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CURRICULUM OF ENVIRONMENTAL AGRONOMY - CYCLE XX

Resistance to graminicides in monocotyledons weeds
Case studies of *Lolium* spp. and *Phalaris paradoxa* in Italy

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

January, 31st 2008 Alberto Collavo

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We are a vulnerable part of
the natural world, subject to the same
damage as the rest of the ecosystem.

Rachel Carson

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RIASSUNTO

In Italia il fenomeno della resistenza agli erbicidi sta evolvendo velocemente nei sistemi culturali caratterizzati da elevata omogeneità, quali ad es. le colture di grano duro e di riso, dove spesso mancano rotazione colturale e rotazione dei meccanismi d'azione degli erbicidi.

In generale la resistenza si sta evolvendo in maniera più veloce nelle specie allogame e ad alta variabilità genetica (ad es. *Lolium*). Nel territorio nazionale si stimano circa 100000 ha infestati da flora resistente, soprattutto *Lolium* spp., *Avena sterilis* e *Papaver rhoeas* in grano duro, mentre in risaia si rinvengono soprattutto *Schoenoplectus mucronatus* e *Cyperus difformis.*

In grano duro la maggior parte dei fenomeni di resistenza si è evoluto nei confronti degli inibitori dell'acetil-coenzima-A-carbossilasi (ACCasi). L'ACCasi è un enzima essenziale per la sintesi degli acidi grassi, nelle piante sono presenti due forme di ACCasi: una localizzata nei plastidi - sito primario della biosintesi degli acidi grassi - e l'altra nel citosol, deputata all'allungamento degli acidi grassi a lunga catena. Nei plastidi di quasi tutte le dicotiledoni è presente un enzima a multisubunità (eteromerico, codificato da più geni) mentre nel citosol è presente l'enzima multifunzionale (omomerico). La forma eteromerica delle dicotiledoni e l'omomerica del citosol sia in dicotiledoni che in graminacee sono insensibili agli inibitori dell'enzima ACCasi. L'enzima omomerico nei plastidi delle graminacee è invece sensibile e su questa caratteristica si basa la selettività dei graminicidi.

Gli obiettivi della ricerca sono stati: (1) monitorare nel territorio nazionale dell'evoluzione della resistenza agli inibitori dell'ACCasi in specie di *Lolium* e *Phalaris* segnalate come possibili resistenti; (2) sviluppare un test rapido ed efficace per determinare la resistenza agli inibitori dell'ACCasi in specie di *Lolium* e *Phalaris*; (3) caratterizzare popolazioni di *Lolium* resistenti a clethodim e pinoxaden usando approcci sperimentali diversi; (4) caratterizzare dal punto di vista agronomico e molecolare popolazioni di *Phalaris* resistenti agli inibitori dell'ACCasi.

Attraverso screening in vaso sono state selezionate popolazioni con diversi pattern e livelli di resistenza successivamente indagati con esperimenti di dose e risposta in ambiente esterno, mediante l'uso e lo sviluppo di marcatori molecolari per rilevare eventuali mutazioni presenti e analizzando l'attività enzimatica in laboratorio. Inoltre è

stato messo a punto un test rapido (in capsule Petri) per lo screening di popolazioni sospettate di resistenza agli inibitori dell'ACCasi.

Dai risultati degli screening, su 41 popolazioni di *Lolium* analizzate, 23 sono risultate resistenti al clodinafop, 19 al sethoxydim e 15 al pinoxaden. Tra le 17 popolazioni di *P. paradoxa,* 3 sono risultate resistenti al clodinafop, una al sethoxydim e una al pinoxaden.

La resistenza al pinoxaden è associata ad un ampio pattern di cross-resistenza tra gli inibitori dell'ACCasi utilizzati.

Il quick-test in capsule Petri è stato sviluppato per trovare una concentrazione di erbicida che discrimini tra popolazioni sensibili e resistenti e una concentrazione paragonabile all'effetto della dose di campo usata negli screening in vaso. Quest'ultima concentrazione è stata validata per ogni erbicida con popolazioni non utilizzate nella fase di set-up. In *Lolium* spp. le dosi scelte sono: clodinafop 1 µM, pinoxaden 0.2 µM, clethodim 0.2 µM e sethoxydim 0.1 µM; per *P. paradoxa*, clodinafop 0.1 µM, pinoxaden 0.05 µM e clethodim 0.1 µM. Il quick-test, paragonato al classico screening in vaso, si è rivelato uno strumento affidabile nel testare campioni numerosi di popolazioni e può essere impiegato come procedura di routine per determinare la resistenza agli ACCasi.

