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Basis of herbicide resistance in two troublesome summer weeds

Echinochloa crus-galli and *Sorghum halepense*

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If you can’t explain it simply,
you don’t understand it well enough.

- A. Einstein

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RIASSUNTO

La resistenza agli erbicidi è un problema a livello mondiale e sta evolvendo velocemente anche in Italia, in particolare nelle zone dove monocoltura e scarsa rotazione di erbicidi con diverso meccanismo d'azione sono pratiche comuni. Inoltre, a causa dell'implementazione dei regolamenti a livello Europeo e dell'ampio utilizzo di gruppi di erbicidi con sito d'azione altamente specifico, sta diminuendo il numero di principi attivi a disposizione, aumentando così il rischio di insorgenza di nuovi casi di resistenza e rendendo più difficoltosa la loro gestione.

La linea guida principale del mio progetto è lo studio multidisciplinare della resistenza agli erbicidi in infestanti poliploidi di colture estive, allo scopo di ottenere informazioni utili per un uso più responsabile degli erbicidi e nuove indicazioni per la gestione della resistenza. In particolare sono state caratterizzate due delle malerbe più problematiche nelle colture estive: *Echinochloa crus-galli* (L.) Beauv., infestante di mais e riso in Nord Italia, e *Sorghum halepense* (L.) Pers., infestante di colture dicotiledoni in Italia e di mais in Ungheria. Entrambe le specie hanno sviluppato resistenza a due classi di erbicidi selettivi: gli inibitori dell'acetil coenzima A carbossilasi (ACCasi) e quelli dell'acetolattato sintasi (ALS). Gli scopi di questo lavoro sono molteplici, a livello pratico in quanto queste due specie stanno diventando un grosso problema nella gestione delle colture infestate e a livello scientifico poiché, vista la difficoltà di lavorarci, sono stati fatti pochi studi su specie poliploidi.

Il progetto è stato sviluppato nel corso dei tre anni, a partire dallo screening delle popolazioni presunte resistenti di ogni specie e la determinazione del pattern e dei livelli di resistenza attraverso degli esperimenti di dose-risposta. I risultati mettono in evidenza come la resistenza agli inibitori dell'ALS in *E. crus-galli* si stia diffondendo rapidamente: sono state confermate diverse popolazioni resistenti agli inibitori dell'ALS (tredici campionate in mais e nove in riso) e, per la prima volta in Europa, dei casi di multi-resistenza agli inibitori dell'ALS e dell'ACCasi (cinque in colture di riso). La situazione del *S. halepense* in Italia è abbastanza stabilizzata (le cinque popolazioni resistenti agli inibitori dell'ACCasi trovate in colture dicotiledoni risalgono al 2007), mentre la resistenza alle solfoniluree (una famiglia degli inibitori

dell'ALS) si sta diffondendo rapidamente in alcune regioni dell'Ungheria, dove dieci popolazioni sono state confermate negli ultimi tre anni. Gli indici di resistenza calcolati per gli inibitori dell'ALS sono risultati molto elevati per tutte le popolazioni, indicando che potrebbe essere coinvolto un meccanismo di tipo target-site. Successivamente sono stati condotti esperimenti diversi per le due specie.

Per quel che riguarda *E. crus-galli* lo studio si è concentrato sulle basi molecolari e fisiologiche della resistenza agli inibitori dell'ALS, attraverso una serie di esperimenti *in vitro*. Il Southern blotting ha chiaramente dimostrato la presenza di copie multiple del gene ALS nel genoma di *E. crus-galli*. Il saggio enzimatico che misura l'attività dell'ALS ha dato alti livelli di resistenza per entrambi gli inibitori dell'ALS testati, confermando che un sito target alterato è responsabile della resistenza a questo gruppo di erbicidi. Un lavoro lungo e difficile, a causa della natura poliploide della specie e della scarsità di informazioni in letteratura, ha permesso di ottenere per la prima volta la sequenza del gene ALS di *E. crus-galli*, che è stata pubblicata nel database GenBank. Su questa sequenza sono stati costruiti primers specifici e, attraverso degli esperimenti di clonaggio, sono state analizzate due o tre piante per ogni popolazione, per arrivare infine alla costruzione di alberi filogenetici utilizzando le sequenze dei cloni. Questa procedura ha permesso di identificare due clusters che sono stati associati a due copie espresse del gene ALS (ALS1 e ALS2). La mutazione forte Trp-574-Leu, una tra le mutazioni più documentate che inducono resistenza agli inibitori dell'ALS in diverse specie, è stata trovata in tutte le piante resistenti, e sempre nella copia del gene ALS1. E' stato poi costruito e validato un marcatore molecolare, chiamato AS-CAPS (Allele Specific - Cleaved Amplified Polymorphic Sequences) per l'identificazione della mutazione 574, in omozigosi o in eterozigosi, in un ampio numero di campioni.

Per quel che riguarda *S. halepense*, geofita che si riproduce anche per seme, sono state eseguite delle analisi di crescita con lo scopo di studiare l'allocazione della biomassa nei due organi riproduttivi di diversi biotipi (suscettibili, resistenti agli inibitori dell'ACCasi e resistenti agli inibitori dell'ALS). Inoltre, sono state parzialmente studiate le basi molecolari della resistenza dei due diversi

gruppi di erbicidi. Analisi molecolari precedenti hanno confermato che in tutte le popolazioni resistenti agli inibitori dell'ACCasi incluse nell'esperimento era presente la mutazione Ile-2041-Asn. L'analisi dei campioni con il marcatore molecolare Cleaved Amplified Polymorphic Sequence (CAPS) 2041 ha confermato la presenza di questa variante allelica in tutte le piante delle popolazioni resistenti. I tre esperimenti di analisi di crescita hanno indicato che la resistenza è associata a diversi pattern di allocazione della biomassa, almeno per quel che riguarda le popolazioni resistenti agli ACCasi che hanno la variante allelica Ile-2041-Asn: il biotipo resistente ha una produzione minore di pannocchie, e allo stesso tempo di semi, e una maggior percentuale di biomassa allocata sotto terra, dovuta alla maggior produzione di rizomi. Non sono state invece osservate chiare differenze fra il biotipo resistente agli inibitori dell'ALS e quello suscettibile, probabilmente a causa del diverso tipo di resistenza coinvolto: infatti sono state trovate due diverse mutazioni, la Trp-574-Leu in una popolazione e la Asp-376-Glu in un'altra. I risultati si sono dimostrati coerenti quando è stato utilizzato lo stesso materiale di partenza. In particolare, gli esperimenti del 2009 e del 2011, dove sono state utilizzate piante cresciute da rizoma, hanno dato risultati molto simili. Invece alcune differenze sono state registrate nell'esperimento del 2010, dove le piante utilizzate sono cresciute da seme.

I risultati mostrano come la situazione di *E. crus-galli* in coltivazioni di riso sia particolarmente preoccupante, a causa della scarsità di erbicidi efficaci con diversi modi d'azione. Quindi è molto importante prevenire almeno l'evoluzione di biotipi multi-resistenti. Questo può essere ottenuto solamente attraverso l'implementazione delle strategie di gestione della resistenza basate su un reale approccio di gestione integrata delle malerbe.

SUMMARY

Herbicide resistance is a worldwide problem and is evolving fast even in Italy, mainly in areas where monoculture and poor rotation of herbicide modes of action are common practices. Furthermore, reduction in number and diversity of active ingredients available due to the implementation of the EU regulations, as well as the widespread use of highly-active herbicide groups, which are target site specific, will increase the risk of resistance and make its management harder.

The main guideline of my project was the multidisciplinary study of herbicide resistance in polyploid summer crop weeds in order to obtain information for a more responsible use of herbicides and new indications for the management. The research focused on two of the most troublesome weeds in summer crops: *Echinochloa crus-galli* (L.) Beauv. infesting maize and rice in northern Italy and *Sorghum halepense* (L.) Pers. infesting dicot crops in Italy and maize in Hungary. Both species have evolved resistance to two groups of selective herbicides, the acetil coenzyme A carboxylase (ACCase) inhibiting herbicides and the acetolactate synthase (ALS) inhibiting herbicides. This study may have two important impacts: at practical level because these two weeds are becoming a big problem in the management of infested crops, and at scientific level because very few studies have been done on polyploid species for the difficulty to work with.

The project evolved during the three years, starting from the screening of the putative resistant populations of each species and the determination of the resistance patterns and levels through dose-response experiments. Twenty two *E. crus-galli* populations resistant to ALS inhibitors (thirteen sampled in maize and nine in rice fields) and five *E. crus-galli* populations multiple-resistant to ALS and ACCase inhibitors in rice crops were confirmed. Furthermore, ten *S. halepense* populations were confirmed to be resistant to ALS inhibitors in maize fields (nine in Hungary and one in Italy) and five to ACCase inhibitors in dicot crops. This means that resistance to ALS inhibitors in *E. crus-galli* is spreading rapidly in Italy and it is particularly worrying where multiple resistance to ALS and ACCase inhibitors is present. Resistance to sulfonylureas, an ALS inhibitors chemical family, in *S. halepense* is instead rapidly spreading in some Hungarian regions. Resistance

indexes calculated for ALS inhibitors resulted very high for all populations, indicating that a target-site resistance mechanism may be involved. Subsequently, the experiments diverged for the two species.

For *E. crus-galli* the focus was the molecular and physiological basis of ALS inhibitors resistance, through several *in vitro* experiments. The Southern blotting analysis clearly showed the presence of multiple copies of the ALS gene in the genome of *E. crus-galli*. ALS enzyme activity bioassay showed high resistance levels to both ALS inhibitors tested, confirming that an altered target site was responsible for conferring resistance to this group of herbicides. A long and tedious work, due to the polyploid nature of the species and the paucity of information in the literature, allowed to obtain the first ALS gene sequence of *E. crus-galli*, which was published in GenBank database. Specific primers were designed on that the sequence and two or three plants for each populations were analyzed through cloning and phylogenetic trees designed on clone sequences. This procedure permitted to identify two clusters that were associated to the expressed ALS genes (ALS1 and ALS2). The “strong” mutation Trp-574-Leu, one of the most documented mutation proved to endow resistance to ALS inhibitors in several weed species, was detected in all the samples belonging the resistant populations, and it was always found in ALS1. A molecular marker, called AS-CAPS (Allele Specific - Cleaved Amplified Polymorphic Sequences), was designed and validated for the rapid detection of the mutation 574, in homozygous or heterozygous status, in a large number of samples.

For *S. halepense*, geophyte which reproduces even by seed, growth analysis experiments were designed in order to study the biomass allocation in these two reproductive organs of different biotypes (susceptible, ACCase inhibitors resistant, and ALS inhibitors resistant). Furthermore, the molecular bases of the two different types of resistance were partially investigated. Previous molecular analyses confirmed that in all of the ACCase inhibitors resistant populations included in the experiments, a single amino acid substitution Ile-2041-Asn had occurred. Analyses with the molecular marker Cleaved Amplified Polymorphic Sequence (CAPS) 2041 confirmed the presence of this allelic variant in all the

plants belonging the resistant populations. The three growth analysis experiments indicated that the resistance status of *S. halepense* is associated with different patterns of dry matter partitioning, at least for the ACCase inhibitors resistant populations having the Ile-2041-Leu allelic variant: the resistant biotype showed a lower panicles production, as well as seeds, and a higher ratio of biomass allocated below ground, due to their higher production of rhizomes. No clear differences were observed between the susceptible and the ALS resistant biotypes, probably due to the different type of resistance involved, i.e. two different mutation were detected, Trp-574-Leu in one population and Asp-376-Glu in another. Results were consistent when the same starting material was used. In particular, 2009 and 2011 experiments, where plants grew from rhizome buds, showed very comparable results. Some differences were, instead, detected in the 2010 experiment, where plants grew from seeds.

Results showed as the overall situation of *E. crus-galli* in Italian rice crops is of great concern, due to the scarcity of effective herbicides with different modes of action available. It is very important to prevent at least the evolution of multiple resistant biotypes. This can only be pursued through the implementation of resistance management strategies based on real integrated weed management approach.

Chapter I

General Introduction

1.1. Herbicide resistance

1.1.1. What are weeds?

Defining a weed as an unwanted plant or a plant out of place does not distinguish plants that are true weeds from those that are only occasional nuisances. These definitions imply that the physical location of a plant is the sole factor determining its potential as a weed. But, a weed is a weed because it possesses certain characteristics that set it apart from other plant species (Baker, 1974). These biological features allow weeds to harm other plants, survive in a variety of environments, generally produce abundant quantity of seeds (and thus produce potentially large populations), and grow and spread rapidly. Because of these features, weeds are better described as “plants that interfere with the growth of desirable plants and are unusually persistent. They damage cropping systems, natural systems, and human activities and as such are undesirable” (Ross and Lembi, 2009). Inside a cropping system, they may interfere also with the quality of the final product, e.g. the main damage produced by red rice is due to the depreciation of rice grain because of the “red grain”.

Why should anyone be interested in learning about weeds? The fact that weeds can have a negative impact on crops yield, quality and interfere with crop management are sufficient reasons to study them. In fact, “weed” is prevalently a concept of humans, not of nature, and, consequently, it depends who says that a plant, at a certain time and place, is objectionable or interferes with our activities. The most common and straight-forward definition still puts humans at the center and considers the weed as “a plant growing in the wrong place at the wrong moment” (Sattin *et al.*, 1995).

The undesirability of weeds most frequently focuses on their deleterious effects on crops. Whether measured by loss of yield (quantity), loss in quality, or the added costs of weed control, the economic impact is relevant difficult to assess. Losses mainly fall into two categories: direct losses, including qualitative and quantitative reduction of agricultural output, and indirect losses due to the higher input required when weeds are present. The first category encompasses

yield reduction due to the competition and lower grain quality through contamination, while the second includes the cost of weed control (herbicides plus application), increased cost of harvesting and land cultivation, and loss in land value. Weed control and economics are inseparable in an agriculture production context (Auld *et al.*, 1987).

1.1.2. Brief herbicide history

Economically, herbicides are the most important category of pesticides, and they have shown the highest average annual increment between 1960 and 1990. Approximately 220 herbicides in over 60 chemical families are assigned to 26 defined mechanism of action categories in the 2007 edition of the Herbicide Handbook of the Weed Science of America. However, the no. of active ingredients available in Europe has been steadily declining in the last decade due to the implementation of the strict EU legislation on pesticides.

An outstanding herbicide is effective at low doses, selective towards the crop, economical to manufacture, has a variety of uses, is safe and easy to handle and apply, and is relatively benign in the environmental. The discovery of herbicidal properties of 2,4-D (2,4-dichlorophenoxyacetic acid), first synthesized in 1941, was the start of modern herbicide technology. 2,4-D proved to be an exceptional herbicide. Its commercial success led to the development in the ‘50s of other herbicides, such as urea herbicides. Glyphosate, a non-selective herbicide introduced in the early ‘70s, provided outstanding control of most perennial grasses and many perennial broadleaved weeds. In the ‘80s, several selective post-emergence treatments in major crops were made available: two families that inhibit acetil coenzyme A carboxylase (ACCase) and destroy meristems of grasses have provided the major post-emergence herbicides for annual and perennial grass control in broadleaf crops of all kinds. Furthermore, a new standard in low herbicide dosage was achieved with the introduction of the sulfonylurea and imidazolinone chemical families. Many of these herbicides provide excellent selectivity on crops and are relatively safe for humans and the environment.

Although herbicide chemistry will continue to foster environmental and human safety, the rate of discovery and development of new chemistries has significantly slowed down. This trend is due, in part to high costs relative to stricter legislations, fewer competitors and shifts away from pesticide development to creation of pest-resistant crop cultivars (first introduced in the ‘90s in USA and still not available in Italy).

1.1.3. Herbicide resistance

Modern agriculture relays on the widespread use of herbicides because they are the most effective and last expensive tool for weed control (Powles and Shaner, 2001). Unfortunately, some weed populations have evolved resistance to one or more herbicides, which represents a major problem in weed management, mainly after the introduction of an increasing number of more selective herbicides with a very specific metabolic target (Heap, 1999). According to the Herbicide Resistance Action Committee (HRAC), herbicide resistance is defined as “the naturally occurring inheritable ability of some weed biotypes within a given weed population to survive a herbicide treatment that would, under normal use conditions, effectively control that weed population”. At the same time, a resistant biotype is a group of individuals that share several physiological characteristics, such as the ability of survive one or more herbicides, belonging a particular group, used under normal conditions. The term resistance is sometimes wrongly used as synonym with the term tolerance, defined by Holt and Le Baron (1990) as “the normal variability of response to herbicides present among plant species”.

Resistance is an evolutive process. The evolution driven by herbicides is due to their characteristic efficacy profile, i.e. some weeds are controlled better than others. As well as this natural variability, some rare individuals are naturally resistant to a specific active ingredient and survive to a herbicide dose that usually control all the individuals of the population. Their frequency vary depending on the species and the resistance mechanism. Subsequent to the occurrence of a resistant mutant, repeated treatments with herbicides having the same mode of action repeatedly remove susceptible individuals whereas the resistant ones may

reproduce and disperse, lead to the evolution, even in short period of time, of a resistant population (biotype). This constrains farmers to use other herbicides, or other methods, which may be more expensive and/or less effective (Sattin and Zanin, 2006).

The European and Mediterranean Plant Protection Organization (EPPO) guidelines on resistance risk analysis precisely distinguish between the resistance selected in the laboratory and that observed under agricultural conditions, introducing the concept of practical resistance: “Although resistance can often be demonstrated in the laboratory, this does not necessarily mean that pest control in the field is reduced, and this is particularly true with fungicides. Practical resistance is the term used for loss of field control due to a shift in sensitivity” (EPPO, 1988). It is widely accepted that a weed population is considered affected by practical resistance when at least 20% of the plants, originated from seeds collected from plants that escaped a herbicide treatment in a field, are not controlled by a treatment done with the same herbicide at the recommended field dose.

The sequence of events which occur since the herbicide is adsorbed by the plant to the final effect caused by the herbicide is called Mode of Action (MoA). Although it is difficult to generalize, a weed population can be resistant to only one herbicide, or to herbicides with the same MoA, i.e. only one resistance mechanism is involved (cross-resistance) or to herbicides with different MoA, i.e. more than one resistance mechanism is involved (multiple resistant) (Hall *et al.*, 1994).

1.1.3.1. Resistance mechanisms

Physiological effects of herbicide action can interfere with cell division, plant growth regulation, photosynthesis and/or respiration and essential metabolic processes such as amino acid biosynthesis. Mechanisms of resistance can be summarized in two groups: target-site resistance and non-target-site resistance.

Target-site resistance is referred as modifications at the herbicide binding site which preclude the herbicide from binding, usually to an enzyme or to a cellular receptor; the gene(s) of the resistant plant is therefore altered/mutated in one (or more) specific points and code for an altered protein.

Non-target-site mechanisms generally reduce the amount of herbicide reaching the target-site, and can imply enhanced metabolism, gene overexpression which leads to overproduction of target proteins, reduced rates of herbicide uptake, translocation and compartmentalization. Enhanced metabolism is due to the presence of detoxifying enzymes that breakdown the herbicide in non-toxic products or reduces toxicity of the herbicide through a molecular alteration which prevents the herbicide to reach its target. The most common detoxifying mechanisms are due to an elevated expression of cytochrome P_{450} mono-oxygenase, which plays an important role in the oxidative metabolism of xenobiotics, and glutathione-S-transferases (Hall *et al.*, 1994). An overexpression of target proteins causes an increase of the number of sites in a plant, which exceed the number of herbicide molecules. As a result, some target proteins remain unaffected by the herbicide and an acceptable level of normal plant function is maintained. Clearly, the overall efficacy is dependent upon the herbicide dose. Less common non-target-site mechanisms are reduced rates of herbicide uptake (acting on the kinetics of herbicide mobility), translocation and compartmentalization (the herbicide is precluded to reach the site of action or enter the cell usually by a mutant carrier or the herbicide is sequestered in vacuoles).

At the same time, two broad cross-resistance categories can be recognized: target-site cross-resistance and non-target-site cross-resistance. Target-site cross-resistance occurs when a change at the biochemical site of action of one herbicide also confers resistance to herbicides belonging to the same chemical class that inhibit the same site of action. Target-site cross-resistance does not necessarily result in resistance to all herbicide chemical families with a similar mode of action or indeed all herbicides within a given herbicide group. For example, it was demonstrated that resistance to FOP herbicides in several biotypes of *A. sterilis* is endowed by resistant forms of the ACCase enzyme. In these cases there are varying degrees of target-site cross-resistance to FOP herbicides, ranging from none to moderate, but not resistance to DIM herbicides (Maneechote *et al.*, 1994). Non-target-site cross-resistance is defined as cross-resistance to dissimilar herbicide chemical families conferred by a mechanism other than resistant enzyme

target sites. For example, a mechanistic basis of non-target-site cross-resistance to ALS inhibitors has been thoroughly investigated in *L. rigidum*: it was resistant as a result of an enhanced rate of herbicide metabolism (catalyzed by Cyt P_{450} enzymes), which endows resistance to certain ALS-inhibiting herbicides (chlorotuluron and chlorsulfuron) (Burnet *et al.*, 1993).

Multiple resistance is defined as the expression (within individuals or populations) of more than one resistance mechanism. Multiple resistant plants may possess from two to many distinct resistance mechanisms and may exhibit resistance to a few or many herbicides, e.g. a biotype of *L. rigidum* (WLR1) selected only with chlorsulfuron is resistant to ALS-inhibiting herbicides and has a resistant ALS enzyme as well as enhanced metabolism of chlorsulfuron (Christopher *et al.*, 1992). The simplest cases are where an individual plant (or population) possesses two or more different resistance mechanisms which provide resistance to a single herbicide, or class of herbicides. More complicated are situations where two or more distinct resistance mechanisms have been selected either sequentially or concurrently by different herbicides and endow resistance to the classes of herbicide to which they had been exposed. The most complicated and difficult to control situations are where a number of resistance mechanisms, involving both target-site and non-target-site resistance mechanisms, are present within the same individual. So far, only simple cases of multiple resistance have been fully documented for a small number of weed species, the majority of cases and the most complicated situations have often been reported for grass weeds as *L. rigidum* (Tardif and Powles, 1994; Owen *et al.*, 2007), *Echinochloa* spp. (Fischer *et al.*, 2000) and *A. myosuroides* (Petit *et al.*, 2010).

1.1.3.2. HRAC herbicide classification

How herbicides are described or categorized is a matter of convenience. It depends largely on those features or aspects that the describer wants to emphasize. In fact, there are at least eight ways in which herbicides commonly and frequently are classified. For our outcomes, the mechanisms by which herbicides kill plants is a useful feature for classification. Another expression, often used as

synonym, is mechanism of action (MoA) which is considered the identification of the specific biochemical target. For example, the lipid synthesis inhibitors at acetyl coenzyme A carboxylase (ACCase) level is the MoA, while the mechanism of action is the binding of the herbicide at the enzyme level.

The Weed Science Society of America (WSSA) and the Herbicide Resistance Action Committee (HRAC) have assigned individual compounds to more than 20 defined MoA groups (www.hracglobal.com). The aim of HRAC is to create a uniform classification of herbicide sites of action in as many countries as possible. The herbicides are classified alphabetically according to their target sites, sites of action, similarity of induced symptoms or chemical classes. If different herbicide groups share the same site of action only one letter is used, e.g. in the case of photosynthesis inhibitors subgroups C_1, C_2 and C_3 indicate different binding behavior at the binding protein D_1. Herbicides with unknown sites of action are classified in group Z as "unknown" until they can be grouped exactly.

1.1.4. Acetil coenzyme A carboxylase (ACCase) inhibitors

Acetyl coenzyme A carboxylase inhibitors (indicated as “group A” in HRAC classification) are a group of commercially important herbicides enabling efficient management of grass weed species. They include three chemical families: the aryloxyphenoxypropionate (AOPP or FOP) and the cyclohexanedione (CHD or DIM) and the phenylpyrazoline (DEN) which includes only one herbicide, pinoxaden (Fig. 1). ACCase catalyzes the first committed step in fatty acid biosynthesis and, in particular, the two-step condensation of acetyl-CoA with bicarbonate to form malonil-CoA. Kinetic studies have indicated that these herbicides inhibit the carboxyltransferase activity of the enzyme (Burton *et al.*, 1991) that leads to inhibition of acyl lipid biosynthesis, eventually resulting in death of the plant.

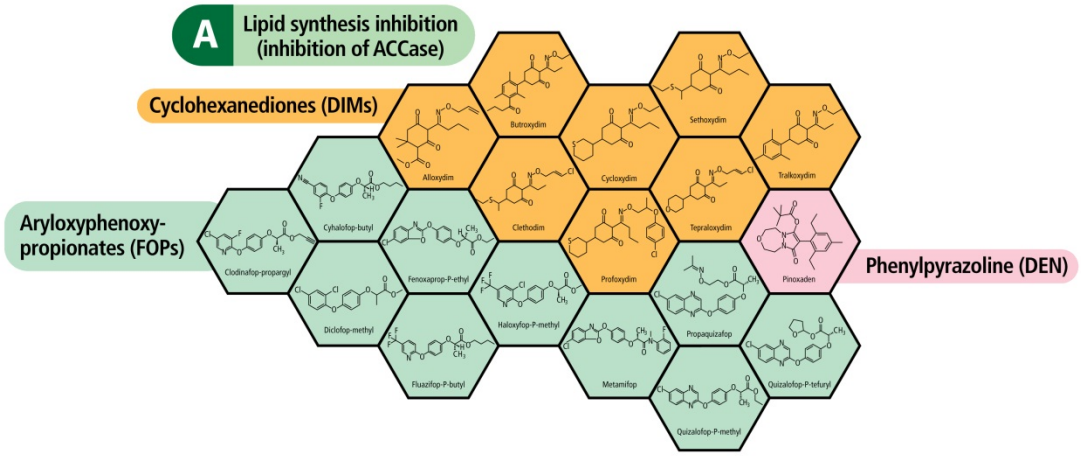

Fig. 1. Section of HRAC panel representing ACCase-inhibiting herbicides divided into families.

At least two Acetil Coenzyme A Carboxylase (ACCase, EC 6.4.1.2) isoforms are present in the cytosol and in the plastids of plants, respectively (Délye, 2005). All of them contain three catalytic domains, namely biotin carboxyl-carrier (BCC) domain, biotin carboxylase (BC) domain and carboxyl transferase (CT) domain (Nikolau *et al.*, 2003). The plastidic ACCase isoform accounts for >80% of the total ACCase activity in leaves (De Prado *et al.*, 2000). In most plant species, it is a heteromeric enzyme composed of four distinct subunits: two for the CT domain and one for each of the BC and BCC domains. In contrast, the ACCase isoforms occurring in the cytosol in all plants and in the plastids and mitochondria in Poaceae (grasses) are large, homomeric enzymes carrying all three functional domains on a single polypeptide (Focke *et al.*, 2003) (Fig. 2). All homomeric ACCase share a high degree of sequence conservation.

FOP and DIM bind to the homomeric ACCase CT domain in competitive way: two molecule of herbicides bind cooperatively inside the two active sites of a CT dimer causing conformational changes in the structure (Zhang *et al.,* 2004). These changes are incompatible with the binding of acetyl CoA and malonyl CoA molecules for catalysis.

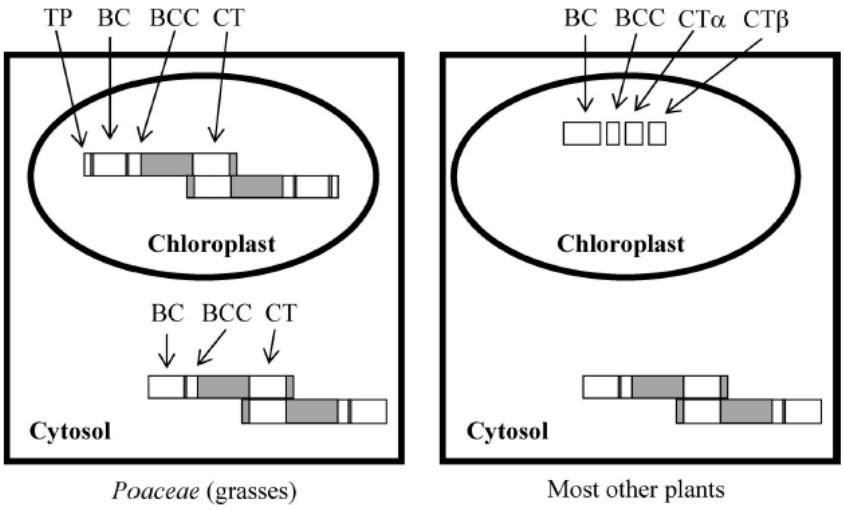

Fig. 2. ACCase isoforms in plants (Délye, 2005).

FOP and DIM strictly target grass species. This can be related to the high sensitivity of chloroplastic homomeric ACCase to these herbicides. On the contrary, cytosolic homomeric and heteromeric ACCase isoforms are consistently found to be less sensitive by several orders of magnitude than the homomeric plastidic isoforms (Alban *et al.*, 1994). Grass crops, such as *Zea mays* L. and *Orysa sativa* L., contain plastidic homomeric ACCase isoforms with sensitivity to FOP and DIM similar to that of plastidic ACCase from grass weeds. However, ACCase-inhibiting herbicides are used to control weeds in these crops. The selectivity is solely based on the capacity of the grass crop to rapidly metabolize the herbicides into inactive products (Cummins and Edwards, 2004).

1.1.4.1. Molecular basis of resistance to ACCase inhibitors

The frequent use of FOP and DIM herbicides has resulted in the development of resistance to these herbicides in 41 species globally (Heap, 2011). Mechanisms of resistance to ACCase-inhibiting herbicides can be divided into two categories: ACCase-related and metabolism-based.

In most cases, the first type predominated and resistance is due to an alteration in the target enzyme, making it less sensitive to inhibition by these

herbicides (Devine and Shukla, 2000). The seven variable amino acids with a role in sensitivity to ACCase-inhibiting herbicides identified so far are listed in table 1. All are located within the active site cavity of the ACCase CT domain (Délye *et al.*, 2005). Results of published studies enable a separation of two groups of cross-resistance patterns conferred by mutant plastidic ACCase isoforms (Table 1). One group showed high resistance to all FOP and DIM assayed and it has been associated with different amino acid substitutions: in *Lolium* spp., this cross-resistance pattern is associated with an Ile-1781-Leu (Yu *et al.*, 2007) or a Cys-2088-Arg, whereas in *P. paradoxa* the Leu in position 1781 is substituted with a Val (Collavo *et al.*, 2010). Instead, in *A. myosuroides* this pattern is associated with an Asp-2078-Gly substitution (Délye *et al.*, 2005). A second group showed high resistance to FOP, but low resistance or no resistance to DIM. This cross-resistance pattern is conferred, in *A. myosuroides*, by a Trp-2027-Cys, an Ile-2041-Asn, or a Gly-2096-Ala substitution (Délye *et al.*, 2003 and 2005). It should be noted that an Ile to Val substitution at position 2041 did not confer high resistance to the FOP clodinafop, in contrast with the Ile to Asn substitution at position 2041 (Délye *et al.*, 2003). Furthermore, a substitution in position 1999 was found in *A. sterilis* (Liu *et al.*, 2007), but it is not possible to associate a pattern of resistance.

Such enzymes enable grass crop to withstand field applications of ACCase-inhibiting herbicides. They also exist in grass weeds. Variation in their regulation, specificity, or both confers metabolism-based resistance to ACCase-inhibiting herbicides in grass weeds. A number of genes are clearly involved in metabolism-based resistance to ACCase-inhibiting herbicides, which renders their study fairly complicated. Besides, cross-resistance patterns associated with metabolism-based resistance are quite unpredictable.

Amino acid residue [b]			Resistance[a]											
			APP [c]						CHD [d]					DEN [e]
Wild-type	Resistant	Weed species	Cd	Cy	Dc	Fx	Fz	Hx	Ct	Cx	Sx	Tk	Te	Pi
Ile_{1781}	Leu	*A. myosuroides*	S		R	R	R	S	S	R	R	R		
	Leu	*A. myosuroides*									R			
	Leu	*A. myosuroides*	ND	R	R	R	ND	ND	ND	ND	ND	ND	R	
	Leu	*A. fatua*	ND		R	ND	ND	ND	ND	ND	R	ND		
	Leu	*A. sterisilis* ssp. *ludoviciana*	R		ND	R	ND	ND	ND	ND	ND	R		
	Leu	*S. viridis*	ND		R	R	ND	ND	ND	ND	R	R		
	Leu	*Lolium* sp.	S/R		R	R	R	R	R	R	R	R		R
	Val	*Phalaris paradoxa*	R		R	R	R	R	R	R	S	R		R
Trp_{1999}	Cys	*A. sterisilis* ssp. *ludoviciana*	ND		ND	R	ND	ND	ND	ND	ND	ND		
Trp_{2027}	Cys	*A. myosuroides*	R		ND		ND	R	S	S	ND	ND		
	Cys	*A. sterisilis* ssp. *ludoviciana*	R		ND	R	ND	R	ND	ND	R	R		
Ile_{2041}	Asn	*A. myosuroides*	R		ND	R	ND	R	S	S	ND	ND		
	Asn	*Lolium* sp.	R		R	ND	ND	R	ND	S	ND	ND		
	Val	*Lolium* sp	S		ND	ND	ND	R	ND	S	ND	ND		
	Asn	*A. sterisilis* ssp. *ludoviciana*	ND		ND	R	ND	R	ND	ND	R	ND		
Asp_{2078}	Gly	*A. myosuroides*	R		R	R	R	R	R	R	R	R		R
	Gly	*A. sterisilis* ssp. *ludoviciana*	ND		ND	R	ND	R	ND	ND	R	R		
	Gly	*Phalaris paradoxa*	R		R	R	R	R	R	R	R	R		R
Cys_{2088}	Arg	*Lolium sp.*	R		R	ND	R	R	R	ND	R	R		R
Gly_{2096}	Ala	*A. myosuroides*	R		ND	R	ND	R	S	S	ND	ND		

Table 1. Amino acid substitutions within plastidic, homomeric ACCase and associated cross-resistance patterns observed at the whole plant level. S and R respectively indicate that plants containing at least one copy of the ACCase mutant allele are sensitive or resistant to the corresponding herbicide either in the field or in bioassays; ND, not determined at the whole plant level; [b] Amino acid number is standardized to *A. myosuroides* plastidic, homomeric ACCase (EMBL accession AJ310767); [c] aryloxyphenoxypropionate: Cd, clodinafop; Cy, cyhalofop; Dc, diclofop; Fx, fenoxaprop; Fz, fluazifop; Hx, haloxyfop; [d] cyclohexanedione: Ct, clethodim; Cx, cycloxydim; Sx; sethoxydim; Tk, tralkoxydim; Te, tepraloxydim: [e] phenylpyrazoline: Pi, pinoxaden.

1.1.5. Acetolactate synthase (ALS) inhibitors

In 1982, chlorsulfuron was the first acetohydroxyacid synthase inhibitor to be commercialized. And, in 1986, the first imizazolinone (imazaquin) was introduced. The introduction of these two classes of herbicides started a new era in herbicide technology. Their unique mode of action coupled with low mammalian toxicity and high potency set new standards. They are active when applied to the soil or foliage and control a broad spectrum of grasses and broadleaves. One of the attractive features of the ALS inhibitors is their low toxicity to non-target organisms including mammals, fish, insects and other invertebrates. These properties are the result of rapid metabolism and excretion by non-target organisms and the fact that there is no target site for these inhibitors in animals. Actually, ALS inhibitors (indicated as "group B" in HRAC classification) include five chemical families (Fig. 3): the sulfonylureas (SU), the imidazolinones (IMI), the triazolopyrimidines (TP), the pyrimidinyl(thio)benzoates (PTB) and the sulfonylamino-carbonyl-triazolinones (SCT).

