreated plots and efficacy of field treatments

215 Plant density recorded in untreated plots gave information on the trend of *Echinochloa*  
216 potential infestation over a three-year period. In the 1<sup>st</sup> year of each trial, plant density ranged  
217 from 35.0 plants m<sup>-2</sup> to 51.0 plants m<sup>-2</sup>. The infestation strongly increased at the 2<sup>nd</sup> year with  
218 values higher than 100 plants m<sup>-2</sup>, while a more moderate increase in plant density was  
219 observed from the 2<sup>nd</sup> to the 3<sup>d</sup> year of trial 1 (Table 2).

220 A low herbicide efficacy was already recorded after only a single application for some of the  
221 tested herbicides, such as penoxsulam. This could arise if there were already present  
222 resistance genes in the populations, as other ALS and ACCase inhibitor herbicides were  
223 applied to control weeds before the experiment began.

224 The ANOVA results showed that herbicide efficacy was, in general, affected by both year and  
225 trial; the efficacy recorded in 2010 and 2011 was higher than that observed in 2012.

226 Furthermore, results from trial 1 indicated that, apart from differences among compared  
227 treatments, average efficacy was similar in 2010 and 2011 (Table 2). Treatment efficacy  
228 among the three trials varied but showed overall reductions in proportion to trial duration.

229 Average 2012 efficacy values in rank order were trial 3 > trial 2 > trial 1.

230 Behavior of penoxsulam and cyhalofop-butyl across the trials was consistent. Across all the  
231 trials, the penoxsulam-treated plots had average efficacy values significantly lower than those  
232 recorded for cyhalofop-butyl, regardless of application rate. In fact, other than the third year  
233 of trial 1, the efficacy in plots treated with penoxsulam ranged between 20% (trial 1, 2<sup>nd</sup> year)  
234 and 67% (trial 2, 1<sup>st</sup> year). Plots treated with cyhalofop-butyl alone always resulted in a  
235 relatively higher efficacy against *Echinochloa* spp. compared to all the treatments, as  
236 demonstrated by values always above 50% and often reaching more than 75% (first year of all  
237 trials and 2<sup>nd</sup> year of trial 1). An exception to this trend occurred during the second year of  
238 trial 2, when efficacy was generally low for all herbicides and the averages for all treatments  
239 (even penoxsulam or cyhalofop-butyl alone) were similar and low (about 30%). The higher  
240 efficacy of cyhalofop-butyl-based treatments compared to penoxsulam-based treatments was  
241 maintained during the 3<sup>rd</sup> year of trial 1, although the values were somewhat lower when  
242 compared to the previous years.

243 Treatment efficacy resulted in significant reductions across years within the same trial after  
244 consecutive years of application of the same herbicides (trial 1 and trial 2). Even though  
245 cyhalofop-butyl efficacy was always higher than that of penoxsulam, it attained the highest  
246 reduction of efficacy after repeated applications during the third year of trial 1. The same  
247 strong reduction in cyhalofop-butyl was also observed in the second year of trial 2.

248 Penoxsulam applied twice (A and B) during the same season at either of two doses (20 g ha<sup>-1</sup>  
249 in treatment 2 or 40 g ha<sup>-1</sup> in treatment 3) failed to demonstrate significant differences in  
250 efficacy compared to a single application of 40 g ha<sup>-1</sup> at timing A (field rate – treatment 1),  
251 with the only exception of trial 2 in 2011 in which lower efficacy was observed in treatment  
252 3. The efficacy at timing A of cyhalofop-butyl applied at higher rate (300 g ha<sup>-1</sup> in treatment  
253 4) was generally similar to that observed for the application at lower rate (150 g ha<sup>-1</sup> in  
254 treatment 5). However, in trial 2, in both years, the application of cyhalofop-butyl at 300 g ha<sup>-1</sup>  
255 in treatment 4 gave a significantly higher efficacy than the application at the lowest rate.