Le indagini molecolari in *Lolium* resistente al clethodim hanno rivelato la presenza di 5 mutazioni (Ile1781Leu, Trp2027Cys, Ile2041Asn, Asp2078Gly e Cys2088Arg), assortite in 12 diversi genotipi nelle 14 popolazioni indagate. La resistenza al pinoxaden è stata associata a 5 mutazioni (Ile1781Leu, Ile2041Val/Asn, Asp2078Gly, Cys2088Arg e Gly2096Ala) con uno specifico stato di etero- o omozigosi delle mutazioni. In *P. paradoxa* sono state descritte due mutazioni associate alla resistenza al pinoxaden (Ile1781Val e Asp2078Gly), sempre ritrovate allo stato omozigote.

Questa ricerca stabilisce che la resistenza agli inibitori dell'ACCcasi dipende dalle dosi degli erbicidi impiegati, da quali mutazioni sono presenti, dallo stato omo- o eterozigote e dallo specifico assortimento dei diversi alleli resistenti. Per comprendere e raccomandare strategie gestionali la conoscenza di tutti questi fattori è determinante. È altresì importante conoscere quali tecniche agronomiche e quali erbicidi sono stati impiegati nel passato.

La situazione della resistenza nelle infestanti del grano duro in Italia indica che non esiste un singolo prodotto chimico in grado di gestire i problemi di resistenza. Perciò gli addetti del settore dovrebbero recepire l'importanza dell'adozione di strategie di gestione integrata delle infestanti (IWM), o meglio di gestione integrata nelle colture (ICM). ICM e IWM richiedono un alto livello tecnologico e una profonda conoscenza delle componenti dei sistemi colturali.

ABSTRACT

Herbicide resistance is evolving fast in Italy, especially in cropping systems characterised by low diversity in space and time. In these systems there is often no rotation of herbicides mode of action (i.e. durum wheat and rice monoculture). Resistance is also evolving faster in cross-pollinating and genetically variable species (i.e. *Lolium* spp.). In Italy about 100,000 ha have been estimated to suffer resistant problems, mainly in durum wheat (*Lolium* spp., *Avena sterilis* and *Papaver rhoeas*) and paddy rice (mostly *Schoenoplectus mucronatus* and *Cyperus difformis*).

ACCase is an essential enzyme in fatty acid biosynthesis in eukaryotes and prokaryotes. In plants, two forms of ACCase have been identified – the first is located in the chloroplast, the primary site of plant fatty acid biosynthesis, and the second in the cytosol. The homomeric ACCase in the cytosol of nearly all plant species and the heteromeric ACCase in the chloroplasts of dicots are insensitive to APP, CHD and DEN herbicides. In contrast, the plastidic homomeric ACCase in nearly all grass species is herbicide-sensitive, and this is the basis for selective control of grass-weeds by ACCase herbicides.

The aims of the research were: (1) to monitor throughout the country the situation of resistance to Acetyl-CoA Carboxylase (ACCase) in *Lolium* and *Phalaris* species following complaints to the agrochemical industries; (2) to develop a reliable Petri dish quick test to detect ACCase-inhibitors resistance in *Lolium* and *Phalaris* species; (3) to characterise clethodim and pinoxaden resistance in *Lolium* spp. using different experimental approaches; (4) agronomic and molecular characterisation of a few populations of *P. paradoxa* resistant to ACCase inhibitors.

Through classic pot screenings, populations with different patterns and levels of resistance have been selected to be studied with different approaches: outdoor dose-response, at molecular level using molecular markers and sequencing and at physiological level through enzyme bioassay. As pot experiments are costly and time consuming, a rapid test in Petri dishes based on survival data has been developed for detecting resistance to ACCase-inhibitors.

Screening results revealed that among 41 tested *Lolium* populations, 23 were resistant to clodinafop, 19 to sethoxydim and 15 to pinoxaden. Among the 17 *P. paradoxa* populations, 3 were resistant to clodinafop, and one to sethoxydim and pinoxaden.

Resistance to pinoxaden is associated to a wide pattern of cross-resistance among ACCase-inhibitors.

The Petri dish seed quick test has been developed in order to find a discriminating herbicide concentration between susceptible and resistant populations; a herbicide concentration ("comparing dose") that gives results comparable to the recommended field dose used in greenhouse pot experiments; and a concentration discriminating between strong and weak resistance. The "comparing doses" has been validated using other populations not included in the set up experiments. The "comparing dose" for *Lolium* spp. have been: clodinafop 1 µM, pinoxaden 0.2 µM, clethodim 0.2 µM and sethoxydim 0.1 µM; for *P. paradoxa*, clodinafop 0.1 µM, pinoxaden 0.05 µM and clethodim 0.1 µM. The Petri dish seed quick test revealed to be reliable with the two species in screening large numbers of samples compared with the costly and time consuming pot experiment. The quick test improves ACCase-resistance detection and can be adopted as a routine facility.

Molecular investigation identified five ACCase mutations (Ile1781Leu, Trp2027Cys, Ile2041Asn, Asp2078Gly and Cys2088Arg) and revealed 12 genotypes in 14 clethodim resistant *Lolium* populations. Pinoxaden resistance has been reported associated to *Lolium* population mutants for five ACCase mutations (Ile1781Leu, Ile2041Val/Asn, Asp2078Gly, Cys2088Arg and Gly2096Ala) with specific homozygous and heterozygous status among mutations. In *P. paradoxa* two mutations have been associated to pinoxaden resistance: Ile1781Val and Asp2078Gly, always recognised at the homozygous status.