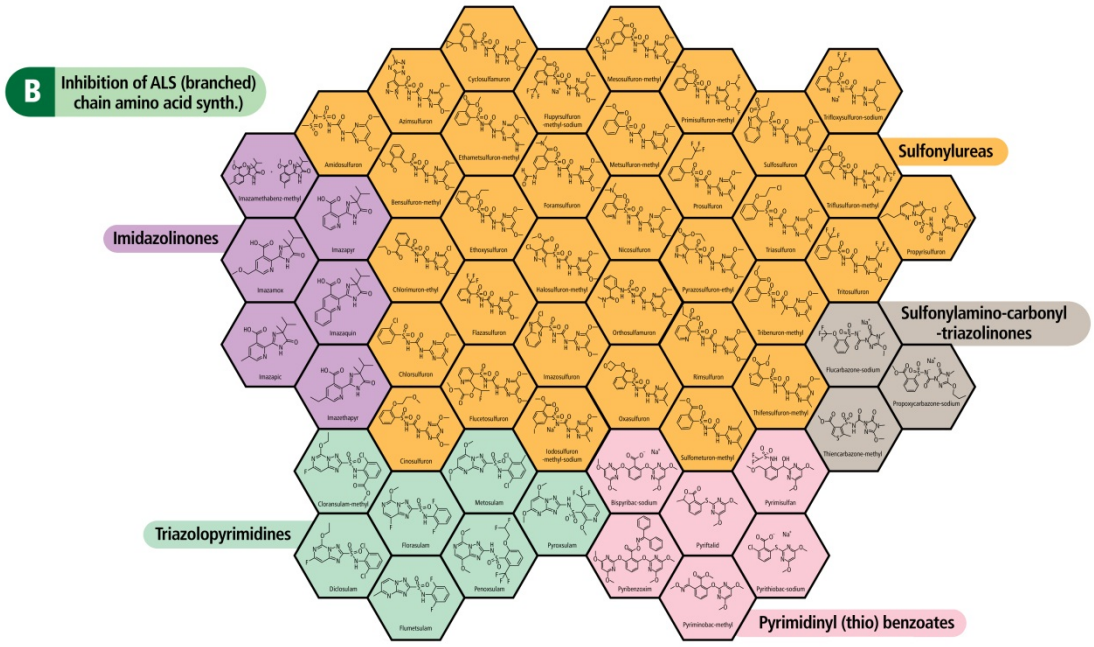

Fig. 3. Section of HRAC panel representing ALS-inhibiting herbicides divided into families.

Acetolactate synthase (ALS or AHAS, EC 2.2.1.6) carries out two parallel sets of reaction in the biosynthesis of branched chain amino acids (Fig. 4). In the first

reaction, ALS condenses two moles of pyruvate to yield acetolactate in the pathway that produces valine and leucine. In the second reaction, ALS uses a mole of pyruvate and a mole of 2-ketobutyrate to yield acetohydroxybutyrate, which is used in parallel pathway that produces isoleucine. The plant ALS uses thiamine pyrophosphate (TPP), flavin adenine dinucleotide (FAD) and Mg^{++} as co-factors in the two condensation reactions (Shaner and Singh, 1997).

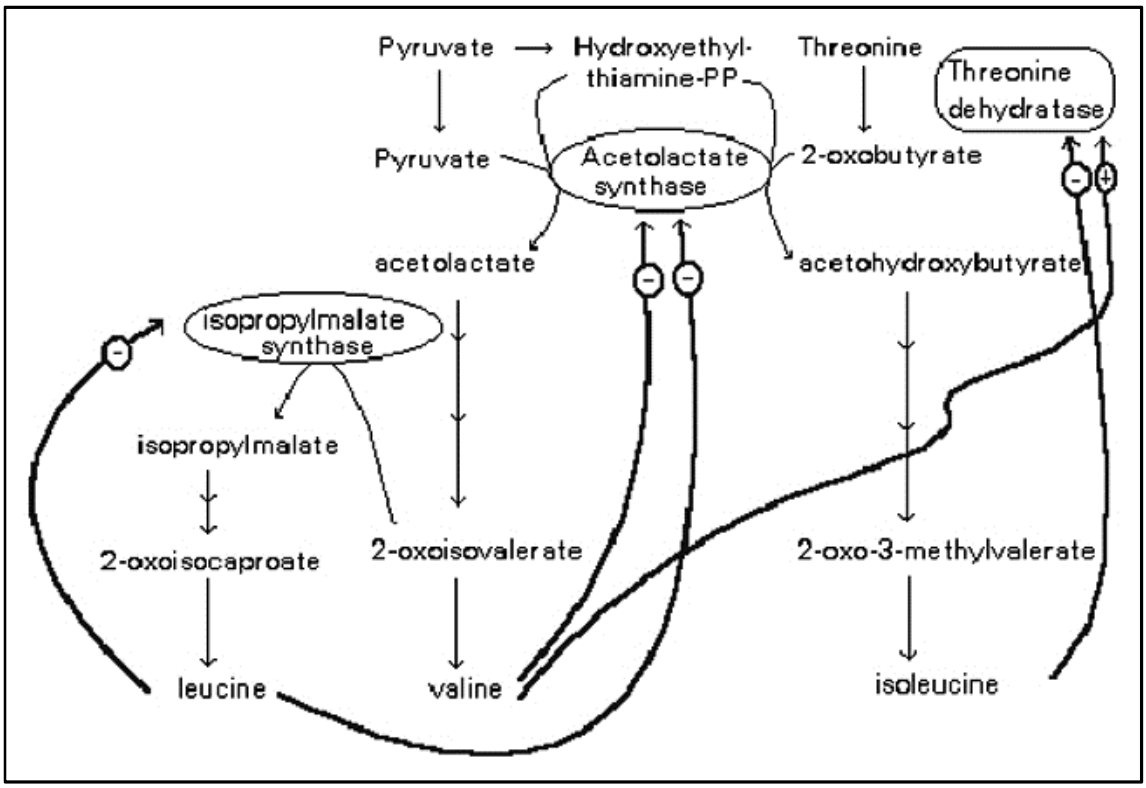

Fig. 4. Pathways of biosynthesis of branched chain amino acids involving the two steps catalyzed by acetolactate synthase.

ALS activity from plants is feedback inhibited by valine, leucine and isoleucine, the end products of the pathway. A synergistic inhibition of the enzyme is observed when a combination of valine and leucine are used, suggesting two separate binding sites for these amino acids. Only a large catalytic subunit of ALS has been detected in plants and at least two regulatory sites with different specificities, leading to a complex pathway, for activity regulation (Chipman *et al.*, 2005). ALS is a nuclear encoded chloroplastic enzyme in plants; all the genes isolated so far contain a putative chloroplast transit peptide sequence consistent with the localization of ALS activity in chloroplasts.

The inhibition of ALS by herbicides is a time-dependent process with an initial weak inhibition followed by a slow transition into a final steady-state where the inhibition is more potent. Both steps are dependent on the concentration of the inhibitor. Whether the inhibition by herbicides is reversible and competitive or non-competitive remains controversial and, probably, the different herbicide families behave differently (Chipman *et al.*, 2005). The location of the herbicide binding site started to become clear when the crystal structure of yeast ALS was published (Pang *et al.*, 2002) and subsequently several structures of yeast ALS with bound sulfonylureas (McCourt *et al.*, 2005) were determined. They has established that these herbicides bind in a channel that leads to the active site, and is lined with amino acids that, when mutated, give rise to sulfonylurea resistance in yeast ALS (Duggleby *et al.*, 2003).

The major advantage of ALS inhibitors as commercial herbicides is due to their selectivity on many of the major crops. There are at least three mechanisms of herbicide selectivity, differential absorption and translocation, differential inhibition at the site of action, and differential metabolism. Different metabolic pathways exist in plant that detoxify ALS inhibitors, including dealkylation, ring hydroxylation, glucose conjugation and ring cleavage. In general, tolerant crops metabolize selective ALS inhibitors within a few hours after application. Another mechanism of selectivity of ALS inhibitors is differential activation of the applied herbicide.

1.1.5.1. Molecular basis of resistance to ALS inhibitors

Predominantly, resistance occurs as a result of reduced sensitivity of the target ALS enzyme to inhibition by the herbicide. A second mechanism of resistance is increased herbicide metabolism resulting in rapid detoxification of the herbicide. One of the most interesting aspects of the evolution of weed populations resistant to ALS inhibitors has been the high frequency of occurrence. Although the degree of dominance varies among plant species or alleles (Hart *et al.*, 1993; Sebastian *et al.*, 1989; Wright and Penner, 1998), resistant (R) alleles are selected even when present in the heterozygous condition. ALS functions in plastids, but it

has a nuclear-encoded gene and follows normal Mendelian inheritance. Resistant ALS alleles are therefore disseminated by both pollen and seed. Thus, simply the genetic of ALS-inhibiting herbicide resistance (i.e. that resistance is conferred by a single, dominant, nuclear-encoded gene) might partially account for the high frequency of its occurrence relative to some other herbicide groups.

Eight single amino acid substitutions in the six conserved domains identified so far, that are sufficient to convert ALS from a herbicide sensitive to a herbicide resistant enzyme, have been identified in all weed biotypes investigated (Table 2). Three of these five amino acids (Ala_{122}, Pro_{197}, and Ala_{205}) are located near the amino-terminal (5') end of ALS, two in the middle (D_{376} and Arg_{377}) and other three (Trp_{574}, Ser_{653} and G_{654}) are located near the carboxy-terminal (3') end.

Although exceptions exist, resistance caused by an altered ALS can be generally classified into three types on the basis of cross-resistance: (1) IMI but not SU resistant, (2) SU and TP resistant, and (3) SU, IMI, TP, and PTB resistant (broad cross-resistance). Substitutions of Ala_{122} or Ser_{653} belong the first group, showing IMI but not SU resistance, whereas substitutions of Pro_{197} belongs the second group, usually resulting in SU but not IMI resistance (Table 2). In some cases, low to moderate levels of IMI resistance have also been observed in biotypes with the Pro_{197} substitution, but the resistance has typically been less than 10-fold and not consistent among various IMI herbicides (Saari *et al.*, 1994). Probably, the greatest cross-resistance to IMI herbicides conferred by a substitution of Pro-197-Leu was reported in *Amaranthus retroflexus*, in which resistances to four IMI herbicides ranged from 4- to 63-fold (Sibony *et al.*, 2001). Substitutions of Trp_{574} showed broad cross-resistance to all the ALS inhibitors chemical families, resulting in high levels of resistance to both IMI and SU herbicides (as well as the TP and PTB herbicides). The one example of a weed biotype with resistance caused by an Ala_{205} ALS substitution also displayed a broad cross-resistance; however, the levels of resistance were much lower (approximately 10-fold) than that observed for biotypes with the Trp_{574} ALS substitution. Resistance to one compound of a particular chemical family of ALS-inhibiting herbicides does not guarantee cross-resistance to all members of that

chemical family. This is particularly true for SU herbicides for which differential resistance has been reported in several biotypes (Hart *et al.*, 1993; Saari *et al.*, 1992).

Table 2. ALS amino acid substitutions that confer herbicide resistance and that were identified in herbicide resistant weed populations. Resistance-conferring ALS mutations that were intentionally selected (i.e., laboratory selections) are not included in this table. [(1)] Amino acid number is standardized to the *Arabidopsis thaliana* sequence; [(2)] S = Susceptible biotype, r = Moderate resistance (< 10-fold relative to sensitive biotype), R = High Resistance (> 10-fold), ND = Not Determined. Modified from Tranel *et al.*, 2011.

Amino Acid Residue[(1)]	**Substitution conferring resistance**	**Weed Species**	**SU[(2)]**	**IMI[(2)]**	**PTB[(2)]**	**TP[(2)]**	**SCT[(2)]**
Ala $_{122}$	Thr	*Xanthium strumarium*	S	R	S	ND	ND
	Thr	*Amaranthus hybridus*	S	R	S	S	ND
	Thr	*Solanum ptycanthum*	S	R	ND	ND	ND
	Thr	*Amaranthus retroflexus*	ND	R	ND	ND	ND
	Thr	*Amaranthus powellii*	S	R	ND	ND	ND
Pro $_{197}$	His	*Lactuca serriola*	R	r	S	r	ND
	Thr	*Kochia scoparia*	R	S	ND	R	ND
	Arg	*Kochia scoparia*	R	ND	ND	ND	ND
	Leu	*Kochia scoparia*	R	ND	ND	ND	ND
	Gln	*Kochia scoparia*	R	ND	ND	ND	ND
	Ser	*Kochia scoparia*	R	ND	ND	ND	ND
	Ala	*Kochia scoparia*	R	ND	ND	ND	ND
	Ala	*Brassica tournefortii*	R	S	ND	R	ND
	Ile	*Sisymbrium orientale*	R	r	ND	R	ND
	Leu	*Amaranthus retroflexus*	R	R	R	R	ND
	Ala	*Raphanus raphanistrum*	R	S	ND	R	ND
	Thr	*Raphanus raphanistrum*	R	S	ND	R	ND
	Ala	*Lindernia dubia*	R	ND	ND	ND	ND
	Ser	*Lindernia dubia var. major*	R	ND	ND	ND	ND
	Gln	*Lindernia micrantha*	R	ND	ND	ND	ND
	Ser	*Lindernia micrantha*	R	ND	ND	ND	ND
	Gln	*Lindernia procumbens*	R	ND	ND	ND	ND
	Ser	*Lindernia procumbens*	R	ND	ND	ND	ND
	Ser	*Amaranthus blitoides*	R	S	r	r	ND
	His	*Raphanus raphanistrum*	R	S	ND	R	ND
	Ser	*Raphanus raphanistrum*	R	S	ND	R	ND
	Thr	*Chrysanthemum coronarium*	R	r	R	r	R
	Ser	*Chrysanthemum coronarium*	ND	ND	ND	ND	ND
	Ser	*Monochoria vaginalis*	R	ND	ND	ND	ND

Amino Acid Residue[(1)]	Substitution conferring resistance	Weed Species	SU[(2)]	IMI[(2)]	PTB[(2)]	TP[(2)]	SCT[(2)]
Pro $_{197}$	Ser	*Helianthus annuus*	R	ND	ND	ND	ND
	His	*Papaver rhoeas*	R	r	ND	S	ND
	Thr	*Papaver rhoeas*	R	r	r	r	ND
	Ser	*Papaver rhoeas*	R	r	r	r	ND
	Ser	*Bromus tectorum*	R	S	ND	ND	R
	Ser	*Sinapis arvensis*	R	S	ND	ND	ND
	Thr	*Lactuca serriola*	R	r	ND	r	ND
	His	*Scirpus juncoides var. ohwianus*	R	S	S	ND	ND
	Ser	*Scirpus juncoides var. ohwianus*	R	S	S	ND	ND
	Leu	*Scirpus juncoides var. ohwianus*	R	S	S	ND	ND
	Ser	*Hordeum leporinum*	R	S	ND	ND	ND
	Leu	*Thlaspi arvense*	R	r	ND	S	ND
	Ser	*Lolium rigidum*	R	S	ND	ND	ND
	Ala	*Lolium rigidum*	R	S	ND	ND	ND
	Leu	*Lolium rigidum*	R	S	ND	ND	ND
	Gln	*Lolium rigidum*	R	S	ND	ND	ND
	Thr	*Alopecurus myosuroides*	R	ND	ND	ND	ND
	Arg	*Lolium rigidum*	R	S	ND	ND	ND
	Leu	*Descurainia sophia*	R	ND	ND	ND	ND
	Thr	*Descurainia sophia*	R	ND	ND	ND	ND
	Arg	*Papaver rhoeas*	R	r	r	r	ND
	Leu	*Papaver rhoeas*	R	R	ND	S	ND
	Ala	*Papaver rhoeas*	R	r	r	r	ND
	Gln	*Stellaria media*	R	ND	ND	S	ND
	His	*Scirpus mucronatus*	R	S	S	R	ND
	Gln	*Salsola iberica*	R	ND	ND	ND	ND
	Ser	*Capsella bursa-pastoris*	R	ND	ND	ND	ND
	Thr	*Apera spica-venti*	R	ND	ND	r	r
	Asn	*Apera spica-venti*	R	ND	ND	r	r
	Ala	*Descurainia sophia*	R	ND	ND	ND	ND
	Ser	*Descurainia sophia*	R	ND	ND	ND	ND
	Leu	*Anthemis cotula*	R	r	ND	r	ND
	Gln	*Anthemis cotula*	R	r	ND	R	ND
	Thr	*Anthemis cotula*	R	r	ND	r	ND
	Ser	*Anthemis cotula*	R	r	ND	R	ND
	Ala	*Conyza canadensis*	R	S	R	R	ND
	Ser	*Conyza canadensis*	R	S	R	R	ND
Ala $_{205}$	Val	*Xanthium strumarium*	r	r	r	r	ND
	Val	*Helianthus annuus*	r	R	ND	ND	ND

Amino Acid Residue[1]	Substitution conferring resistance	Weed Species	SU[2]	IMI[2]	PTB[2]	TP[2]	SCT[2]
Ala 205	Val	*Amaranthus retroflexus*	S	R	ND	ND	ND
	Val	*Solanum ptycanthum*	r	R	ND	S	ND
Asp 376	Glu	*Amaranthus hybridus*	R	R	R	R	R
	Glu	*Kochia scoparia*	R	ND	ND	ND	ND
	Glu	*Amaranthus powellii*	R	R	R	R	R
	Glu	*Conyza canadensis*	R	r	R	R	ND
Arg 377	His	*Apera spica-venti*	R	ND	ND	R	R
Trp 574	Leu	*Xanthium strumarium*	R	R	R	R	ND
	Leu	*Amaranthus tuberculatus (syn. rudis)*	R	R	ND	R	ND
	Leu	*Amaranthus hybridus*	R	R	ND	R	ND
	Leu	*Kochia scoparia*	R	R	ND	ND	ND
	Leu	*Sisymbrium orientale*	R	R	ND	R	ND
	Leu	*Ambrosia artemisiifolia*	R	R	ND	R	ND
	Leu	*Ambrosia trifida*	R	R	ND	R	ND
	Leu	*Raphanus raphanistrum*	ND	ND	ND	ND	ND
	Leu	*Amaranthus blitoides*	R	R	R	R	ND
	Leu	*Camelina microcarpa*	R	R	ND	R	R
	Leu	*Amaranthus retroflexus*	R	R	ND	ND	ND
	Leu	*Amaranthus powellii*	R	R	ND	ND	ND
	Leu	*Sinapis arvensis*	R	R	ND	R	R
	Leu	*Lolium rigidum*	R	R	ND	ND	ND
	Leu	*Scirpus juncoides var. ohwianus*	R	R	R	ND	ND
	Leu	*Alopecurus myosuroides*	R	ND	ND	ND	ND
	Leu	*Bidens subalternans*	R	R	R	R	ND
	Leu	*Stellaria media*	R	ND	ND	R	ND
	Leu	*Salsola iberica*	R	ND	ND	ND	ND
	Leu	*Scirpus mucronatus*	R	R	R	R	ND
	Leu	*Papaver rhoeas*	R	ND	ND	ND	ND
	Leu	*Apera spica-venti*	R	ND	ND	R	R
Ser 653	Thr	*Amaranthus powellii*	S	R	ND	ND	ND
	Thr	*Amaranthus retroflexus*	S	R	ND	ND	ND
	Asn	*Amaranthus tuberculatus (syn. rudis)*	S	R	ND	S	ND
	Thr	*Amaranthus tuberculatus (syn. rudis)*	S	R	ND	S	ND
	Asn	*Amaranthus hybridus*	r	R	R	r	ND
	Ile	*Setaria viridis*	r	R	R	ND	r
	Asn	*Setaria viridis*	r	R	R	ND	r
	Thr	*Setaria viridis*	r	R	S	ND	r
Gly 654	Asp	*Setaria viridis*	r	R	S	ND	r

1.1.6. Resistance evolution: worldwide and Italian situation

Herbicide resistance was first reported in 1968 in the state of Washington, where a triazine resistant population of *Senecio vulgaris* was identified (Ryan, 1970). Between 1970 and 1977 one new case per year was signaled. Since 1978, the improve of new resistant species remained constant with an average of nine new cases per year worldwide (Heap, 2000). In the following two decades, more than 110 weed species were reported to be resistant to one or more of 15 classes of herbicides and this trend continues (Fig. 5).

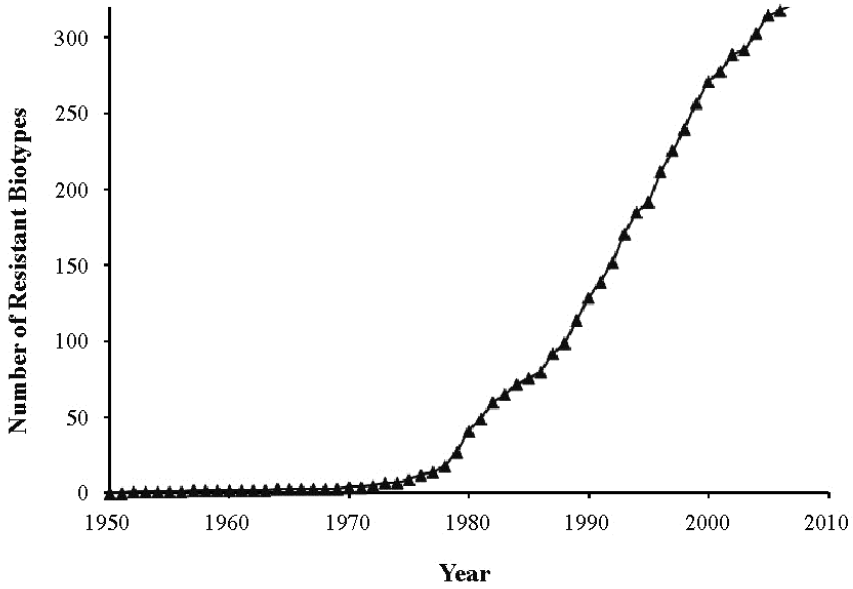

Fig. 5. The chronological increase in the number of herbicide-resistant weeds worldwide through 2010 (Heap, 2011).

By 2011, 370 biotypes of 200 species (115 dicots and 85 monocots) were reported to be resistant worldwide (Fig. 6), with 20 known modes of action (The International Survey of Herbicide Resistant Weeds is continually updated at www.weedscience.org). Resistance has developed mainly to three mode of action: ALS inhibitors, PSII-inhibiting herbicides and ACCase inhibitors (Fig. 7). Among these, ALS inhibitors are the most prone to select for resistance due to their high efficacy and very specific target (Saari *et al.*, 1994).

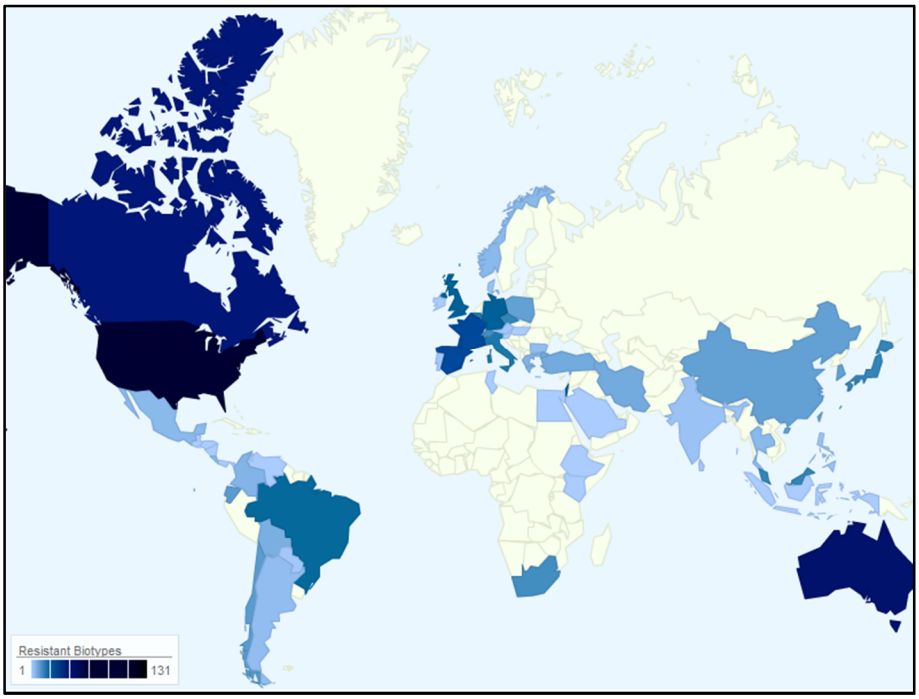

Fig. 6. Distribution of herbicide resistance biotypes globally (Heap, 2011).

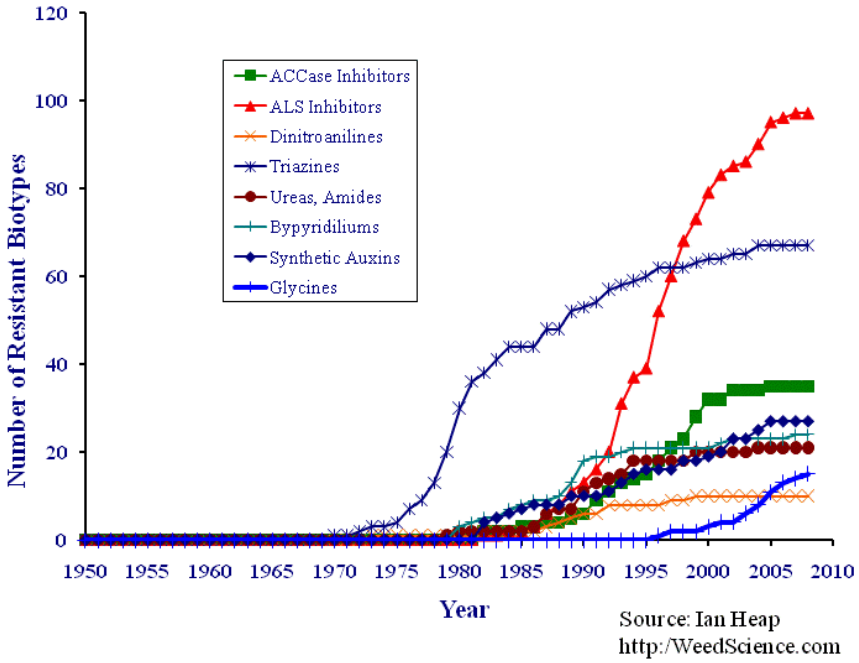

Fig. 7. Evolution of resistance biotypes worldwide sorted by herbicide mode of action (Heap, 2011).

The development of herbicide resistance in weeds is an evolutionary process. In response to repeated treatments with a particular family or group of herbicides, weed populations change in genetic composition such that the frequency of resistance alleles and resistant individuals increase. In this way, weed populations become adapted to the intense selection imposed by herbicides (Jasieniuk *et al.*, 1996). Numerous factors influence evolutionary processes in natural populations of plants and animals, the main are: (1) the initial frequency of the resistance trait in unselected populations; (2) the genetic bases of resistance (number of alleles involved); (3) the selection pressure; (4) the relative fitness of resistant weeds; (6) the soil seed bank; (7) the seed production by resistant weeds and (8) the residual activity of herbicides (Covalleri and Onofri, 2001).

Genetic variation for resistance must be present in a susceptible weed population for the evolution of herbicide resistance to occur. The major source of genetic variation in an area where resistance has not been detected previously is likely to be gene mutation. In general, gene mutations conferring resistance to a specific herbicide family are not induced by application of the herbicide, but rather are believed to occur spontaneously. The initial frequency of resistant plants in an unselected population has been estimated to vary roughly between 10^{-3} and 10^{-15} depending on species and the herbicide considered (Gressel, 1991). Rates of mutation to herbicide resistance are unknown for any weed species and indications are very variable, although a few estimates have been made. For example, mutation rates in the ALS gene are typically estimated to be 10^{-6} per nucleotide base-pair per generation (Gressel, 2002), but is known that the variability of ALS is not the same in all weed species (Tranel and Wright, 2002).

Because herbicide treatments generally result in such a high mortality of susceptible plants, mathematical models have shown that selection pressure by herbicide is the most important factor determining the rate of enrichment of herbicide resistance in a weed population (Maxwell *et al.*,1990). Selection pressure is defined as the ratio between the rate of resistant plants surviving the herbicide treatment and the survival rate of susceptible plants (Gressel, 1991). With this process an environment tends to eliminate an organism, and thus its genes, or to

give it an adaptive advantage, in this specific case. The higher the intensity of selection against susceptible seedlings imposed by a particular herbicide, the faster the expected rate of evolution and spread of resistance. ALS inhibitors have been demonstrated to select for resistance just after four generations (Moss, 2007).

Seed dynamic in the soil plays an important role in the resistance evolution. Less long-lived are the seeds and more concentrated the emergences, faster will be the appearance of the phenomenon. In fact, species with a transitory seed stock are selected faster from an herbicide selection than species with persistent stock. This is linked also with agronomical practices to delay or prevent the weed emergence (Covalleri and Onofri, 2001).

The fitness is defined as the survival and reproductive success in a specific environment. The relative fitness of two genotypes, different only for a resistance allele, may be calculated as the ratio between their contribution to the next generation, i.e. fitness is a parameter that describes the relative advantage of a genotype to another. If fitness of resistant individuals is lower than fitness of susceptible ones, in the evolution and without a selective factor, the frequencies inside a population may be back to susceptible individuals.

Finally, the reproduction system may play an important role in the resistance evolution: the natural selection strongly favors the dominant mutations in allogamous species.

In Italy, between the end of ‘70s and the begin of ‘80s, herbicide resistance was a marginal problem involving only *Solanum nigrum, Amarantus* spp. and *Chenopodium album* resistant to atrazine in maize field. This type of resistance had a low practical impact because of both the availability of several alternative products and the lower fitness of the resistant plants. In 1986, atrazine use was limited, and then banned, and the absence of the herbicide selective pressure permitted to reinstate the equilibrium to susceptible populations. Until mid-‘90s, no new herbicide resistance cases were found. Since the introduction of very high specific site of action herbicides (e.g. SU inhibiting ALS and graminacides inhibiting

ACCase), the first signaling of ALS inhibitors resistance appeared in rice weeds, *Alisma plantago-aquatica* (1994) and *Schoenoplectus mucronates* (1995), and durum wheat weed, *Papaver rhoeas* (1998). Instead, the first cases of resistance to ACCase inhibitors were found in some Poaceae (*Avena sterilis* in 1992, *Lolium* spp. in 1995 and *Phalaris paradoxa* in 1998). Actually, 27 biotypes have been documented involved 18 species (5 dicots and 13 monocots), and 13 regions are interested (Fig. 8). Species and areas involved in the resistance phenomenon are increasing both to ALS and to ACCase inhibitors. In particular, the first cases of *Sorghum halepense* and *Digitaria sanguinalis* populations resistant to ACCase inhibitors in dicot crops (soybean, tomato and melon) were detected. Furthermore, some *Echinochloa crus-galli* populations resistant to ALS inhibitors in paddy rice and *Sinapis arvensis* resistant to ALS inhibitors in wheat were documented. It is very recent the detection of the first cases of *Lolium* spp. resistance to glyphosate in olive grove and vineyard.

The Italian situation is constantly monitored by GIRE (Gruppo Italiano Resistenza Erbicidi) and updated in its website (www.resistenzaerbicidi.it). From the foundation of GIRE, in 1997, an average of 1.2 new biotypes per year were detected (Sattin, personal communication).

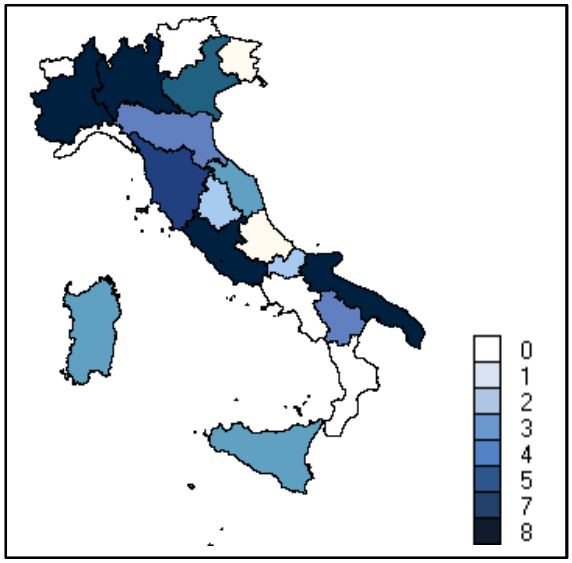

Fig. 8. Distribution of herbicide resistance biotypes in Italy.

1.1.6.1. Integrated Weed Management (IWM)

The most important cause of resistance is the standardization of cropping systems (i.e. the lack of crop and herbicide rotation, repeated use of a single herbicide, or use of several herbicides with the same site of action) that provides continuing selection pressure for herbicide resistance. The approval and implementation of the EU Pesticide package will have a significant impact on herbicide resistance appearance, diffusion and management. The further reduction in number and diversity of active ingredients available due to the process of re-registration imposed by the Directive 91/414/CEE that will go on with the Regulation (EC) No 1107/2009, as well as the widespread use of highly-active herbicide groups, which are target site specific (i.e. ALS and ACCase inhibitors), will increase the risk of resistance and make its management harder (Sattin, 2011). Another aspect is that no new herbicide modes of action are expected to be marketed in the next few years and therefore it is fundamental to preserve the efficacy of those already available. To keep the good herbicides that currently exist, their current use must be a high priority. Practices to prevent and manage herbicide resistant weeds are now being developed and employed.

To reduce the selection pressure on a weed population by continue use of a single herbicide, Integrated Weed Management (IWM) should be a priority for all farmers. IWM is defined as the use of a range of control techniques, embracing physical, chemical and biological methods in an integrated fashion without excessive reliance on any one method (Powles and Matthews, 1992). The tactic is to practice a continuum of different disturbances to the infesting flora such as (1) crop rotation, (2) cultural techniques and (3) chemical control.

(1) Crop rotation, in which herbicide chemistry and other control methods differ for each crop, can delay the build-up of resistance;
(2) alternating non-chemical control measures with herbicides helps reduce herbicide selection pressure. Available options include crop rotation, pre-plant tillage, selective in-crop tillage, mowing, cover crops, allelopathic mulches, stale seedbed, grazing and hand weeding;

(3) mixing herbicides with different MoA will delay the development of resistance, provided that both herbicides have activity on the same target weed. What one herbicide misses, the other will kill. Therefore, very few resistant survivors will remain. The HRAC classification allows a user who wishes to avoid resistance to choose herbicides from different groups, minimizing the selection of herbicide resistant weed populations.

For the Italian situation, general and specific guidelines for resistance management are reported on the GIRE website.

1.2. Two troublesome summer weeds

Objects of the study were two of the most troublesome weeds in summer crops evolved resistance in the last few years in Italy, *Echinochloa crus-galli* (L.) Beauv. and *Sorghum halepense* (L.) Pers.

1.2.1. *Echinochloa crus-galli*

Echinochloa spp. include several hygrophanous species with annual life cycle, typical rice weeds, but that may infest even other cultures and producing a large number of seeds (Sparacino and Sgattoni, 1993). A lot of these *Echinochloa* species are very difficult to distinguish due to the great morphological and phenological variability. The most dangerous species infesting rice crop were identified in *E. crus-galli* and *E. colona* (Tabacchi, 2003), but *E. crus-galli* (barnyardgrass) is the most widespread. It is a polyploid species, not rhizomatous, warm-season annual weed. Barnyardgrass reproduces by seed, it is predominantly self-pollinating, has a C4 photosynthetic cycle, is very successful competitor and is a prolific seed producer which often result in important soil seed bank (Holm *et al.*, 1977), i.e. a healthy plant can produce up to more than 400,000 seeds (Norris, 1992). Seeds are water dispersed and their viability in soil is variable.

In the recent years, the management of this troublesome species is of great concern because of the selection of populations resistant to major herbicides used

in rice, such as molinate, propanil, quinclorac, thiobencarb, butachlor, fenoxaprop, penoxsulam, byspyribac-sodium (Carey *et al.*, 1995; Lovelace *et al.*, 2000). In Italy, it has become resistant to propanil, quinclorac, and some *Echinochloa* species have also evolved multiple resistance to propanil and quinclorac (Tabacchi *et al.*, 2004). The occurrence of *E*. *crus-galli* populations resistant to ALS inhibitors in recent years in different rice cropping areas may put at risk the sustainability of the rice cropping system.

Another cropping system involved in *E. crus-galli* infestation is maize. So far, only resistance to atrazine (an herbicide belongs the PSII-inhibiting herbicide group) was detected, whereas, no case of ALS inhibitors resistance has been documented.

1.2.1.1. Rice crop and resistant weeds

Paddy rice is a cropping system where diversity is particularly low and weed control relies on heavy herbicide input. In paddy rice cultivation in several north-western areas of Italy, where monoculture is widespread, prevention and control of resistance is becoming difficult due to the effects of EU regulations. Since 1959, with the introduction of broad spectrum herbicide propanil, use of this, and other herbicides, is the basic weed control technology in rice (Smith, 1961). Actually, the exacerbation of EU regulations led to exclusion of some of them, e.g. quinclorac was forbidden in Italy two years ago, whereas the use of others (e.g. propanil) is limited to very low doses usually not enough for an efficient control. Thereby, the weed control is mainly achieved using ALS inhibitors, and in part ACCase inhibitors, synthetic auxins and thiocarbamates.

The continuous use of ALS-inhibiting herbicides without rotation with other mode of action (MoA) is increasing the evolution of new weed resistance cases. Under these conditions herbicide resistance has become a major and widespread problem in many areas, involving several ALS chemical families (Valverde and Itoh, 2001). The worst cases in Europe involve three species, *Alisma plantago-aquatica*, *Schoenoplectus mucronatus* and *Cyperus difformis* infesting rice crops in southern and western European countries (Calha *et al.*, 1999; De Prado *et al.*,

1997; Sattin *et al.*, 1998). Furthermore, a population of *Echinochloa erecta* proved to be multiple-resistant to quinclorac and propanil in 2004 (Tabacchi *et al.*, 2004). Several thousand hectares are infested in Italy, Portugal, Spain and Turkey by *A. plantago-aquatica* with the worst situation being in Italy where the first cases appeared in the mid-'90s and it is now estimated that about 15,000 ha are affected, about 6% of the total area of rice fields, but is quite stabilized. Italian rice fields are the only involved in *S. mucronatus* ALS inhibitors resistance into Europe. The first case was confirmed in 1994 and actually is estimated that between 10,000 and 15,000 ha are affected. *C. difformis* infested a few hundred hectares in Italy and Turkey, while only one case was found in Spain. The first case in Italy was documented since 1999 and is still spreading (Heap, 2011). In Italy, it is estimated that ALS inhibitors resistance exists in more than 25,000 ha rice fields, with *S. mucronatus* and *C. difformis* showing the highest rate of increase. Among Poaceae, all over the world, *Echinochloa* spp. are the most common weed in rice fields (see above).