256 Two applications of cyhalofop-butyl at different rates (treatment 6 vs treatment 7) were able  
257 to give a similar efficacy in controlling *Echinochloa* spp. However, the double application of  
258 this herbicide showed higher efficacy than a single application at 150 g ha<sup>-1</sup> (treatment 5) in  
259 the first year of both trial 2 and 3. A single application of cyhalofop-butyl at 300 g ha<sup>-1</sup>  
260 (treatment 4) was more effective than a double application (treatments 6 and 7) at the second  
261 year of trial 2.

262 In trial 1, the efficacy of alternate year rotation of cyhalofop-butyl and penoxsulam  
263 application (treatment 8) was lower when penoxsulam was applied compared to when  
264 cyhalofop-butyl was applied. In trial 2 the efficacy was low with either herbicide. Two  
265 treatments considered the effect of penoxsulam and cyhalofop-butyl applied in mixture at the  
266 field rate at timing A (treatment 9) *versus* application of penoxsulam alone at timing A  
267 followed by cyhalofop-butyl alone at timing B (treatment 10). Whether penoxsulam and  
268 cyhalofop-butyl in mixture were applied at timing A only (treatment 9) or at timings A and B  
269 (treatment 10) no significant differences in efficacy were evident in any trials. The overall  
270 efficacy of treatments 9 and 10 was higher than that attained with penoxsulam alone only at  
271 the first year of both trial 2 and 3, but was more similar to the efficacy achieved in treatments  
272 with cyhalofop-butyl alone; an exception was treatment 5 in the 1<sup>st</sup> year of trial 2 in which  
273 cyhalofop-butyl, applied at 150 g ha<sup>-1</sup>, showed a significant lower efficacy compared to  
274 treatments 9 and 10. Overall, treatments in which cyhalofop-butyl was present were the most  
275 effective treatments compared to all the others.

### 276 ***Confirmation of herbicide resistance in greenhouse***

277 Plants grown in greenhouse from seeds of plants that survived treatments of trial 1 showed a  
278 significantly lower sensitivity to the herbicides, used in the field and applied at the label rate,  
279 compared to plants coming from untreated check plots (Table 3). In fact, plants from field  
280 untreated plots showed values of efficacy, compared to untreated plants in greenhouse, of  
281 about 62% for penoxsulam and about 76% for cyhalofop-butyl.

282 The lowest efficacy (2%) to penoxsulam was recorded from plants that in the field underwent  
283 treatments with penoxsulam and cyhalofop-butyl alternated every year (treatment 8). A level  
284 of efficacy ranging from 25% to about 32% was recorded from plants that derived from seeds  
285 collected in plots treated in the field with penoxsulam 20 g ha<sup>-1</sup> fb penoxsulam 40 g ha<sup>-1</sup>  
286 (treatment 2), penoxsulam at 40 g ha<sup>-1</sup> (treatment 1) and on plants deriving from seeds  
287 collected in plots treated once with a mixture of penoxsulam and cyhalofop-butyl (treatment  
288 9). The highest efficacy on field treated plants to penoxsulam treatment in greenhouse was  
289 recorded in plants arose from plots treated with penoxsulam at 40 g ha<sup>-1</sup> fb 40 g ha<sup>-1</sup> (treatment  
290 3), with an efficacy slightly above 36%, and from plots treated with penoxsulam 40 g ha<sup>-1</sup> fb  
291 cyhalofop-butyl 300 g ha<sup>-1</sup> (treatment 10) with an efficacy of about 38%. However, treatment  
292 1 and 9 were not statistically different in terms of efficacy to treatments 3 and 10.