The research has established that resistance to ACCase herbicides depends on the specific resistant allele(s), on the homo/heterozygous status of plants for the specific resistant allele(s), and on the combinations of different resistant alleles, as well as herbicide rates. To understand and devise resistance management strategies, knowledge of all these factors coupled with field records of herbicide and agronomic techniques used is essential.

The overall situation of grass resistance in Italian durum wheat crops indicates that there is no single chemical that can solve all resistance problems. Therefore all stakeholders should be aware that IWM (or better ICM) is needed to properly manage resistance in the field.

It must also be clear that IWM or ICM requires a higher technological level coupled with a deeper knowledge of all components of the cropping system.

1. INTRODUCTION

Modern agriculture relay on a massive use of herbicides. Often they are the most effective and last expensive tool in weeds control (Powles and Shaner, 2001). The impact of herbicides has been so considerable that now their use has overcome that of fungicides and insecticides combined. One of the negative sides of this efficient technologic tool is the appearance of resistant weeds, mainly after the introduction of an increasing number of more selective herbicides with a very specific metabolic target (Heap, 1999).

1.1 Resistance to herbicides

1.1.1 Definitions

According to (Herbicide Resistance Action Committee - HRAC) **herbicide resistance** is "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. Selection of resistant biotypes may result in control failures". Another definition provided by Heap and LeBaron (2001) states as herbicide resistance "the evolved capacity for a previously herbicide-susceptible weed population to withstand a herbicide and complete its life cycle when the herbicide is used at it normal rate in an agricultural situation". Resistance is defined by the European and Mediterranean Plant Protection Organisation (EPPO) as: "The naturally occurring, inheritable adjustment in the ability of individuals in a population to survive a plant protection product treatment that would normally give effective control" (Anonymous, 2001). EPPO guidelines 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) and 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.

Different is the situation of a naturally tolerant plant which, according to the Weed Science Society of America (WSSA), is officially defined as follows: "**herbicide tolerance** is the inherent ability of a species to survive and reproduce after herbicide treatment. This implies that there was no selection or genetic manipulation to make the plant tolerant; it is naturally tolerant". The difference with resistance is based on absence of selectivity in tolerant species.

The problem of herbicide resistance is well known and has risen (Figure 1) a few years later the introduction of triazines in the early 1970s (Ryan, 1970) although the first herbicide to be used over large areas were 2,4D and MCPA. Fortunately, these latter compounds are not prone to easily select for resistance (Powles and Shaner, 2001).