In most of the country producing rice, all over the world, *E. crus-galli* resistant to several herbicide groups was detected from 1986, when the first case of *E. crus-galli* resistant to propanil was detected in Greece. In the following years the problem has spread from Brazil (multiple resistance to ALS inhibitors and auxine) to China (resistance to quinclorac), passing through USA, when resistance to propanil, quinclorac and, more recently, ACCase inhibitors and clomazone, is very widespread. Furthermore, many underdeveloped country - such as Philippine, Sri Lanka, Thailand - showed *E. crus-galli* control problems, mainly to propanil. In Europe, the unique cases documented so far of ALS inhibitors resistance in *E. crus-galli* was in Yugoslavia in 1992 and several sites were found infested to ACCase and ALS inhibitors multiple resistant *E. crus-galli* in Turkey in 2009.

1.2.2. *Sorghum halepense*

S. halepense (jonsongrass) is a tetraploid, geophyte warm-season grass (Poaceae family) that reproduces by both seeds and rhizomes. It grows to a height of 120 to 250 cm and has a large, open panicle type of inflorescence. The

rhizomes are about 1.3 cm in diameter and have tan to black scale-like leaves enriching the nodes. Two characteristics of *S. halepense* that separate it from most other perennial grasses are its prolific seed production (a single plant may produce more than 80,000 seeds in a single growing season), that provides the plant with mechanisms for both long-term survival and spread, and its highly competitive seedlings, those compete effectively with most summer annual crops and acquire perennial characteristics by midseason. *S. halepense* competes vigorously with crop plants largely because of the plants that emerge from its extensive rhizome system (a single plant can produce up to more than 8 m of rhizomes) (McWhorter, 1989). They establish more quickly in the spring than seedlings do. Rhizome growth begins when plant reaches the seven-leaf stage, but its development is slow until flowering occurs in the summer. Most rhizomes produced during a growing season can overwinter, but few live more than one year. Late-season re-sprouting of buds is suppressed by apical dominance and cool temperatures. Cutting the rhizome into pieces removes apical dominance and permits sprouting of lateral buds; rhizome pieces with a single node can sprout into new plants.

1.2.2.1. Non-chemical control of S. halepense

Preventing *S. halepense* from becoming established in new areas is the best available control method, because the weed spreads in so many ways. Because it is a perennial weed, single cultural control measures or herbicide applications rarely provide adequate control. To reduce johnsongrass infestations with herbicides, it will be necessary to use an integrated approach consisting of soil-applied herbicides, post-emergence herbicides, crop rotation and tillage. In particular, the control programs should: prevent spread of rhizomes from infested to un-infested areas; kill or weaken established plants and their underground rhizome system; prevent production of seed and its spread to new areas; use fall tillage to bring rhizomes to soil surface, where they may be killed by winter conditions. Summer annual crops as corn and soybean also can compete actively when properly managed. Crop rotation is important to disrupt the physical environment that johnsongrass and many other weeds live in and will help to

minimize seed and rhizome production. It can reduce the losses caused by moderate infestations and rotation with crops in which *S. halepense* can be controlled and rhizome formation prevented will provide substantial control for the subsequently planted crop. When properly executed even mowing can prevent seed formation and often can reduce the vigor of established stands.

Simplistically translocate post-emergence herbicides introduced since 1980 (i.e. ACCase and ALS inhibitors) have allowed growers to improve control and reduce the amount of tillage needed in *S. halepense* infested fields.

1.2.2.2. Herbicide control and herbicide resistant S. halepense

Herbicide rotation is important to minimize selection pressure for herbicide resistant biotypes. The first advance involved pre-plant soil incorporated herbicides to control germinating seeds. In USA, glyphosate is the most popular and consistent of the non-selective post-emergence, translocate herbicides that move into the rhizomes when applied to the shoots of established plants and, therefore, it is used to control seedling and some plants from rhizomes. A commonly used program for intensively tilled maize includes a pre-emergence treatment with S-metolachlor plus atrazine, but they control seedlings from seeds but not the ones from rhizomes. The most significant breakthrough was the introduction of selective herbicides that control *S. halepense* when applied over the top of established broadleaved and grass crops. These are FOP and DIM in soybean, cotton and other broadleaved crops and SU in maize. The most recent advance has been the introduction of genetically modified crop cultivars resistant to glyphosate (maize, soybean and cotton) and imidazolinones (corn and rice); they cannot be used in Italy.

Unfortunately, several herbicides resistant cases of *S. halepense* where recorded worldwide in different cropping systems. Since 1991, when the first case of *S. halepense* resistant to ACCase inhibitors was detected in cotton crops in USA, the same problem was found in Greek and Israeli cotton fields and in American and Italian soybean fields. Even resistance to glyphosate and other pre-plant herbicides (i.e. pendimenthalin) in soybean and cotton fields, respectively,

was detected in USA and Argentina (Heap, 2011). Instead, in maize crops the problem is linked to ALS inhibitors and it was detected in USA, Mexico and Venezuela from 2005. Actually, no similar case has been documented in Europe.

1.3. Aims of the research

This research, aimed at characterizing ALS inhibitors resistant *E. crus-galli* and ALS and ACCase inhibitors resistant *S. halepense*. In particular:

- to confirm and characterize ALS and ACCase inhibitors resistance in some European populations;
- to elucidate the molecular bases of target-site resistance in the two polyploid species;
- to verify if ALS or ACCase inhibitors resistance in *S. halepense* is associated with variations in biomass partitioning among plant organs.

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Chapter II

Preliminary screenings and dose-response experiments

2.1. Introduction

Sorghum halepense and *Echinochloa crus-galli* samples were collected by industries technicians in fields were chemical control resulted poor or ineffective. The samples analyzed came from Italian and Hungarian areas, thanks to the collaboration with Syngenta, Enterisi, Dow Agroscience, SIPCAM and BASF.

This survey do not give the real situation since samples has not randomly collected, but it is a complain monitoring based on the identification of the worst cases where resistance was already evolved, and probably missed most of the situation where resistance had just begun to evolve. Populations were cataloged and tested in greenhouse screenings. In this research a sample coming from a specific field is referred as population. The population code is formed from two number, the year of the sampling (e.g. 08, 09, etc.) and a progressive number identifying the farm of provenience. The field, that has a own history derived from the strategies that have been adopted (i.e. his treatment, crop rotation), is another criteria to identify a population. When fields of a same farm have different histories, the samples are ascribed to different populations adding a letter after the second number (e.g. 09-44a and 09-44b).

The aims of the screenings were to confirm the resistance and to determine the resistance pattern and level. Sometimes, there are not real resistance cases, but only cases of treatments performed with the wrong doses or too late, i.e. at the wrong plant growth stage. Several herbicides belonging different groups were tested in order to determine the cross- or multiple resistance pattern.

Some populations were selected for each species based on the resistance level determined in the preliminary screenings and dose-response experiments were carried out in order to calculate dose-response curves. From these curves it is possible to extrapolate the inflection point that represent the I_{50} (the herbicide dose causing 50% of response), for survival and fresh weight, and to calculate the R.I. (Resistance Indexes), i.e. the ratio between the I_{50} of the tested population and the I_{50} of the susceptible check (a sample which has never treated). R.I. may give indications about the mechanism of resistance involved that will be investigated and discussed in the following chapters.

All the experiments were carried out in the greenhouse of the Institute of Agro-environmental and Forest Biology (IBAF) - CNR located into the “Azienda Agricola Sperimentale L. Toniolo” at Agripolis, Legnaro (PD), Italy (45° 21' N, 11° 58' E).

2.2. Materials and methods

2.2.1. Plant material

Seven *S. halepense* putative resistant populations were sampled from 2005 to 2007 in Veneto and Lombardy regions, five in dicot crops (soybean and tomato) and two in maize fields. Furthermore, thirty six populations were sampled in Hungarian maize fields.

Thirty one *E. crus-galli* putative resistant populations were sampled from 2005 to 2010, fifteen in maize field and sixteen in rice fields, in several regions of northern Italy (Piedmont, Lombardy, Emilia Romagna, Sardinia and Tuscany).

Historical field data, including previous field treatments, were collected from farmers and used to plan the greenhouse experiments. Populations of both species sampled in maize fields were harvested from plants surviving an ALS-inhibiting herbicide treatment, usually with a sulfonylureas (SU) or a triazolopyrimidines (TP), whereas for *E. crus-galli* harvested in rice fields the selective agent was an imidazolinones (IMI) or, more rarely, a TP. Instead, *S. halepense* harvested in dicot crops was selected by ACCase inhibitors treatments.

2.2.2. Preliminary screenings

Preliminary screenings were carried out in the greenhouse. The populations were tested for resistance to ALS- and ACCase-inhibiting herbicides and an inhibitor of cell division, depending to their field history. The experimental layout was a completely randomized design with two replicates for each of the two doses used: recommended field dose (1x) and three times that (3x). The list of herbicides used are reported in table 1. Seeds were chemically scarified in concentrated

sulfuric acid (97%) - twenty and five minutes for *E. crus-galli* and *S. halepense*, respectively - and rinsed with water. They were hence placed into plastic boxes containing 80 mL of agar medium 0.6% (wt/V) with KNO_3 0.2% (wt/V) and transferred in a germination cabinet. *S. halepense* seeds were subjected to a hot scarification (see Harrington method) at the following conditions: 4 hours at 45 °C and 20 hours at 24 °C for three days, 12 hours photoperiod with neon tubes providing a Photosynthetic Photon Flux Density (PPFD) of 15-30 µmol m^{-2} s^{-1}. Subsequently, temperature was 25/15 °C (day/night). *E. crus-galli* seeds were placed straight to 25/15 °C conditions. After one day, germinated seeds to be used for the pre-emergence treatment were transplanted into plastic trays, while those for the post-emergence treatments remained in the germination cabinet. Trays (325 x 265 x 95 mm) were filled with a standard potting mix (60% silty loam soil, 15% sand, 15% perlite and 10% peat) and were transferred in the greenhouse, where the temperature ranged from 15 to 19 °C and from 26 to 33 °C night/day, respectively. Light was supplemented using 400 W metal-halide lamps, which provided a PPFD of about 450 µmol m^{-2} s^{-1}, a 16-hour photoperiod and placed in large tubs full of water until field capacity was reached. The day after planting, pre-emergence herbicide was applied as commercial formulations (Table 1), 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). They were hence transferred in smaller saucers and watered, from both above and below in order to maintain the herbicide in the area where the seeds are placed.

For post-emergence treatments, 15-18 germinated seedlings at very similar growth stage, were transplanted after six days. Trays were placed inside the greenhouse and watered daily to maintain the substrate at field capacity. Approximately 10 days after the transplanting, when the plants were around 2-3 leaves stage (i.e. growth stage 12-13 of the Extended BBCH scale (Hess *et al.*, 1997)), they were sprayed as reported for the pre-emergence treatment.

Survival and visual estimate of biomass (VEB) in relation to the untreated check were recorded three weeks after treatment (WAT) for ACCase inhibitors and

four WAT when an ALS inhibitor was used. The efficacy of the treatment was evaluated using a susceptible check (S). Plants were assessed as being dead if, regardless of color, they showed no active growth. VEB was determined giving a score of 10 to the untreated check and 0 to replicates where all plants were clearly dead. Survival records have been expressed as percentage of no. of plants treated and standard error was calculated per each mean value. Populations were ascribed to four categories: S when less than 5% of plants survived the dose 1x, SR when survivors ranged from 5% to 20% at dose 1x, R when more than 20% of plants survived the dose 1x and RR when survivors were more than 20% at dose 1x and more than 10% at dose 3x (Sattin *et al.*, 2001).

Table 1. Details of herbicide treatments. In bold herbicides tested only in the dose-response experiments (see text).

Mode of action	**Commercial product**	**Active ingredient (g L^{-1} (or kg^{-1}))**	**Surfactant (L ha^{-1})**	**Field dose (1x) (mL ha^{-1})**	**Time of the treatment**
Inh. of cell division	Dual Gold [a, b]	S-metolachlor (960)	-	1250	Pre-emergence
ALS inhibitors	Ghibli [a, b]	nicosulfuron (40)	-	1500	Post-emergence
	Gulliver [a]	azimsulfuron (500)	Trend 2%	40	Post-emergence
	Equip [b]	**foramsulfuron (22.5)**	**-**	**2700**	**Post-emergence**
	Altorex [a]	imazamox (40)	Dash 0.5%	900	Post-emergence
	Viper [a]	penoxsulam (20.4)	-	2000	Post-emergence
	Nomine [a]	byspiribac-Na (414)	Biopower (1)	75	Post-emergence
ACCase inhibitors	Fusilade Max [a, b]	fluazifop (125)	-	2000	Post-emergence
	Stratos [b]	cicloxydim (200)	Mineral oil (1)	800	Post-emergence
	Aura [a]	profoxydim (100)	Dash (0.8)	500	Post-emergence

[a] herbicides used for *E. crus-galli* treatments and [b] herbicides used for *S. halepense* treatments

2.2.3. Dose-response experiments

Populations with different resistance patterns, different levels of resistance to ALS and ACCase inhibitors and peculiar field history were chosen for two outdoor dose-response pot experiments, one for each species. At least one S check was included. Seeds germination and herbicide treatments were performed as explained above for the preliminary screenings. After germination, seven seedlings were transplanted into 16 cm diameter pots and placed outside in a semi-controlled environment. The pots were provided of saucers and watered two times a day with an automatic irrigation system to maintain the substrate at field capacity. Both the experiments were carried out during the summer 2010, below details for each one.

2.2.3.1. S. halepense dose-response

Three putative resistant populations harvested in Hungarian maize fields were tested, two showed high resistance levels in the preliminary screenings (08-16 and 09-20) and one showed intermediate resistance levels (09-21). Two susceptible checks (one from Italy, 08-10, and one from Hungary, 08-19) were included. Four ALS-inhibiting herbicides were tested (Table 1), two belonging SU chemical family (foramsulfuron and nicosulfuron), one IMI (imazamox) and one pyrimidinyl(thio)benzoates (PTB) (byspiribac-Na). The herbicide doses were calculated using a geometric progression based on the preliminary screening results, different for putative resistant and susceptible populations (Table 2).

Table 2. Range of doses used in *S. halepense* dose-response for each population. In bold the susceptible populations included in the experiment.

Pop.	NT	1/16x	1/8x	1/4x	1/2x	1x	2x	4x	8x	16x
08-10	X	X	X	X	X	X	X			
08-19	X	X	X	X	X	X	X			
09-21	X		X	X	X	X	X	X		
08-16	X				X	X	X	X	X	X
09-20	X				X	X	X	X	X	X

2.2.3.2. E. crus-galli dose-response

Five putative resistant populations harvested in rice fields were selected (09-42, 09-44a, 09-44c, 09-45 and 09-46) and one susceptible check (07-16) was included. Three herbicides were tested, an ACCase inhibitor (profoxydim) and two ALS inhibitors (penoxsulam and imazamox) (Table 1). The herbicide doses tested were calculated using a geometric progression with different ranges for S and R populations, from 1/32x to 2x and from 1/4x to 16x, respectively.

For both experiments, the design was a completely randomized block with two pots and three replicates for each of the seven (eight for *E. crus-galli*) doses used for each population. Plants were sprayed at the 12-13 BBCH stage in the same conditions explained for the preliminary screenings. Survival and fresh weight were recorded after four WAT and expressed as a percentage of the untreated control. Plants were assessed to be dead if they showed no active growth, regardless of color: the fresh weight of all plants assessed as alive and weight of residual death plant material were recorded.

2.2.4. Statistical analyses

Doses giving the 50% response, i.e. LD_{50} (based on survival data) and GR_{50} (based on fresh weight data), and relative standard errors, were calculated using non-linear regression based on a log-logistic equation to fit the data (Seefeldt *et al.*, 1995):

$$Y = C + \frac{(D - C)}{1 + \left(\frac{x}{I_{50}}\right)^{b}}$$

where *Y* is the fresh weight or survival, *C* and *D* are the lower and upper asymptotes at the highest and zero dose respectively, I_{50} is the dose giving the 50% response, *b* is the slope, and *x* is the herbicide rate. The data analysis was performed using the macro BIOASSAY®, developed by Onofri (2004) and running in Windows Excel® environment.

The data were first analyzed separately as single curve to estimate the parameters and then all curves designed for a specific herbicide were regressed

together. For biological reasons and to improve the estimates of the parameters, the upper and lower asymptotes were forced to 100 and zero, respectively, when survival data were analyzed, but not for fresh weight. The complex model was then compared with progressively simplified models having common parameters among curves. The lack-of-fit F-test was performed at each step, and the simplification stopped when a significant lack of fit occurred. Resistance indexes (R.I.) were calculated as ratio between the LD_{50} (or GR_{50}) of each resistant population and the LD_{50} (or GR_{50}) of the susceptible check(s).

2.3. Results and discussion

2.3.1. Preliminary screenings

2.3.1.1. S. halepense screenings

Results of preliminary screenings for *S. halepense* populations are reported in table 3. S checks (reported in bold in table 3) were adequately controlled by all herbicides tested. All populations tested were adequately controlled by S-metolachlor, herbicide used in pre-emergence that interferes with the cell division.

Five populations harvested in Italian dicot crops were selected by ACCase-inhibiting agent, always a FOP, and in fact all resulted highly resistant to the FOP but not to the DIM used. Two populations harvested in Italian maize fields, selected by ALS inhibitors, were tested for the resistance to both ALS and ACCase inhibitors; they were adequately controlled by fluazifop and only one resulted highly resistant to nicosulfuron. It was the first case of *S. halepense* resistant to ALS inhibitors in Italy and no new case was registered afterwards. In figure 1 is reported a map including the cases of resistance in *S. halepense* registered in Italy so far.

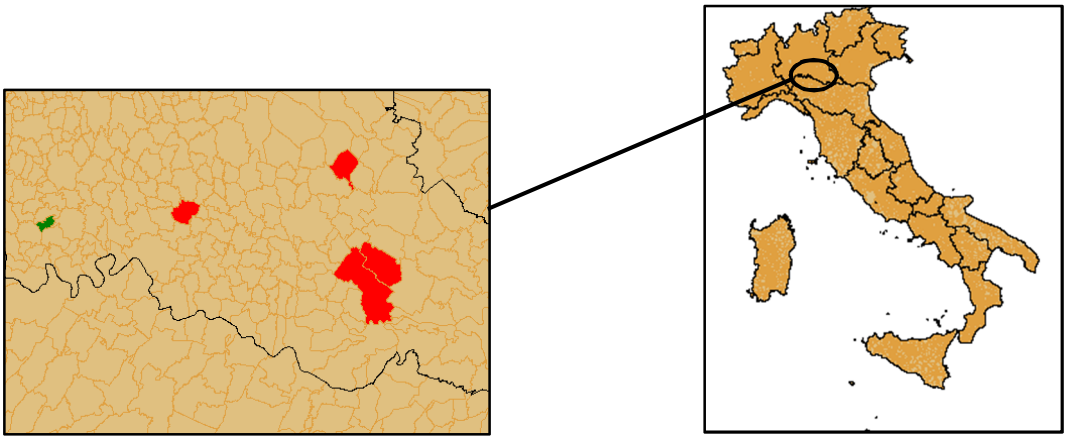


Fig. 1. Map of the municipalities where at least one case of resistant *S. halepense* was documented. In red cases of resistance to ACCase inhibitors and in green the first case of resistance to ALS inhibitors in Italy.

All Hungarian populations were harvested in maize fields where they showed an inadequate control using ALS inhibitors and, in particular, to the herbicides belonging to the SU chemical family. Only the first year two ACCase inhibitors (one FOP and one DIM) and an inhibitor of the cell division were tested, and all populations were adequately controlled. In the afterwards screenings, one ACCase inhibitor and one ALS inhibitor were tested and, while all populations were controlled by the first one, ten populations resulted highly resistant to nicosulfuron with little effect concerning the herbicide dose used (data not shown). Nine of these populations were harvested in the Tolna county, while one was harvested in Bàcs county at south-east of Tolna county (Fig. 2). Twelve populations had a survival comprised between 3% and 10% to nicosulfuron at dose 1x, with relatively high variability between replicates, but they were completely controlled by nicosulfuron at dose 3x. This may be the beginning of an infestation that will become worse if a correct management to avoid the widespread and evolution of resistance will not be adopted. Only one of these populations (09-21) showed a plant survival of about 10% at nicosulfuron 3x and it was selected for further investigation (see dose-response experiment).

Fig. 2. Hungary map with resistant samples reported; for samples 08-16, 08-17 and 08-18 the exact city was not known, they are reported inside the county of origin.

2.3.1.2. E. crus-galli screenings

The S check, 07-16, was adequately controlled in all screenings and by all different group of herbicides used. Among the 31 populations tested, 15 were harvested in maize fields and selected by treatment with SU, whereas 16 where harvested in rice fields and selected by treatment with an IMI or a PTB.

In table 4 are reported the survival data at field dose for all populations tested with the different herbicides. In figure 3 and 4 (a and b) are reported the percentage of populations ascribed to the different resistance level grouped into populations harvested in maize and rice fields, respectively.

Nine of the fifteen populations harvested in maize fields were highly resistant to nicosulfuron and the sum of resistant populations (R+RR) was 87% of tested (Fig. 3). Imazamox was tested only with samples harvested in 2010 (Table

4), none sample resulted as being RR and 60% resulted still adequately controlled. Four populations resulted resistant to SU herbicide at dose 1x with plant survival ranging from 30% to 70%, but adequately controlled at dose 3x and by the IMI herbicide. This may indicate a non-target-site resistance mechanism involved. All populations were completely controlled by ACCase-inhibiting herbicide tested (fluazifop).

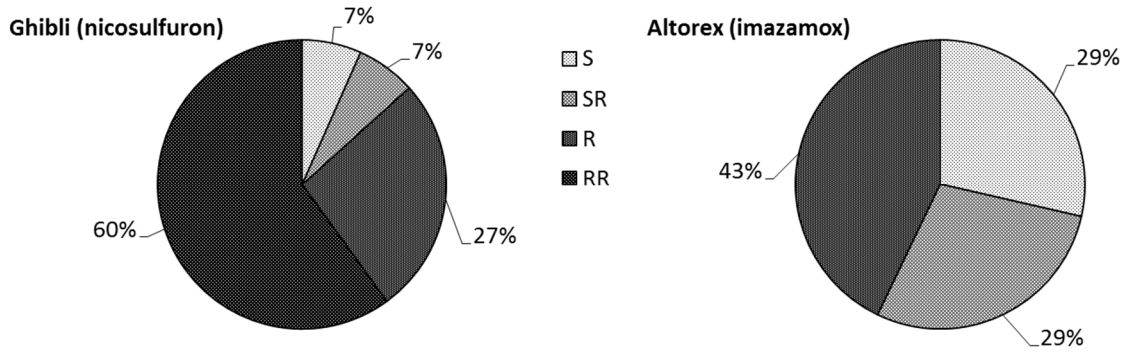


Fig. 3. Screening of *E. crus-galli* populations harvested in maize fields with two ALS inhibitors. Percentage of populations showed certain resistance level: S, survival <5% at dose 1x; SR, survival 5-20% at dose 1x; R, survival >20% at dose 1x; and RR, survival >20% at dose 1x and >10% at dose 3x.

Among the 16 populations sampled in rice fields, three biotypes may be recognized: seven populations resulted as being highly cross-resistant to all ALS inhibitors tested, two resistant only to the SU and five multiple resistant to ALS and ACCase inhibitors. Only two populations resulted as being controlled to all herbicide groups tested. Detailed results are reported in table 4 and in figure 4 (a and b). More than 70% of populations tested resulted resistant (R+RR) to SU, IMI and PTB, whereas the rate of populations still controlled by TP is around 50%. No population RR to the ACCase inhibitor profoxydim has been detected (Fig. 4b).

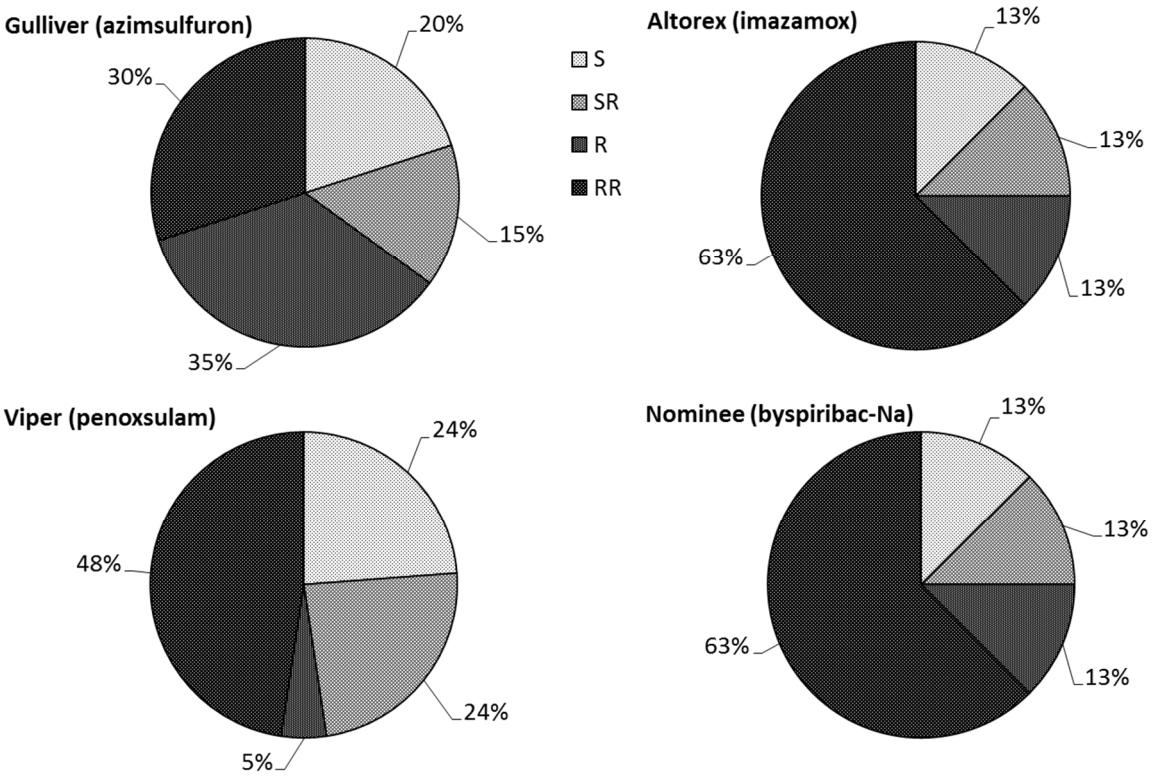


Fig. 4a. Screening of *E. crus-galli* populations harvested in rice fields with four ALS inhibitors. Percentage of populations showed certain resistance level: S, survival <5% at dose 1x; SR, survival 5-20% at dose 1x; R, survival >20% at dose 1x; and RR, survival >20% at dose 1x and >10% at dose 3x.

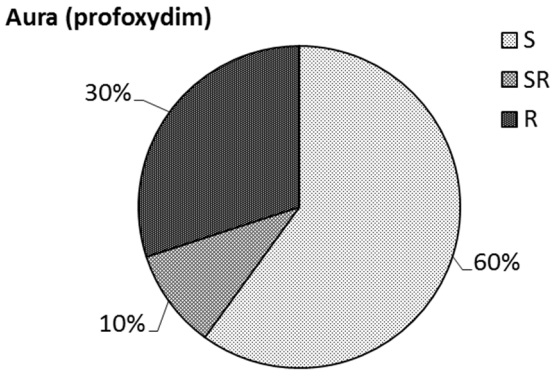


Fig. 4b. Screening of *E. crus-galli* populations harvested in rice fields with one ACCase inhibitor. Percentage of populations showed certain resistance level: S, survival <5% at dose 1x; SR, survival 5-20% at dose 1x; and R, survival >20% at dose 1x.

Field histories indicate that, generally, Italian rice crops have been receiving at least 2-3 herbicide treatments every year, with at least one ALS application. In recent years ALS treatments always involved herbicides controlling *Echinochloa* spp., whereas ACCase treatments were sporadic. In all cases the last selecting agent was either penoxsulam or imazamox.

All multiple resistant cases were recorded in Piedmont, while a few cases of cross-resistance to ALS inhibitors were found also in central Italy, Emilia Romagna and Sardinia (Fig. 5) (Panozzo *et al.*, 2011). Their distribution in a few Italian regions suggests that the majority of them were independently selected and they have not never appeared to be associated with an intense use of ACCase inhibitors.

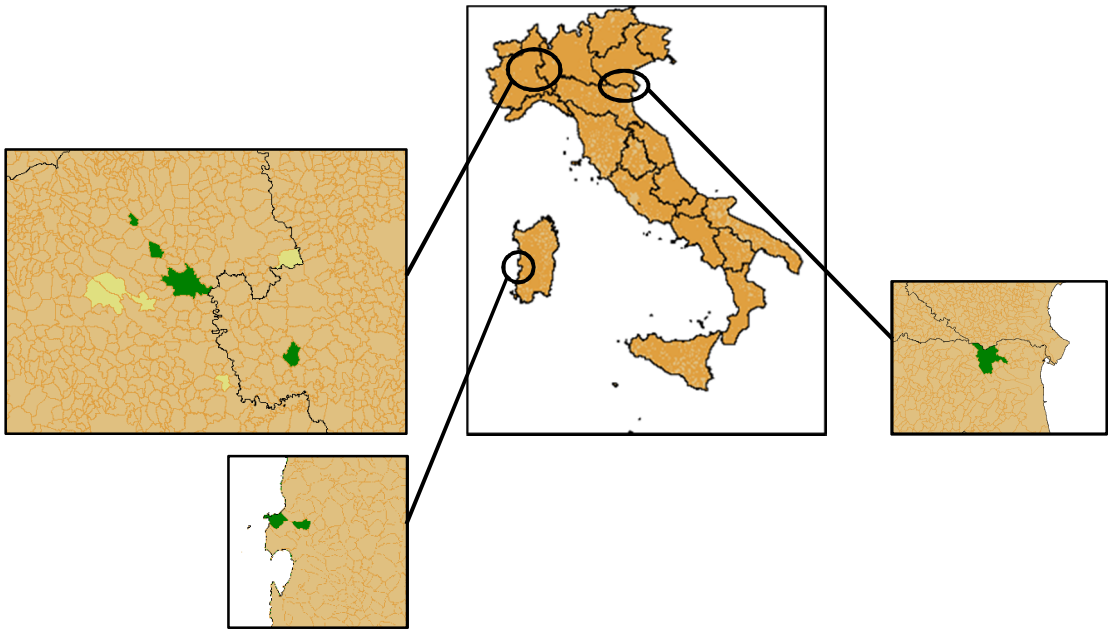


Fig. 5. Map of the municipalities where at least one case of resistant *E. crus-galli* was documented. In green the cases of resistance to ALS inhibitors and in yellow the cases of multiple resistance to ALS and ACCase inhibitors in Italy.

Table 3. Results of *S. halepense* screenings divided into populations coming from Italy (top) and from Hungary (bottom). For each population is reported the city of origin (county for Hungarian samples), the culture at the moment of the sampling, the last treatment (selecting agent) and the % of survival at the field dose (1x) for herbicides tested. In bold the S checks. Standard errors are reported in brackets.

Population code	Municipality	Crop	Selecting agent	Resistance pattern (% of survival at dose 1x)			
				ACCase inhibitors		ALS inhibitors	Inh. of cell division
				fluazifop	cicloxydim	nicosulfuron	S-metolachlor
05-1	Camisano Vicentino (VI)	-	Check S	0 (0)	0 (0)	0 (0)	2.5 (2.50)
06-10	Legnaro (PD)	-	Check S	0 (0)	0 (0)	0 (0)	0 (0)
05-2	Corte de' Frati (CR)	Soybean	Fluazifop	88.2 (0.69)	8.8 (2.94)	0 (0)	2.5 (2.50)
05-3	Occhiobello (RO)	Maize	Foramsulfuron	0 (0)	0 (0)	0 (0)	0 (0)
05-4	Marcaria (MN)	Soybean	Quizalofop	79.8 (8.40)	0 (0)	0 (0)	7.5 (2.50)
05-6	Castellucchio (MN)	-	Fluazifop	88.2 (0.69)	9.0 (3.47)	0 (0)	15.0 (5.00)
06-9	Castellucchio (MN)	-	-	100 (0)	12.6 (0.78)	0 (0)	7.5 (2.50)
07-12	Medole (MN)	Tomate	Fluazifop	83.3 (3.33)	0 (0)	0 (0)	0 (0)
07-13	Cavacurta (LO)	Maize	Nicosulfuron	0 (0)	0 (0)	93.9 (0.18)	0 (0)

Population code	County	Crop	Selecting agent	Resistance pattern (% of survival at dose 1x)			
				ACCase inhibitors		ALS inhibitors	Inh. of cell division
				fluazifop	cicloxydim	nicosulfuron	S-metolachlor
08-19	Komàron	-	Check S	0 (0)	0 (0)	0 (0)	2.5 (2.50)
08-14	Bàcs	Maize	Nicosulfuron	0 (0)	0 (0)	5.6 (5.56)	0 (0)
08-15	Fejér	Maize	Nicosulfuron	0 (0)	0 (0)	0 (0)	0 (0)
08-16	Tolna	Maize	Nicosulfuron	0 (0)	0 (0)	96.7 (3.33)	0 (0)
08-17	Tolna	Maize	Cicloxydim	0 (0)	0 (0)	94.4 (5.56)	0 (0)
08-18	Tolna	Maize	Nicosulfuron	0 (0)	0 (0)	91.7 (2.78)	0 (0)
09-20	Tolna	Maize	Cicloxydim	0 (0)	-	100 (0.00)	-
09-21	Tolna	Maize	Nicosulfuron	0 (0)	-	10.0 (4.71)	-
09-22	Baranya	Maize	Nicosulfuron	0 (0)	-	5.6 (5.56)	-
09-23a	Békés	Maize	Nicosulfuron	0 (0)	-	3.1 (3.13)	-
09-23b	Békés	Maize	Nicosulfuron	0 (0)	-	2.9 (2.94)	-
09-24	Békés	Maize	Nicosulfuron	0 (0)	-	0 (0)	-
09-25	Csongràd	Maize	Nicosulfuron	0 (0)	-	0 (0)	-

Population code	County	Crop	Selecting agent	Resistance pattern (% of survival at dose 1x)			
				ACCase inhibitors		ALS inhibitors	Inh. of cell division
				fluazifop	cicloxydim	nicosulfuron	S-metolachlor
09-26	Szabolcs	Maize	Rimsulfuron	0 (0)	-	3.3 (3.33)	-
09-27	Szabolcs	Maize	Rimsulfuron	0 (0)	-	5.6 (0.00)	-
09-28	Pest	Maize	Nicosulfuron	0 (0)	-	0 (0)	-
09-29a	Pest	Maize	Florasulam	0 (0)	-	0 (0)	-
09-29b	Pest	Maize	Florasulam	0 (0)	-	8.3 (8.33)	-
09-29c	Pest	Maize	Florasulam	0 (0)	-	3.8 (3.84)	-
10-31	Tolna	Maize	Nicosulfuron	0 (0)	-	89.7 (3.09)	-
10-32b	Tolna	Maize	Nicosulfuron	0 (0)	-	92.8 (0.51)	-
10-33	Tolna	Maize	-	0 (0)	-	61.7 (11.67)	-
10-34	Tolna	Maize	-	0 (0)	-	64.3 (7.11)	-
10-35	Tolna	Maize	Nicosulfuron	0 (0)	-	35.7 (14.28)	-
10-36	Tolna	Maize	Nicosulfuron	0 (0)	-	20.9 (7.62)	-
10-37	Baranya	Maize	-	0 (0)	-	3.3 (3.33)	-
10-38a	Somogy	Maize	Nicosulfuron	0 (0)	-	0 (0)	-
10-38c	Somogy	Maize	Nicosulfuron	0 (0)	-	0 (0)	-
10-39	Baranya	Maize	Nicosulfuron	0 (0)	-	0 (0)	-
10-40	Bàcs	Maize	-	0 (0)	-	90.3 (1.39)	-
10-41	Bàcs	Maize	-	0 (0)	-	0 (0)	-
10-42	Bàcs	Maize	Foramsulfuron	0 (0)	-	3.8 (3.84)	-
10-43a	Baranya	Maize	Nicosulfuron	0 (0)	-	9.6 (3.73)	-
10-43b	Baranya	Maize	Nicosulfuron	0 (0)	-	0 (0)	-
10-44	Somogy	Maize	-	0 (0)	-	0 (0)	-
10-45	Baranya	Maize	-	0 (0)	-	0 (0)	-
10-46	Bàcs	Maize	Nicosulfuron	0 (0)	-	0 (0)	-

Table 4. Results of *E. crus-galli* screenings. For each population is reported the city of origin, the culture at the moment of the sampling, the last treatment (selecting agent) and the % of survival at the field dose (1x) for the herbicides tested. In bold the S check. Standard errors are reported in brackets.