293 Treatment with cyhalofop-butyl in greenhouse on plants that came from plots treated with the  
294 same herbicide in the field showed lower efficacy compared to plants coming from untreated  
295 plots (Table 3). Unexpectedly, double treatment (timing A and B) with half the field rate  
296 (treatment 7) showed the lowest efficacy (about 16%). While intermediate efficacy of about  
297 30% was recorded on plants from plots treated in the field at 150 g ha<sup>-1</sup> fb 300 g ha<sup>-1</sup>  
298 (treatment 6), for plants treated once at 300 g ha<sup>-1</sup> (treatment 4) and for those treated with  
299 penoxsulam 40 g ha<sup>-1</sup> fb cyhalofop-butyl 300 g ha<sup>-1</sup> (treatment 10). The efficacy among these  
300 treatments was not statistically significant probably because of the variable response to this  
301 herbicide among plants treated in the greenhouse.

302 The highest efficacy (from about 46% to more than 55%) on plants already treated in the field  
303 was recorded in *Echinochloa* plants coming from plots previously treated with cyhalofop-  
304 butyl at 150 g ha<sup>-1</sup> in the field (treatment 5) and from those that underwent field treatments  
305 with both herbicides alternated every year (treatment 8) or distributed all at once (treatment  
306 9).

307 *Echinochloa* plants that came from plots that underwent field treatments 8 and 9 with both  
308 herbicides exhibited generally a higher efficacy to the greenhouse treatment with cyhalofop-  
309 butyl compared to penoxsulam. Only plants that arose from plots treated in the field with 40  
310 g ha<sup>-1</sup> penoxsulam fb 300 g ha<sup>-1</sup> cyhalofop-butyl (treatment 10) had a more similar level of  
311 efficacy to penoxsulam and cyhalofop-butyl treatments in greenhouse.

## 312 **Discussion**

313 The study evaluated the effect of repeated use of ALS and ACCase-inhibitor herbicides for  
314 the control of *Echinochloa* spp. under field conditions. The field study showed, in general, a  
315 significant reduction of herbicide efficacy after continuous application of the same herbicides  
316 on the same plots over three years. In the first year of each trial, which occurred during  
317 different growing seasons (2010 for trial 1, 2011 for trial 2, and 2012 for trial 3), the same  
318 herbicides exhibited variable efficacy underlying a year-to-year variability. Moreover, the  
319 level of efficacy was already low after the first application, as was the case of penoxsulam,  
320 confirming the initial presence in the field of a certain degree of resistance in *Echinochloa*  
321 populations; the presence of resistant plants before the experiment was then later confirmed  
322 by the test conducted in greenhouse. Some previous studies have also found that penoxsulam  
323 was ineffective for control of most of the tested *Echinochloa* biotypes (Matzenbacher et al.  
324 2013). In fact, a study conducted in Turkey showed penoxsulam applied at the field rate failed  
325 to control about 80% of the tested *Echinochloa oryzoides* accessions, as opposed to a 38%  
326 population failure rate by cyhalofop-butyl applied at twice the field rate (Altop et al. 2014).  
327 On the other hand, other studies have reported that penoxsulam performed at a higher level of  
328 efficacy than cyhalofop-butyl, with values as high as 100% efficacy (Damalas et al. 2008).  
329 Pacanoski and Glatkova (2009) and Ottis et al. (2003) recorded similar efficacy levels, even  
330 at rates below the field rates. These inconsistent results with some of the most common  
331 herbicides used in rice fields on barnyardgrass may arise from the presence of different

332 *Echinochloa* species- or biotype-specific herbicide sensitivities (Damalas et al. 2006), from  
333 the environmental conditions that occurred in the fields at the moment of treatment and from  
334 herbicide resistance occurrence. Indeed, extreme variability in the response to the same  
335 herbicides has been noted in different *Echinochloa* spp. populations collected previously in  
336 Italy (Vidotto et al. 2007). Similarly, studies conducted in Spain (Del Busto et al. 1997;  
337 Lopez-Martinez 1999), and in Greece (Damalas et al. 2006; Damalas et al. 2008) have also  
338 reported variable efficacy among some of the most used herbicides (quinclorac, cyhalofop-  
339 butyl, penoxsulam, and others) on different species or biotypes of *Echinochloa* spp. In the  
340 present study, spontaneously grown *Echinochloa* spp. plants were characterized by different  
341 morphological traits. The potential that they might have differed in sensitivity to herbicides as  
342 well might in part explain their different responses to the herbicides across the trials.