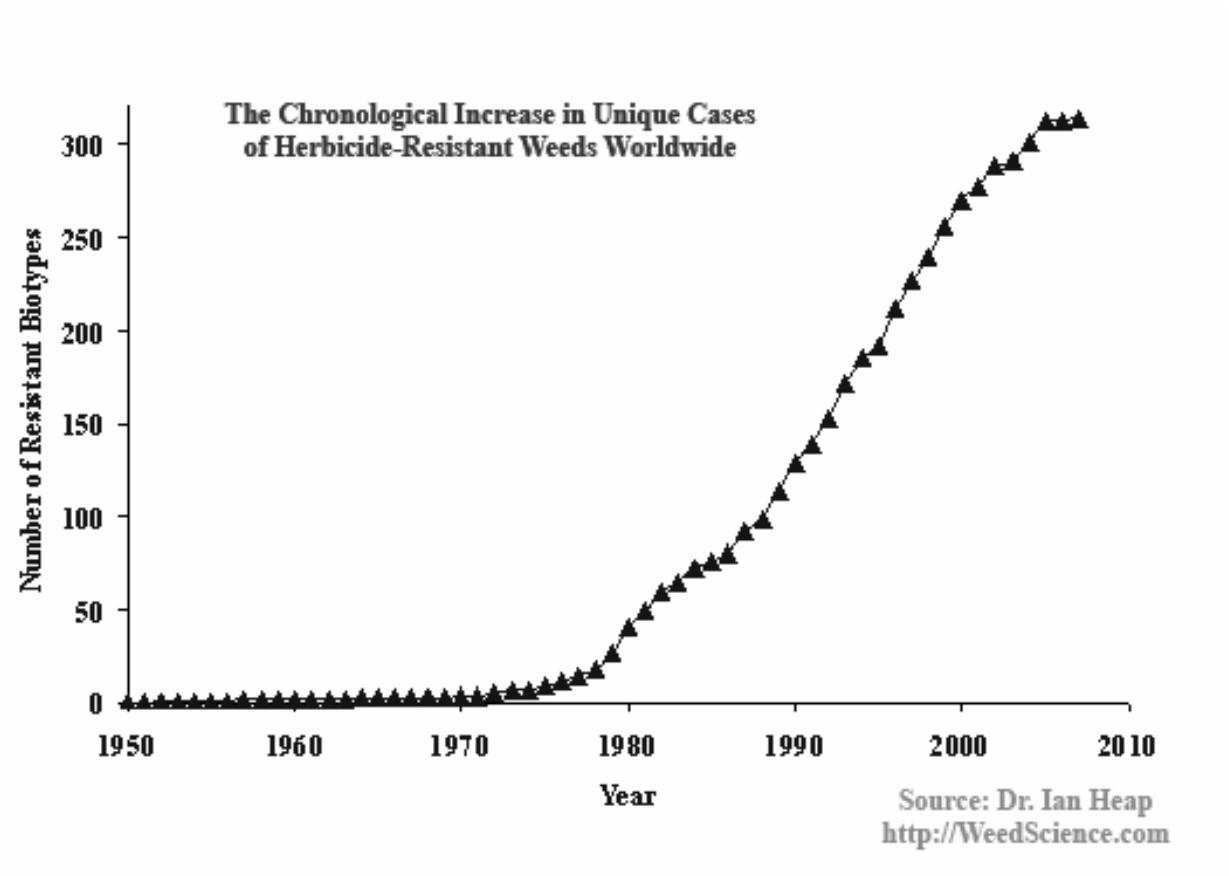


Figure 1. Evolution of resistant biotypes worldwide - 315 biotypes, 182 species: 110 dicotyledonous and 73 monocotyledonous, (Heap, 2008).

Although it is difficult to generalise, a weed population can be resistant to only one

herbicide, or to herbicides with the same mode of action, i.e. only one resistance mechanism is involved, or to herbicides with different modes of action, i.e. more than one resistance mechanism is involved. When a plant is resistant to several herbicides with the same resistance mechanism the phenomenon is recognised as **cross resistant**, while when a plant is resistant to several herbicides through different resistance mechanism is defined as **multiple resistant** (Hall et al., 1994).

The **mode of action** (MoA) of a herbicide is represented by the sequence of events which occur since the herbicide is adsorbed by the plant to the final effect caused by the herbicide. The **mechanism of action** is considered the identification of the specific biochemical target (i.e. the lipid synthesis inhibitors at acetyl coenzyme A carboxylase – ACCase – is the MoA, while the mechanism of action is the binding of the herbicide at the enzyme level). However, the two expressions are often used as synonyms.

Not only weeds can be resistant to herbicide. Since the escalation of resistant weeds to an increasing number of herbicides, herbicide **resistant crops** have been developed in attempt to improve the management strategy. Firstly, traditional crop improvement techniques have been used, such as selective breeding (i.e. triazine-resistant canola) and seed mutagenesis (i.e. terbutryn-resistant wheat; sulfonyl urea-resistant soybean and imidazolinone-resistant rice and wheat. Then techniques such as cell culture selection (sulfonyl urea-resistant canola and atrazine-resistance in soybean) and genetic engineering (sulfonyl urea-resistance in cotton; glufosinate resistant rice and canola; glyphosate resistance in cotton, soybeans, maize and wheat; bromoxynil-resistant cotton and subclover and 2,4-D resistant cotton) were applied in attempt to give crop plants the ability to tolerate currently marketed herbicides (for a review see Connor, 1995). Generally, induced herbicide tolerance in crops developed under laboratory conditions is primarily based on herbicide deactivation and reduced sensitivity (Duke, 2005; Dill, 2005).

1.1.2 Resistance mechanisms

Physiological effects of herbicide action usually consist in cell division inhibition, plant growth regulation, photosynthesis and/or respiration inhibition or interruption of essential metabolic processes. Mechanisms of resistance can be summarised 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 genes of the resistant plant code for an altered protein.

The second typology groups all the **non target-site mechanisms,** which reduce the amount of herbicide reaching the target-site , such as enhanced metabolism, gene over expression which leads to overproduction of target proteins, reduced rates of herbicide uptake, translocation and compartmentalisation.

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 mechanism are due to an elevated expression of cytochrome P450 monooxygenasi (P450s), which plays an important role in the oxidative metabolism of xenobiotics, and glutathione-S transferases (GSTs) transferase (Hall et al., 1994).

An **over expression** of target proteins causes an increasing of the number of target-site s in a plant which exceeds 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 effectiveness is dependent upon the herbicide dose.

Less common non target-site mechanisms are reduced rates of herbicide **uptake** (act on the kinetics of herbicide mobility), **translocation** and **compartmentalisation** (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).