Code	Municipality	Crop	Selecting agent	Resistance pattern (% of survival at dose 1x)						
				ACCase inhibitors		ALS inhibitors				
				profoxydim	fluazifop	azimsulfuron	nicosulfuron	penoxsulam	imazamox	byspiribac-Na
07-16L	Marzano (PV)	Rice	Check S	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
05-30	Dronero (CN)	Maize	Nicosulfuron	-	0 (0)	-	92.9 (7.14)	-	-	-
05-31	Cona (VE)	Maize	Nicosulfuron	-	0 (0)	-	69.1 (2.38)	-	-	-
05-32	Chioggia (VE)	Maize	Nicosulfuron	-	0 (0)	-	96.9 (3.13)	-	-	-
06-33	Maccarese (RM)	Maize	Nicosulfuron	-	0 (0)	-	0 (0)	-	-	-
06-34	Massarosa (LU)	Maize	nicosulfuron	-	0 (0)	-	86.4 (6.43)	-	-	-
06-35	Dronero (CN)	Maize	Nicosulfuron	-	0 (0)	-	92.3 (0.00)	-	-	-
07-41	Cavarzere (VE)	Maize	Nicosulfuron	-	0 (0)	-	100 (0.00)	-	-	-
09-42	Vercelli (VC)	Rice	Penoxsulam	-	0 (0)	80.0 (6.67)	65.9 (19.8)	85.6 (1.03)	75.1 (1.79)	93.3 (6.67)
09-43	Ottobiano (PV)	Maize	Nicosulfuron	-	0 (0)	-	28.6 (28.6)	-	-	-
09-43b	Ottobiano (PV)	Rice	Penoxsulam	5.0 (5.00)	-	90.0 (10.00)	-	55.0 (20.6)	56.7 (3.33)	24 (19.41)
09-44a	Bianzè (VC)	Rice	Imazamox	38.1 (4.76)	-	-	-	100 (0.00)	100 (0.00)	100 (0.00)
09-44b	Bianzè (VC)	Rice	Imazamox	0 (0)	-	100 (0.00)	-	100 (0.00)	100 (0.00)	94.4 (5.56)
09-44c	Bianzè (VC)	Rice	Imazamox	30.9 (9.91)	-	100 (0.00)	-	87.5 (12.5)	100 (0.00)	100 (0.00)
09-45	San Vero Milis (OR)	Rice	Penoxsulam	0 (0)	-	91.7 (8.33)	-	100 (0.00)	91.7 (8.33)	100 (0.00)
09-46	Ferrara (FE)	Rice	Penoxsulam	0 (0)	-	100 (0.00)	-	100 (0.00)	100 (0.00)	100 (0.00)

Code	County	Crop	Selecting agent	Resistance pattern (% of survival at dose 1x)						
				ACCase inhibitors		ALS inhibitors				
				profoxydim	fluazifop	azimsulfuron	nicosulfuron	penoxsulam	imazamox	byspiribac-Na
10-47	Argenta (FE)	Maize	-	-	-	-	72.2 (2.67)	-	33.3 (33.33)	-
10-48	Gropparello (PC)	Maize	Nicosulfuron	-	-	-	9.4 (6.00)	-	0	-
10-49	Torre S.Giorgio (CN)	Maize	-	-	-	-	69.1 (11.67)	-	8.3 (8.33)	-
10-50	Mesola (FE)	Maize	Foramsulfuron	-	-	-	25.8 (2.81)	-	16.2 (3.67)	-
10-51	Savigliano (CN)	Maize	Foramsulfuron	-		-	87.6 (5.82)	-	100 (0)	-
10-52	Ronsecco (VC)	Rice	Penoxsulam	33.3 (0.00)	-	97.1 (2.94)	-	85.8 (2.45)	83.3 (5.56)	-
10-53	Giussago (PV)	Rice	Imazamox	0 (0)	-	8.3 (8.33)	-	0 (0)	11.1 (11.11)	-
10-54	Villanova Biellese (BI)	Rice	Penoxsulam	0 (0)	-	100 (0)	-	77.4 (5.95)	80.6 (2.78)	-
10-55	Bianzè (VC)	Rice	Imazamox	13.9 (13.89)	-	64.9 (29.58)	-	0 (0)	0 (0)	-
10-56	Livorno Ferraris (VC)	Rice	Penoxsulam	75.7 (0.74)	-	100 (0)	-	100 (0)	94.1 (5.88)	-
10-57	Cerano (NO)	Rice	Penoxsulam	22.9 (10.40)	-	94.4 (0)	-	0 (0)	19.4 (19.44)	-
10-59	Casanova Elvo (VC)	Rice	Imazamox	0 (0)	-	92.9 (7.14)	-	100 (0)	92.3 (7.69)	-
10-60	Pornaro Monferrato (AL)	Rice	Penoxsulam	27.8 (5.56)	-	51.8 (12.91)	-	0 (0)	30.6 (13.89)	-
10-61	Gudo Visconti (MI)	Rice	Penoxsulam	0 (0)	-	16.7 (3.33)	-	0 (0)	0 (0)	-
10-62	Pianezze (VI)	Maize	Nicosulfuron	-	0 (0)	-	93.7 (6.2)	-	81.7 (4.00)	-
10-67	Albettone (VI)	Maize	-	-	0 (0)	-	39.2 (14.2)	-	0 (0)	-

2.3.2. Dose-response experiments

2.3.2.1. S. halepense dose-response

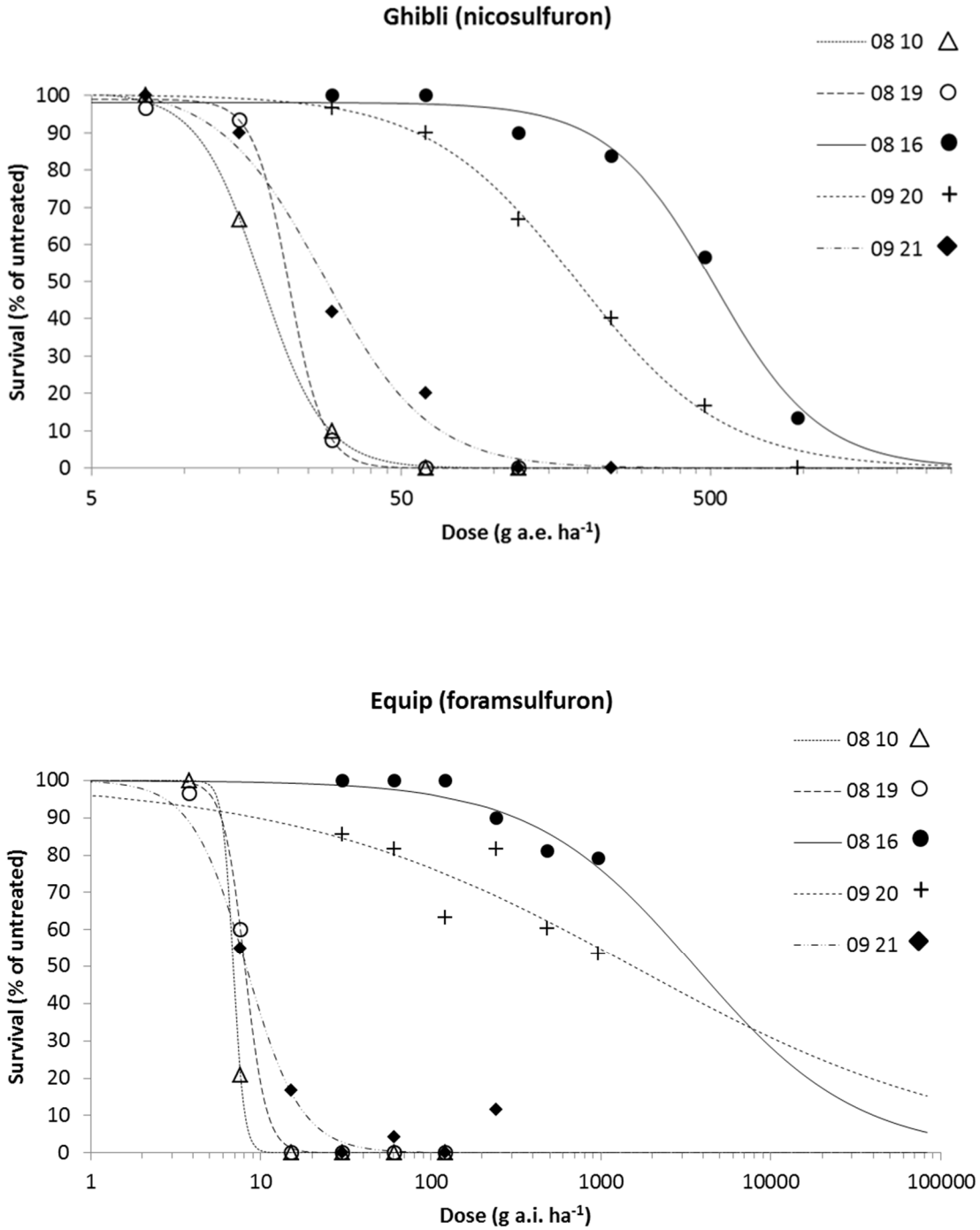
Standard errors calculated with the non-linear regressions are <10% for all the LD_{50} calculated, indicating that the log-logistic equation fitted the data accurately without any data transformation (λ=1) and the range of the doses was appropriate. As showed in the figure 6, the two S checks behaved similarly, even if the Hungarian check resulted less susceptible than the Italian one to all herbicides considered. Byspiribac-Na was only partially effective: survival at 62 g a.i. ha^{-1} (two times the field dose) was higher than 20%, but the fresh weight was hardly influenced already at the field dose. The other three herbicides adequately controlled S checks at field dose. Very high efficacy was reported using foramsulfuron.

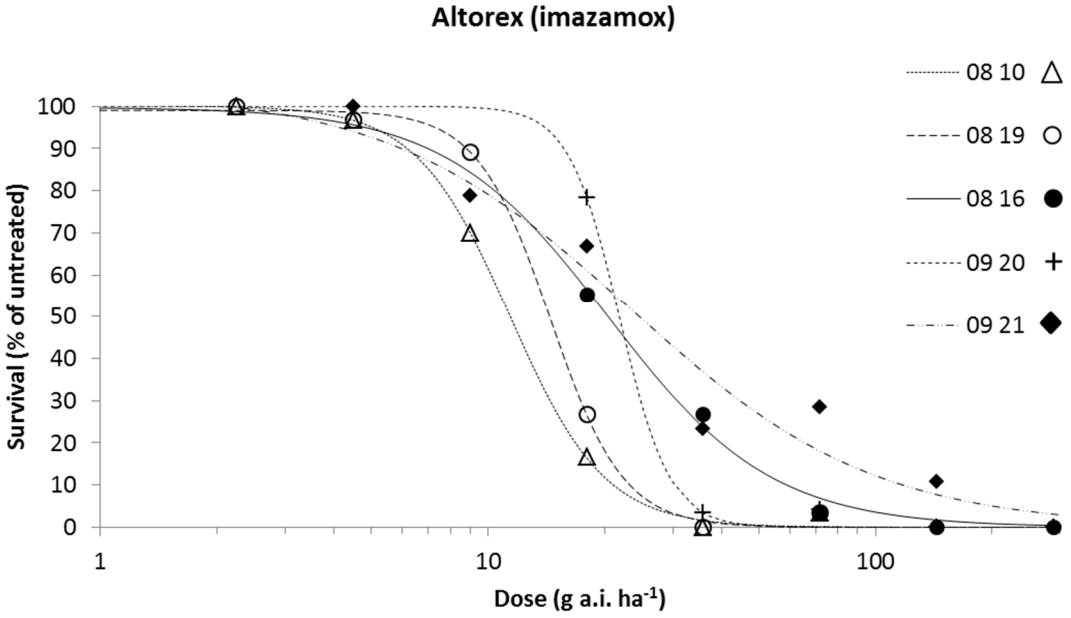
Populations 08-16 and 09-20 responded similarly, showing high resistance to both SU, mainly foramsulfuron where LD_{50} and GR_{50} calculated were higher than the maximum herbicide dose tested (Table 5). R.I. of population 09-20 were, in general, lower than R.I. of population 08-16 and visual observations highlighted that plants of population 09-20 died more slowly, possibly indicating a shift in susceptibility. The IMI herbicide resulted still effective on both populations with R.I. never higher than 2 for survival data and ranged from 2.5 to 3.1 for fresh weight. Very likely the resistance mechanism is related to an altered ALS enzyme, although different mutations/substitutions may be involved.

Population 09-21 behaved differently from the other populations: it was still controlled by sulfonylureas, both foramsulfuron (R.I. = 1.2), and nicosulfuron (R.I. = 1.6). On the opposite, it was the only population with some survivors at doses 2x (1x = 72 g a.i. ha^{-1}) and 4x of imazamox, showing a R.I. slightly higher (2.1), but plants were highly damaged (R.I. = 1.7). Therefore, population 09-21 was somehow in between S and R population.

Multiple curves analyses and the lack-of-fit test on survival and fresh weight (where possible) indicated that it was not possible to simplify the regression to a model with common slopes for all populations, even if only the resistant populations were considered; therefore, a single curve approach was preferred.

Fig. 6. Dose-response curves of susceptible (08-10 ∆ and 08-19 ○) and putative resistant (08-16 ●, 09-20 + and 09-21 ♦) populations to four ALS-inhibiting herbicides using the log-logistic model. Lines are the response curves predicted from non-linear regression; symbols represent percentage of mean survival, based on the untreated controls, of two pots for each of the three replicates.




Altorex (imazamox)
Survival (% of untreated)
Dose (g a.i. ha-1)
08 10
08 19
08 16
09 20
09 21

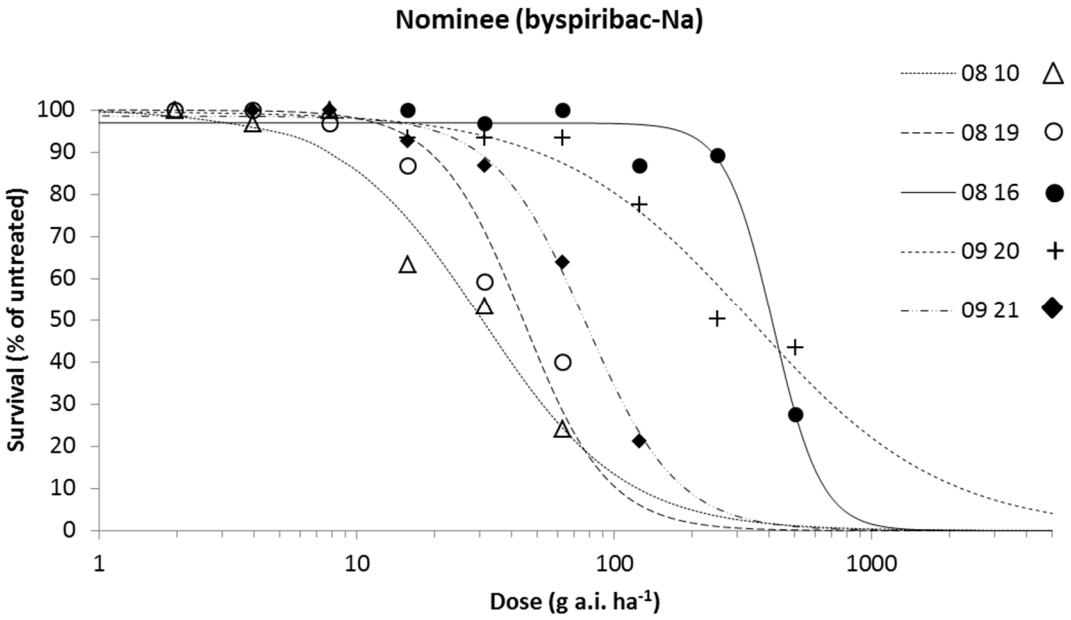

Nominee (byspiribac-Na)
Survival (% of untreated)
Dose (g a.i. ha-1)
08 10
08 19
08 16
09 20
09 21

Table 5. *S. halepense* dose-response data elaborated by the log-logistic model. Reported LD_{50}, GR_{50} and Resistance Indexes (RI), calculated on the basis of both S checks 08-10 and 08-19; LD_{50} and GR_{50} are the herbicide doses causing 50% reduction in survival and fresh weight, respectively; standard errors are given in brackets.

Survival												
Pop.	byspiribac-Na			foramsulfuron			imazamox			nicosulfuron		
	LD_{50} (g a.i. ha^{-1})	RI 08-10	RI 08-19	LD_{50}	RI 08-10	RI 08-19	LD_{50}	RI 08-10	RI 08-19	LD_{50}	RI 08-10	RI 08-19
08-10	31 (4.3)	-	0.69	6.9	-	0.9	11 (0.7)	-	0.8	18 (0.7)	-	0.8
08-19	44 (7.5)	1.44	-	8.0	1.2	-	14 (0.9)	1.3	-	22 (0.8)	1.2	-
08-16	414 (27.4)	13.5	9.8	>972	> 141	>121	20 (1.5)	1.8	1.4	514 (34.8)	29.2	23.7
09-20	339 (48.1)	11.1	7.7	>972	>141	>121	22 (1.2)	1.9	1.5	185 (19.2)	10.5	8.6
09-21	77 (6.1)	2.5	1.8	8.2	1.2	1.0	23 (5.8)	2.1	1.6	28 (2.1)	1.6	1.3

Fresh weight												
Pop.	byspiribac-Na			foramsulfuron			imazamox			nicosulfuron		
	GR_{50} (g a.i. ha^{-1})	RI 08-10	RI 08-19	GR_{50}	RI 08-10	RI 08-19	GR_{50}	RI 08-10	RI 08-19	GR_{50}	RI 08-10	RI 08-19
08-10	23 (5.3)	-	0.7	-	-	-	7 (0.8)	-	0.9	15 (1.5)	-	0.7
08-19	35 (7.0)	1.5	-	-	-	-	8 (1.2)	1.1	-	21 (2.3)	1.4	-
08-16	382 (51.9)	16.8	11.0	-	-	-	22 (10.8)	3.1	2.7	415 (47.4)	27.6	19.7
09-20	197 (41.7)	8.7	5.7	-	-	-	21 (0.9)	2.9	2.5	175 (34.0)	11.6	8.3
09-21	67 (19.0)	3.0	1.9	-	-	-	12 (3.1)	1.7	1.5	25 (36.7)	1.7	1.2

2.3.2.2. E. crus-galli dose-response

LD_{50} and GR_{50} of the susceptible population (07-16) were 26.0±1.52 g a.i. ha^{-1} (i.e. 0.64x) and 24.1±1.39 g a.i. ha^{-1} (i.e. 0.59x), respectively, for penoxsulam; whereas for imazamox, LD_{50} was 21.9±1.22 g a.i. ha^{-1} (i.e. 0.59x) and GR_{50} was 10.3±1.0 g a.i. ha^{-1} (i.e. 0.29x) (Table 6). The low values of all the standard errors of the parameters indicate that the log-logistic equation fitted the data accurately without any data transformation (λ=1) and the range of doses was appropriate.

The five putative resistant populations (09-42, 09-44a, 09-44c, 09-45 and 09-46) were confirmed as being highly cross-resistant to both ALS inhibitors, and even at the highest herbicide doses plant survival and fresh weight never reached the 50% value of the untreated control. It was therefore impossible to fit the data with the log-logistic equation and LD_{50} and GR_{50} were assigned as being higher than the maximum herbicide dose used (Table 6). Only two ALS inhibitors were tested, but given the clear results of the preliminary screenings, it is trustworthy to extend the results to all chemical families of ALS inhibitors. This pattern suggests an ALS target-site mediated resistance mechanism for all the populations.

Populations 09-44a and 09-44c, that resulted multiple resistant to ALS and ACCase inhibitors in the preliminary screening were tested with profoxydim in the dose-response experiment. They were harvested in the same farmer and differed only for the historical field treatments which they were submitted in the last two years. The two experiments gave consistent results, therefore, it is possible to confirm the first documented case of *E. crus-galli* multiple resistant to ALS and ACCase inhibitors in rice in Europe. Similar cases of multiple resistance involving ALS and ACCase inhibitors were registered, but not adequately documented, in South Korea in 2008 and in Turkey in 2009 (Heap, 2011). The dose-response curves for profoxydim, based on survival, are reported in figure 7. The lack-of-fit test on survival and fresh weight indicated that it was not possible to simplify the regression to a model with common parameters for all the populations, even if only the resistant ones were considered; therefore, a single curve approach was preferred.

GR_{50} and LD_{50} of the two resistant populations proved to be well above the highest profoxydim recommended field dose for controlling *E. crus-galli* at the onset of tillering (BBCH stage 21-22). Plant survival and fresh weight gave similar results for both populations with R.I. higher than 2.2 and standard errors always below 10%. Only GR_{50} resulted a little lower for population 09-44a, indicating an higher biomass damage in plants of that population using the same herbicide dose. Therefore, for the further experiments the two populations were considered as a unique population. The low survival R.I. values for profoxydim suggest that the ACCase enzyme of the R biotypes was not much less susceptible to this herbicide than that of the S biotype, hence it is difficult to speculate the resistance mechanism involved, but probably may not be related to an altered target site.

Table 6. *E. crus-galli* dose-response data elaborated by the log-logistic model. Reported LD_{50}, GR_{50} and Resistance Indexes (RI), calculated on the basis of the S check 07-16; LD_{50} and GR_{50} are the herbicide doses causing 50% reduction in survival and fresh weight, respectively; standard errors are given in brackets. For fresh weight, only the data of populations 09-44a and 09-44c are reported.

	Survival					
	ACCase inhibitor		**ALS Inhibitors**			
	profoxydim		**penoxsulam**		**imazamox**	
Pop.	**LD_{50} (g a.i. ha^{-1})**	**RI $_{07-16}$**	**LD_{50} (g a.i. ha^{-1})**	**RI $_{07-16}$**	**LD_{50} (g a.i. ha^{-1})**	**RI $_{07-16}$**
07-16	74 (4.4)	-	26 (1.5)	-	22 (1.2)	-
09-42	-	-	>652	>25	>576	>26
09-44a	171 (3.9)	2.3	>652	>25	>576	>26
09-44c	181 (6.8)	2.4	>652	>25	>576	>26
09-45	-	-	>652	>25	>576	>26
09-46	-	-	>652	>25	>576	>26

	Fresh weight					
	ACCAse inhibitor		**ALS Inhibitors**			
	profoxydim		**penoxsulam**		**imazamox**	
Pop.	**GR_{50} (g a.i. ha^{-1})**	**RI $_{07-16}$**	**GR_{50} (g a.i. ha^{-1})**	**RI $_{07-16}$**	**GR_{50} (g a.i. ha^{-1})**	**RI $_{07-16}$**
07-16	73 (5.7)	-	24 (1.4)	-	10 (1.0)	-
09-44a	171 (3.9)	2.2	>652	>27	>576	>56
09-44c	181 (6.8)	2.9	>652	>27	>576	>56

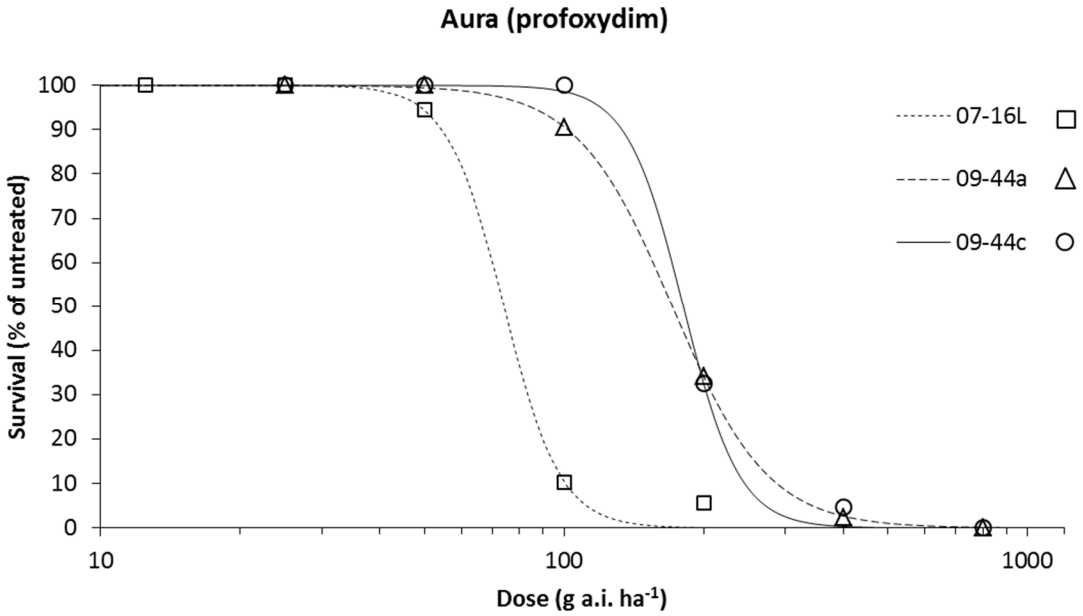


Fig. 7. Dose-response curves of susceptible (07-16L □) and resistant (09-44a Δ and 09-44c ○) biotypes to profoxydim using the log-logistic model. Lines are the response curves predicted from non-linear regression, symbols represent mean survival, based on the untreated controls, of two pots for each of the three replicates.

2.4. Conclusions

Results from greenhouse screenings are in keeping with outdoor dose-response experiments. Resistance to ALS inhibitors in *E. crus-galli* is evolving quickly in Italy: twenty two populations were confirmed in the last three years, thirteen in maize fields and nine in rice fields. At the same time, the first cases of multiple resistance to ALS and ACCase inhibitors in rice crops were detected.

While in Italy the resistance to ALS inhibiotrs in *S. halepense* is quite stabilized, in fact no new case was signaled in the last three years (the last population analyzed was sampled in 2007, 07-13), in Hungary, resistance to sulfonylureas is evolving fast, in particular in Tolna county, with ten cases confirmed from 2008 to 2010.

R.I. may give indication of the resistance mechanism involved: high R.I. usually indicate a target-site mechanism, whereas low R.I. indicate a non-target-site mechanism (metabolic, compartmentalization, translocation, etc.). Dose-

response experiments confirmed that the levels of resistance to all ALS inhibitors chemical families are very high in *E. crus-galli*, suggesting that a target-site mediated resistance mechanism may be involved. Moreover, the dose effect observed in some *E. crus-galli* populations resistant to nicosulfuron, suggests that even the occurrence of a non-target-site mechanism of resistance may not be excluded, probably covered by the target-site resistance mechanism. In fact, due to the polyploid nature of *E. crus-galli*, it is plausible to speculate that more than one mechanism may be involved as previously observed in ALS inhibitors resistant *E. phyllopogon* (Osuna *et al.*, 2002).

For *S. halepense*, dose-response confirmed that the problem is confined to just one ALS inhibitors chemical family (SU) and it is possible to control the infestation with the other families of ALS inhibitors or using other mechanism of action, i.e. ACCase inhibitors. Even in dicot crops, the DIM seems to be still effective in *S. halepense* control.

The problem is more serious for *E. crus-galli* control in rice fields where no active ingredient are still effective. Information coming from stakeholders and the number of putative resistant samples collected very recently indicate that herbicide resistance is evolving rapidly in *E. crus-galli*. This is of great concern for rice growers, especially when multiple resistance to ACCase and ALS inhibitors is present. The widespread occurrence of *E. crus-galli*, the intensive use of ALS inhibitors to control it, and the lack of alternative MoA available in the Italian market combine to seriously threaten the sustainability of the current rice cropping system, which is based on a lack of crop rotation. The resistance situation and the new European legislation on plant protection products highlight the need for more sustainable cropping systems. Were feasible, crop rotation, broad-spectrum herbicides (e.g. glyphosate and clomazone), false seed bed preparation and delayed rice sowing should be introduced into current rice production systems.

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Chapter III

Molecular bases of resistance to acetolactate synthase inhibitors in *Echinochloa crus-galli*

3.1. Introduction

Target-site resistance to ALS inhibitors in all weed biotypes investigated so far is caused by substitution(s) of one of eight amino acids within the six conserved domains of the ALS gene (Tranel *et al.*, 2011). Resistant alleles are totally or partially dominant, varying among plant species; therefore they are selected even when present at the heterozygous status (Wright and Penner, 1998).

To date 116 species have developed resistance to at least one ALS inhibitor and the relatively high frequency of discovery of new ALS resistant biotypes (Fig. 1) has triggered interest in the development of various diagnostic tests. Many of these tests are now DNA-based so that suspected resistant biotypes can be rapidly identified and controlled within the growing season to minimize the proliferation of resistant populations (Corbett and Tardif, 2006).

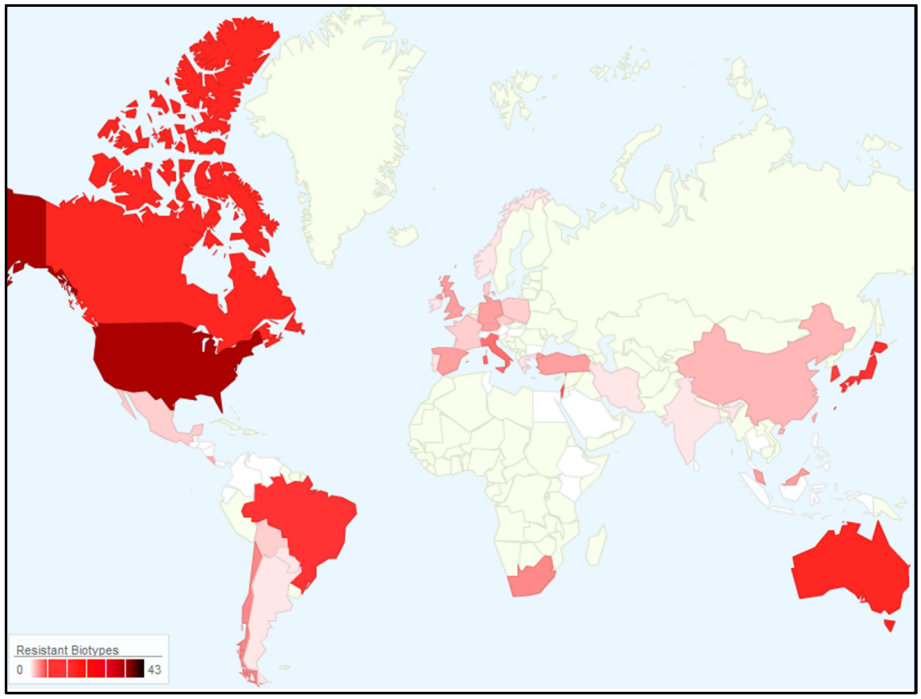

Fig. 1. Global distribution of ALS inhibitors resistant weeds (Heap, 2011).

3.1.1. Enzyme-based tests

Plants with target-site resistance have an ALS enzyme that is less affected by an ALS inhibitor than ALS enzyme from wild-type populations. Numerous assays that measure extracted ALS activity in the presence of an ALS inhibitor

have been developed. ALS enzyme can be extracted from any plant tissue, but in most cases the enzyme is extracted from newly developed parts of the plant, as the enzyme is most active in meristematic tissues. Most *in vitro* ALS assays measure acetoin accumulation as a function of ALS activity to detect ALS inhibitor resistance. ALS catalyzes the condensation of two pyruvate molecules to form one acetolactate molecule. The next enzyme in the pathway is ketoacid reductoisomerase (KARI), which uses NADPH to reduce acetolactate to dihydroxy isovalerate (Fig. 2). Extracted ALS enzyme solution does not contain KARI, therefore acetolactate cannot be converted to dihydroxy isovalerate and is instead decarboxylated to acetoin (Wittenbach and Abell, 1999). Acetoin absorbs light at approximately 525 nm and the amount of acetoin produced can be quantified with a spectrophotometer. The amount of acetoin corresponds to the activity of ALS, because acetoin cannot be produced without acetolactate, and acetolactate cannot be produced if ALS is inhibited.

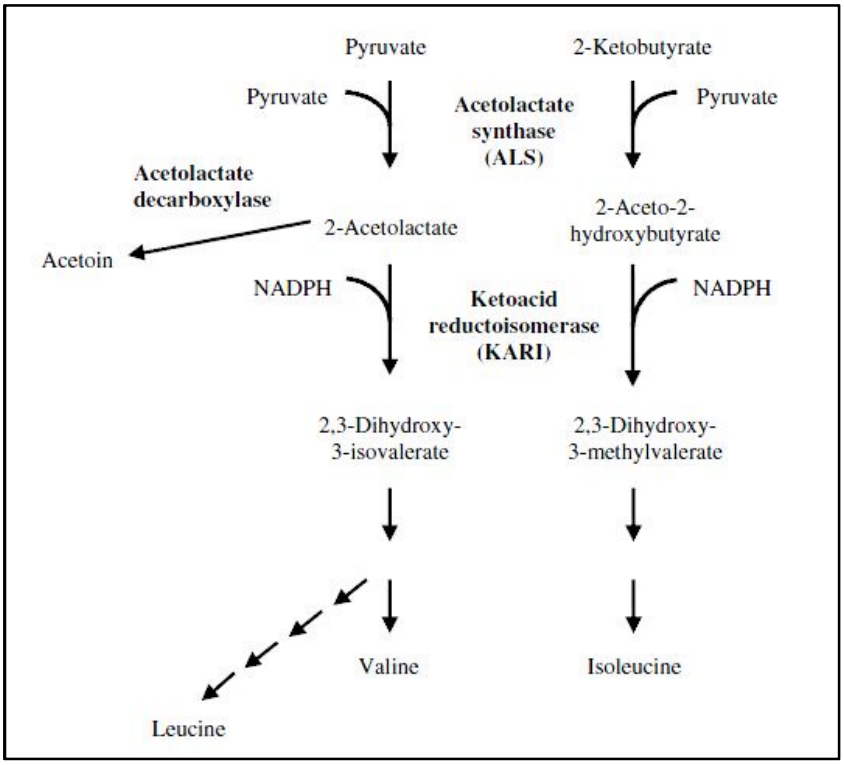

Fig. 2. Role of the ALS enzyme in the synthesis of branched chain amino acids. ALS catalyzes two parallel reactions: the condensation of two pyruvate molecules leads to valine and leucine, while the condensation of pyruvate with 2-ketobutyrate leads to isoleucine (Corbett and Tardif, 2006).

Production of acetolactate by herbicide susceptible ALS is dramatically reduced by an ALS inhibitor, therefore the absorption of the sample is minimal. Resistant ALS is able to form acetoin because the enzyme is insensitive to herbicide binding, and so the absorption is much greater. This method has been used to detect resistant weed populations from several weed species (Hinz and Owen, 1997; Hall *et al.*, 1998).

3.1.2. DNA-based tests for mutation detection

ALS gene sequencing for the identification of mutations responsible for ALS target-site resistance is a widely used approach, but its prohibitive expense, expertise and time required can be minimized by exploiting only the salient differences between resistant and susceptible DNA plants. Therefore, if DNA sequencing may be useful for a preliminary study, techniques that detect unique differences in a nucleotide sequence have been used for the quick and massive test of a large number of samples. These techniques include PCR-RFLP (Restriction Fragment Length Polymorphism) and PASA (PCR Amplification of Specific Alleles).

In PCR-RFLP, regions of the ALS gene are PCR amplified and digested with one or more restriction endonucleases. These enzymes are able to recognize a specific short nucleotide sequence and then cleave DNA at that site. The single nucleotide substitutions that confer resistance may result in the gain or loss of a recognition site for specific enzymes. The PCR product that lacks any recognition sites will not be cut when digested with that enzyme. The lengths of the resulting fragments can be visualized by gel electrophoresis. Instead, PASA is a modified PCR that has also been referred to as ARMS (Amplification Refractory Mutation System) (Newton *et al.*, 1989) or AS-PCR (Allele-Specific PCR). These techniques rely on a primer that precisely matches a resistant (or susceptible) sequence and differs from the wild-type (or mutated) sequence by a 3’ end nucleotide mismatch. When the allele-specific primer is well designed and the PCR conditions are appropriate, only DNA that precisely matches the 3’ end nucleotide is amplified into a PCR fragment, and the two genotypes can be differentiated.

Many molecular investigations have been conducted in the last decade to detect the mutations conferring ALS resistance. Most documented cases concern diploid species, while studies involving polyploid weeds are rare (Fortune *et al.*, 2008) even if their involvement in ALS resistance is important (Kolkman *et al.*, 2004; Scarabel *et al.*, 2009; Lamego *et al.*, 2009). 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 (Grula *et al.*, 1995).