343 Moreover, before the start of the experiment, the fields in which the study was conducted  
344 underwent herbicide treatments with other ALS and ACCase inhibitor herbicides not used in  
345 the study, which have already started a selection pressure against *Echinochloa* populations  
346 present in the field, resulting in an initial level of resistance, as shown in the greenhouse trial,  
347 and in a consequent scarce treatment efficacy during the study. Previous studies have  
348 demonstrated that the evolution of resistance in a population is driven by both the strength of  
349 the selection pressure of the herbicides and by the initial frequency of resistant individuals  
350 (Preston and Powles 2002); thus, the fast increase level of resistance in *Echinochloa*  
351 population found in this study could be linked to the high initial number of resistant plants as  
352 shown by the relatively low herbicide efficacy measured prior to the experiment. Using a  
353 model approach, Diggle and Neve (2001) predicted that the presence of an initial high rate of  
354 resistance genes in a population induces a rapid development of resistance in *Lolium rigidum*  
355 populations after only a few years, giving an initial resistance gene frequency of 1/100,  
356 absence of gene flow, resistant gene dominance, and annual application of herbicides. Under  
357 these conditions, the model predicted that the population would be constituted of 50%

358 resistant individuals after just two generations (Diggle and Neve 2001). A similar fast  
359 resistance increase reported for *L. rigidum* has been observed in our study for *Echinochloa*  
360 spp. Moreover, some SOAs, such as ALS inhibitors, have shown to rapidly evolve resistance  
361 in few generations in some species; for example, the most rapid resistance evolution has been  
362 recorded in *Alopecurus myosuroides* in 1982 after only two years following the  
363 commercialization of ALS inhibitor herbicides (Moss and Cussans 1991; Burgos et al. 2018).  
364 Environmental conditions may be linked to the efficacy variability observed in this study.  
365 Temperature and soil moisture have been shown to affect herbicide efficacy mainly by  
366 altering adsorption and translocation of plant molecules, and by causing plant stress that then  
367 influences leaf cuticle composition and foliar penetration of the herbicide (Willingham et al.  
368 2008).

369 Regardless of variation in the response to herbicides at the start of each trial, the study  
370 confirmed the hypothesis that repeated application of the same herbicides over time results in  
371 lower efficacy against *Echinochloa* spp. and thus in a fast increase of the level of resistance.  
372 This reduced efficacy was more evident during the third study year and in cyhalofop-butyl-  
373 based treatments, although it also occurred with penoxsulam but to a lower degree as its initial  
374 efficacy was already low. Weed populations are known to gain resistance to different families  
375 of herbicides, as has been extensively proved for the ALS and ACCase inhibitor herbicides  
376 (Riar et al. 2013; Heap 2014). Moreover, *E. crus-galli* and *E. colona* have been labelled the  
377 two and five “worst herbicide-resistant weeds,” respectively, on the globe among twenty  
378 species identified as such. Their rank on the list is derived from their spread of resistant  
379 populations to different countries and cropping systems and because they have overtaken a  
380 number of SOAs (Heap 2014).

381 For this study, different control strategies were simulated by employing practices usually  
382 considered as triggers for resistance development in weeds, such as the use of herbicides with  
383 many reports of resistance, frequent applications of effective herbicides to induce high