1.1.3 Evolution of resistance worldwide

Rachel Carson predicted the phenomenon in her 1962 book “Silent Spring” by analysing the technologies (including chemical control) that conventional agriculture had adopted, while Harper emphasised the future impact of herbicide resistance in 1977. Since then the first reported case of resistance was in 1970 (Ryan) in a *Senecio vulgaris* population from Washington resistant to triazine (HRAC code C1: Photosynthetic inhibitors at Photosystem II, Site A - these chemicals interfere with photosynthesis and disrupt plant growth, ultimately leading to death). Later on more than 30 species have been reported as triazine-resistant and now 315 biotypes involving 182 species (110 dicotyledonous and 73 monocotyledonous) with over 290,000 fields have been reported (Heap, 2008 - Figure 1). Resistance has developed mainly to three mode of action: ALS-inhibitors, PSII inhibiting herbicides and ACCase-inhibitors (Figure 2). Among these, ALS-inhibitors are the those most prone to select for resistance due to their high efficacy and very specific target (Saari et al., 1994). Considering the situation in Europe, the number of populations, triazines are still the group which selected the highest number of resistant populations, but considering the last years, ALS and ACCase-inhibitors are the ones which reported the greatest increment in resistant populations (Sattin, 2005).

ACCase- and ALS-inhibitors are the most important herbicides for the control of grass weeds in cereal crops, therefore the herbicides belonging to these groups need an appropriate risk analysis regarding resistance selection.

The main factors influencing the evolution of herbicide resistance are: (1) the initial frequency of the resistance trait in unselected populations; (2) the genetic bases of resistance (number of alleles involved); (3) selection pressure; (4) relative fitness of resistant weeds; (6) soil seed bank; (7) seed production by resistant weeds and (8) residual activity of herbicides.

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. Selection pressure, with which an environment tends to eliminate an organism, and thus its genes, or to give it an adaptive advantage, in this specific case it is defined as the ratio between the rate of resistant plants surviving the herbicide treatment and the survival rate of susceptible plants (Gressel, 1991). Selection pressure is considered the most important factor in determining the rate of resistance evolution. ALS-inhibitors

have been demonstrated to select for resistance just after four generations (Moss, 2007), this implies that the initial resistant trait for ALS-inhibitors resistance is higher compared to other forms of resistance. The seed bank can slow down the evolution of resistance in species with persistent seeds (i.e. dormant seeds like *Papaver rhoeas*) by exerting a strong buffering activity, but cannot avoid resistance evolution.

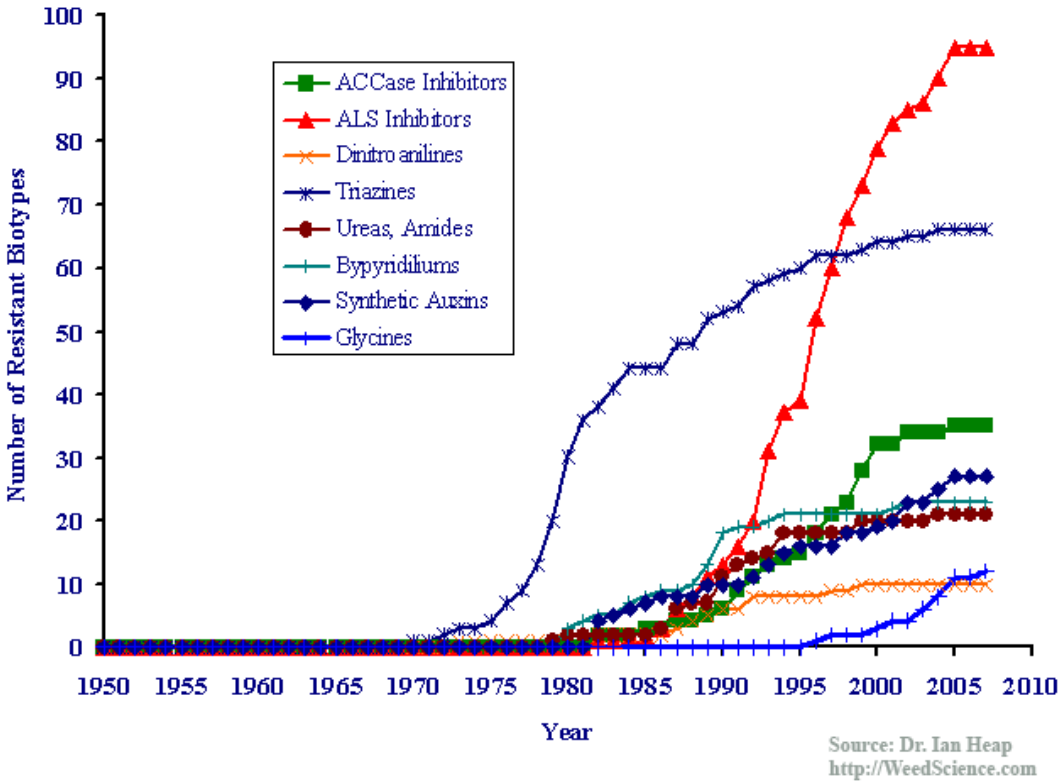


Figure 2. Evolution of resistant biotypes worldwide sorted by herbicide mode of action (Heap, 2008).