To our knowledge, no molecular studied has been done on the polyploid species *E. crus-galli* resistant to ALS inhibitors, therefore this study addresses to characterize the target-site resistance in some populations of this species and to develop a DNA-based test for the detection of ALS inhibitors resistance due to a particular point mutation.

3.2. Materials and methods

3.2.1. Southern blotting

3.2.1.1. Genomic DNA extraction and probe synthesis

In order to detect the presence of multiple ALS genes, Southern blot analyses were carried out. Genomic DNA was isolated from two susceptible (S), 07-16 and 05-27, and two resistant (R), 09-45 and 09-46, *E. crus-galli* populations. A method reported in Kobayashi *et al.* (1998) was used with slight modifications. One gram of the youngest leaf tissue (pooled from 10 plants) was grinded in liquid nitrogen using mortar and pestle. Before tissue thawed, 20 mL of Buffer 1 [50 mM Tris-HCl (pH 8.0), 5 mM ethylenediaminetetraacetic acid (EDTA), 350 mM Sorbitol, 10% Polyethylen-glycol 6000, 0.1% mercaptoethanol (added just before use)] were added. The mixture was mixed thoroughly and centrifuged at 12,000 rpm for 25 min at 4 °C. The pellet was re-suspended in 10 mL of Buffer 2 [50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 350 mM Sorbitol, 1% Sodium Dodecyl Sulfate (SDS), 710 mM NaCl, 0.1% citrimonium bromide (CTAB), 0.1% mercaptoethanol (added just

before use)] and incubated at 65 °C, adding 30 µL of RNase (Roche), for 10 min. In order to separate the nucleic acids from the other plant components, 10 mL of chloroform-isoamyl alcohol (24:1 V/V) were added, mixed thoroughly and centrifuged at 12,000 rpm for 15 min at room temperature. The aqueous phase was transfer to a new tube, two-third volume of cold isopropanol was added, and the tubes were placed at -20 °C overnight. The day after, the precipitated DNA was centrifuged at 12,000 rpm for 15 min at 4 °C. The pellet was washed with ethanol 70%, centrifuged at 12,000 rpm for 10 min and drained to eliminate all the ethanol residues. DNA was re-suspended in 400 µL of double distilled (dd) H_2O, concentration was determined using a Nano Drop spectrophotometry (Applied Biosystem) and the DNA quality was checked in 1% agarose gel stained with SYBR® Safe DNA gel stain (Invitrogen) using the 1Kb Plus DNA ladder (Invitrogen) for reference.

Digoxigenin (DIG) - labeled probe was generated by amplification of an ALS fragment from genomic DNA of susceptible plants (population 07-16) using PCR DIG Probe synthesis Mix (Roche) and SB-F2 and SB-R2 primers (Table 1). Go Taq® Flexi DNA Polymerase (Promega) was used for the amplification. In a final volume of 50 µL, the following reagents were added: 10 µL 5x colorless Go Taq® Flexi buffer, 2 µL $MgCl_2$ solution 25 mM, 5 µL PCR DIG Probe synthesis Mix (Roche), 2 µL of each primer 10 µM, 2.5 U of Go Taq® DNA polymerase and 200 ng of genomic DNA. Amplification was conducted using the following program: DNA denaturation for 2 min at 95 °C, and 30 cycles of 30 s at 95 °C, 30 s at 54 °C, and 40 s at 72 °C. Finally, samples were subjected to a 5 min extension time at 72 °C. The amplification was checked in 1% agarose gel. PCR product was purified using MinElute PCR purification kit (Qiagen) and stored at -20 °C.

Table 1. Primers used for the DIG-labeled probe synthesis.

Primer name	Primer sequence (5'-3')	Amino acid position
SB-F2	TGA GTT GGA TCA GCA GAA GAG	442
SB-R2	AAG ACC TTC ACT GGG AGG TTC	554

3.2.1.2. Experiments design

Five 6-nucleotide-recognizing restriction endonucleases that did not cut within the probe sequence (see below) were selected to perform Southern blotting on four *E. crus-galli* populations. Three experiments were carried out: (1) genomic DNA of plants from populations 07-16 and 09-45 was digested with two restriction endonucleases (HindIII and EcoRV); (2) the same populations of the first experiment were used, and genomic DNA was digested with three other restriction endonucleases (XbaI, KpnI and BamHI); (3) genomic DNA of all the four populations reported above was digested with two restriction endonucleases alredy used, HindIII and BamHI, in order to confirm the results.

30 µg of genomic DNA was digested overnight at 37 °C with restriction endonucleases in a total volume of 400 µL in 1x buffer specific for each restriction enzyme. The digestion was checked in 1% agarose and stopped when the genomic DNA was completely digested. DNA was precipitated and re-suspended in a smaller volume of ddH_2O. Digested DNA and an undigested control were run on a 0.8% agarose gel in TBE buffer overnight using the DNA molecular weight market III DIG-labeled (Roche) for reference.

3.2.1.3. Blotting and detection

The gel was denatured in HCl 0.25 M stirring for 15 min at room temperature, followed by 20 min in denaturation buffer [0.5 M NaOH, 1.5 M NaCl]. It was then washed in neutralization buffer [0.5 M –Tris-HCl pH 7.5, 1.5 M NaCl] for 30'. DNA on gel was transferred to positively charged nylon membrane (Roche) by capillary transfer in 20x SSC buffer [3 M NaCl, 0.3 M sodium citrate, pH 7 and autoclaved] overnight.

The day after, DNA was fixed on the membrane using the Stratalinker® UV Crosslinker with the appropriate program and the hybridization was carried out following the manufacturer's instructions. DIG-labeled probe was used as a non-radioactive probe hybridizing at 49 °C overnight to detect the homologous DNA fragments on the DNA blot in DIG easy Hyb (Roche).

The membrane was washed two times in low stringency wash buffer [2x SSC, 0.1% SDS] and two times in high stringency wash buffer [0.5x SSC, 0.1% SDS] for 5 and 15 min, respectively. The blot was rinsed using DIG wash and block buffer set (Roche) and the chemiluminescent detection was carried out with CSPD ready to use kit (Roche) according to manufacturer's instruction. Signal was detected on X-ray film using different exposure time.

3.2.2. ALS enzyme *in vitro* bioassay

3.2.2.1. ALS extraction

Proteins were extracted from plants of four *E. crus-galli* populations (two R, 09-44c and 09-45, and two S, 07-16 and 05-27) as well as from two populations of *Amaranthus tuberculatus* (waterhemp), one R (ACR) and one S (WCS) to ALS inhibitors (Patzoldt and Tranel, 2007). *Amaranthus tuberculatus* was included as a diploid control species.

Proteins were extracted from fresh leaf tissue as described by Wright *et al.* (1998) with slight modifications. Five grams of youngest leaves (pooled from 40 to 50 plants) were homogenized with liquid nitrogen in a mortar and suspended in 35 mL of ice-cold homogenization buffer [100 mM potassium phosphate buffer (pH 7.2), 5 mM Na-pyruvate, 5 mM $MgCl_2$, 10 µM flavin adenine dinucleotide (FAD), 1 mM thiamine pyrophosphate (TPP), 1 mM dithiothreitol (DTT), 10% (V/V) glycerol] and 1% (wt/wt) polyvinylpolypyrrolidone (PVPP). The homogenate was filtered through four layers of cheesecloth, and then centrifuged at 20,000 x *g* for 20 min at 4 °C. The supernatant was decanted to a new centrifuge tube and an appropriate quantity of ammonium sulfate was added to create a 45% (wt/V) solution. The mixture was stirred gently for one hour on ice, and then centrifuged at 20,000 x *g* for 20 min at 4 °C. The resultant pellet was dissolved in 3 mL of re-suspension buffer [50 mM potassium phosphate buffer (pH 7.2), 5 mM $MgCl_2$, 1 mM TPP and 10 µM FAD]. The solution was desalted using Disposable PD-10 Desalting Columns (GE Healthcare). Proteins concentration of the extract was determined using a Nano Drop (Applied Biosystem).

3.2.2.2. ALS activity assay

ALS enzyme assays were performed as described by Schmitzer *et al.* (1993). Enzyme activity was determined based on the amount of acetoin formed from acetolactate using the method of Westerfeld (1945). Each reaction contained 50 µL of protein extract (4.0 µg μL^{-1}), 50 µL of the standard reaction buffer [50 mM potassium phosphate buffer (pH 7.0), 100 mM Na-pyruvate, 5 mM $MgCl_2$, 1 mM TPP, 10 µM FAD] and 50 µL of various concentrations of ALS-inhibiting herbicides. A positive control without herbicide and a blank with sulfuric acid (see below) were included. Each population was assayed with two ALS-inhibiting herbicides (penoxsulam and imazamox), in three replications per concentration per two set (i.e. a total of two different protein extractions per population). Herbicide concentrations ranged from 10^{-4} to 10^{5} nM for penoxsulam and from 10^{-1} to 10^{6} nM for imazamox in 10-fold increments. The mixtures were incubated at 37 °C for 90 min. The reactions were stopped by adding 25 µL of 3.5% (V/V) sulfuric acid, and then incubated at 60 °C for 20 min. The amount of acetoin formed was determined by incubating the mixture with 150 µL of 0.55% (wt/V) creatine, 5.5% (wt/V) α-naphthol and 1.375 N NaOH at 37 °C for 40 min. Acetoin concentration was measured by spectrophotometry at a wavelength of 530 nm.

3.2.2.3. Statistical analyses

ALS enzyme assay data were analyzed using the macro BIOASSAY® developed by Onofri (2004) and running in Windows Excel® environment. I_{50} and standard errors were calculated using a non-linear regression analysis based on a log-logistic equation to fit the data (Seefeldt *et al.*, 1995):

$$Y = C + \frac{(D - C)}{1 + \left(\frac{x}{I_{50}}\right)^{b}}$$

where *Y* is the fresh weight or survival, *C* and *D* are the lower and upper asymptotes at highest and zero dose respectively, I_{50} is the dose giving the 50% response, *b* is the slope and *x* the herbicide rate. Data were analyzed separately as single curve and none of the parameters was forced. R/S ratio was calculated

as ratio between the I_{50} of each R populations and the S population(s) of each species considered.

3.2.3. ALS gene sequencing

3.2.3.1. RNA extraction and cDNA synthesis

Total RNA was extracted from 100 mg of one single plant fresh young leaf tissue using the commercial kit RNeasy Plant mini kit (Qiagen). RNA was extracted from a few plants of two populations, one from the S population 07-16 and one from the R population 09-45 selected by herbicide treatment with penoxsulam (see chapter II for treatment details). A DNA digestion step, using the RNase-Free DNase Set (Qiagen), was included to eliminate DNA contamination. cDNA was synthesized using the ImProm-II™ Reverse Transcriptase System (Promega) in a total volume of 20 µL including 1µg of RNA, 2.5 µM oligo dT_{18}, 3.75 mM $MgCl_2$, 0.5 mM dNTP mix, 1 µL of Improm-II™ Reverse Transcriptase with 1x concentration of supplied buffer. The reactions were incubated for 5 min at 25 °C, 1 h at 42 °C and 10 min at 70 °C as recommended by the manufacturer in a T1 Thermocycler (Biometra).

3.2.3.2. cDNA amplification and sequence analysis

Because no information regarding the ALS gene sequence of any *Echinochloa* species was available, nucleotide sequences (both susceptible and resistant biotypes were selected when available) of nine Poaceae species available in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) were aligned: *Alopecurus myosuroides* (AJ437300), *Bromus tectorum* (AF487459, AF488771), *Hordeum vulgare* (AF059600), *Hordeum leporinum* (EF540587, EF540590), *Lolium multiflorum* (AF310684), *Lolium rigidum* (EF411170, EF411171), *Oryza sativa* (AB049823, AB049822), *Triticum aestivum* (AY210405) and *Zea mays* (BT042814, X63553). MEGA 5.05 software was used for sequence alignment and several degenerate primers for PCR were designed in the most conserved areas of the ALS gene using “Oligo Calc: Oligonucleotide Properties Calculator” software (http://www.basic.northwestern.edu/biotools/oligocalc.html)

and synthesized by Invitrogen. Several commercial PCR amplification kits, amplification conditions and primer combinations were tried. Lastly, PCR amplification was conducted using the Advantage® 2 PCR kit (Clontech) in a 50 µL mixture of 1x Advantage® 2 SA PCR Buffer, 1x dNTP mix, 0.2 µM of each primer, 1x Advantage® 2 Polymerase Mix and 100 ng cDNA. Amplification was conducted using the following program: DNA denaturation for 1 min at 95 °C, and 35 cycles of 30 s at 95 °C, 30 s at 60 °C, and 100 s at 68 °C. Finally, samples were subjected to a 3 min extension time at 68 °C. The primer combination ECH-5F and ECH-3R (Fig. 3, Table 2) produced a cDNA fragment from the central part of the ALS gene. PCR product was analyzed in 1% agarose gel. The amplified fragment was purified using MinElute Gel Extraction Kit (Qiagen) following the manufacturer's instructions, and sequenced from both senses. Sequencing was performed by BMR Genomics (Padova, IT) using an ABI 3730XL sequencer. Sequences were edited with FinchTV 1.4.0 software and nucleotide sequences were manipulated using DNASTAR® software.

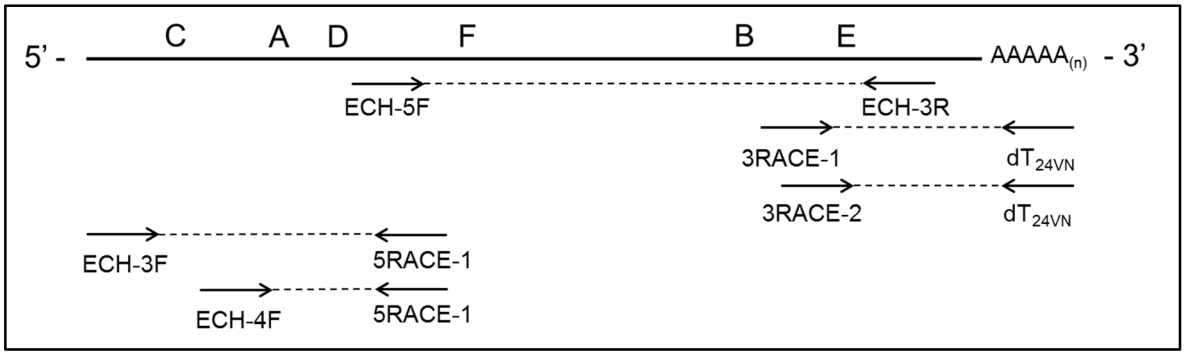

Fig. 3. Schematic representation of the ALS gene amplification procedure. Primer names are as described in Table 2. Letters C, A, D, F, B, and E represent the known conserved domains where mutations endowing herbicide resistance have previously been found.

Table 2. List of primers used to amplify different parts of the ALS gene and their target

Primer name	Primer sequence (5'-3')	Target
ECH-5F	AGG TCA CSC GCT CCA TCA CCA	Amplification of central part
ECH-3R	TCC TGC CAT CAC CHT CCA KGA	Amplification of central part
3RACE-1	CCA ACC GAG CAC ACA CAT AC	3'RACE forward
3RACE-2	TTC CAG CAG TCC GTG TGA CAA	3'RACE nested
ECH-3F	CCG CAA GGG CKC SGA CAT CCT	Targeted gene walking forward
ECH-4F	ACC AAC CAC CTY TTC CGS CAC GA	Targeted gene walking forward
5RACE-1	GCC GCG ACT CAC CAA CAA GA	Targeted gene walking reverse
ECH_F	TCG CAA GGG CGC GGA CAT CCT CGT	Specific amplification at 5' end

3.2.3.3. 3'-Rapid Amplification of cDNA end (RACE)

The RACE procedure was used to obtain the 3' end of the ALS gene (Fig. 3). RNA extraction was performed as explained above and the synthesis of first cDNA strand was conducted using Promega reverse transcriptase starting from 1 µg of total RNA and 0.5 µM of a oligo dT_{24VN} at 56 °C annealing temperature. The gene-specific forward primer for the RACE amplification (3RACE-1; Table 2) was designed on the fragment previously amplified with primers ECH-5F and ECH-3R. The RACE PCR was carried out using the Advantage® 2 PCR kit (Clontech) as explained above, with slight modifications. Since the fragment amplified was not very abundant, a nested PCR amplification was conducted using a second specific forward primer (3RACE-2). The PCR product of the RACE procedure was purified as described above and used as template for a second amplification step following the same conditions. PCR fragment obtained was excised from the gel, purified and sequenced.

3.2.3.4. Targeted gene walking PCR

To obtain the 5' end of the ALS gene, several specific reverse primers were designed on the fragment previously obtained and several degenerate forward primers were designed based on ALS nucleotide sequences available in the GenBank database as described above. 5RACE-1 primer and forward primers (ECH-3F, ECH-4F) produced the expected amplicons (Fig. 3, Table 2). The amplification was carried out adding an enhancing agent for PCR (Henke *et al.*, 1997). This fragment was purified using the NucleoSpin Extract II (Macherey-

Nagel), cloned into a plasmid vector and transformed into competent *E. coli* bacterial cells using the TOPO TA Cloning® kit (Invitrogen). A few positive colonies were chosen for a PCR colonies using GoTaq® Hot Start Master Mix (Promega) and the universal oligonucleotides M13 reverse and M13 forward. Liquid cultures were grown starting from the colonies in LB medium plus kanamycin antibiotic (100 µg mL^{-1}) and stirred overnight at 37 °C. Plasmids were extracted using PureYield™ Plasmid Miniprep System (Promega), quantified in 1% agarose gel and sequenced with universal M13 primers.

The amplicons obtained with the different approaches were overlapped using DNASTAR® software in order to obtain a consensus sequence of the *E. crus-galli* ALS gene. The partial sequence has been deposited in the GenBank database with the accession number JQ319776. The sequence was then aligned with the ALS sequences of other species and a phylogenetic analyses was carried out using MEGA 5.05 software.

3.2.3.5. ALS isolation on genomic DNA

To test for the presence of introns, genomic DNA was extracted from the same samples, as those used for RNA extraction, using the CTAB (citrimonium bromi) method (Aras *et al.*, 2003). Specific primers were designed to amplify the ALS sequence obtained with the above explained procedure, but due to the great variability of the ALS sequence in the 3' region, only the mix of one specific (ECH_F) and one degenerate primer (ECH_3R) produced the desired amplicon. The amplification protocol, DNA fragment extraction from agarose gel and sequencing were the same as described above for cDNA.

3.2.4. ALS gene cloning

Total RNA was extracted from 100 mg of one single plant fresh young leaf tissue using the commercial kit RNeasy Plant mini kit (Qiagen). RNA was extracted from a few plants of six populations harvested in rice fields, two S populations 07-16 and 05-27 and four R populations: 09-42, 09-44, 09-45 and 09-46.

Two cloning experiments were carried out: in the first one the genotyping of the ALS gene was performed, i.e. the full length sequences of the ALS gene were cloned from several populations and aligned in order to detect the Single Nucleotide Polymorphisms (SNP) and mutations endowing resistance, whereas in the second one only a part of the gene was amplified and cloned and the clone sequences were analyzed by a phylogenetic analysis in order to characterize the part of the gene including the mutation endowing herbicide resistance.

cDNA was synthetized using the ImProm-II™ Reverse Transcriptase System (Promega) and the different parts of the ALS gene were amplified using primer pairs ECH_F/ECH_3R and ECH_5F/ECH_3R, respectively, as explain above. PCR products were purified on column using MinElute PCR purification kit (Qiagen) and cloned using the TOPO TA Cloning® kit (Invitrogen) following the manufacturer's instructions. In the first experiment, 10-20 colonies were selected for each plant, plasmids were extracted from *E. coli* bacterial cells using PureYield™ Plasmid Miniprep System (Promega), quantified in 1% agarose gel and sequenced with universal M13 primers and a forward primer positioned in the middle of the gene (ECH-5F). In the second experiment, only the M13 reverse primer was used for the sequencing. The nucleotide sequences were manipulated using DNASTAR® software and a step by step phylogenetic analysis were carried out in order to examine the potential phylogenetic information contained in the SNP, analyzing the sequence in a hierarchical way: in the first step sequences of each plant were analyzed together, then those of each population and then all together. The phylogenetic analysis was performed using MEGA 5.05 software.

3.2.5. Allele Specific – Cleaved Amplified Polymorphic Sequence 574

For the quick analysis of a large number of samples, a molecular marker for the detection of the mutation 574 (the amino acid position refers to the ALS *Arabidopsis thaliana* sequence) was developed. The aim was pursued using a mix of an AS-PCR (Ye *et al.*, 2001; Bundock *et al.*, 2006), in order to amplify only the copy of the gene including the 574 mutation, and a Cleaved Amplified Polymorphic Sequence (CAPS) method (Neff *et al.*, 1998), in order to distinguish the mutated

from the wild type allele through a restriction endonuclease digestion. The CAPS is based on the same principles of the RFLP.

3.2.5.1. Allele-Specific PCR

The alignment of clone sequences obtained in the previous experiments highlighted SNP able to distinguish the different copies of the ALS gene, which were used to design allele specific primers. Allele-specific reverse primers, including a mismatch to improve the specificity as explained in You *et al.* (2008), were designed (Table 3). They were tested for the specific amplification of the gene copy including mutation 574 using the plasmidic DNA derived from the cloning experiments. Finally, the primers F_526 and R1_590 gave the desired amplicon and were tested on cDNA. The PCR was performed using the Go Taq® Flexi DNA Polymerase (Promega) in a final volume of 25 µL adding the requested reagents. Amplification was conducted using the following program: DNA denaturation for 2 min at 95 °C, and 30 cycles of 30 s at 95 °C, 30 s at 55 °C, and 25 s at 72 °C. Finally, samples were subjected to a 5 min extension time at 72 °C. The amplification was checked in 1% agarose gel.

Table 3. Primers used for the Allele-Specific PCR and its validation. In bold the mismatch including to improve the specificity.

Primer name	Primer sequence (5'-3')	Amino acid position
F_526	CCC AGG TGT TAC AGT TGT TGA CAT CGA	526
R1_590	GGA TAT ATC TCG CTC TCA TTC TCT **T**GA	590
R_621	ACT TCG CTC TTC TTT GTC ACA CGG A	613

3.2.5.2. Cleaved Amplified Polymorphic Sequence

The part of the ALS gene sequence including the mutation 574 was analyzed with dCAPS Finder Software (Neff *et al.*, 2002) in order to detect the presence of a cleaved site for a restriction endonuclease to distinguish the mutated from the not-mutated allele. The target sequence for the endonuclease BtsI was found in the sequence of the wild type, i.e. in the sequence of the allele with a Trp in position 574. Different digestion time, quantity of PCR product and enzyme concentration were tested in order to define the best conditions. Finally, 5 µL of

PCR product were digested using 3 U of BtsI (NEF) enzyme in a total volume of 20 µL with the specific buffer (Buffer 4 provided by NEF) for 2 h at 55 °C followed by 20 min at 80 °C for the enzyme inactivation. The digestion was checked in 2% agarose gel.

3.3. Results and discussion

3.3.1. Southern blotting

Southern blotting analysis to quantify the number of ALS loci in *E. crus-galli* genome, revealed that barnyardgrass had more than one copy of ALS gene. The Digoxigenin (DIG)-labeled DNA probe of 360 bp corresponds to a conserved region near the region B of the ALS gene and was used as a probe to detect the homologous fragments on genomic DNA blots.

Results of hybridization of the DNA fragments digested with HindIII (first experiment) and with XbaI (second experiment) revealed that there are 3 or 4 copies of ALS gene in the genome of S population 07-16, whereas 2 or 3 copies are present in the genome of the R population 09-45 (data not shown). Results were not clear for the other endonucleases tested. In order to clarify these results, a third experiment was performed and the results are reported in figure 4.

DNA-blotting analysis revealed differences in the ALS gene locus number in the different populations investigated. The results of the hybridization of DNA fragments cut with HindIII were consistent with those obtained in the first experiment and showed that 2 copies of the ALS gene are present in the genome of the S population 05-27 and in the genome of the R populations 09-45 and 09-46, whereas one more copy was detected in the genome of the S population 07-16. The visible band at 21226 bp is not considered because corresponds to the genomic DNA residues. This suggests that the copy number of the ALS gene is not linked with the susceptible or resistant status of the populations. The fact that a different copy number of the ALS gene may be present in different populations of the same species may be due to the heterogeneity of *Echinochloa* species and the

difficulty to distinguish them (Tabacchi, 2003). A number of evolutionary stages could affect the reorganization of the genome and therefore the selection of a copy gene rather than another. The Southern analysis clearly showed the presence of multiple copies of the ALS gene in the genome of *E. crus-galli*. However, to know exactly the number of copies, it would be appropriate to make a genomic library. This would also help to understand how the complex genome of this polyploid specie is organized.

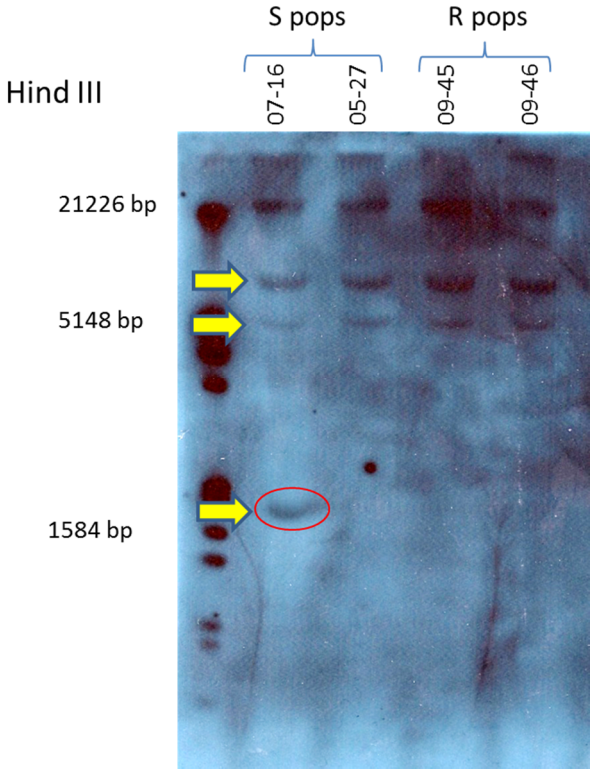

Fig. 4. Southern blotting analysis of *E. crus-galli* genomic DNA extracted from leaf tissue. DNA samples of different populations (reported on the top) were digested with the enzyme HindIII. The number on the left side showed DIG-labeled molecular ladder. Arrows indicate the copies of the ALS gene and the circle highlights the more copy in the population 07-16.

3.3.2. ALS enzyme bioassay

ALS activity assays were performed to determine if an altered target site was responsible for ALS resistance in R populations tested. Two R populations were chosen from the ones previously analyzed, 09-44c and 09-45. The *in vitro* measurements of acetolactate synthase activity indicated a sigmoidal response to increasing doses of penoxsulam and imazamox and the data were well fitted by the

log-logistic equation (Fig. 5, A and B). The herbicide concentrations required to reduce the ALS activity by 50% are reported in table 4. Two S biotypes (07-16 and 05-27) were tested. They had similar curves and I_{50} values, with slight differences: population 07-16 was more sensitive to penoxsulam, while population 05-27 was more sensitive to imazamox. R/S ratio is calculated as the ratio between the I_{50} value obtained for the R population and the I_{50} value obtained for the S population, therefore two different R/S ratios were calculated based on the two S populations tested. Enzyme assays from the two resistant populations evaluated indicated that both had target-site resistance, with I_{50} values at least 5-fold higher relative to sensitive populations. For both herbicides, however, ALS activity of population 09-45 was about 5-fold less sensitive than that of population 09-44c.

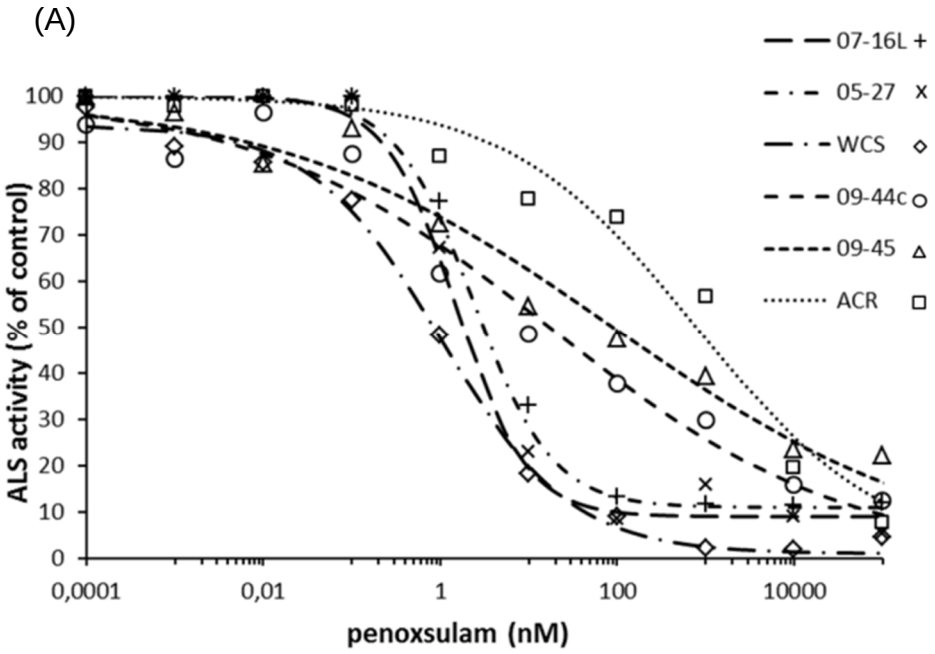

Fig. 5A. Inhibition curves by penoxsulam of ALS extracted from four populations of *E. crus-galli* (07-16 +, 05-27 x, 09-44c ○ and 09-45 Δ) and two populations of *A. tuberculatus* (WCS ◊ and ACR □) obtained using BIOASSAY® macro. Lines are the response curves predicted from non-linear regression; symbols represent percentage of mean survival, based on the untreated controls, of three replicates.

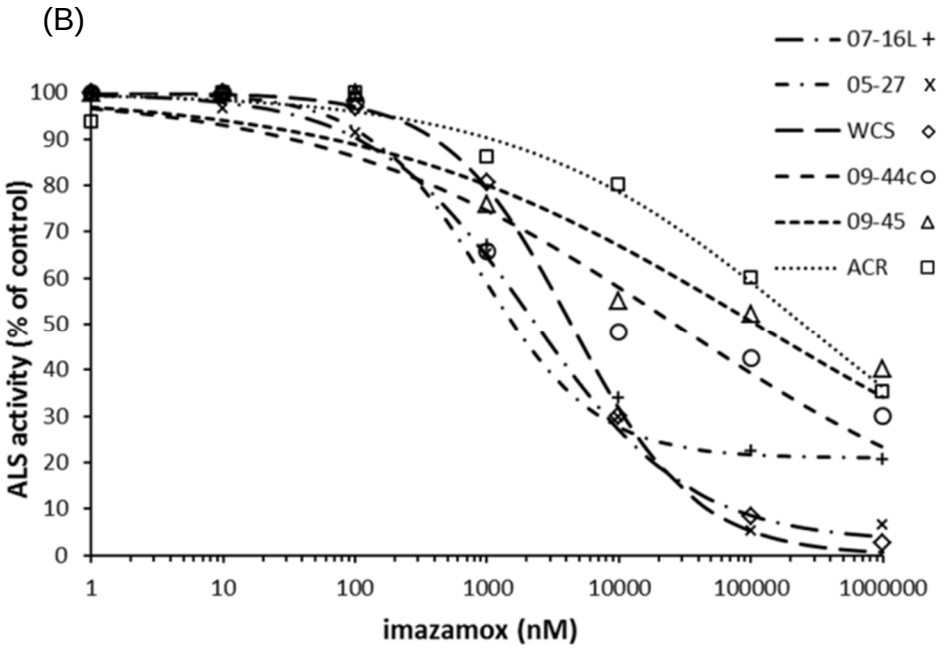

Fig. 5B. Inhibition curves by imazamox of ALS extracted from four populations of *E. crus-galli* (07-16 +, 05-27 x, 09-44c ○ and 09-45 Δ) and two populations of *A. tuberculatus* (WCS ◊ and ACR □) obtained using BIOASSAY® macro. Lines are the response curves predicted from non-linear regression; symbols represent percentage of mean survival, based on the untreated controls, of three replicates.

Results obtained from the enzyme assay were not completely consistent with whole-plant herbicide efficacy data. Considering the high LD_{50} values obtained at the whole plant level, one would have expected to have higher I_{50} values for the R populations at the enzyme level. Analyses conducted on some species with different ALS gene copies indicate that a negative correlation exists between the copy number and resistance level (Zheng, 2007). For this reason, the diploid *A. tuberculatus* was included in the experiment. As expected, for penoxsulam the R/S ratio (744) of enzyme extracts from waterhemp ACR biotype (which has the W574L substitution), calculated on the basis of the WCS (S population) I_{50} value, was much higher than that of *E. crus-galli* resistant biotypes (Table 4). For imazamox, however, the difference between *A. tuberculatus* and *E. crus-galli* was not as striking, and depended on which S population was used for comparison. Regardless, the enzyme activity data indicated that both population 09-44c and 09-45 likely had target-site-based resistance to both penoxsulam and imazamox.

Table 4. Herbicide concentrations causing 50% inhibition (I_{50}) and R/S ratios obtained from curves shown in figure 3 for penoxsulam and imazamox on partially purified acetolactate synthase from four populations of barnyardgrass (ECHCG) and two populations of common waterhemp (AMATA).

Population	Penoxsulam			Imazamox		
	I_{50} (nM)	**R/S $_{07-16}$**	**R/S $_{05-27}$**	**I_{50} (nM)**	**R/S $_{07-16}$**	**R/S $_{05-27}$**
ECHCG 07-16 (S)	1.8(0.23)	1	0.59	2,352(295)	1	1.49
ECHCG 05-27 (S)	3.1(0.17)	1.68	1	1,580(153)	0.67	1
ECHCG 09-44c	17.2(7.53)	9.48	5.63	26,513(12,787)	11.3	16.8
ECHCG 09-45	89.8(40.2)	49.4	29.3	108,997(55,674)	46.3	69.0
	I_{50} (nM)	**R/S $_{WCS}$**		**I_{50} (nM)**	**R/S $_{WCS}$**	
AMATA WCS (S)	1.1(0.20)	1		4,327(256)	1	
AMATA ACR	784(270)	744		248,215(75,397)	57.4	

3.3.3. ALS gene sequencing

The alignment of the ALS gene sequences of nine Poaceae available in GenBank showed highly conserved regions that allowed the design of primers for the amplification of the ALS gene. The combination of degenerate primers ECH-5F and ECH-3R initially amplified a DNA fragment of 1270 bp that was queried using Blastx (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) analysis, resulting in 96-98% identity with the ALS gene sequence from Poaceae species. Nucleotide ALS sequences from susceptible plant (population 07-16) and from penoxsulam-resistant plant (population 09-45) were then aligned to identify specific primers for further PCR amplifications and to perform the RACE-PCR. The 3'-RACE procedure consists of a gene sequence isolation strategy to overcome problems with DNA amplification through regular PCR at the extreme parts of a gene. It produced a fragment of 325 bp at the 3' end of the gene that overlapped with the first fragment obtained, resulting in a 1460 bp ALS gene sequence. As the RACE procedure did not work on the 5' end of the ALS gene, a "targeted gene walking PCR" procedure was adopted. This procedure is used to amplify an unknown sequence adjacent to a known sequence of the gene of interest (Merotto *et al.*, 2009) and successfully produced a 400 bp fragment including the three domains not yet amplified with the

other methods. Finally, a 1870 bp consensus sequence, including all the known conserved domains, was obtained (Fig. 6).