384 selection pressure, the use of residual herbicides (penoxsulam has some residual activity), and  
385 repeated use of the same herbicides over years in a field that already showed some plants  
386 escaped to the treatments (Heap 2014). Indeed, the weed control techniques adopted in this  
387 study probably accelerated resistance development already in 3 years as repeated applications  
388 of the same herbicides resulted in a reduction of herbicide efficacy, and induced a selection of  
389 plants able to survive the treatments. A model developed to predict the evolution of herbicide  
390 resistance for a generic weed, estimates a rapid resistance development after just five years of  
391 continued application of the same herbicide, if no sensitive populations were introduced into  
392 the system (Maxwell et al. 1990). In this experiment, in addition to the fact that the number of  
393 plants able to escape the treatments increased, it is also probable that a low amount of pollen  
394 from sensitive plants was introduced in the plots throughout the years due to *Echinochloa*  
395 species characteristics. Indeed, these species are mainly self-pollinating and have a low  
396 outcrossing rate, which probably limit the gene flow via pollen (Tsuji et al. 2003). Moreover,  
397 gene flow at long-distance is unlikely to occur in *Echinochloa* species (Norsworthy et al.  
398 2012; Bagavathiannan et al. 2014).

399 The low efficacy of the herbicides used in the field study was also confirmed in the  
400 greenhouse test on the *Echinochloa* progeny that came from seeds developed from plants  
401 survived the field treatments. The study led to the hypothesis of a fast increase, in a short  
402 time, in the level of resistance to penoxsulam and cyhalofop-butyl on the *Echinochloa*  
403 populations after the field study as the ability of the plants to survive the treatments was  
404 inherited by the progeny. Moreover, the level of efficacy to both penoxsulam and cyhalofop-  
405 butyl in greenhouse treatments on field treated plants was significantly lower than that  
406 recorded for plants left untreated in the field. The test conducted in greenhouse was performed  
407 by spraying the plants only at the field rate of each herbicide; the use of a single herbicide rate  
408 (usually the field rate) in resistance screening is considered the first step of a resistant

409 confirmation test; a further step could include a dose-response test to define the level of  
410 resistance and the discriminating rate (Burgos et al. 2013).

411 The present experiment also tested some weed control strategies to prevent resistance, such as  
412 herbicide mixing and rotation of herbicides with different modes of action. In general, in this  
413 study, the adoption of herbicide mixing and herbicide rotation showed higher efficacy when  
414 compared to application of penoxsulam alone, but a similar efficacy was record for the  
415 application of cyhalofop-butyl alone. The most effective technique was sequential application  
416 of penoxsulam and cyhalofop-butyl at two different timings within the same growing season.  
417 This result was evidenced by the highest efficacy after three years as compared to the other  
418 treatments, even though repeated application significantly lowered it. In addition, the progeny  
419 continued to exhibit low efficacy under greenhouse application of penoxsulam and cyhalofop-  
420 butyl on plants previously treated with sequential rotation and a mixture of herbicides.

421 Previous studies have found that herbicide applied in sequence (multiple applications in the  
422 same year), in rotation, and in mixture were able to delay the development of resistance,  
423 particularly if resistance is target site-based (Beckie 2006). In this study, even these  
424 techniques were unable to limit or delay significantly the increase of resistance.

425 Moreover, it has been suggested that as both ALS and ACCase inhibitors are considered  
426 herbicides at high risk for selection of resistant populations, they should be applied less  
427 frequently in rotation or sequence, relative to other herbicide groups (Beckie 2006). The  
428 annual herbicide rotation could be a helpful but not sufficient strategy to prevent resistance:  
429 by applying rotation, weed populations are subjected to the herbicide effect of a mechanism of  
430 action at a time. This results in a potential possibility for the progeny of the resistant plants to  
431 survive and increase the resistance through gene flow before the succeeding herbicide  
432 treatment (Norsworthy et al. 2012).

433 This study found agreement with the above results; the time interval between applications of  
434 the same herbicide was key to capturing the benefits of rotation, sequencing, and mixing  
435 strategies.

#### 436 **Conclusions**

437 The study highlighted that repeated applications of ALS and ACCase inhibitor herbicides can  
438 accelerate resistance selection in *Echinochloa*, in particular if resistance is already established  
439 in a population. Moreover, even the techniques that are in general suggested to delay  
440 resistance occurrence (i.e. herbicide rotation and mixing) could be only partially effective,  
441 especially if applied at time intervals of insufficient length. Thus, additional preventive  
442 methods should be undertaken to avoid resistance development, such as application of pre-  
443 emergence herbicides, rotation with low risk herbicides, and above all, adoption of integrated  
444 weed management techniques (Owen 2016). The use of these techniques able to prevent the  
445 fast development of resistance have to be strongly encouraged among farmers as once the  
446 resistance level of a weed population has become high, its control could be very difficult, as  
447 in the case of the *Echinochloa* populations of the present study.

