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Target-site resistance to ALS inhibitors in the polyploid species *Echinochloa crus-galli*

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ABSTRACT

Acetolactate synthase (ALS) inhibitors are widely used herbicides in rice and their recurrent use has resulted in several resistant weed populations. Recent reports from Italian rice growers indicated that resistance to ALS inhibitors evolved in the polyploid species *Echinochloa crus-galli* (L.) Beauv. (barnyardgrass), which is the most noxious weed infesting Italian rice fields. Fourteen *E. crus-galli* populations were confirmed to be resistant to at least one ALS-inhibiting herbicide. Three patterns of herbicide resistance were identified: seven populations were highly cross-resistant to ALS inhibitors, two were resistant to a sulfonylurea but not to an imidazolinone and five were multiple resistant to ALS and the ACCase inhibitor profoxydim. The level of resistance to the latter herbicide was low. Molecular analyses yielded the first reported consensus sequence for *E. crus-galli* ALS gene, encompassing all known mutation sites conferring herbicide resistance. The nucleotide substitution of a G with a T, giving a Trp to Leu change at amino acid 574 was detected in plants of five resistant populations analyzed, confirming an ALS target-site-mediated resistance mechanism. The W574L is a common ALS mutation endowing cross-resistance to all ALS inhibitor chemical families, as confirmed by the high levels of resistance observed for ALS inhibitors at both whole-plant and enzyme activity levels. ALS-resistant, and especially ALS- and ACCase multiple resistant barnyardgrass are threatening the sustainability of Italian rice crops due to the lack of alternative post-emergence herbicides.

1. Introduction

Italy is the main rice producing country in Europe, with about 250,000 ha that account for 50% of the total European rice area. Most of the production comes from paddy rice fields and is concentrated in the regions of Piedmont and Lombardy in the northwest of the country (www.enterisi.it). Weeds are considered the worst pest affecting rice production in Europe and ineffective weed control can result in severe reduction in crop yield and quality [1] due to species such as *Echinochloa* spp., weedy rice, and *Cyperus* spp.

Herbicides, especially in the continuous paddy rice system, are the most effective tools to control weeds, but their intense use led to environmental issues and problems of weed control due to weed shifts and the increasing number of resistance cases [2]. Nowadays, herbicide-resistant weeds in rice crops are of major concern also because of the progressive reduction in active ingredients (a.i.s) available due to the strict European legislation on plant protection products, the lack of commercialization of herbicides with new modes of action (MoAs) and the widespread use of highly-active herbicide groups that are target-site specific (i.e. acetolactate synthase – ALS- and acetyl coenzyme A carboxylase – ACCase-inhibitors).

ALS inhibitors are by far the most widely used herbicides in rice and it is estimated that around 90% of Italian rice fields are treated with these herbicides. Since the first sulfonylurea (bensulfuron-methyl) was marketed in 1988 for rice crops in Italy, many new a.i.s with the same MoA have been introduced [sulfonylureas (SUs), triazolopyrimidines (TPs), pyrimidinyl thiobenzoates (PTBs), imidazolinones (IMIs), and sulfonylamino-carbonyl-triazolinones (SCTs)] [3], and their repeated use has selected several resistant biotypes [4]. The recent introduction of the Clearfield® technology in Italy, i.e. imidazolinone tolerant rice varieties, is further reducing the diversity of MoAs used in rice crops [5]. It is estimated that 40,000 ha are now cropped with these varieties. Since 1994, three species (*Schoenoplectus mucronatus*, *Alisma plantago aquatica*, *Cyperus difformis*) have evolved resistance to ALS inhibitors in Italy [6,7] and the Italian Herbicide Resistance Working Group (GIRE) [8] estimates that more than one third of the rice fields are infested with resistant populations.

ALS is the first enzyme that is common to the biosynthesis of the branched-chain amino acids isoleucine, valine and leucine [9]. It is the target site of the five ALS-inhibiting herbicide chemistries. Widespread and persistent use of ALS-inhibiting herbicides has consequently resulted in the rapid evolution of many ALS

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herbicide-resistant weed populations. Predominantly, resistance occurs as a result of reduced sensitivity of an altered target ALS enzyme to the inhibition exerted by the herbicide [10]; however enhanced rates of herbicide metabolism have also been reported [11,12]. Several single amino acid substitutions have been identified that are sufficient to convert ALS from a herbicide-sensitive to a herbicide-resistant enzyme [12]. Target-site resistance to ALS inhibitors in all weed biotypes investigated so far has been caused by a substitution(s) of one of eight amino acids [3,13]. Resistant alleles are totally or partially dominant, at normal herbicide use rates; therefore, they are selected even when present in the heterozygous state [14]. Although exceptions exist, resistance caused by an altered ALS can be generally classified in three types according to the resistance pattern: SU and TP resistant, IMI and PTB resistance, and broad cross-resistance. For example, substitution of Ala_{122} or Ser_{653} [numbered after *Arabidopsis thaliana* (L.) Heynh.] results in IMI but not SU resistance [15], whereas substitution of Pro_{197} usually results in SU but not IMI resistance [16]. Substitution of Trp_{574} endows high resistance levels to both IMI and SU herbicides, as well as the TP and PTB herbicides [17].

Many molecular investigations have been conducted in the last decade to detect the mutations conferring resistance to ALS inhibitors [18,19]. Most documented cases concern diploid species, while studies involving polyploid weeds are rare [20] even though several polyploid weeds are known to be resistant to ALS inhibitors [21–24]. Depending on the level of ploidy, higher plants may have a variable number of ALS genes. The organization of the ALS gene family has predominantly been studied in cultivated polyploid species, and it appears to be more complex than in diploid organisms [25,26].