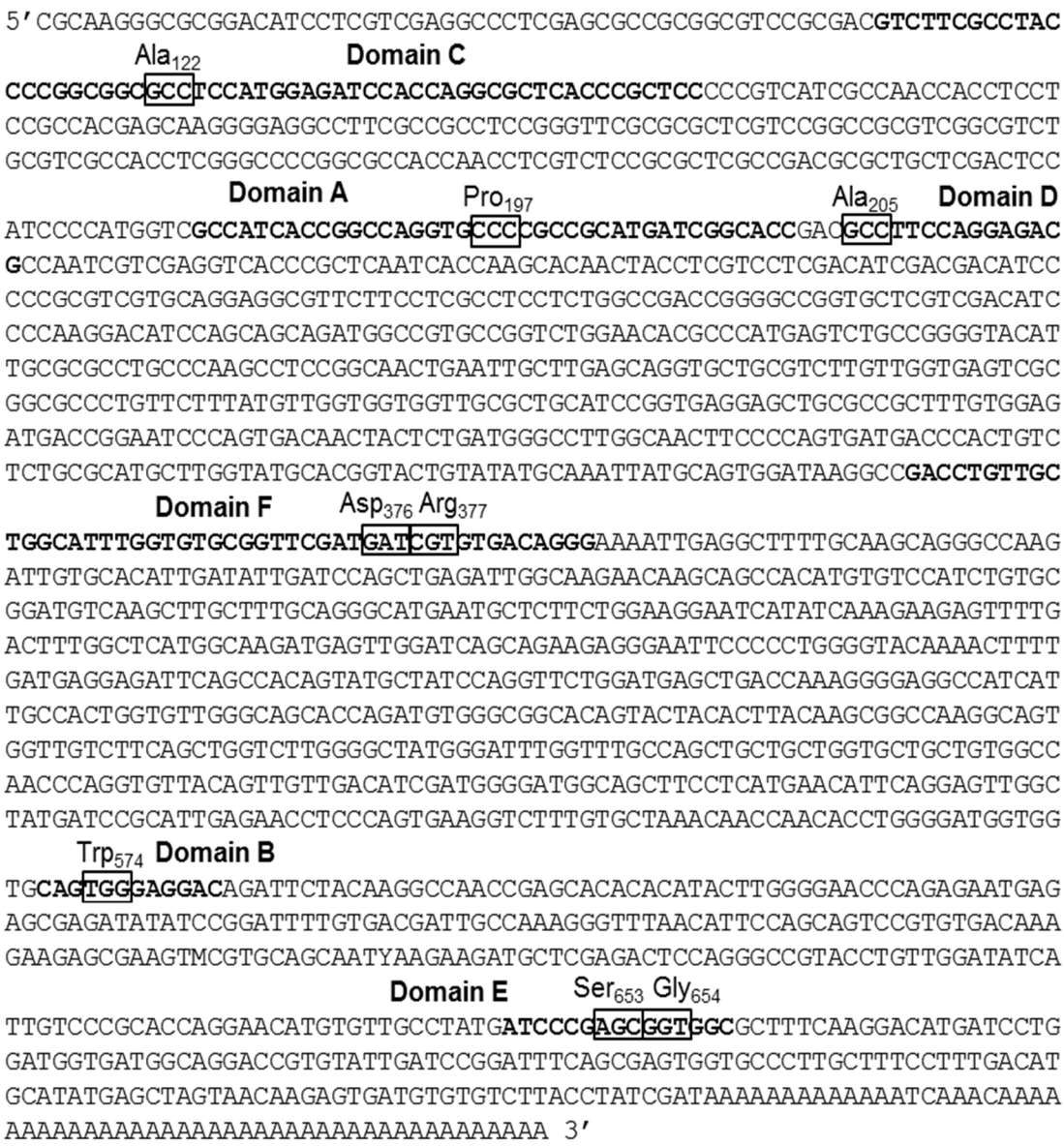

Fig. 6. The 1870 bp nucleotide sequence of *E. crus-galli* ALS gene from cDNA (GenBank A.N. JQ19776). In bold, the six conserved domains are shown and for each of them the amino acid position where mutations responsible for resistance have been detected so far (Tranel *et al.*, 2011) are reported.

The *E. crus-galli* ALS gene sequence was aligned with the sequences used for the primers design and the phylogenetic analysis indicated that it clustered well

with all the Poaceae used, in particular with *Zea mays* (Fig. 7). On the opposite, two clear clusters are obtain if ALS gene sequences from dicot species are included in the alignment (data not shown) as it was also seen in Tan *et al.* (2007).

Amplification of both cDNA and genomic DNA gave the same amplicons (data not shown) and confirmed the absence of introns in the ALS gene of *E. crus-galli*. However, since amplification of the *E. crus-galli* ALS gene starting from genomic DNA was not consistently successful, the ALS gene was amplified from cDNA in subsequent investigations.

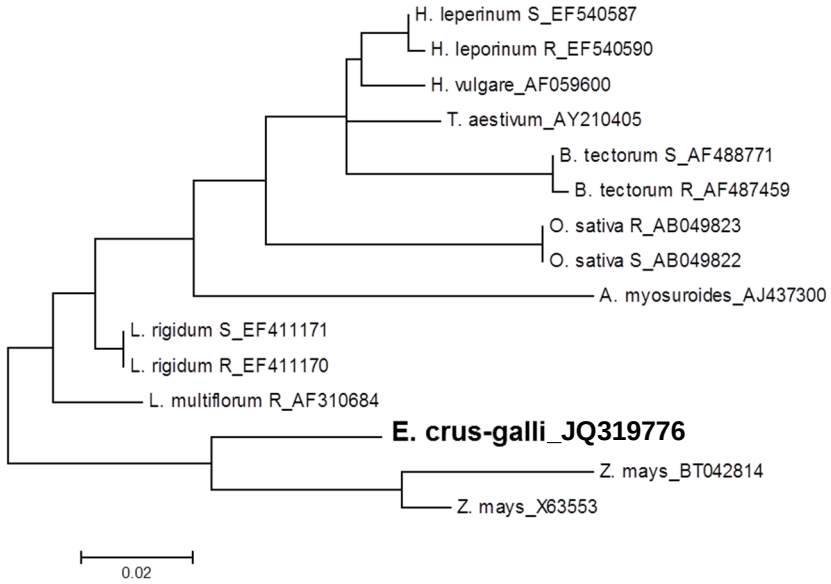

Fig. 7. Cladogram obtained from maximum-likelihood estimation (MLE) of ALS sequences using MEGA 5.05. *E. crus-galli* ALS gene sequence is in bold. Each taxon is displayed with the GenBank accession number, species name and status (S or R), when available.

No known SNP endowing resistance was found in the sequences of the S population, whereas the SNP resulting in substitution of a G with a T, giving a Trp to Leu change at amino acid 574, was detected in the sequences of the R population. The heterozygous status may be detected already at PCR product level as a double overlapped peaks (Fig. 8).

This is consistent with the resistance pattern found in the whole-plant experiments. In fact, the Trp-574-Leu mutation is one of the most frequent mutations detected in ALS inhibitors resistant weeds endowing cross-resistance to

all ALS inhibitors chemical families (SU, TP, IMI, PTB and SCT) (Devine and Shukla, 2000).

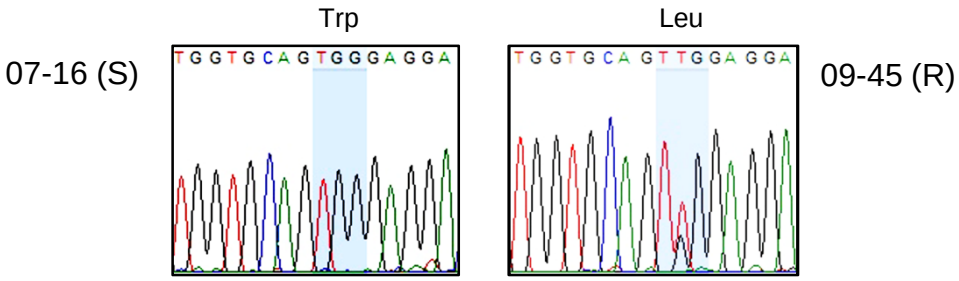

Fig. 8. Partial sequencing chromatograms of ALS fragments amplified starting from cDNA, of a susceptible (left) and resistant (right) plant of *E. crus-galli* populations 07-16 and 09-45, respectively. The codon highlighted indicates position 574 of the ALS gene (the amino acid position refers to the ALS *Arabidopsis thaliana* sequence), the G to T change causes the substitution of Trp to Leu in domain B.

3.3.4. ALS gene cloning

The analysis of the cDNA full length ALS cloned sequences has identified a large number of SNP (data not shown). They may be associated to the presence of homologous copies of the gene that could be subjected to natural modification during the evolution. Furthermore, Advantage 2 Polymerase Mix (Clontech) exhibits an error rate of 25 errors per 100,000 bp after 25 PCR cycles. Hence, it was impossible to determine which SNP were ascribable to the first category and which ones were sequencing errors. Therefore, it was necessary to determine specific motifs into the sequences able to distinguish the homologous gene copies.

ALS gene from one plant of each of the six populations analyzed was cloned and partially sequenced (800 bp starting from 3' end) and cloned sequences were analyzed through a phylogenetic analysis using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method in order to group the sequences belonging to the same ALS gene copy. This has led to identify two clusters (Fig. 9, A and B), which were called ALS1 and ALS2. For each plant analyzed, the tree designed showed the same clustering (data not shown) and it was observed that all clones having the mutation Trp-574-Leu, detected in all the R samples, clustered in the group called ALS1. At the same time, this amino acid substitution

has never been detected in the sequences of the S samples (07-16 and 05-27), both clustered in ALS1 or ALS2 groups. The results are reported in table 5.

Table 5. Results of the 800 bp ALS sequence cloning starting from 3' end. Several information are reported, from left to right: no. of clones analyzed, no. of clones belonging to ALS1 and ALS2, no. of clones mutated in position 574, aa positions that can distinguish ALS1 from ALS2.

Sample	no. of clones	ALS1	ALS2	ALS1 W574L	Pos 590	Pos 621	Pos 625
					ALS1/ALS2		
07-16-1 (S)	18	11	7	-	T/C	C/A	T/C
05-27-1 (S)	9	7	2	-	T/C	C/A	T/C
09-45-4A	18	10	8	10	T/C	C/A	T/C
09-46-2	11	6	5	6	T/C	C/A	T/C
09-44c-2	10	9	1	7	T/C	C/A	T/C
09-42-2	9	9	-	9	T	C	T

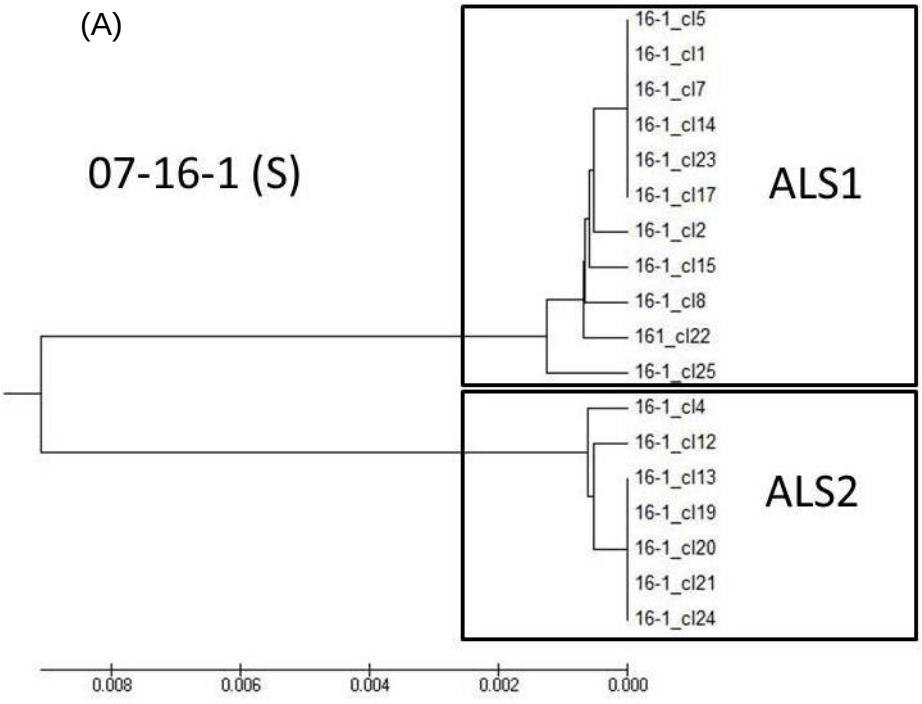

Fig. 9A. Cladogram obtained from UPMGA estimation of eighteen partial clone sequences of the S sample 07-16-1 using MEGA 5.05. Two clusters (called ALS1 and ALS2) are highlighted.

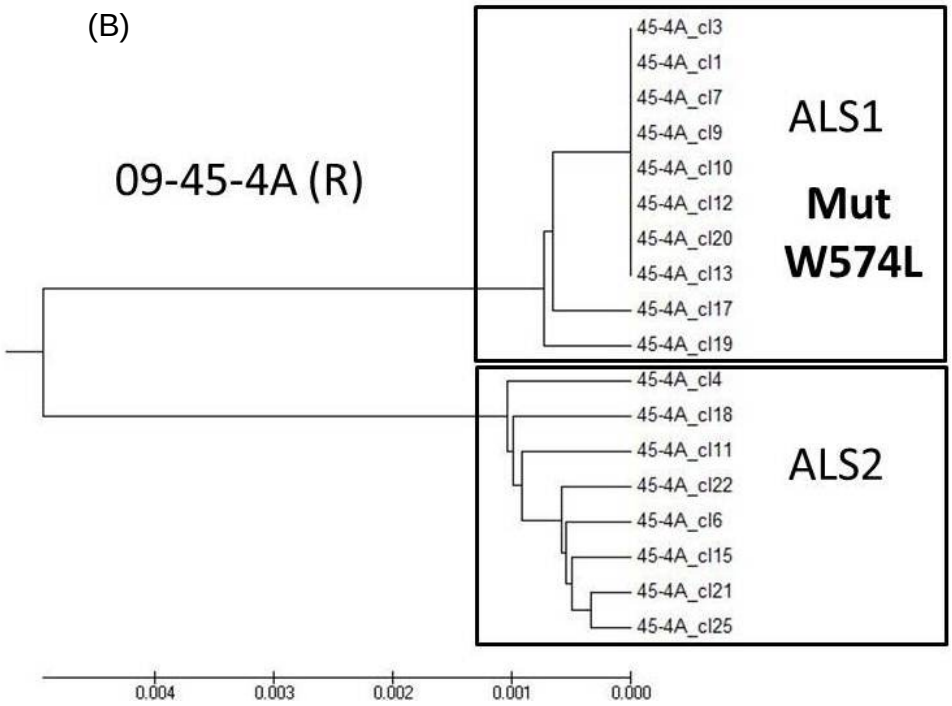

Fig. 9B. Cladogram obtained from UPMGA estimation of eighteen partial clone sequences of the R sample 09-45-4A using MEGA 5.05. Two clusters (called ALS1 and ALS2) are highlighted. In all the sequences of the ALS1 of R sample the mutation W574L was detected.

Furthermore, some clones for each group (ALS1 and ALS2) of the samples 07-16-1 and 09-45-4A were completely sequenced in order to check if clusters were maintained even at whole gene level (Fig. 10). A third cluster including the sequences from the S sample 07-16 was observed.

The seventy five partial sequences of the different populations were analyzed together (Fig. 11). Even in this case, a third cluster was observed and all the sequences in the third cluster belong to the S sample 07-16-1. In three out of four resistant plants analyzed, all the sequences belong to ALS1 were mutated in position 574, whereas in the sample 09-44c-2 two sequences that clustered in ALS1 were not mutated in that position (Table 5), meaning that the substitution may be present in both homozygous and heterozygous status. However, even a single resistant allele allows the plant to survive the herbicide treatment.

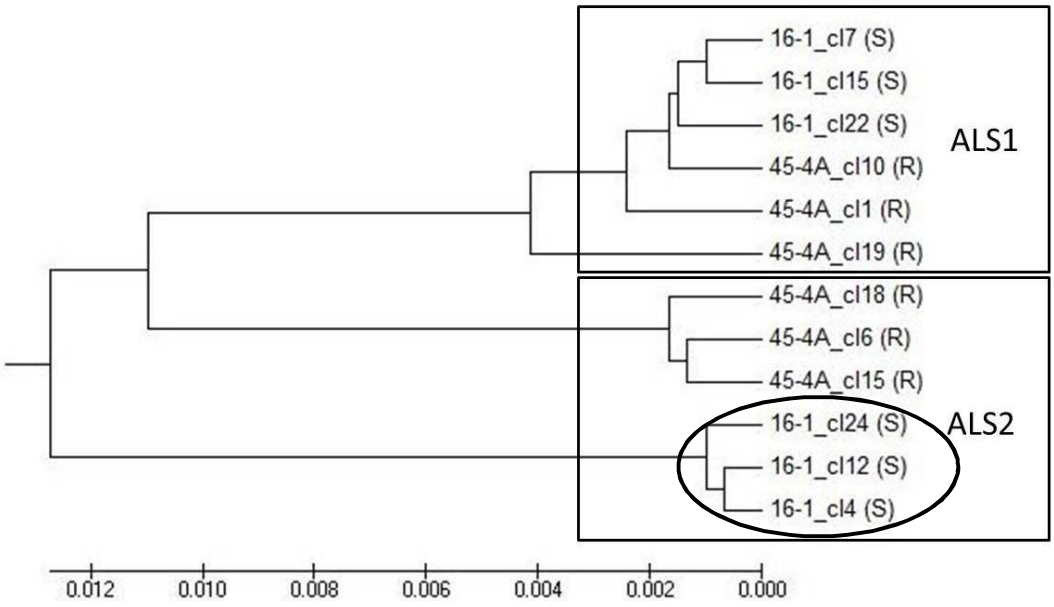

Fig. 10. Cladogram obtained from UPMGA estimation of twelve ALS complete clone sequences, six of the S sample 07-16-1 and six of the R sample 09-45-4A, using MEGA 5.05. ALS1, ALS2 and the three sequences of sample 07-16-1 that formed the third cluster are highlighted. In all the sequences of the ALS1 of R sample the mutation W574L was detected.

Population 07-16 showed three copies of the ALS gene in the Southern analysis (see section 3.3.1), suggesting that the separation of its sequences in a different cluster may be associated with the presence of a different expressed allele. As Southern blotting is done on the genomic DNA, not all the copies of the gene detected could be expressed in the cells. Instead, the cloning is done on cDNA and all samples analyzed showed two copies of the ALS gene expressed represented from the two clusters (Fig. 9). Thus, population 07-16 may express one gene common to the other populations and one gene different from them, whereas the third copy of the gene may be silent.

The sequence analyses have identified several SNP intra the gene copy and a few SNP extra gene copy useful to distinguish ALS1 from ALS2 (independently from the presence of the third cluster). Three of the latter where identify in position 590, 621 and 625 and were used to designed allele-specific primers (Table 5).

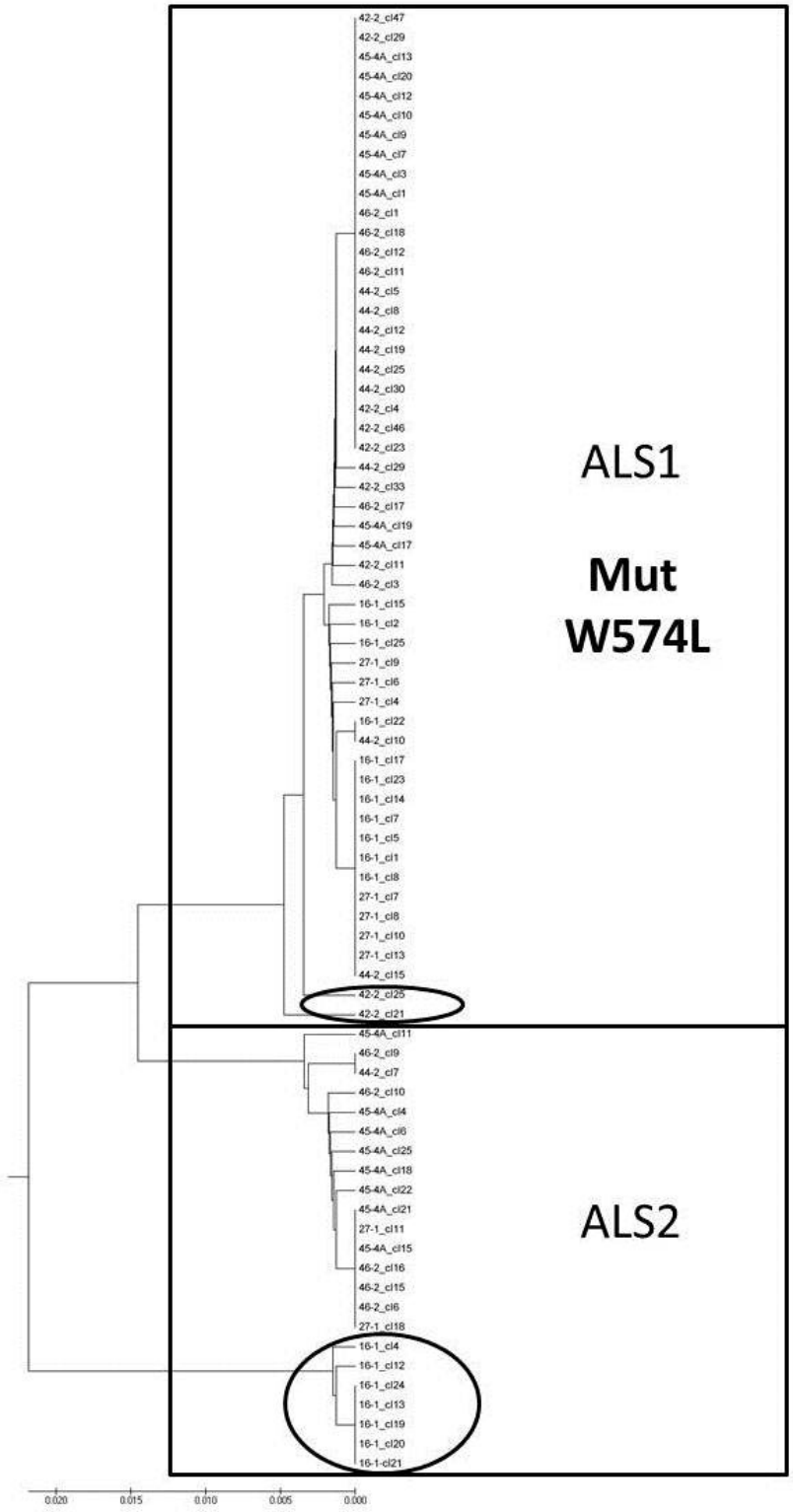

Fig. 11. Cladogram obtained from UPMGA estimation of ALS sequences using MEGA 5.05. Seventy five partial sequences from different S and R samples are reported, see table 5 for details. The two sequences of sample 09-44c-2 that clustered in ALS1 but do not belong mutation W574L (top) are highlighted and also the seven sequences of sample 07-16-1 that formed the third cluster (bottom).

3.3.5. AS-CAPS 574

An AS primer is specific to one of two alleles of a SNP located at the 3' end of primers and specifically amplifies one of the two alleles. If a common forward primer is used in the reaction, the reaction is called allele-specific PCR (AS-PCR). Several ALS1 reverse specific and forward unspecific primers were designed in order to set an AS-PCR. To enhance the specificity in the AS-PCR reaction, an additional mismatch may be deliberately introduced at the third or other position from the 3' end of each of the AS primer (Ye *et al.*, 2001). Rules for selection of a nucleotide for the mismatch are summarized in You *et al.* (2008). The aa position 590 (Table 5) was selected after several tests: in that position a T was present in ALS1 gene copy, whereas a C was present in ALS2 gene copy. Primers used are reported in table 3.

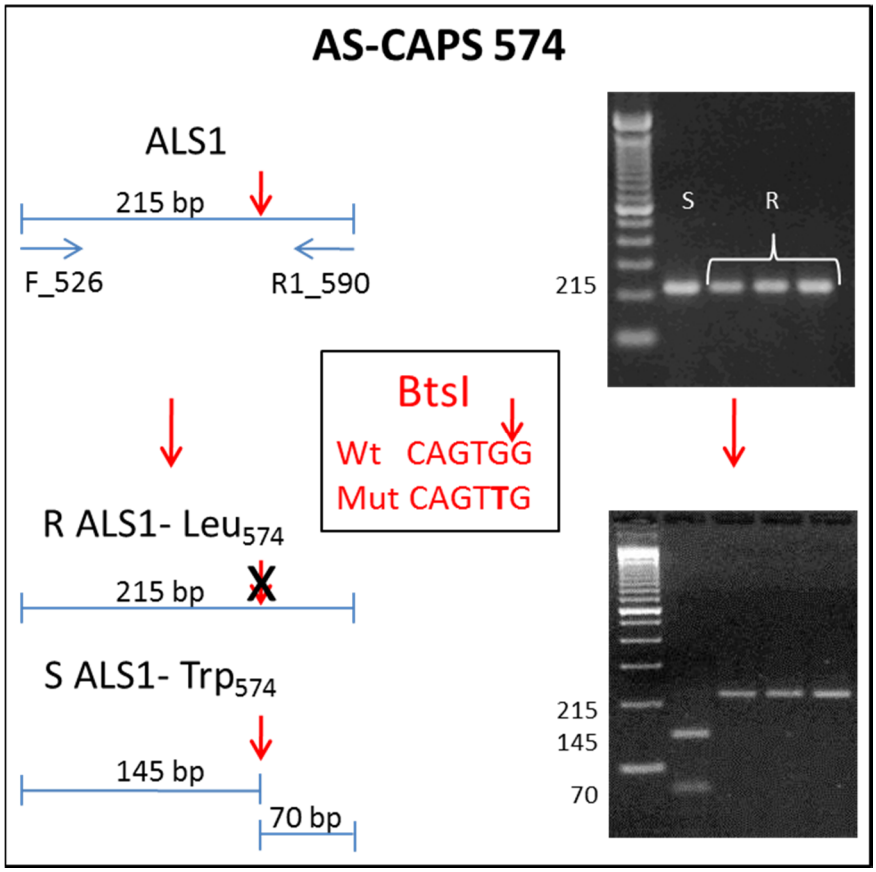

Fig. 12. Scheme of the DNA-based molecular marker AS-CAPS 574. Above is represented the allele-specific amplification producing an amplicon of 215 bp. The digestion with the endonuclease BtsI produced two bands if the ALS1 is not mutated in position 574.

The specificity of the primers was tested using plasmidic DNA where the nature of the ALS clone held was known (data not shown). The scheme of the molecular marker is reported in figure 12. The amplification with primers F_526 and R1_590 produced an amplicon of 215 bp. If the ALS1 has a triplet TGG, coding for a Trp, in position 574 the digestion with the endonuclease BtsI produced two bands. Therefore, any other nucleotide in that position block the cut by BtsI and not only the nucleotide substitution resulting in a Leu will be detected, even if actually no different nucleotide substitution were detected in that position (Tranel *et al.*, 2011). Furthermore, even the heterozygous status may be detected, resulting in a pattern of three band on the gel: an uncut band of 215 bp and two cut bands of 145 and 70 bp, respectively (Fig. 12).

The molecular marker was validated on cDNA through amplification using the unspecific forward primer (F_526) and, alternatively, specific (R1-590) and unspecific (R_621) reverse primer. PCR products were sequenced with the reverse primer, specific and unspecific for ALS1, respectively, and results were reported in table 6. The same samples were analyzed with the AS-CAPS 574 (Fig. 13).

Table 6. Validation of AS-CAPS 574. Results of molecular marker: S indicates that two bands were presented, R indicates the presence of one uncut band, R/S indicates that the uncut band was brighter than the cut bands and *vice versa*. Sequencing results: the SNP in positions 574 and 590 are reported in brackets; the first reported nucleotide into brackets was the highest peak in the chromatogram. In bold the nucleotide associated with the mutated aa in position 574.

Sample	**AS- CAPS 574**	**Seq. with ALS1 specific primer (R1_590)**		**Seq. with unspecific primer (R_621)**	
		574	**590**	**574**	**590**
16-2	S	TGG	AAT	-	-
44c-2R	R	T**T**G	AAT	-	-
16-4	S/**R**	T(G/**T**)G	AAT	TGG	AA(C/T)
27-3	S	TGG	AAT	TGG	AA(C/T)
44-3R	**R**/S	T(**T**/G)G	AAT	T(G/**T**)G	AA(T/C)
45-3R	**R**/S	T(**T**/G)G	AAT	T(G/**T**)G	AA(T/C)
46-3R	**R**/S	T(**T**/G)G	AAT	T(G/**T**)G	AA(T/C)

The specificity of the R1_590 primer is confirmed from results of the sequencing (see columns 590 in table 6): when the ALS1 specific primer was used only the nucleotide T was detected in the chromatrogram, i.e. only ALS1 was amplified; instead the PCR with the unspecific primer R-621 individuate a double peak in all samples in position 590, i.e. both ALS1 and ALS2 were amplified. At the same time, the intensity of the peaks T/G in position 574 was inverted, depending on the primers used for the amplification, indicating a prevalence of not mutated alleles in the unspecific amplification due to the high presence of ALS2, whereas only the ALS1 was amplified using the specific primer. This highlights the presence of the heterozygous status as previously observed in the phylogenetic trees (Fig. 8, sample 09-44-2).

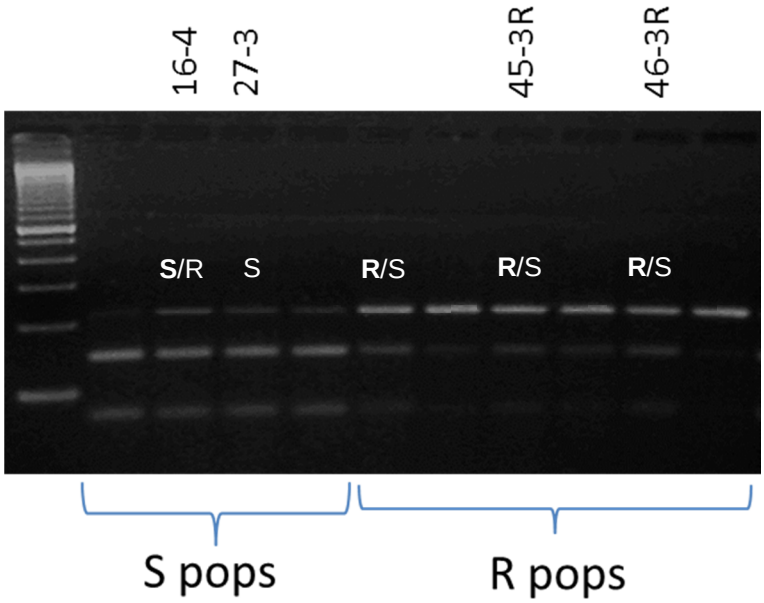

Fig. 13. AS-CAPS 574 on cDNA samples. It is possible to distinguish susceptible samples (on the left) from resistant samples (on the right) and the heterozygous status of, at least, three of them (indicated with R/S). For some samples, results may be compared with sequencing data reported in table 6.

3.4. Conclusions

Results from both molecular analyses and ALS enzyme bioassay were in keeping with the whole plant experiments (see chapter II) and proved that resistance to ALS inhibitors is due to an altered enzyme. The lower than expected R/S ratio based on the *in vitro* bioassay may be linked with the polyploid status of *E. crus-galli*. The identification of multiple ALS genes was expected, as the ALS probe hybridized to several fragments on Southern blotting. It revealed that at least two copies of the ALS gene are presented in the genome of barnyardgrass. The first ALS gene sequence of *E. crus-galli* was obtained and the presence of the Trp-574-Leu amino acid substitution was highlighted. This allele variant, as it has been observed in the whole plant pot experiments and already demonstrated in several other weed species, endows a broad cross-resistance pattern to ALS inhibitors. This implies that all the classes of ALS inhibitors are involved in a lack of sensitivity at the target enzyme. Furthermore, its presence was detected always in the same copy of the ALS gene, which is consistently expressed in all the biotypes. This led to develop a DNA-based molecular marker useful for the quick detection of the broad cross-resistance to ALS inhibitors associated with the presence of mutation 574.

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Chapter IV

Growth analysis of herbicide resistant and susceptible *Sorghum halepense*

4.1. Introduction

Predictions based on evolutionary theory suggest that the adaptive value of evolved herbicide resistance alleles may be compromised by the existence of fitness costs. There have been many studies quantifying the fitness costs associated with herbicide resistance alleles, reflecting the importance of fitness costs in determining the evolutionary dynamics of resistance. However, many of these studies have incorrectly defined resistance or used inappropriate plant material and methods to measure fitness (Vila-Aiub *et al.*, 2009).

When a herbicide resistance allele confers a fitness cost, there are at least three explanations for the origin of this phenomenon. First, fitness costs may result when novel, resistance-conferring mutations in herbicide target enzymes (target-site resistance) also compromise or interfere at some level with normal plant function or metabolism. Second, resource-based allocation theory predicts a trade-off between plant reproduction, growth and defense functions. Third, fitness costs may arise as a consequence of altered ecological interactions (Purrington, 2000).

Several factors should be considered to evaluate the effective fitness costs associated to herbicide resistance, as genetic background of the individuals that should be similar except for the alleles endowing herbicide resistance. For this, where possible, it is desirable to characterize the biochemical and molecular bases of resistance before conducting a fitness study. Furthermore, plant fitness may be defined as the relative number of offspring contributing to future generations by one form compared with another (Primack and Hyesoon, 1989). This focus on plant reproduction as a measure of fitness has led to many fitness studies that have compared seed production between herbicide resistant and herbicide susceptible individuals (Menchari *et al.*, 2008). However, the amount of seeds produced is the main but not the unique criteria to evaluate fitness costs, especially when “agronomic” fitness, rather than biological fitness is being evaluated (Parks *et al.*, 1996; Vila-Aiub *et al.*, 2005) and when species that reproduces not only by seeds are considered (Arriola and Ellstrand, 1997). According to Harper (1977), there are trade-offs between different life history stages and changes in one component of plant fitness, which may involve compromises in other traits. For

example, seed production is a crucial determinant of fitness which will integrate and be influenced by other life history variations between resistant and susceptible plants.

It has been shown that some herbicide resistance alleles have no observable impact on plant fitness (Vila-Aiub *et al.*, 2009). An understanding of the fitness consequences of herbicide resistance alleles in the presence and absence of a herbicide is important for predicting the evolutionary dynamics of herbicide resistance (Neve *et al.*, 2003) and, therefore, in conceiving strategies by which fitness costs can be manipulated to result in selection against resistance alleles (resistance management).

Although there are many cases of non-target-site herbicide resistance, there are thus far only a few studies of fitness costs of non-target-site resistance mechanisms caused by enhanced rates of herbicide metabolism and transport, like demonstrated in *Lolium* resistant to glyphosate correlates with reduced rates of glyphosate translocation to active meristematic root and shoot tissues (Powles and Preston, 2006).

However, the majority of fitness cost studies in the literature are on target-site herbicide resistance. There have been a wealth of studies unequivocally documenting a fitness cost associated with target site resistance to triazine herbicides (Holt and Thill, 1994).

Regarding ACCase inhibitors, studies evaluating several fitness related traits in *L. rigidum* and *A. myosuroides* with the Ile-1781–Leu mutation have shown no physiological or ecological resistance costs and no detectable reduction in plant productivity (Vila-Aiub *et al.*, 2005). Menchari *et al.* (2008) evaluated fitness costs in several *A. myosuroides* populations with Ile-2041-Asn and Asp-2078-Gly ACCase resistance-endowing mutations. In a two-year field experiment in which plants were grown in competition with wheat, there was clear evidence that Ile-2041-Asn was not associated with any adverse pleiotropic effects on vegetative and reproductive plant traits. The ACCase resistance mutation Cys-2088-Arg has been identified in *L. rigidum* (Yu *et al.*, 2007). The ACCase activity of plants with this mutation is only one-half of that of wild type plants, and this impaired ACCase

activity is probably responsible for the compromised growth observed at the whole plant level. No indications are reported in literature about the other ACCase endowing resistance mutations known.

Regarding ALS inhibitors herbicide resistance, fitness consequences have only been examined for the Pro-197-His substitution in *Lactuca serriola*, where it was demonstrated a reduction in vegetative biomass of resistant compared with susceptible individuals growing under competitive conditions (Alcocer-Ruthling *et al.*, 1992). Furthermore, strong pleiotropic effects on plant morphology and anatomy, leading to a fitness cost, have been described in field evolved, ALS resistant *Amaranthus powellii* with the Trp-574-Leu ALS mutation (Tardif *et al.*, 2006). For other resistance-endowing ALS mutations, their impact on plant fitness remains unknown.

However, resistance costs are not universal and thus must be assessed on a case-by-case basis. (Vila-Aiub *et al.*, 2009).

Sorghum halepense (johnsongrass) is a tetraploid, rhizomatous spring-summer monocot weed belonging to the *Poaceae* family. It is a very plastic weed that can cause heavy yield losses in a large range of situations, different climate or cropping system. It is predominantly self-pollinated and is a geophyte which reproduces also by seed, therefore cropping practices may interfere with the spread and evolution of the resistant biotypes. Several *S. halepense* populations resistant to ACCase-inhibiting herbicides were found in northern Italy from 2005 to 2007 (see chapter II) in summer dicot crops, mainly soybean and tomato. Only one case of resistance to ALS-inhibiting herbicides, in particular to sulfonylureas (SU), was detected in Italian maize fields so far. Instead, several cases were found in Hungary in the last three years, where the problem in maize crops is evolving quite fast (see chapter II).

Molecular analysis confirmed that in all of the ACCase inhibitors resistant populations a single amino acid substitution Ile-2041-Asn (amino acid position corresponds to ACCase coding sequence of *Alopecurus myosuroides* [Genbank AC: AJ 310767]) had occurred in the ACCase gene (Pignata *et al*., 2008; Scarabel and Sattin, 2010), whereas no information were available about the molecular

basis of the resistance in ALS inhibitors resistant populations, even if whole plant experiments showed very high level or resistance suggested a target-site mediated mechanism involved.