448 Presently, in Italy, the only available herbicides to be used post-emergence against these  
449 species belong to ALS and ACCase inhibitors, if we exclude the few molecules authorized for  
450 emergency use (which often is granted to control resistant weeds), and those that are no more  
451 authorized but are in their grace period. Thus, the only possibility to control these weeds is to  
452 apply pre-emergence herbicides with different SOAs, such as pendimethalin, clomazone,  
453 flufenacet and quinclorac, or total herbicides, such as glyphosate, which is often combined  
454 with false seedbed technique. Further studies are needed to define the best weed management  
455 strategies in different environments and on different *Echinochloa* populations that will both  
456 control weeds and mitigate the emergence of herbicide resistance.

457

458 **Disclosure statement**

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464

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623

624 **Table 1.** Herbicide treatments compared in the study.

Treatment	Timing***	Herbicide*	Field rate active ingredient (g ha <sup>-1</sup> )
1	A	penoxsulam	40
2	A	penoxsulam fb	20
	B	penoxsulam	40
3	A	penoxsulam fb	40
	B	penoxsulam	40
4	A	cyhalofop-butyl	300
5	A	cyhalofop-butyl	150
6	A	cyhalofop-butyl fb	150
	B	cyhalofop-butyl	300
7	A	cyhalofop-butyl fb	150
	B	cyhalofop-butyl	150
8**	A	penoxsulam/	40
	A	cyhalofop-butyl	300
9	A	penoxsulam+	40
	A	cyhalofop-butyl	300
10	A	penoxsulam fb	40
	B	cyhalofop-butyl	300
11		untreated check	-

625 \* wetting agent Astrol Nuovo was added at 2 l ha<sup>-1</sup> with each occurrence of cyhalofop-butyl.

626 \*\* penoxsulam (at 2 l ha<sup>-1</sup>) or cyhalofop-butyl (1.5 l ha<sup>-1</sup>) was applied every other year in the same  
627 plots.

628 \*\*\* timing A: 23-25 days after seeding, timing B: 33-35 days after seeding; fb: followed by.

629 **Table 2.** Average *Echinochloa* spp. plant density in untreated plots and efficacy of the applied treatments on the basis of plant density (%  
630 compared to untreated) against *Echinochloa* spp. in each trial of a different duration of years (first, second, and third year) at 28 days after  
631 treatment at timing B (DATB).