1.1.4 The Italian situation

Herbicide resistance in Italy had a marginal impact until the mid-90' (Sattin, 2005), with only three atrazine-resistant biotypes (*Solanum nigrum, Amaranthus* spp. and *Chenopodium* – Zanin et al., 1981) that at their peak infested about 8-10% of maize fields (Porceddu et al., 1997), However these populations did not cause big agronomic problems because of their fitness penalty and because they were easily controlled with herbicides having different mode of action.

Since mid-90' the situation has been evolving fast. There are now 16 different species that have evolved resistant populations and these infest approximately 100,000 ha (Table 1). Two the major cultural systems are affected: rice in the north and wheat, mostly durum wheat in central and southern Italy. In rice the most problematic weeds are *Schoenoplectus mucronatus, Cyperus difformis* (Sattin, 2005) and *Alisma plantago-aquatica* resistant to ALS-inhibitors. Other species which evolved resistance are *Echinochloa cruss-galli* and *E. erecta* (Tabacchi et al., 2004). In wheat the most widespread cases are involving *Lolium* spp., located in central Italy (mainly along Tyrrhenian and Adriatic coast – Figure 3) and *Avena sterilis* in southern Italy (mainly Apulia and Sicily – Figure 3) resistant to ACCase-inhibitors (Sattin et al., 2001; Campagna et al., 2006; Collavo et al., 2007a and 2007b); some of them are multiple resistant to ACCase- and ALS-inhibitors (Campagna et al., 2006). A few populations belonging to *Phalaris* spp. have also been reported (Figure 4).

Among dicotyledonous species, 26 ALS-resistant *P. rhoeas* populations have been detected (Figure 4), some of them multiple resistant to 2,4-D (Sattin et al., 2006) as well as a few ALS-resistant populations of *Sinapis arvensis* (Figure 4). Among these species, resistant *A. sterilis*, *S. arvensis* and marginally *P. rhoeas* are concentrated in areas with an arid or semi-arid climate according to the aridity index, which is the ratio between precipitations and potential evapotranspiration (Collavo et al., 2007b).

The two worst cases in wheat are *Lolium* spp. and *P. rhoeas*: among populations of *Lolium* spp. tested since 1998 (Figure 5), 60% have been proved to be resistant at least to one ACCase-inhibitor and with highly variable resistance level, 36% cross-resistant to ACCase-inhibitors with a wide resistance pattern and 25% resistant at least to one ALS-inhibitor, usually with a low resistance level. While in *P. rhoeas* the cumulative trend of ALS-inhibitors and 2,4D resistance shows that 59% of the tested populations are resistant

at least to one ALS-inhibitor with high resistance level to sulfonylureas, a wide cross resistance among sulfonylureas and a low level of cross resistance to triazolopyrimidines; 38% of the populations are resistant to 2,4D with low resistance level and 23% of the populations are multiple resistant.

Recently an *Amaranthus retroflexus* population from northern Italy has been found resistant to ALS-inhibitors (sulfonylureas and imidazolinones). Even if it has not practical impact, as it is controlled by other mode of actions, is worth of note that care has to be taken in those crops, such corn and soybean, where the use of ALS-inhibitors is increasing.

It is now estimated that about 20% of rice paddies (Tabacchi et al., 2004) and 2-3% of durum wheat fields are infested by ALS- and/or ACCase-resistant populations, respectively. To manage the increasing resistance problems in paddy rice, the Italian Herbicide Resistance Working Group (GIRE) was funded in 1997. Now the group deals with all cases of herbicide resistance. As reported by Sattin (2005), the group is formed by agrochemical companies which are directly or potentially involved in herbicide resistance, plus academic, research and extension personnel. The mission is to facilitate herbicide resistance management through cooperation and communication between public and private stakeholders in order to 1) encourage a responsible attitude to herbicide use; 2) improve knowledge on herbicide resistance in Italy, including monitoring, causes and consequences; 3) effectively communicate and disseminate resistance management strategies; 4) stimulate collaboration between public and private research, especially in the area of devising and implementing resistance management strategies. There is a regular exchange of information within the Herbicide Resistance Working Group of the European Weed Research Society (EWRS). GIRE has regularly published updates and guidelines for resistance management of ALS-inhibiting and other rice herbicides (Sattin et al., 2004).

Table I. Chronological history of resistant specie in Italy. For each species are reported: year of the first occurrence, no. of resistant populations and no. of multiple resistant populations, herbicides or herbicide group involved in resistance, HRAC classification and crops affected by resistance problems (updated from Sattin et al., 2006 and Sattin, personal communication).