There was no information in the literature about possible fitness penalties related to Ile-2041-Asn ACCase resistance-endowing mutation in *S. halepense*. Regarding ALS inhibitors resistant populations, the molecular investigation were performed in order to evaluate possible fitness costs on the bases of the resistance mechanism involved.

The main aim of the growth analysis experiments was to identify if there was a different development and biomass allocation among the biotypes, susceptible (never treated), ACCase inhibitors resistant and ALS inhibitors resistant.

4.2. Materials and method

Considering the results of preliminary screenings and the dose-response experiments (see Chapter II and Pignata *et al.*, 2008), ten *S. halepense* populations were chosen (Table 1).

Table 1. Details of populations investigated in the different growth analysis experiments.

Population code	Origin	Infested crop	Resistance status
05-1	Camisano Vic. (VI)	-	S check
06-10	Legnaro (PD)	-	S check
08-19	Komàron (H)	-	S check
05-2	Corte dei Frati (CR)	Soybean	ACCase inhibitors
05-4	Marcaria (MN)	Soybean	ACCase inhibitors
05-6	Castellucchio (MN)	Tomato	ACCase inhibitors
07-12	Medole (MN)	Tomato	ACCase inhibitors
08-16	Tolna (H)	Maize	ALS inhibitors
09-20	Tolna (H)	Maize	ALS inhibitors
07-13	Cavacurta (LO)	Maize	ALS inhibitors

4.2.1. Growth analysis

Three growth analysis outdoor pot experiments, in a semi-controlled environment, were carried out (Table 2) from 2009 to 2011.

Table 2. Details of the three-year growth analysis pot experiments. Starting material, populations involved and no. of samplings.

Year	Starting material	No. of populations tested			No. of samplings
		S	R ACCase	R ALS	
2009	Rhizomes	2	4	1	5
2010	Seed	3	4	3	4
2011	Rhizomes	3	4	3	1

Rhizome buds were used as starting material in the first and the third year, whereas the second year the experiment started from seeds. Ten populations were included: three susceptible populations (two harvested in Italy and one in Hungary), four populations resistant to ACCase inhibitors (harvested in Italian dicot crops) and three populations resistant to ALS inhibitors (one harvested in Italy and two in Hungary) (Table 1). The first two years, phenological and destructive samplings were performed periodically during plants life cycle, whereas the third year only the destructive final sampling was done.

4.2.1.1. 2009 experiment

Seven populations were included in the experiments: two susceptible checks (06-10 and 05-1), the four ACCase resistant populations and the one ALS resistant population sampled in Italy (07-13) (Table 1).

Growth analysis was performed in plants grown from rhizomes. Rhizomes were obtained from plants grown in large pots outdoor that survived an ACCase or ALS inhibitors treatment with fluazifop (125 g a.i. L^{-1}) and nicosulfuron (40 g a.i. L^{-1}), respectively, in the summer 2008. At the end of the season, shoots were removed and the pots containing the substrate with roots and rhizomes were left outside.

In late spring 2009, rhizomes were extracted from pots, washed from soil, cut and transplanted into pots containing silty-loam soil. Four plants were considered for each population. For each plant, three clones (i.e. three rhizome buds) were planted (one clone per pot) in 30-litre pots (30 cm Ø), which were watered daily and fertilized three times with Nitrofosca (15-9-15 N-P-K).

The plants development was studied during all the life cycle from seedling stage to seed maturity. Classical approach was used: plant growth was followed through a series of samplings of plants weight relatively far in time (Hunt, 1981; Sattin and Tei, 2006). Five samplings were performed (approximately 30 days apart) at temperature sums (°C d) 58, 263, 555, 955 and 1530 (temperature sum 0 was set when 50% emergence was recorded). Temperature sum is calculated based on Growing-Degree Day equation:

$$GDD = \frac{Tmax - Tmin}{2} - Tbase$$

where T_{max} and T_{min} are the daily maximum and minimum temperatures, respectively, and T_{base} is the base temperature, estimated to be 11.8 °C for S. halepense (Masin *et al.*, 2010).

Depending on the development stage, the main characteristics were counted (height, tillage number, panicles number), above ground part of plants was divided into main and secondary stems, panicles and seeds, and below ground part was washed from soil and divided into rhizomes and roots. The plant material was put in a dryer for 36 h at 105 °C and weighted. Classical growth analysis, calculating mean indexes between consequently samplings, AGR (Absolute Growth Rate) and RGR (Relative Growth Rate), was performed. ANOVA and Fisher LSD analyses for the main characteristics to evaluate biomass allocation and allometric relationships, considering both populations and type of resistance (S, R ACCase and R ALS) as statistical unit, were performed.

Methods to store *S. halepense* rhizomes are not available in the literature. Therefore, in order to develop a preservation method a few tests were performed. Rhizomes for each type of resistance (one R ACCase, one R ALS and one S) were washed, cut and conserved in three ways: (1) fresh and under vacuum, (2) dried and under vacuum, (3) fresh into sand with 5% of constant moisture. All the samples were placed at 4°C. Every month for a total of six samplings and after one year, ten rhizomes for each treatment were transplanted and the percentage of budding was detected.

4.2.1.2. 2010 experiment

All ten populations reported in table 1 were included in the experiment. In spring 2010, seeds were chemically scarified in concentrated sulfuric acid (97%) and placed in 14 cm diameter pots containing silty-loam soil and perlite (15%), without previously germination. The pots were placed in the greenhouse where the temperature range was 14/19 °C and 24/37 °C night/day, respectively. To make sure that all plants included in the growth analysis experiment were resistant, at 2-3 leaf stage, seedlings were sprayed with a selective herbicide (fluazifop for the ACCase inhibitors resistant populations and nicosulfuron for the ALS resistant ones). Susceptible populations were not sprayed. Herbicides were applied as commercial formulations (Table 3), 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). Pots were hence transferred outside and, two week after treatment (WAT), resistant plants at a very similar growth stage were transplanted into 30-litre pots (the same was done for susceptible populations), which were watered daily and fertilized two times with Nitrofosca (15-9-15 N-P-K). Six plants were considered for each population at each of the four samplings (approximately 30 days apart) at temperature sums (°C d) of 454, 957, 1260 and 1395 (temperature sum 0 was set when 50% emergence was recorded). Depending on plants life stage, several characteristics were recorded: height, no. of tillers and panicles per plant, leaf area were measured and dry weight were determined for different plant parts divided in stems, leaves (dried and green), panicles and seeds for the above ground part and in roots and rhizomes for the below ground part. The plant material was put in a dryer for 36 h at 105 °C and weighted. In the last sampling, seeds were separated from rachis to evaluate the effective seed production. Classical growth analysis was performed and all indexes were calculated and valuated through ANOVA and Fisher LSD analysis. Furthermore, ANOVA and Fisher LSD analysis were performed for the main characteristics to evaluate biomass allocation and allometric relationships.

Table 3. Details of herbicide treatments.

Mode of action	Commercial product	Active ingredient (a.i.)	a.i. concentration (g L^{-1})	Field dose (1x) (mL ha^{-1})
ALS inhibitors	Ghibli	Nicosulfuron	40	1500
ACCase inhibitors	Fusilade Max	fluazifop	125	2000

4.2.1.3. 2011 experiment

All populations were included in the experiment. Rhizomes obtained from plants selected for herbicides resistance in 2010 were used. Four plants and two replicate (two buds per plant) were considered. Rhizomes buds were transplanted in 30-litre pots filled with silty-loam soil and perlite (15%) in late spring, watered daily and fertilized two times with Nitrofosca (15-9-15 N-P-K). A unique sampling was performed at the end of vegetative season at temperature sum 1395 °C d. Height was measured, no. of tillers and panicles per plant were counted and dry weight was determined for different plant parts: stems, leaves and panicles for the above ground part, roots and rhizomes for the below ground part. The plant material was put in a dryer for 36 h at 105 °C and weighted. ANOVA and Fisher LSD analysis were performed for the main characteristics to evaluate biomass allocation and allometric relationships and the results were compared with those of the other two experiments.

4.2.2. Molecular analyses

4.2.2.1. ACCase inhibitors resistant populations

Every year, a small young leaf of each plant belonging R ACCase inhibitors populations was sampled for the analysis with a molecular method called CAPS (Cleaved Amplified Polymorphic Sequence) already used in our laboratory for *Lolium* spp. (Kaundun and Windass, 2006; Scarabel *et al.*, 2011), and modified for *S. halepense*, for the detection of the mutation 2041. This method allow to detect the presence or the absence of the mutation 2041 through a fast and simple PCR amplification with specific primers and an endonuclease analysis.

Genomic DNA was extracted using the CTAB method (Aras *et al.*, 2003) and quantify using a spectrophotometry. Specific primers for *S. halepense* ACCase gene were designed into the conserved CT domain sequence:

SORG-For-2027 (5'-CAGCTTGATTCCCATGAGCGATC-3)'
SORG-2-REV (5'-TTCATGCTTTGCTCCCTGGAGT-3')

PCR was carried out using Go Taq® Flexi DNA Polymerase (Promega). In a final volume of 25 µL, the following reagents were added: 5 µL 5x colorless Go Taq® Flexi buffer, 1 µL $MgCl_2$ solution 25 mM, 0.5 µL PCR nucleotide mix 10 mM each, each primer to a final concentration of 0.8 µM, 1 U of Go Taq® DNA polymerase and 50 ng of genomic DNA. Amplification was performed using the following program: DNA denaturation for 2 min at 95 °C, and 35 cycles of 30 s at 95 °C, 30 s at 56 °C, and 60 s at 72 °C. Lastly, samples were subjected to a 5 min extension time at 72 °C. The amplification was checked in 1% agarose gel and amplicon was purified using the NucleoSpin Extract II (Macherey-Nagel). The PCR product (3 µL) was digested with 2 U of the endonuclease EcoRI (Promega) in a total volume of 20 µL, adding the specific buffer H and BSA 10 µg μL^{-1}, for 75 min at 37 °C. The digestion was checked in 2% agarose gel.

4.2.2.2. ALS inhibitors resistant populations

As described in the chapter III for *E. crus-galli*, the ALS gene of *S. halepense* was sequenced, cloned and analyzed in order to determine the presence of mutation(s) endowing herbicide resistance. One or two samples were analyzed for each population (R: 07-13, 09-20, 08-16; S: 08-19). Degenerate primers were used to amplify the central part of the gene and a 3' RACE was carried out to obtain the 3' end of the sequence. The ALS gene sequence was completed at the 5' end using the targeted gene walking approach. The partial segments were overlapped in order to obtain the full length sequence and specific primers were designed to amplify it. The ALS gene so obtained was cloned into TOPO® vector (Invitrogen) and the construct was transformed in *E. coli* bacterial cells. A few clones for each sample were completely sequenced and aligned in order to detect the SNP. Data were analyzed using DNASTAR® software. Lastly, a phylogenetic tree was design using MEGA 5.05 software.

4.3. Results and discussion

4.3.1. Growth analysis

Data were analyzed through ANOVA considering clones (where presented), plants and populations. Both clones and plants did not give significant differences. To evaluate the differences between susceptible and resistant categories, the mean values grouping populations for “type of resistance” (S, R ACCase and R ALS) were considered. Populations belonging to the same “type of resistance” group showed similar results, mostly without significant differences (Panozzo and Sattin, 2010), therefore only data grouping types of resistance are reported.

Analysis of dry weights, including final total weight and weight of above and below ground plant parts, showed a few significant differences between S and R populations during all life cycle. In 2009 (Fig. 1) data showed some differences between the biotypes. Since only one ALS population was included in the experiment, the results for this group are not very robust. R ACCase and S populations showed no statistical differences in the total weight, but they showed complementary weights between above e below ground parts, with a higher biomass allocated in the below ground part for R ACCase populations. In 2010, no significant differences were observed among of types of resistance for all plant parts (Fig. 2). In 2011 (data not shown), results were in keeping with the first year, which may be linked with the starting material used.

Significant differences between R ACCase and S populations were recorded for panicles number (data not shown) and weight during the whole life cycle in 2009 experiment (Fig. 1) and in the unique sampling of 2011 experiment, whereas differences were less clear in 2010 even if a higher production of seeds in the S populations is observable in the last sampling, in particular respect to R ACCase populations (Fig. 2) (Panozzo and Sattin, 2011). Panicles weight was measured for the sake of simplicity but, in the last sampling of the 2010 experiment, seeds were separated from rachis. The results showed that the ratio was fairly constant (Fig. 3) and the two variables were highly correlated (Fig. 4). Therefore, it was plausible to use the panicles weight to evaluate the plant reproductive effort.

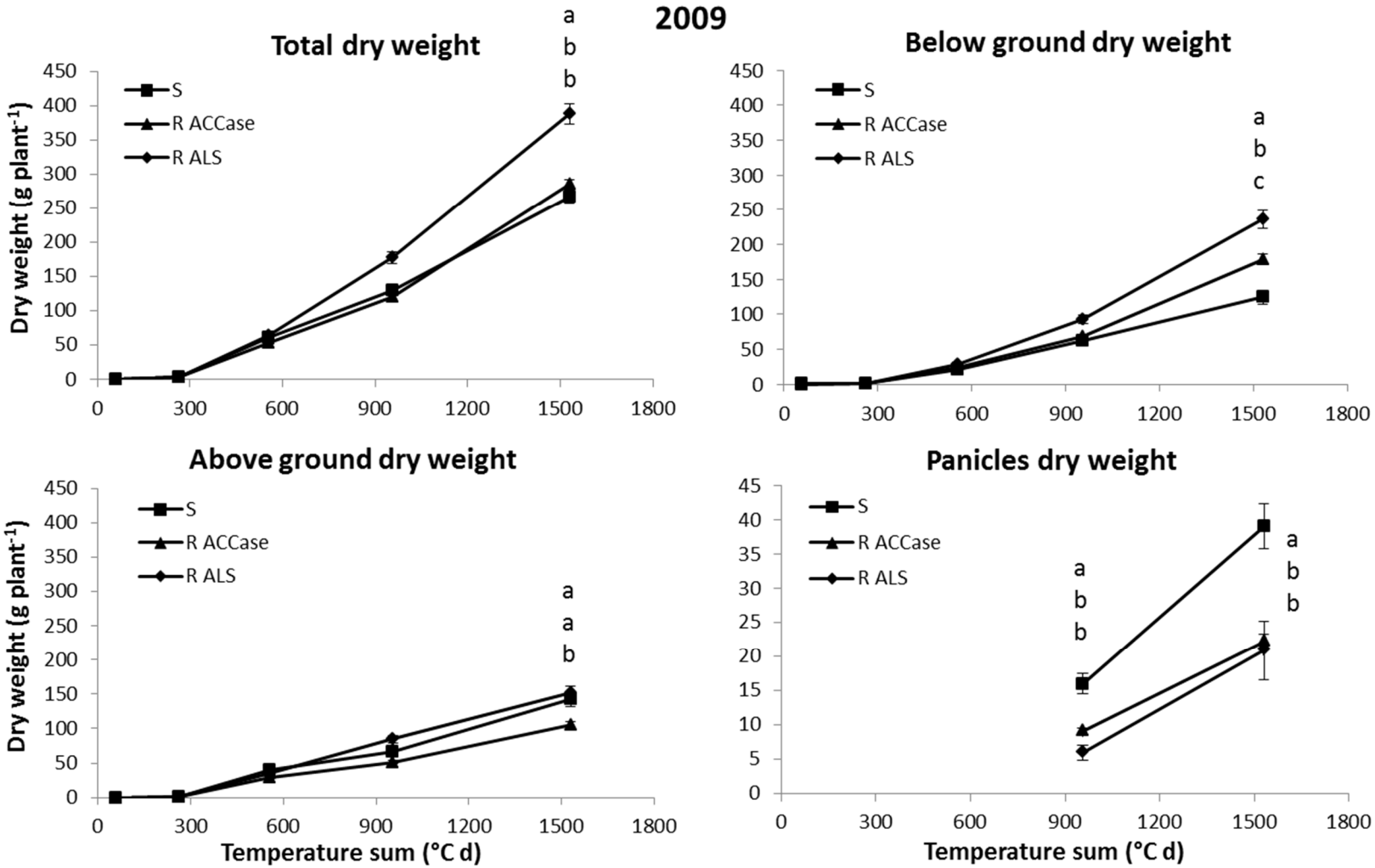

Fig. 1. Total dry weigh and dry weight of the different plant parts divided into above e below ground, and panicles dry weight of 2009 experiment data divided for type of resistance: susceptible (■), resistant to ACCase inhibitors (▲), and resistant to ALS inhibitors (♦). Vertical bars represent standard errors. Different letters indicant significant differences (P≤ 0.05) in dry weight mean values, separated by Fisher's LSD test.

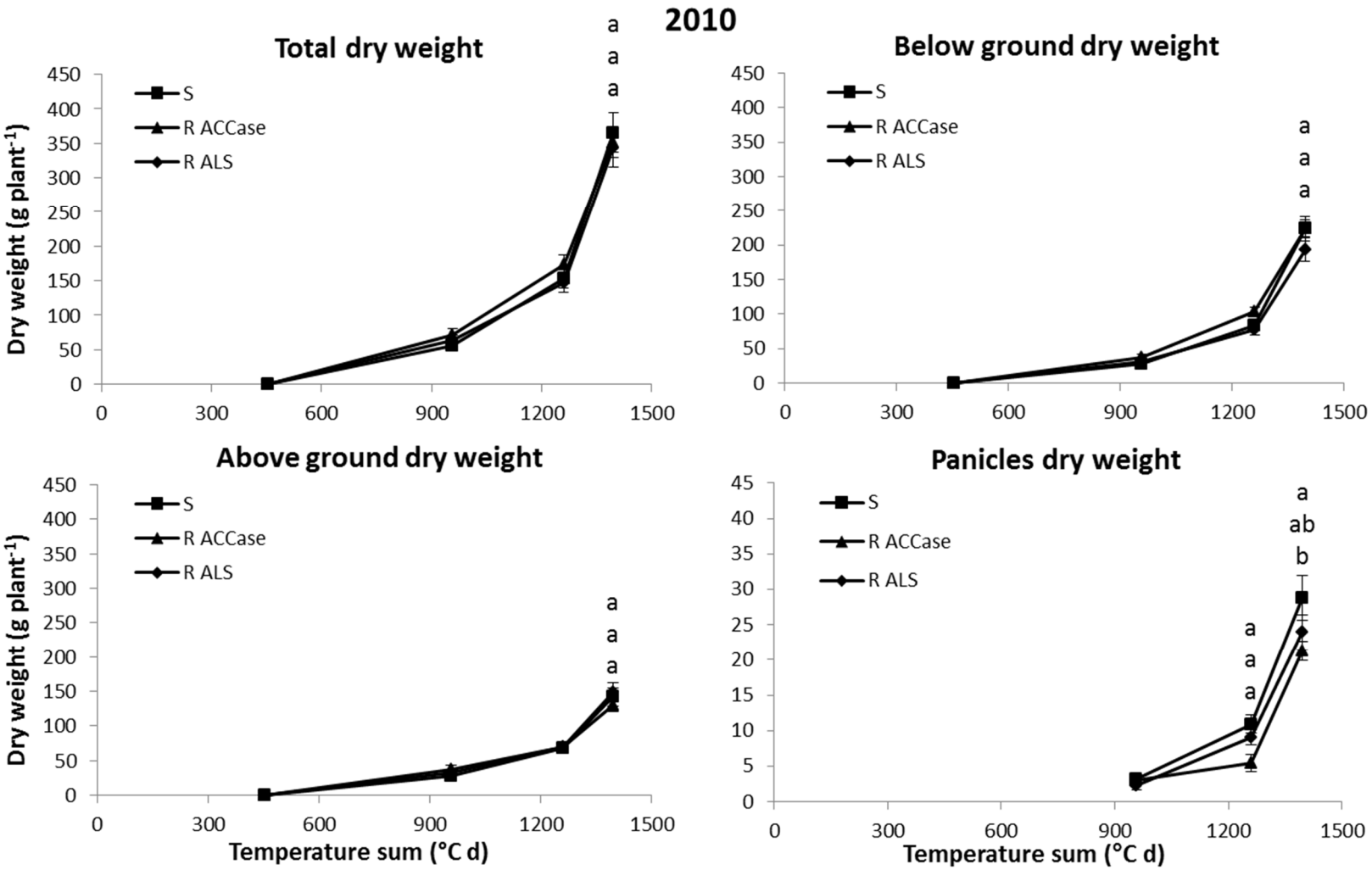

Fig. 2. Total dry weigh and dry weight of the different plant parts divided into above e below ground, and panicles dry weight of 2010 experiment data divided for type of resistance: susceptible (■), resistant to ACCase inhibitors (▲), and resistant to ALS inhibitors (♦). Vertical bars represent standard errors. Different letters indicant significant differences (P≤ 0.05) in dry weight mean values, separated by Fisher's LSD test.

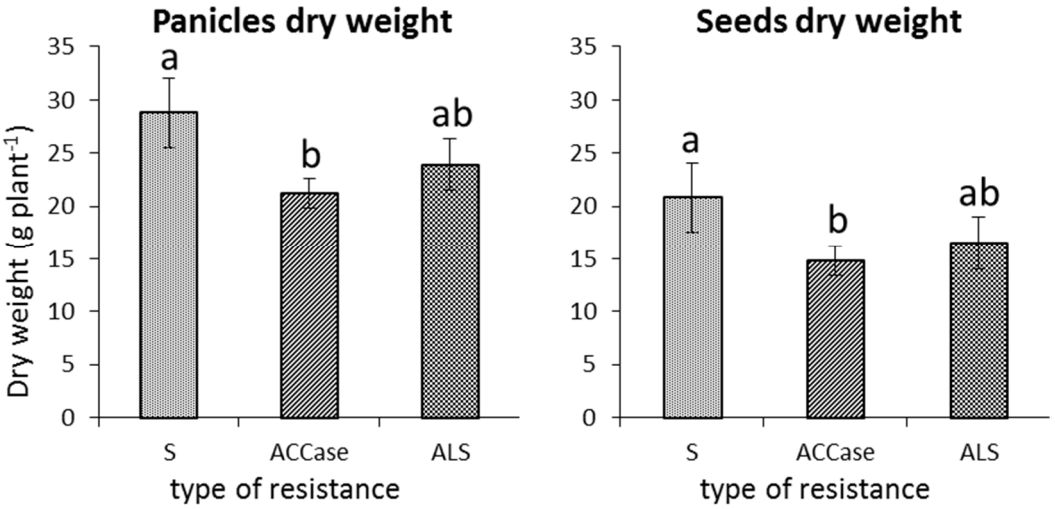

Fig. 3. Panicles and seeds dry weight of the last sampling of 2010 experiment data divided for type of resistance. Vertical bars represent standard errors. Different letters indicant significant differences (P≤ 0.05) in dry weight mean values, separated by Fisher's LSD test.

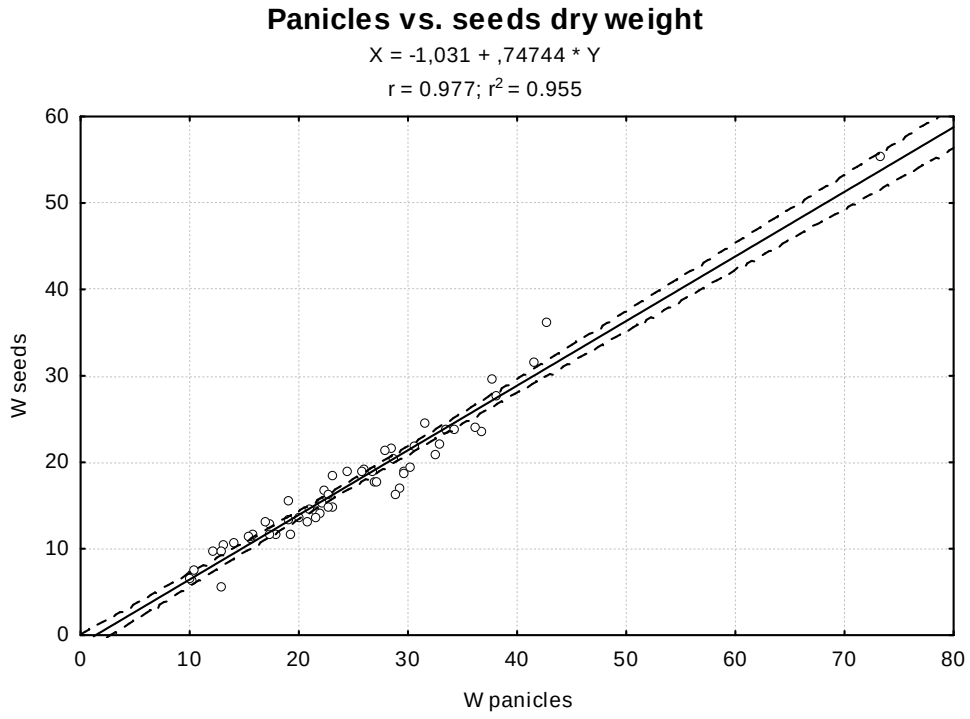

Fig. 4. Correlation between panicles and seeds dry weight evaluated with data harvested in the last sampling of the 2010 experiment.

The test on how to efficiently store *S. halepense* rhizomes showed that after six months only the rhizomes conserved in wet sand were viable. It was estimated that, in normal conditions, 68% and 78% of the rhizome nodes budded for susceptible (06-10) and resistant populations (05-6 and 07-13), respectively. After the first month, buds conserved both fresh and under vacuum, and fresh into wet sand showed a good percentage of germination. From the second to the sixth sampling, only the buds kept in wet sand exhibit a medium-high percentage of germination (from 50% to 70% for resistant biotypes and from 30% to 50% for the susceptible one). After one year, in the last sampling, no rhizome budded, indicating that the rhizomes conserve their viability for a limited time.

Sand maintained at a relatively constant moisture (i.e. above 3%) proved to be the best method to preserve the viability of *S. halepense* rhizomes.

4.3.1.1. Biomass allocation and allometric ratios

Ratio between dry weight of different plant parts and their total weight confirmed that ACCase resistant populations showed a significant different pattern of biomass allocation between above and below ground plant parts, with more biomass allocated below ground for R ACCase populations (Fig. 5). This is due to a significantly higher biomass allocated to the rhizomes, i.e. the ratio between rhizome and total dry weight was higher for both the resistant biotypes, whereas the ratio between root and total dry weight was very similar for all biotypes (Fig. 7). Results were consistent in 2009 and 2011 when rhizomes were used as starting material, whereas no significant differences were found in below ground biomass allocation in 2010 (when the starting material was seed), where biomass allocated to roots for R ACCase populations was higher than for S populations, but that allocated to rhizomes was similar.

Significant differences between R ACCase and S populations were recorded for panicles number (data not shown). At the same time, data of biomass allocation in panicles clearly showed that S populations proportionally allocated more biomass in panicles than R ACCase populations, independently from the starting material (Fig. 6). R ALS populations seem to have intermediate values, no

significant different from neither S nor R ACCase populations, both in 2010 and in 2011.

Root shoot ratio was calculated considering the complex of roots and rhizomes, so it will be called Root System Shoot Ratio (RSSR). It showed significant differences between S and ACCase resistant biotypes in 2009 and 2011 with higher values for R ACCase populations, whereas in 2010 R ACCase showed higher values as well, but not statistical different from S populations in the last sampling (Fig. 8).

As mentioned above, the results of 2009 and 2011 growth analyses, when plants grew from rhizomes, were consistent, showing a different and complementary development of rhizomes and roots in R ACCase and S populations, especially when the ratio between dry weight of rhizome and root was considered (Fig. 9b). Intermediate and variable values were recorded for R ALS populations, no significant different from the other two biotypes. The root/panicle weight ratio showed very variable values through the different experiments (Fig. 9a). Instead, the rhizome/panicle weight ratio showed a significant difference between S and R ACCase biotypes in all experiments (Fig. 9b). This is mainly due to the significant lower panicle production of the R ACCase populations consistent in all the experiments: the S populations showed values of about 45%, 25% and 20% higher in 2009, 2010 and 2011, respectively (Fig. 6).

The results of 2010 growth analysis, when plants grew from seeds, partly confirmed the results of the other experiments, although the differences were less evident. However, the rhizome/root weight ratio showed a different behavior (Fig. 9b), with the ratio significantly higher for S populations, due to the higher root production of the R ACCase populations observed only in the 2010 experiment (Fig. 7).

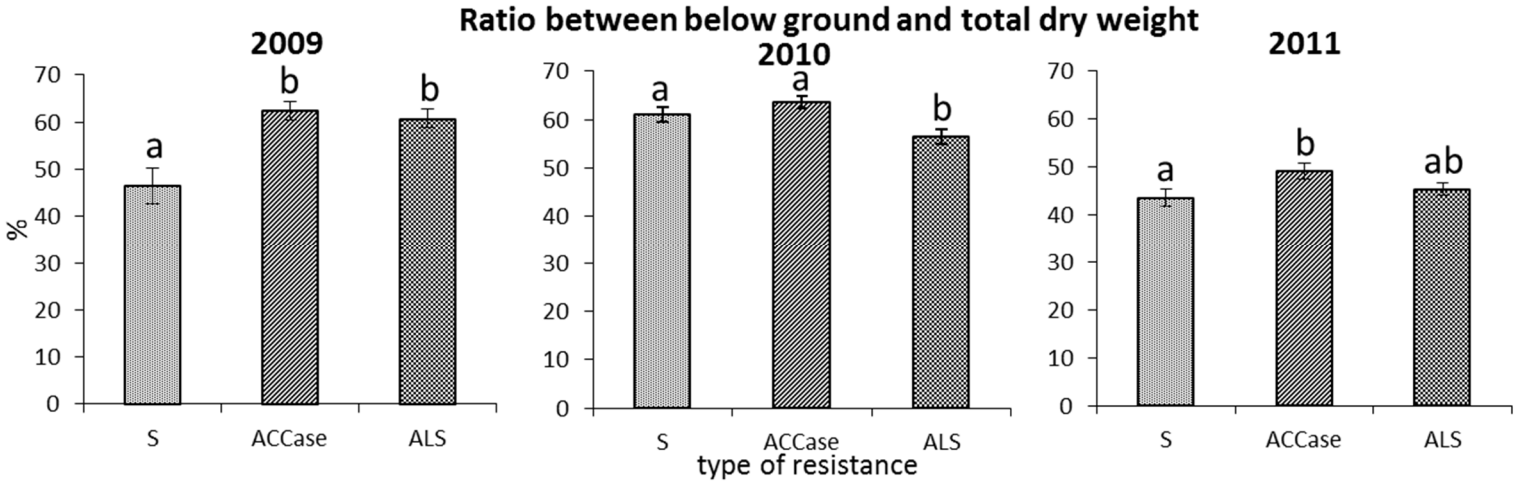

Fig. 5. Ratio between below ground and total dry weight, expressed in percentage, of the three-year experiments divided for type of resistance. Vertical bars represent standard errors. Different letters indicant significant differences (P≤ 0.05) separated by Fisher's LSD test.

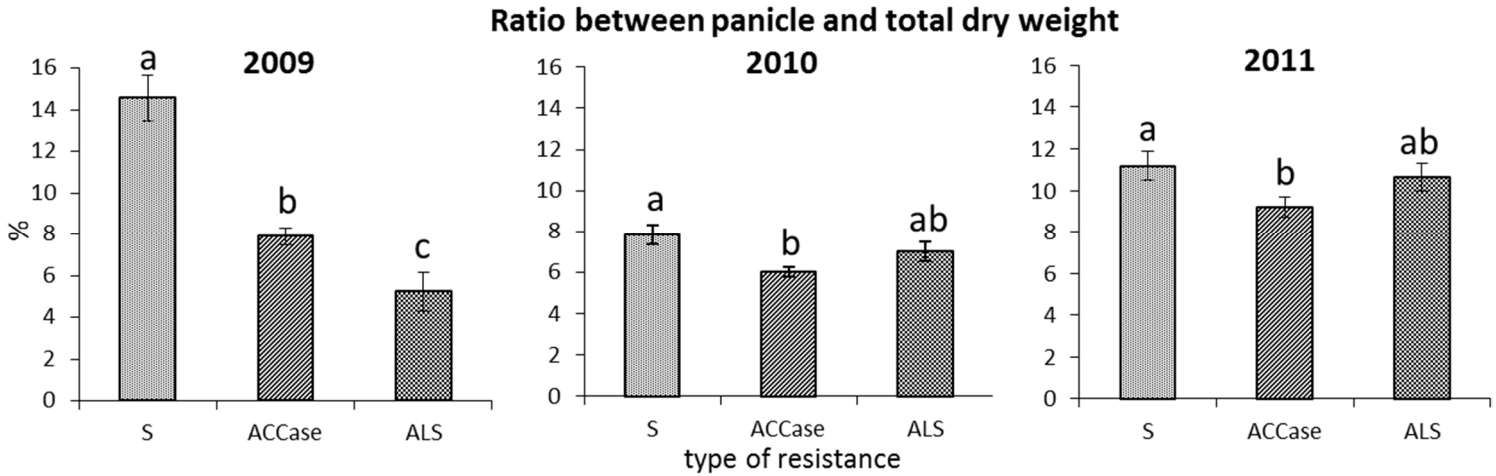

Fig. 6. Ratio between panicle and total dry weight, expressed in percentage, of the three-year experiments divided for type of resistance. Vertical bars represent standard errors. Different letters indicant significant differences (P≤ 0.05) separated by Fisher's LSD test.

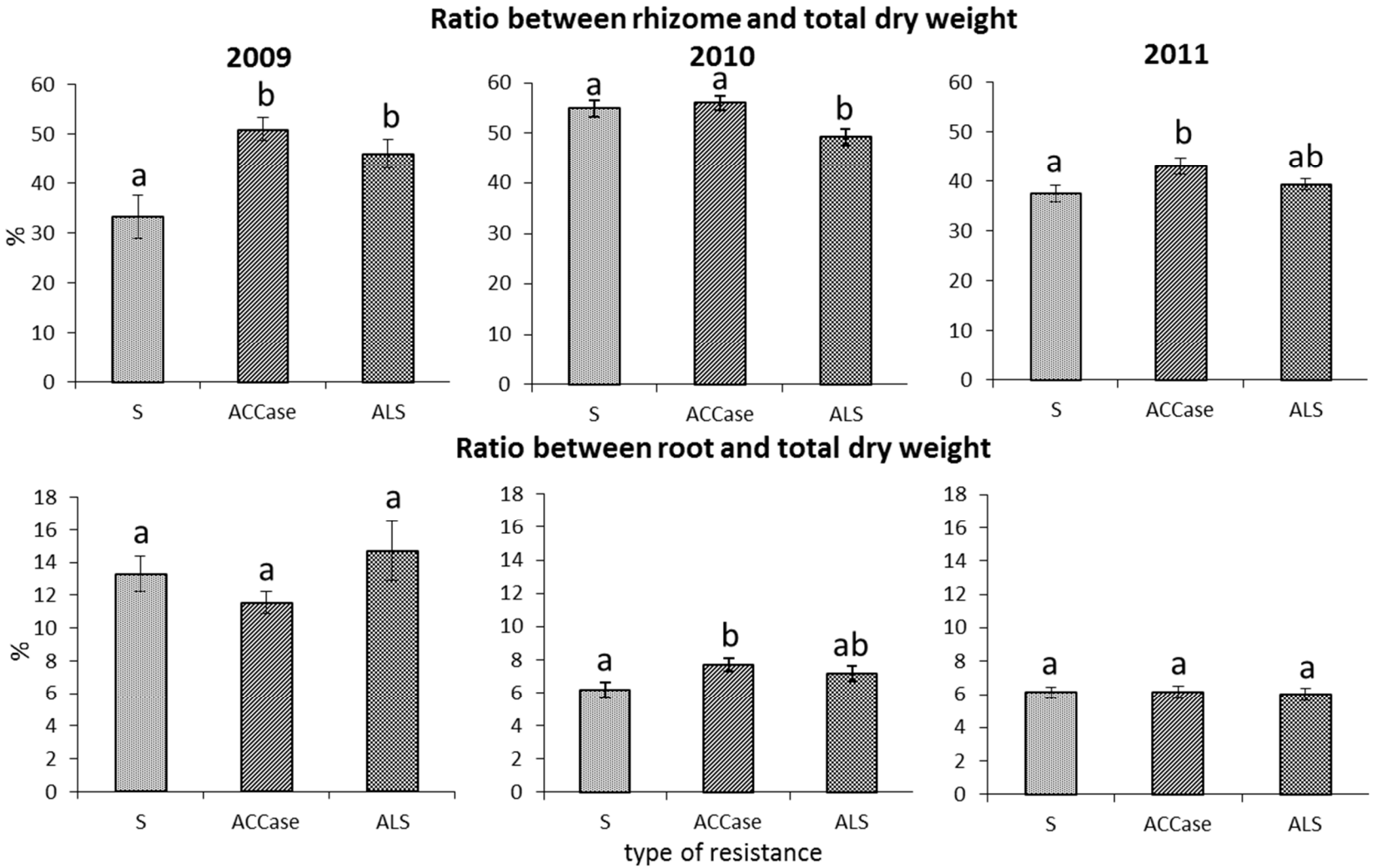

Fig. 7. Ratio between rhizome (top), and root (bottom), and total dry weight, expressed in percentage, of the three-year experiments divided for type of resistance. Vertical bars represent standard errors. Different letters indicant significant differences (P≤ 0.05) separated by Fisher's LSD test.

