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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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Abstract

Weed control in rice fields has become particularly difficult because of the increased occurrence of herbicide resistance. The objective of the study was to assess in field conditions, in Italy, if repeated applications of the same herbicides on *Echinochloa* spp. populations already showing an initial level of resistance would increase their level of resistance over a short time. Repeated applications of penoxsulam and cyhalofop-butyl were performed at different rates, either alone, in combination, or in sequence for one, two or three consecutive years (2010-2012) in the same plots. Confirmation of resistance was performed in greenhouse on plants derived from untreated plots and from those that survived the field treatments. Penoxsulam efficacy was lower than that obtained with cyhalofop-butyl. The average efficacy across herbicide treatments declined over time as it was 52%, 32%, and 13% at 28 days after treatments in plots treated for one, two and three years, respectively. The treatments performed in greenhouse confirmed the low herbicide efficacy observed in the field. Study results highlighted that repeated applications of ALS and ACCase inhibitor

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

Keywords: barnyardgrass, herbicide resistance, ALS-inhibitor herbicides, ACCase inhibitor herbicides

Introduction

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

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

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

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

ALS and ACCase inhibitor herbicides, and their continuous use has resulted in resistant populations (Vidotto et al. 2007; Altop et al. 2014; Peterson et al. 2018).

In Italy, rice weed control has mirrored global and EU history, with a few additional considerations. Italian rice is often cultivated in monoculture across a large area located mostly in the northern regions of Piemonte, Lombardia, and Veneto (Bordiga et al. 2014). The territory represents about 90% of the total Italian rice area (about 219,300 ha in 2018) (Ente Nazionale Risi 2019) and is characterized by common environmental conditions, and by farms with specialized labor organizations and equipment (Ferrero and Vidotto 2010). The common environment, lack of crop rotation, and adoption of few, widely used herbicides in Italy results in its rice fields having similar herbicide-based weed control programs.

Knowledge of the response of *Echinochloa* populations to repeated field application of different herbicides is of primary importance to understand resistance evolution and consequently to plan appropriate weed management strategies (Vidotto et al. 2007). In particular, we tested the hypothesis that *Echinochloa* populations, already showing some individuals escaping herbicide treatments with ALS and ACCase inhibitors, would increase their level of resistance over a short time if herbicides with same SOA (site of action) were continuously applied over three years. We also tested the opposite hypothesis, in which alternating SOAs would maintain or lower the resistance level of the populations. To this end, we conducted a field study over three years (2010, 2011, and 2012) by repeating the application of the same herbicides in naturally occurring infestations of *Echinochloa* spp.

Materials and Methods

Field trial

Experimental site

The study was carried out during 2010-2012 in a rice field located in Vercelli province, in northwestern Italy (45.37421° N, 8.27557° E; WGS84). The field had a long history of rice cultivation in monoculture. At the start of the study, the abundance of *Echinochloa* spp. and other rice weeds was deemed representative of average infestation level found in the rice area. The majority of individuals (>90%) were classified as *E. crus-galli* (L.) Beauv. while the remaining as *E. erecta* (Pollacci) Pignatti. The *Echinochloa* complex present in the field was characterized by high morphological variability, even within a same species, as already observed previously in this and other weeds (Andres et al. 2015; Claerhout et al. 2016). The field was chosen as it was infested by *Echinochloa* populations already showing an initial level of resistance to both ALS- and ACCase inhibiting herbicides due to the use of these SOAs for many years, even though penoxsulam and cyhalofop-butyl, the two herbicides used in this study, have never been used in the field. In the year preceding the experiment, the efficacy of ALS-inhibitors was about 63%, while the efficacy of other ACCase herbicides was 75% (data not shown). Every year, the field was chisel-ploughed twice (autumn and spring) to a depth of 25 cm and then harrowed about 10 days before seeding. The field was sown each year in the first ten days of May.