Echinochloa spp. are polyploid species belonging to the Poaceae [27] and are the most common weeds in Italian rice fields, as in many part of the world, but they may also infest other crops. All *Echinochloa* species have a C_4 photosynthetic pathway and are very successful competitors. They can produce a high number of seeds [28], which often results in significant soil seed banks [29]. *Echinochloa* species are very difficult to distinguish due to the wide morphological and phenological variability [30]. The most troublesome species infesting rice were identified as *E. crus-galli* (L.) Beauv. and *Echinochloa colona* (L.) Link. In recent years, the management of these species has been of concern because of the selection of populations resistant to major herbicides used in rice, including molinate, propanil, quinclorac, thiobencarb, butachlor, fenoxaprop, penoxsulam, bispyribac-Na and cyhalofop-butyl [31–33]. In Italy, resistance to propanil evolved in a few populations of *E. crus-galli* (barnyardgrass) and *E. colona* [34] and one population of *Echinochloa erecta* showed multiple resistance to propanil and quinclorac. Several Italian farmers in different rice growing areas have complained about the poor control of *Echinochloa* spp. by ALS inhibitors.

The aims of the present research were to: confirm resistance to ALS inhibitors in multiple populations of *E. crus-galli*, determine the level of resistance and cross-resistance to ALS inhibitors, verify whether a target-site resistance mechanism is involved and, if so, identify the mutation(s) endowing resistance.

2. Materials and methods

2.1. Plant material

Seeds of *E. crus-galli* were collected from 2008 to 2010 in rice fields located in several Italian regions. Seeds were harvested by GIRE members from plants that had survived an ALS-inhibiting herbicide treatment, mainly penoxsulam or imazamox, following reports regarding poor *E. crus-galli* control by these herbicides used in rice crops, i.e. through complaint monitoring and not a random survey. A population was defined as pooled seeds from at least 20 plants collected from a single field. A total of 16 populations were sampled from different municipalities (sampled fields were separated by at least 10 km) in the major Italian rice growing area (northern and northern-central Italy) (Table 1). It is therefore likely that they derive from independent selections. Historical records of herbicide use in the sampled fields were collected from farmers (Table 1). All greenhouse experiments were carried out at the Institute of Agro-environmental and Forest Biology (IBAF) - CNR located at Agripolis, Legnaro (PD), Italy (45° 21′N, 11° 58′E).

2.2. Preliminary screenings

Greenhouse pot experiments were performed to test for resistance in all 16 populations. Plants of each population were tested with four ALS- and one ACCase-inhibiting herbicides. Four ALS inhibitors belonging to different chemical classes were tested: azimsulfuron (SU), penoxsulam (PTB), imazamox (IMI) and bispyribac-Na (PTB) (Table 2). The latter herbicide was not tested in 2011 because it is the least used ALS inhibitor in paddy rice in Italy. The ACCase inhibitor (profoxydim) was introduced starting from 2009, after the first report of poor control by ACCase inhibitors. For each population, an untreated control was included as well as a susceptible check (S) 07-16S. The experimental layout was a completely randomized design with two replicates (18 seedlings per replicate) and two herbicide doses: recommended field dose in rice in Italy (1×) and three times that (3×). Seeds were chemically scarified in concentrated sulfuric acid (97%) for twenty minutes, rinsed with water and sown in plastic boxes containing agar medium 0.6% (wt/V) and KNO_3 0.2% (wt/V). Boxes were then placed in a germination cabinet at 15/25 °C (night/day) and 12 h photoperiod with neon tubes providing a Photosynthetic Photon Flux Density (PPFD) of 15–30 μmol m^{-2} s^{-1}. Eighteen seedlings, at very similar growth stage, were transplanted into plastic trays (325 × 265 × 95 mm) with a standard potting mix (60% silty loam soil, 15% sand, 15% perlite and 10% peat). The trays were placed in the greenhouse where the temperature ranged from 15 to 19 °C and from 26 to 33 °C night/day, respectively, and watered daily to maintain the substrate at or near field capacity. When the plants were at the 2–3 leaf stage (i.e. growth stage 12–13 of the Extended BBCH scale [35]), they were sprayed. Herbicides were applied as commercial formulations (see Table 2), with recommended surfactants, using a precision bench sprayer delivering 300 L ha^{-1}, at a pressure of 215 kPa, and a speed of 0.75 m s^{-1}, with a boom equipped with three flat-fan (extended range) hydraulic nozzles (TeeJet®, 11002). Survival and Visual Estimated Biomass (VEB) in relation to the untreated control were recorded three and four weeks after the treatment (WAT) for ACCase and ALS inhibitors, respectively. Mean VEB based on whole tray was estimated; it was determined by giving a score ranging from 10 for the untreated check to 0 for replicates where all plants were clearly dead. Plants were assessed as being dead if, regardless of color, they showed no active growth. Survival records were expressed as percentage of no. of plants treated and standard error was calculated per each mean value. Populations were ascribed as resistant when the absolute value of plant survival minus S.E. is more than 20% when treated at the 1× dose [36].

2.3. Dose-response experiment

Based on the screening results, four resistant populations (09-44a, 09-44c, 09-45 and 09-46) and the susceptible one (07-16S) were included in an outdoor dose-response pot experiment. Seed germination, transplanting and herbicide treatments were done as explained in Section 2.2, with the following differences. After