Species	First year report	No. resistant populations	No. multiple resistant populations	Herbicides or herbicide group	HRAC group	Crops
Amaranthus cruentus	1978	(*)	-	Atrazine	C1	Maize
Solanum nigrum	1978	(*)	-	Atrazine	C1	Maize
Chenopodium album	1982	(*)	-	Atrazine	C1	Maize
Avena sterilis	1992	51 18	16	ACCase-inhibitors ALS-inhibitors	A B	Durum wheat
Alisma plantago-aquatica	1994	54	-	ALS-inhibitors	B	Rice
Lolium spp	1995	49 12	12	ACCase-inhibitors ALS-inhibitors	A B	Durum wheat
Schoenoplectus mucronatus	1995	68	-	ALS-inhibitors	B	Rice
Papaver rhoeas	1998	26 11	6	ALS-inhibitors ormonici	B O	Durum wheat
Phalaris paradoxa	1998	12	-	ACCase-inhibitors	A	Durum wheat
Amaranthus retroflexus	1999	1	-	Terbuthylazine, Metamitron	C1	Maize
Cyperus difformis	2000	20	-	ALS-inhibitors	B	Rice
Echinochloa crus-galli	2000	7 3		Propanil ALS-inhibitors	C2 B	Rice Maize
Echinochloa erecta	2003	2 2	2	Propanil Quinclorac	C2 O	Rice
Amaranthus retroflexus	2003	1	-	ALS-inhibitors	B	Soybean
Digitaria sanguinalis	2006	1	-	ACCase-inhibitors	A	Soybean
Sinapis arvensis	2006	4	-	ALS-inhibitors	B	Durum wheat
Sorghum halepense	2006	4	-	ACCase-inhibitors	A	Soybean

(*) During the mid-80s, these resistant populations infested about 10% of Italian maize field (Cantele et al., 1985).

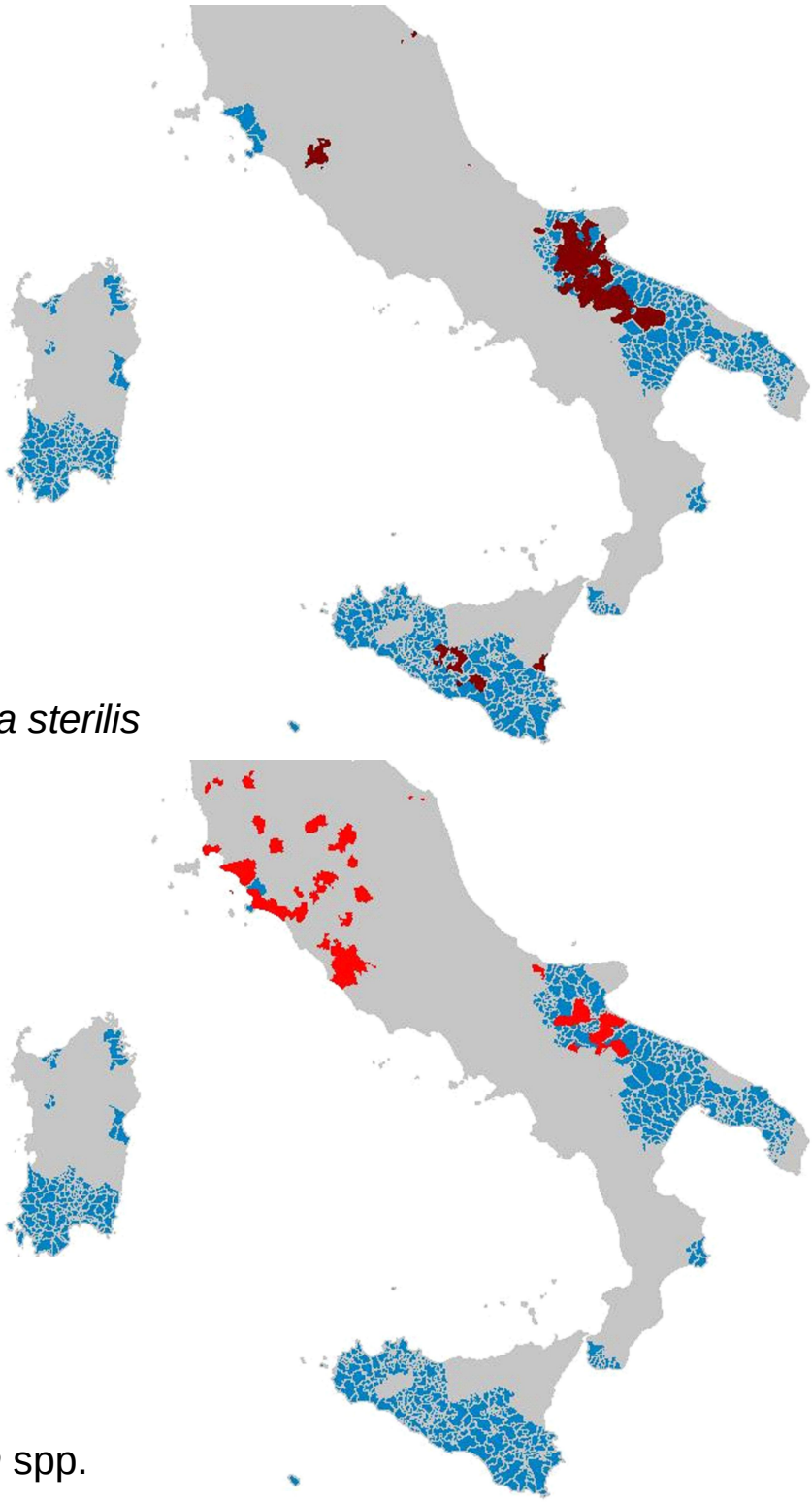
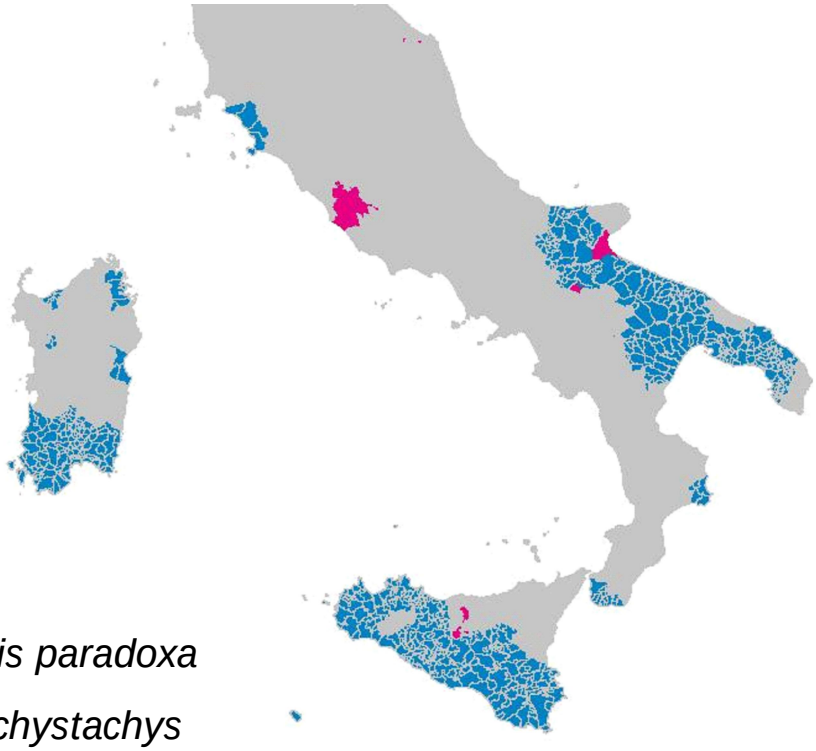