Experimental layout

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

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

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

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

Thus, among the three growing seasons, trials were performed for one, two, or three years. This layout was chosen to distinguish the year effect from the herbicide efficacy effect. Each area hosting one of the three trials was further subdivided into 44 plots, 5.5 × 10 m in size.

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

Herbicide treatments

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

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

Application methods and timing

The herbicides were applied using an experimental backpack sprayer equipped with three flat fan nozzles, calibrated to deliver 300 L ha^{-1} at a pressure of 203 kPa. Depending on the treatment, the herbicides were sprayed in one or two timings (timing A and timing B). First applications (timing A) were performed between 23 (2012) and 25 (2010 and 2011) days after seeding, approximately at the beginning of June each year, while second applications (timing B) were done between 33 (2012) and 37 (2010) days after seeding, corresponding to 10-12 days after timing A in mid-June. The growth stage of *Echinochloa* spp., according to the BBCH scale, ranged from 12 to 22 at timing A and from 13 to 24 at timing B (Lancashire et al. 1991).

Assessments and data analyses

Efficacy of field treatments

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

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

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

Confirmation of herbicide resistance in greenhouse

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

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

Herbicide treatments

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

Assessments and data analyses

The effects of the applied products on the plants were assessed at 21 days after treatment by cutting them with scissors just above the soil level and measuring the fresh biomass. Fresh biomass data of the plants pertaining to each treatment were expressed as efficacy (percentage reduction of plant biomass compared to the untreated control plants). Efficacy of penoxsulam and cyhalofop-butyl treatments on plants coming from different field treatments were tested separately by using ANOVA analysis and the REGWF test ($p \leq 0.05$). As the test conducted in greenhouse was repeated twice, two separate analyses (ANOVA) were conducted on the data. The results of the ANOVA did not find any significant differences among the results of the two tests and thus the data were averaged. All the analyses were performed with the statistical package SPSS version 22.

Results

Field trial

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

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

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

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

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

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

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

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

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

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

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

Confirmation of herbicide resistance in greenhouse

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

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

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

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

Echinochloa plants that came from plots that underwent field treatments 8 and 9 with both herbicides exhibited generally a higher efficacy to the greenhouse treatment with cyhalofop-butyl compared to penoxsulam. Only plants that arose from plots treated in the field with 40 g ha^{-1} penoxsulam fb 300 g ha^{-1} cyhalofop-butyl (treatment 10) had a more similar level of efficacy to penoxsulam and cyhalofop-butyl treatments in greenhouse.

Discussion

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

Echinochloa species- or biotype-specific herbicide sensitivities (Damalas et al. 2006), from the environmental conditions that occurred in the fields at the moment of treatment and from herbicide resistance occurrence. Indeed, extreme variability in the response to the same herbicides has been noted in different *Echinochloa* spp. populations collected previously in Italy (Vidotto et al. 2007). Similarly, studies conducted in Spain (Del Busto et al. 1997; Lopez-Martinez 1999), and in Greece (Damalas et al. 2006; Damalas et al. 2008) have also reported variable efficacy among some of the most used herbicides (quinclorac, cyhalofop-butyl, penoxsulam, and others) on different species or biotypes of *Echinochloa* spp. In the present study, spontaneously grown *Echinochloa* spp. plants were characterized by different morphological traits. The potential that they might have differed in sensitivity to herbicides as well might in part explain their different responses to the herbicides across the trials. Moreover, before the start of the experiment, the fields in which the study was conducted underwent herbicide treatments with other ALS and ACCase inhibitor herbicides not used in the study, which have already started a selection pressure against *Echinochloa* populations present in the field, resulting in an initial level of resistance, as shown in the greenhouse trial, and in a consequent scarce treatment efficacy during the study. Previous studies have demonstrated that the evolution of resistance in a population is driven by both the strength of the selection pressure of the herbicides and by the initial frequency of resistant individuals (Preston and Powles 2002); thus, the fast increase level of resistance in *Echinochloa* population found in this study could be linked to the high initial number of resistant plants as shown by the relatively low herbicide efficacy measured prior to the experiment. Using a model approach, Diggle and Neve (2001) predicted that the presence of an initial high rate of resistance genes in a population induces a rapid development of resistance in *Lolium rigidum* populations after only a few years, giving an initial resistance gene frequency of 1/100, absence of gene flow, resistant gene dominance, and annual application of herbicides. Under these conditions, the model predicted that the population would be constituted of 50%