Treatment	----- Trial 1 -----			----- Trial 2 -----		-Trial 3-
	1 <sup>st</sup> year (2010)	2 <sup>nd</sup> year (2011)	3 <sup>rd</sup> year (2012)	1 <sup>st</sup> year (2011)	2 <sup>nd</sup> year (2012)	1 <sup>st</sup> year (2012)
untreated check (plant density m <sup>-2</sup> )	51.0	127.3	164.5	46.4	151.3	35.0
	treatment efficacy (%)					
1. penoxsulam 40 g ha <sup>-1</sup>	37.5abc B	37.5abc B	2.5a A	67.5b B	20.0ab A	20.0ab
2. penoxsulam 20 g ha <sup>-1</sup> fb penoxsulam 40 g ha <sup>-1</sup>	38.8abcd B	35.0ab B	3.8a A	65.0b B	35.0abc A	11.3a
3. penoxsulam 40 g ha <sup>-1</sup> fb penoxsulam 40 g ha <sup>-1</sup>	26.3a B	20.0a B	7.5ab A	51.3a B	26.3ab A	16.3ab
4. cyhalofop-butyl 300 g ha <sup>-1</sup>	75.0cd B	68.8bcd AB	27.5b A	92.5d B	47.5c A	67.5cd
5. cyhalofop-butyl 150 g ha <sup>-1</sup>	58.8abcd B	51.3abcd AB	18.8ab A	78.8c B	22.5ab A	33.8abc
6. cyhalofop-butyl 150 g ha <sup>-1</sup> fb cyhalofop-butyl 300 g ha <sup>-1</sup>	76.3d AB	80.0d B	18.8ab A	97.5d B	27.5abc A	81.3d
7. cyhalofop-butyl 150 g ha <sup>-1</sup> fb cyhalofop-butyl 150 g ha <sup>-1</sup>	72.5bcd AB	80.0d B	11.3ab A	97.5d B	26.3ab A	78.8d
8. penoxsulam 40 g ha <sup>-1</sup> or cyhalofop-butyl 300 g ha <sup>-1</sup>	35.0ab AB	77.5d B	16.3ab A	45.0a B	16.3a A	51.3bcd
9. penoxsulam 40 g ha <sup>-1</sup> +cyhalofop-butyl 300 g ha <sup>-1</sup>	70.0abcd AB	73.8cd B	8.8ab A	93.8d B	27.5abc A	58.8cd
10. penoxsulam 40 g ha <sup>-1</sup> fb cyhalofop-butyl 300 g ha <sup>-1</sup>	72.5cd B	72.5cd B	26.3ab A	93.8d B	46.3bc A	70.0cd

632 Values sharing the same lower case letter are not significantly different according to REGWF test ( $P \leq 0.05$ ) for the comparisons among  
633 treatments within each trial and each year of experiment. Values sharing the same upper case letter are not significantly different  
634 according to Sidak comparison among years of experiment within the same trial (for trial 1 and 2).

635 **Table 3.** Treatment efficacy (in terms of plant biomass reduction compared to untreated plants)  
 636 evaluated in greenhouse of penoxsulam and cyhalofop-butyl at the field rate on *Echinochloa* plants  
 637 coming from seeds collected in Trial 1 of the field trial. The efficacy of penoxsulam and cyhalofop-  
 638 butyl on *Echinochloa* plants derived from seeds collected in untreated check plots in the field is also  
 639 shown. Efficacy were compared among treatments separated for penoxsulam and cyhalofop-butyl.  
 640 Values sharing the same letter are not significantly different according to the REGWF test ( $P \leq 0.05$ ).

641

Field treatments	Greenhouse treatments	
	penoxsulam efficacy (%)	cyhalofop-butyl efficacy (%)
untreated check	62.04 d	75.66 d
1. penoxsulam 40 g ha <sup>-1</sup>	32.30 bc	-
2. penoxsulam 20 g ha <sup>-1</sup> fb penoxsulam 40 g ha <sup>-1</sup>	25.00 b	-
3. penoxsulam 40 g ha <sup>-1</sup> fb penoxsulam 40 g ha <sup>-1</sup>	36.58 c	-
4. cyhalofop-butyl 300 g ha <sup>-1</sup>	-	30.59 b
5. cyhalofop-butyl 150 g ha <sup>-1</sup>	-	55.61 c
6. cyhalofop-butyl 150 g ha <sup>-1</sup> fb cyhalofop-butyl 300 g ha <sup>-1</sup>	-	31.87 b
7. cyhalofop-butyl 150 g ha <sup>-1</sup> fb cyhalofop-butyl 150 g ha <sup>-1</sup>	-	16.18 a
8. penoxsulam 40 g ha <sup>-1</sup> or cyhalofop-butyl 300 g ha <sup>-1</sup>	2.08 a	46.56 c
9. penoxsulam 40 g ha <sup>-1</sup> + cyhalofop-butyl 300 g ha <sup>-1</sup>	30.59 bc	55.70 c
10. penoxsulam 40 g ha <sup>-1</sup> fb cyhalofop-butyl 300 g ha <sup>-1</sup>	38.44 c	33.83 b

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