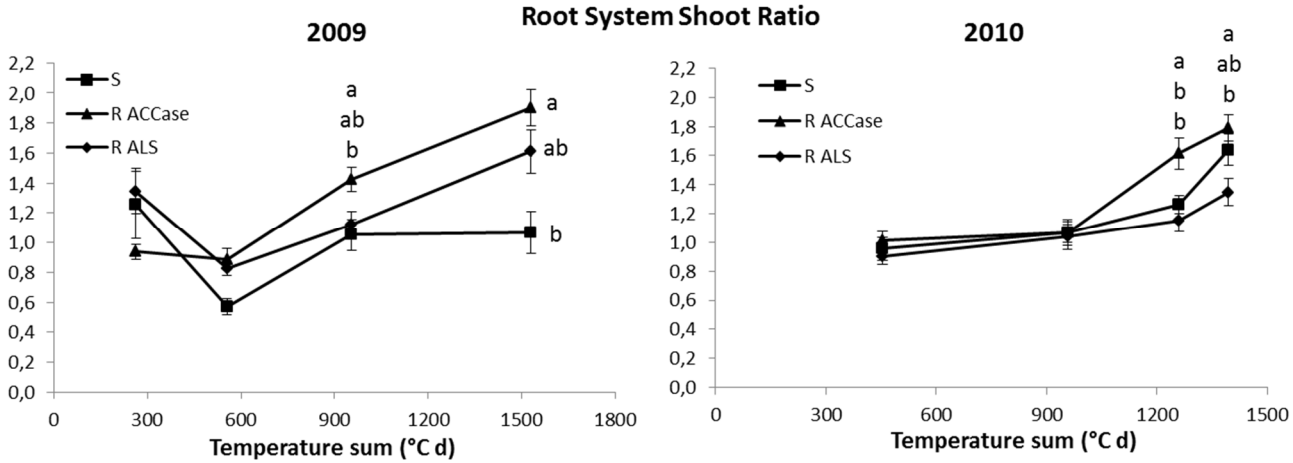

Fig. 8. Root System Shoot Ratio of 2009 and 2010 experiments data divided for type of resistance: susceptible (■), resistant to ACCase inhibitors (▲), and resistant to ALS inhibitors (♦). Vertical bars represent standard errors. Different letters indicant significant differences (P≤ 0.05) separated by Fisher's LSD test.

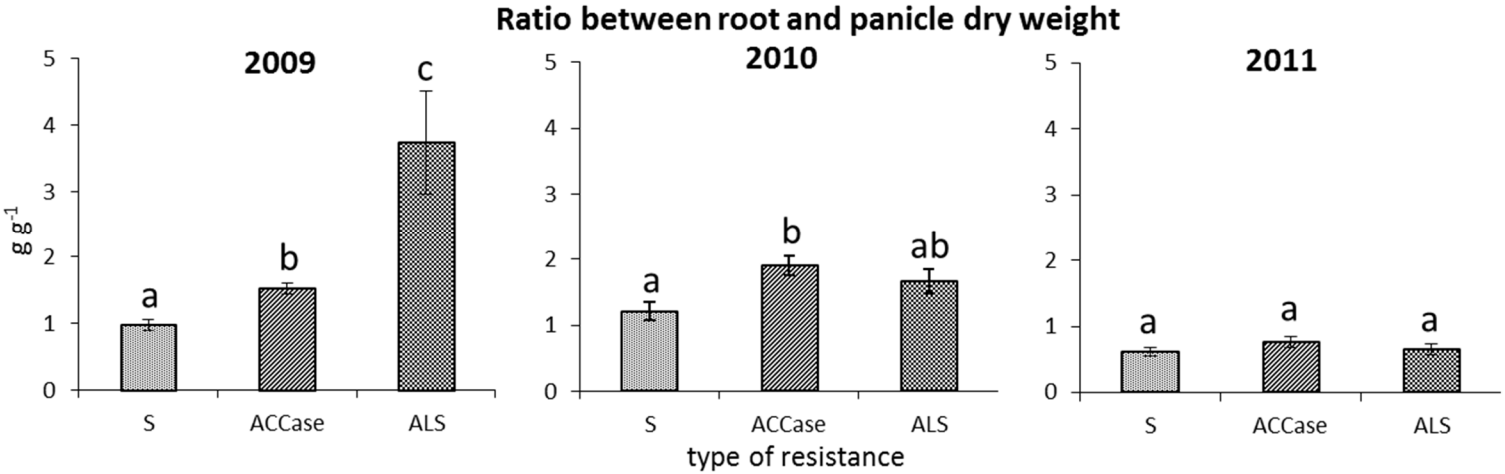

Fig. 9a. Ratio between root and panicle dry weight of the three-year experiments divided for type of resistance. Vertical bars represent standard errors. Different letters indicant significant differences (P≤ 0.05) separated by Fisher's LSD test.

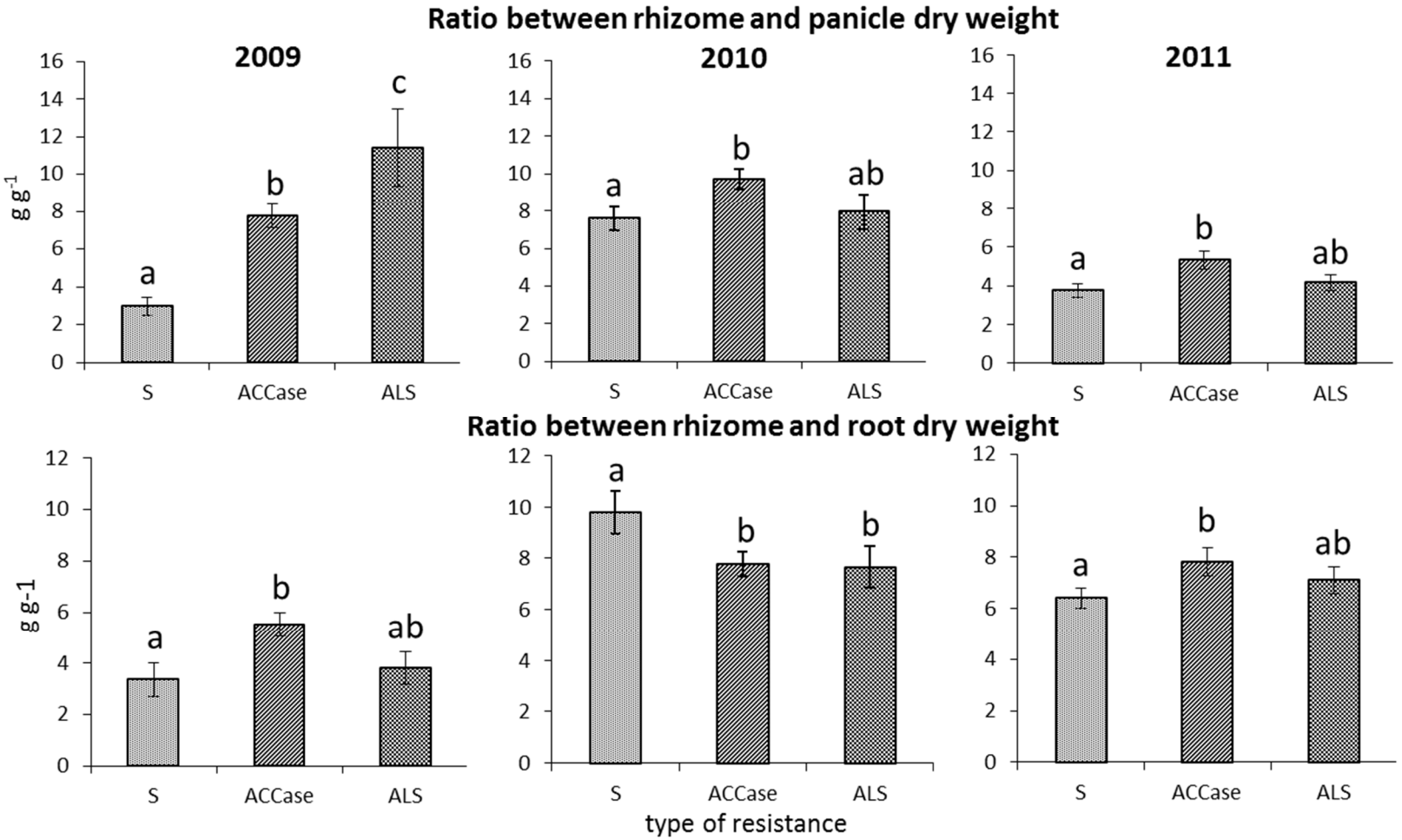

Fig. 9b. Ratio between rhizome and panicle dry weight (top) and between rhizome and root dry weight (bottom) of the three-year experiments divided for type of resistance. Vertical bars represent standard errors. Different letters indicant significant differences (P≤ 0.05) separated by Fisher's LSD test.

4.3.1.2. Classical growth analysis, 2009 and 2010 experiments

Data recorded in 2009 led to calculate absolute and relative growth rate using classical growth analysis (Table 4 and 5). The R ACCase populations grew more slowly than the other biotypes in the second period, where plants started to produce rhizomes, suggesting that plants probably invested more resources to produce rhizomes (see Fig. 7). R ALS populations show the highest values in the third and fourth periods, during phase of flowering, i.e. plants invested more resources in producing biomass, according to the low seeds production (Fig. 6). The growth efficiency is significantly higher for R ACCase populations in all periods, a part in the third one, whereas it is very similar for S and R ALS populations.

Table 4. Absolute Growth Rate (AGR) (mg (°C d)$^{-1}$) of 2009 experiment data divided for type of resistance: susceptible (S), resistant to ACCase inhibitors (R ACCase), and resistant to ALS inhibitors (R ALS). Different letters indicant significant differences (P≤ 0.05) separated by Fisher's LSD test.

	Period			
Type of resistance	**1°**	**2°**	**3°**	**4°**
S	9,99 (1.404) a	205,16 (7.364) a	169,45 (13.208) a	240,78 (17.096) a
R ACCase	11,62 (0.665) a	170,39 (6.618) b	166,15 (6.356) a	289,35 (10.803) a
R ALS	11,04 (1.230) a	208,47 (15.034) a	283,76 (21.316) b	353,34 (36.234) b

Table 5. Relative Growth Rate (RGR) (mg g^{-1} (°C d)$^{-1}$) of 2009 experiment data divided for type of resistance: susceptible (S), resistant to ACCase inhibitors (R ACCase), and resistant to ALS inhibitors (R ALS). Different letters indicant significant differences (P≤ 0.05) separated by Fisher's LSD test.

	Period			
Type of resistance	**1°**	**2°**	**3°**	**4°**
S	7.50 (0.542) a	11.32 (0.413) a	1.83 (0.126) a	1.25 (0.070) a
R ACCase	10.07 (0.374) b	10.23 (0.170) b	2.08 (0.077) a	1.52 (0.047) b
R ALS	6.90 (0.604) a	10.52 (0.491) ab	2.60 (0.214) b	1.34 (0.139) ab

Data harvested in 2010 included leaf area (Fig. 10), therefore all the classical growth analysis indexes were calculated. Only data calculated for the mean values for resistance type are reported, because populations behaved similarly.

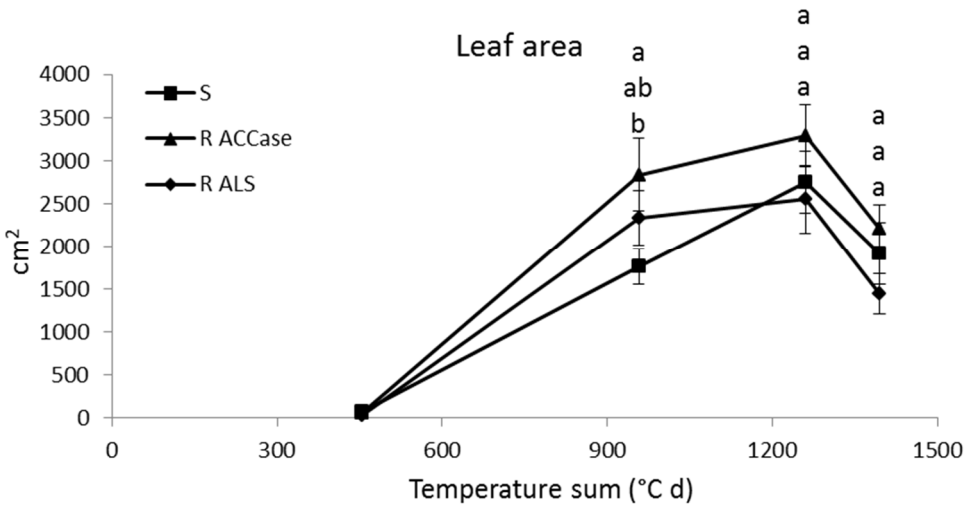

Fig. 10. Leaf area divided for type of resistance: susceptible (■), resistant to ACCase inhibitors (▲), and resistant to ALS inhibitors (♦). Vertical bars represent standard errors. Different letters indicant significant differences (P≤ 0.05) separated by Fisher's LSD test.

AGR and RGR did not show significant differences among biotypes during all life cycle (Table 6 and 7), as well as leaf area, a part at temperature sum 955 where R ACCase populations showed values significantly higher than S populations.

Table 6. Absolute Growth Rate (AGR) (mg (°C d)$^{-1}$) of 2010 experiment data divided for type of resistance: susceptible (S), resistant to ACCase inhibitors (R ACCase), and resistant to ALS inhibitors (R ALS). Different letters indicant significant differences (P≤ 0.05) separated by Fisher's LSD test.

	Period		
Type of resistance	**1°**	**2°**	**3°**
S	1338 (167.0) a	3352 (493.1) a	9670 (1417.0) a
ACCasi	1794 (237.7) a	3254 (571.5) a	8142 (4819.8) a
ALS	1555 (191.3) a	2869 (552.1) a	8882 (1159.2) a

Table 7. Relative Growth Rate (RGR) (mg g^{-1} (°C d)$^{-1}$) of 2010 experiment data divided for type of resistance: susceptible (S), resistant to ACCase inhibitors (R ACCase), and resistant to ALS inhibitors (R ALS). Different letters indicant significant differences (P≤ 0.05) separated by Fisher's LSD test.

	Period		
Type of resistance	**1°**	**2°**	**3°**
S	97.1 (3.61) a	36.3 (4.08) a	40.57 (5.37) a
R ACCase	104.8 (3.03) a	32.4 (5.15) a	33.1 (18.86) a
R ALS	125.7 (6.72) b	31.1 (5.98) a	39.4 (5.05) a

The Net Assimilation Rate (NAR) (Table 8) showed similar values in the first and second period with a sensitive improve in the third period. This is not physiologically plausible, but may be explained assuming an under-estimate of the leaf area due to experimental errors: erroneous selection of dry and fresh leaves, due to the presence of fungi (rust) and the fact that the shoot area was not considered. The large part of the photosynthetic apparatus is located in fresh tillers, constituted mainly by shoots, therefore they play an important role and may contribute to the under-estimate of the green apparatus in the last sampling, led to a substantial improvement of the production efficiency in the last period. Another factor may be the continuous tiller production of *S. halepense*. In this way plants always have fresh leaves, which are much more efficient than the older ones.

Table 8. Net Assimilation Rate (NAR) (mg cm^{-2} (°C d)$^{-1}$) of 2010 experiment data divided for type of resistance: susceptible (S), resistant to ACCase inhibitors (R ACCase), and resistant to ALS inhibitors (R ALS). Different letters indicant significant differences (P≤ 0.05) separated by Fisher's LSD test.

	Period		
Type of resistance	**1°**	**2°**	**3°**
S	0.20 (0.011) a	0.16 (0.022) a	0.90 (0.155) ab
R ACCase	0.20 (0.007) a	0.13 (0.224) a	0.58 (0.426) a
ALS	0.26 (0.015) b	0.26 (0.127) a	0.94 (0.126) b

As Specific Leaf Area (SLA) (Table 9) and Leaf Area Ratio (LAR) (Table 10) are based on leaf area, as well as NAR, they had the same problems of the latter and the significant differences observed in the third period may be virtual.

Regarding the first two periods, it seems that S populations had leaves significantly thinner than R populations, which transmitted more light, indicating a higher ability to accumulate resources useful for plants development, whereas no substantial differences were observed in the index that describes the leafiness of the plants.

Table 9. Specific Leaf Area (SLA) (cm^2 g^{-1}) of 2010 experiment data divided for type of resistance: susceptible (S), resistant to ACCase inhibitors (R ACCase), and resistant to ALS inhibitors (R ALS). Different letters indicant significant differences (P≤ 0.05) separated by Fisher's LSD test.

	Period		
Type of resistance	**1°**	**2°**	**3°**
S	217.2 (4,77) a	188.1 (3.98) a	180.2 (6.17) a
R ACCase	205.1 (4.61) a	180.0 (3.49) ab	168.8 (24.4) ab
ALS	190.1 (5.86) b	175.5 (4.26) b	163.6 (6.00) b

Table 10. Leaf Area Ratio (LAR) (cm^2 g^{-1}) of 2010 experiment data divided for type of resistance: susceptible (S), resistant to ACCase inhibitors (R ACCase), and resistant to ALS inhibitors (R ALS). Different letters indicant significant differences (P≤ 0.05) separated by Fisher's LSD test.

	Period		
Type of resistance	**1°**	**2°**	**3°**
S	56.4 (2.19) a	24.5 (0.86) a	11.1 (0.95) a
R ACCase	55.9 (1.76) a	28.2 (0.97) b	12.9 (3.81) a
ALS	53.0 (2.15) a	25.7 (1.48) a	10.6 (0.93) a

Leaf Weight Ratio (LWR) (Table 11) is significantly higher for R biotypes in the first and second periods, indicating a higher investment of these plants to the leaf apparatus, fundamental for photosynthesis. This confirms the hypothesis of the under-estimate of the leaf area, in particular in the period when a higher quantity of dry leaves were presented.

Table 11. LWR (mg g^{-1}) for type of resistance. S = susceptible populations; ACCase = populations resistant to ACCase inhibitors; ALS = populations resistant to ALS inhibitors. Letters indicate significant differences (P ≤ 0,05) followed LSD Fisher's test. Time indicates period between samplings.

	Period		
Type of resistance	**1°**	**2°**	**3°**
S	248.2 (8.78) a	132.1 (4.08) a	59.7 (4.44) a
R ACCase	267.7 (7.42) ab	160.7 (5.76) b	69.8 (16.28) a
ALS	275.2 (7.72) b	153.0 (4.92) b	60.9 (4.95) a

Classical growth analysis did not highlight significant differences among the types of resistance. Perhaps, a functional analyses approach (Hunt, 1981), starting from a different experimental design with samplings closer in time, may be more adequate to highlight the differences among the biotypes.

4.3.2. Molecular analyses

4.3.2.1. CAPS 2041 on ACCase gene

Two different nucleotide substitutions at position 2041 of the ACCase gene endowing herbicide resistance has been detected in several species. The A to G nucleotide transition at the first position of codon 2041 of the plastidic ACCase gene determines an Ile to Val change, while a T to A transversion at the second position of the codon determines an Ile to Asn change. Both these mutations determine the loss of an EcoRI restriction site in the gene sequence (Zhang and Powles, 2006). The CAPS assay did not discriminate 2041-Val from 2041-Asn ACCase allele, but in *S. halepense* the allele 2041-Val has never detected (Pignata *et al.*, 2008).

The combination of primers SORG-For-2027 and SORG-2-REV was used to amplify a 360 bp fragment, followed by EcoRI digestion. The homozygous wild type plants with 2041-Ile allele had two bands of 190 and 170 bp (not visible on gel), whereas mutant plants, with at least one 2041 mutated allele, displayed one undigested band (Fig. 11). Plants with both alleles should show all three fragments, case never verified in our samples. This leaves different interpretation: (1) primers are very selective for the mutated allele in the resistant samples, (2) only the

mutated allele is expressed in the resistant samples or it is expressed much more than the not mutated allele, (3) the mutation 2041 is present in homozygous status in the resistant plants. These hypothesis will need more investigations to be confirmed, first the detection of the number of ACCase gene present in the genome of *S. halepense* and how many of them are expressed in the cells.

For the purpose of this research, it was enough to detect the presence of the mutation 2041 and CAPS analysis confirmed its presence in all plants of the R populations, whereas it has never been detected in the ACCase gene of susceptible populations (Table 12).

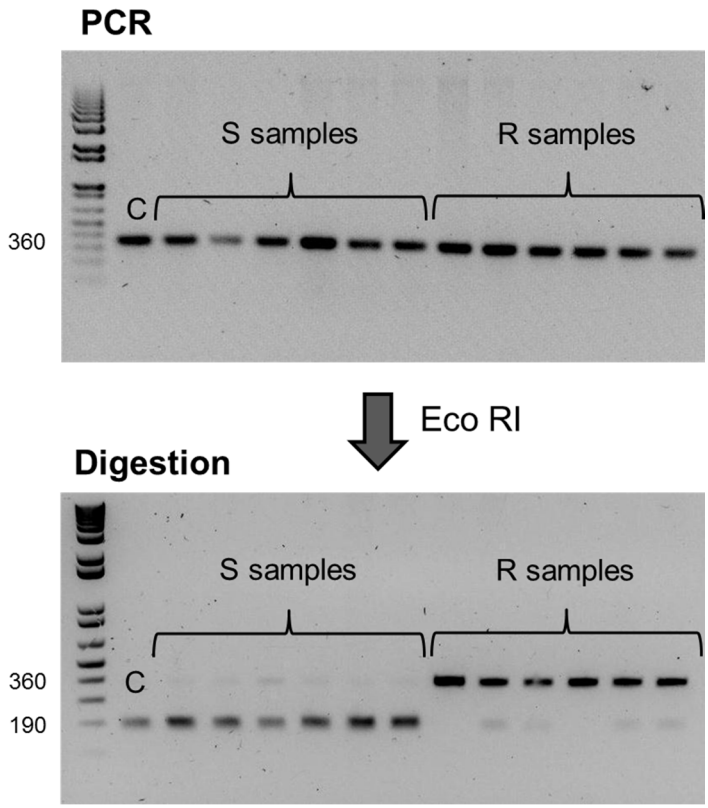

Fig. 11. CAPS 2041. PCR produces an amplicon of 360 bp which, through an EcoRI digestion, is cut if a 2041-Ile allele is presented. All the S samples were cut, where all the R samples were not cut. C represents a susceptible control.

Table 12. Results of CAPS on samples analyzed in the three years. The total no. of samples analyzed and the no. of samples with or without mutation 2041 found in pops S and R, respectively, are reported.

Year	Samples analyzed	Mut S	Not mut S	Mut R	Non mut R
2009	24	0	8	16	0
2010	41	0	17	24	0
2011	26	0	11	15	0

4.3.2.2. ALS genotyping

In the population 07-13 the mutation Trp-574-Leu was detected, whereas in the population 09-20 was detected the mutation Asp-396-Glu. Only one sample of the population 08-16 was analyzed, and it did not present known mutations (other analyses will be carried out). This could explain the different and variable behavior of the populations belonging this type of resistance, as well as the different pattern of resistance detected in the preliminary whole plant pot experiments (Chapter II, table 13).

Table 13. Details of populations resistant to ALS inhibitors analyzed: origin, resistance pattern, no. of plants analyzed through molecular tools, no. of clones sequenced and mutation detected.

Population	Origin	Resistance pattern	No. of samples analyzed	No. of clones analyzed	Mutation detected
08-19 (S)	Hungary	-	2	11	-
09-20	Hungary	SU, no IMI	1	6	Asp-376-Glu
08-16	Hungary	SU, no IMI	1	1	-
07-13	Italy	Cross-R	3	13	Trp-574-Leu

Phylogenetic analysis led to identify three clusters, called ALS1, ALS2 and ALS3 (Fig. 12). All clusters included sequences from both susceptible and resistant populations. All three sequences of population 07-13 having the 574 mutation clustered in ALS1, whereas all five sequences of population 09-20 having the 376 mutation clustered in ALS2. Whereas not-mutated sequences are spread in the

other clusters without a specific order. Other investigations are necessary to clarify how many ALS alleles are present and/or expressed in *S. halepense* genome and if distribution of mutations in different clusters is associated with different copies of the gene.

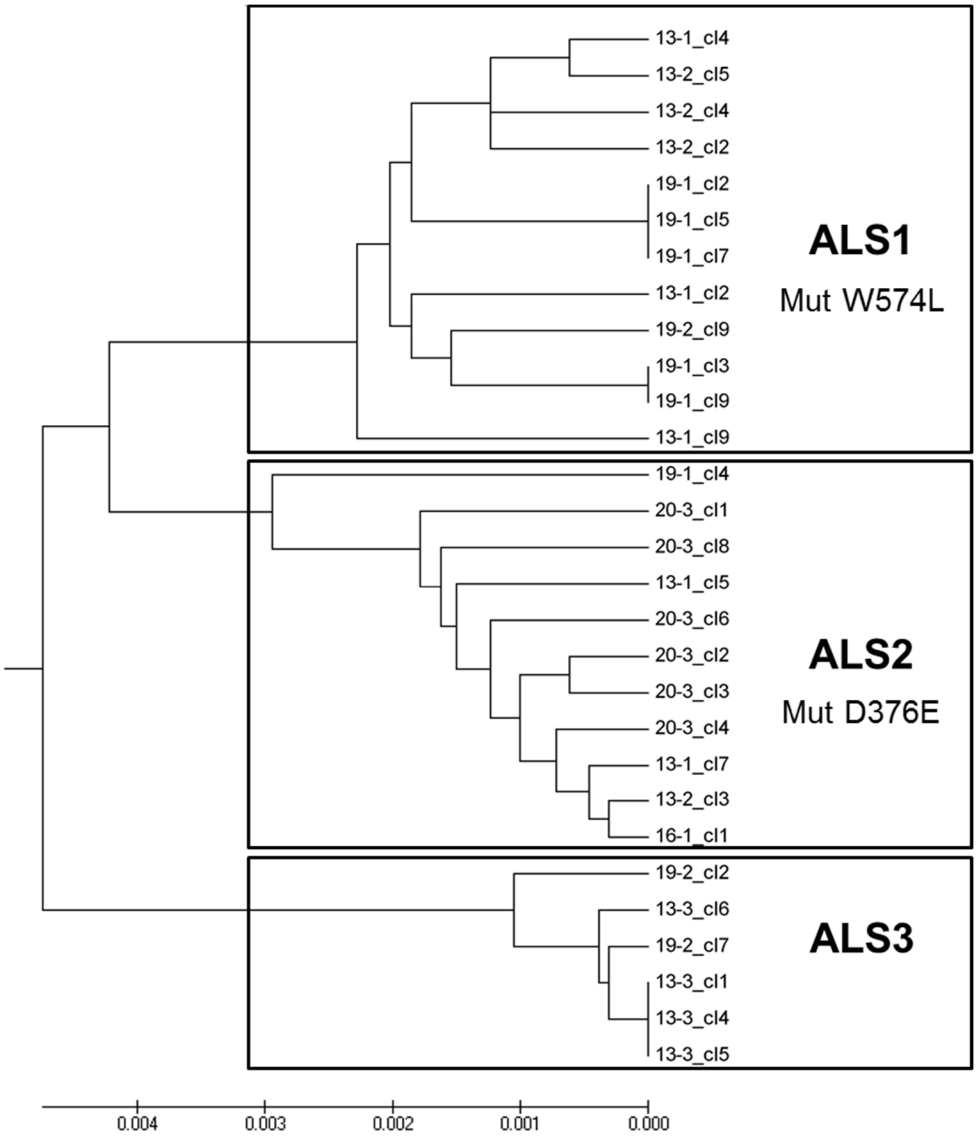

Fig. 12. Cladogram obtained from UPMGA estimation of ALS sequences using MEGA 5.05. Twenty nine full length clone sequences were included in the analysis. Three clusters (called ALS1, ALS2 and ALS3) are highlighted. In some sequences of R samples belong ALS1 the mutation W574L was detected, whereas in some sequences of R samples belong ALS2 the mutation D376E was detected.

Actually, these results let to hypothesize an association between the lack of fitness costs observed in R ALS group and the lack of a similar herbicide resistance molecular basis, one of the fundamental requisite requests for a robust fitness costs analysis (Vial-Aiub, 2009).

4.4. Conclusions

The three experiments indicated that the resistance status of *S. halepense* seems to be associated with a different pattern of dry matter allocation, at least for the ACCase inhibitors resistance, predominantly due to a lower panicles (and therefore seeds) production. A different biomass allocation was detected between above e below ground plants part, with a higher proportion of biomass allocated below ground for R ACCase populations, due to their higher production of rhizomes. Results were more consistent when the same starting material was used, i.e. 2009 and 2011 experiments, where plants grew from rhizome buds. Differences were, instead, detected in the 2010 experiment, where plants grew from seeds. The different pattern of biomass allocation between R ACCase and S biotypes can have a significant impact on plant reproduction and therefore on resistance evolution under ACCase inhibitors selection pressure. Reproduction through rhizomes seems to be favored in R ACCase plants. It is worth mentioning that most herbicides do not effectively control *S. halepense* originated from rhizomes.

The CAPS results lead to associate the presence of the mutation 2041 with the results obtained in the growth experiments. This is the first report of a possible fitness variation associated with the mutation 2041, in fact previously fitness studies in several *A. myosuroides* populations with Ile-2041-Asn did not show any adverse pleiotropic effects on vegetative and reproductive plant traits (Menchari *et al.* 2008).

The analysis for R ALS populations have not shown any significant differences from S populations, likely because of the data variability and limited number of populations considered in the first year. Molecular analyses and preliminary whole plant pot experiments highlighted that the populations used behave differently. In fact, two different ALS mutations endowing resistance were

detected: the Ile-574-Leu in the population 07-13 harvested in Italy (cross-resistant to all ALS inhibitors) and the Asp-376-Glu in the population 09-20 harvested in Hungary (resistant to SU but not to IMI herbicides). The dissimilar target-site resistance mechanism, i.e. with different mutations involved, and the different behavior against ALS inhibitors chemical families, suggest that the choice of populations could not be proper in order to evaluate possible fitness cost at type of resistance level.

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Chapter V

Overall conclusions

5.1. *S. halepense* characterization

In Italy ALS inhibitors resistance in *S. halepense* is quite stable, with only one case found four years ago, whereas in Hungary resistance to sulfonylureas is evolving fast, especially in Tolna county. In this area continuous maize cropping system is frequent and fields are treated only with post-emergence herbicides (Torma *et al.*, 2007). Pre-emergence herbicides have some drawbacks: they have a poor effect against *S. halepense* grown from rhizome and their performance is affected by many factors, not least a minimum of precipitation within two weeks after application. In Hungary, the weather conditions are generally dry in spring so pre-emergence herbicide applications do not provide acceptable weed control.

Dose-response experiment on *S. halepense* confirmed that resistance is confined to only one ALS inhibitors chemical family (SU) with high resistance levels. Therefore alternatives are still available, especially if crop rotation is implemented: use of pre-emergence herbicides (where possible) to control at least the infestation of *S. halepense* from seed, ALS inhibitors different from SU (i.e. IMI in IMI tolerant maize crops), post-emergence herbicides with different MoA (i.e. ACCase inhibitors, DIM seem to be still effective in *S. halepense* control*)*.

S. halepense selected in dicot crops showed high levels of resistance to ACCase inhibitors, suggesting a target-site mediated mechanism, confirmed from the detection of the allelic variant Ile-2041-Asn by Pignata *et al.* (2008). Molecular analysis, performed through the CAPS-2041 method, showed that in all ACCase inhibitors resistant plants used in *S. halepense* growth analysis experiments a single amino acid substitution in position 2041 was presented in the ACCase gene. The three experiments indicated that the ACCase inhibitors resistance status is associated with a different pattern of dry matter allocation between above and below ground plants part, with a proportionally higher percentage of biomass allocated to rhizomes for R ACCase populations, whereas S populations showed a higher seeds production. Results were similar when the starting material was rhizome buds, whereas differences were detected when plants grew from seeds. This is the first report of a possible fitness cost associated with the mutation 2041,

in fact previously fitness studies in several *A. myosuroides* populations with Ile-2041-Asn did not show any adverse pleiotropic effects on vegetative and reproductive plant traits (Menchari *et al.*, 2008). However, the assumption that a genotype that produces many seeds is fitter than a genotype producing fewer seeds is only true if seed dispersal, germination and colonization rates, seed longevity, seedling vigor and resistance to pathogens, diseases or herbivores are identical (Hanley, 1998). Results from these experiments may be useful for the design of weed management strategies to exploit those traits that result in reduced ecological performance (Vila-Aiub, 2009). The different reproduction pattern observed between R and S biotypes may play a role on the resistance evolution under herbicide selection pressure, depending on the cropping (and weed) management, i.e. several herbicides (mainly pre-emergence ones) are not effective against *S. halepense* from rhizomes.

The great number of studies quantifying the fitness costs associated with novel herbicide resistance alleles, indicate the importance of this knowledge for understanding and predicting the dynamics of resistance evolution and management. However, Vila-Aiub (2009) highlighted that many of these studies have incorrectly defined resistance or used inappropriate plant material and methods to measure fitness. This could be the case of our study on ALS inhibitors resistant biotype. Data from populations resistant to ALS inhibitors were highly variable and no significant differences from susceptible populations were detected. Origin of populations, together with preliminary whole plant pot experiments that showed different resistance patterns, could suggest very different genetic background for the populations selected.

5.2. *E. crus-galli* characterization

Resistance to ALS inhibitors in *E. crus-galli* is evolving quickly in Italian summer crops and the first cases of multiple resistance to ALS and ACCase inhibitors in paddy rice are showing up. Apart from two cases reported in Heap's

global resistance database (Heap, 2011), but not adequately documented, to our knowledge these are the first cases of *E. crus-galli* multiple resistant to ALS and ACCase inhibitors (Panozzo *et al.*, 2012).

Dose-response experiments on *E. crus-galli* showed that resistance levels to all ALS inhibitors chemical families are high, suggesting a target-site mediated resistance mechanism involved. Moreover, the dose effect observed in some populations resistant to nicosulfuron suggests that even the occurrence of a non-target-site mechanism of resistance may not be excluded, probably "masked" by the target-site one. In fact, due to the polyploid nature of *E. crus-galli*, it might be speculated that more than one mechanism may be involved as previously observed in ALS inhibitors resistant *E. phyllopogon* (Osuna *et al.*, 2002). The same may be speculate for resistance to ACCase inhibitor profoxydim where resistance levels were low in *E. crus-galli*, suggesting that a non-target-site resistance mechanism may be involved.

ALS enzyme *in vitro* bioassay on *E. crus-galli* confirmed that the resistance mechanism is related to an altered target enzyme and the lower than expected R/S ratio calculated may be linked with the polyploid status of *E. crus-galli* (Zheng, 2007). The first ALS gene sequence was obtained and the "strong" Trp-574-Leu amino acid substitution was constantly detected in resistant plants (Panozzo *et al.*, 2012). Southern blotting analysis confirmed the presence of multiple ALS genes (at least two) and, despite the polyploid nature of *E. crus-galli*, ALS gene cloning showed that probably two copies of the ALS gene are expressed and it behaves likely a diploid. The mutation 574 was detected always in the same copy of the ALS gene, which is consistently expressed in all the biotypes. This led to the successful development of a new AS-CAPS marker to detect the Trp-574-Ile allele in *E. crus-galli* plants, which will be useful for large-scale genotyping because of the limited cost of the restriction enzyme. This allele variant, as it has been observed in the whole plant pot experiments and already demonstrated in several others weed species (Tranel *et al.*, 2011), endows a broad cross-resistance pattern to ALS inhibitors. This implies that all the classes of ALS inhibitors are involved in a lack of sensitivity at the target enzyme.

Information coming from stakeholders and the number of putative resistant samples collected very recently indicate that herbicide resistance in *E. crus-galli* is of great concern for rice growers, especially when multiple resistance to ACCase and ALS inhibitors is present. The widespread occurrence of *E. crus-galli* and the intensive use of ALS inhibitors to control it, especially after the introduction of the Clearfield® technology in Italy (it is estimated that 40,000 ha are now cropped with these varieties) is exacerbating the problem. The lack of alternative herbicide MoA available in the Italian market seriously threaten the sustainability of the current rice cropping system, which is based on the lack of crop rotation. The resistance situation and the new European legislation on plant protection products highlight the need of more sustainable cropping systems, where feasible, based on a real integrated weed management: rotation of crops and herbicide MoA wherever possible, use of broad-spectrum herbicides (e.g. glyphosate and clomazone), false seed bed preparation and delayed rice sowing.

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