resistant individuals after just two generations (Diggle and Neve 2001). A similar fast resistance increase reported for *L. rigidum* has been observed in our study for *Echinochloa* spp. Moreover, some SOAs, such as ALS inhibitors, have shown to rapidly evolve resistance in few generations in some species; for example, the most rapid resistance evolution has been recorded in *Alopecurus myosuroides* in 1982 after only two years following the commercialization of ALS inhibitor herbicides (Moss and Cussans 1991; Burgos et al. 2018).

Environmental conditions may be linked to the efficacy variability observed in this study. Temperature and soil moisture have been shown to affect herbicide efficacy mainly by altering adsorption and translocation of plant molecules, and by causing plant stress that then influences leaf cuticle composition and foliar penetration of the herbicide (Willingham et al. 2008).

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

For this study, different control strategies were simulated by employing practices usually considered as triggers for resistance development in weeds, such as the use of herbicides with many reports of resistance, frequent applications of effective herbicides to induce high selection pressure, the use of residual herbicides (penoxsulam has some residual activity), and repeated use of the same herbicides over years in a field that already showed some plants escaped to the treatments (Heap 2014). Indeed, the weed control techniques adopted in this study probably accelerated resistance development already in 3 years as repeated applications of the same herbicides resulted in a reduction of herbicide efficacy, and induced a selection of plants able to survive the treatments. A model developed to predict the evolution of herbicide resistance for a generic weed, estimates a rapid resistance development after just five years of continued application of the same herbicide, if no sensitive populations were introduced into the system (Maxwell et al. 1990). In this experiment, in addition to the fact that the number of plants able to escape the treatments increased, it is also probable that a low amount of pollen from sensitive plants was introduced in the plots throughout the years due to *Echinochloa* species characteristics. Indeed, these species are mainly self-pollinating and have a low outcrossing rate, which probably limit the gene flow via pollen (Tsuji et al. 2003). Moreover, gene flow at long-distance is unlikely to occur in *Echinochloa* species (Norsworthy et al. 2012; Bagavathiannan et al. 2014).

The low efficacy of the herbicides used in the field study was also confirmed in the greenhouse test on the *Echinochloa* progeny that came from seeds developed from plants survived the field treatments. The study led to the hypothesis of a fast increase, in a short time, in the level of resistance to penoxsulam and cyhalofop-butyl on the *Echinochloa* populations after the field study as the ability of the plants to survive the treatments was inherited by the progeny. Moreover, the level of efficacy to both penoxsulam and cyhalofop-butyl in greenhouse treatments on field treated plants was significantly lower than that recorded for plants left untreated in the field. The test conducted in greenhouse was performed by spraying the plants only at the field rate of each herbicide; the use of a single herbicide rate (usually the field rate) in resistance screening is considered the first step of a resistant confirmation test; a further step could include a dose-response test to define the level of resistance and the discriminating rate (Burgos et al. 2013).

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

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

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

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

Conclusions

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

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

Disclosure statement

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Table 1. Herbicide treatments compared in the study.

Treatment	Timing***	Herbicide*	Field rate active ingredient (g ha^{-1})
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	-

* wetting agent Astrol Nuovo was added at 2 l ha^{-1} with each occurrence of cyhalofop-butyl.

** penoxsulam (at 2 l ha^{-1}) or cyahalofop-butyl (1.5 l ha^{-1}) was applied every other year in the same plots.

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

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

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

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

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

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