Figure 3. Central and Southern Italy municipalities where resistant *Avena sterilis* (top – brown spots) and *Lolium* spp. (bottom – red spots) populations have been detected. In blue arid and semi-arid areas of Italy.



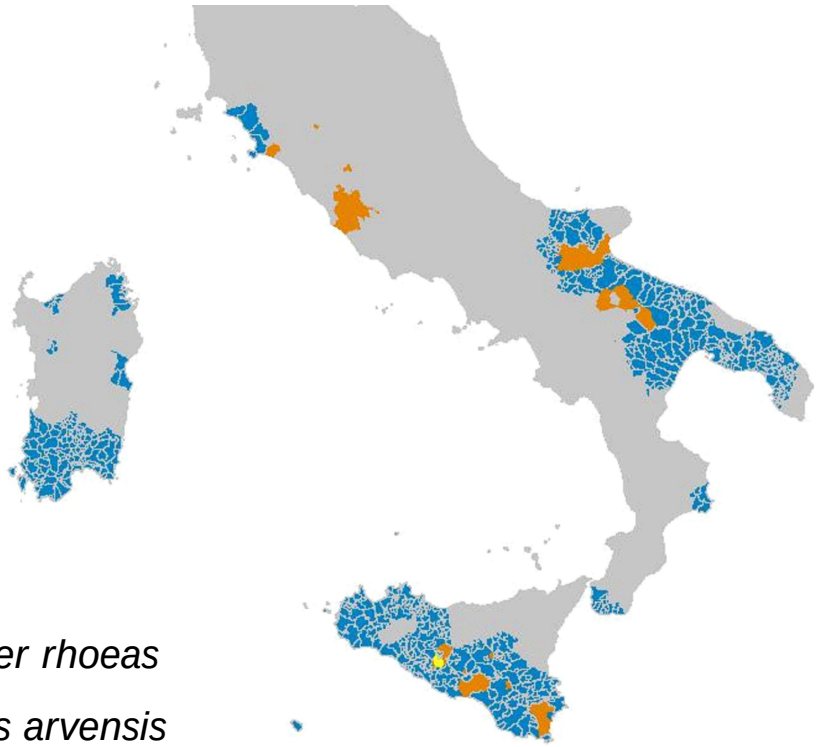


Figure 4. Central and Southern Italy municipalities where resistant *Phalaris paradoxa* and *P. brachystachys* (top – violet spot) and the dicotyledonous (bottom) *Papaver rhoeas* (brownish spots) and *Sinapis arvensis* (yellow spot in Sicily) populations have been detected. In blue arid and semi-arid areas of Italy.

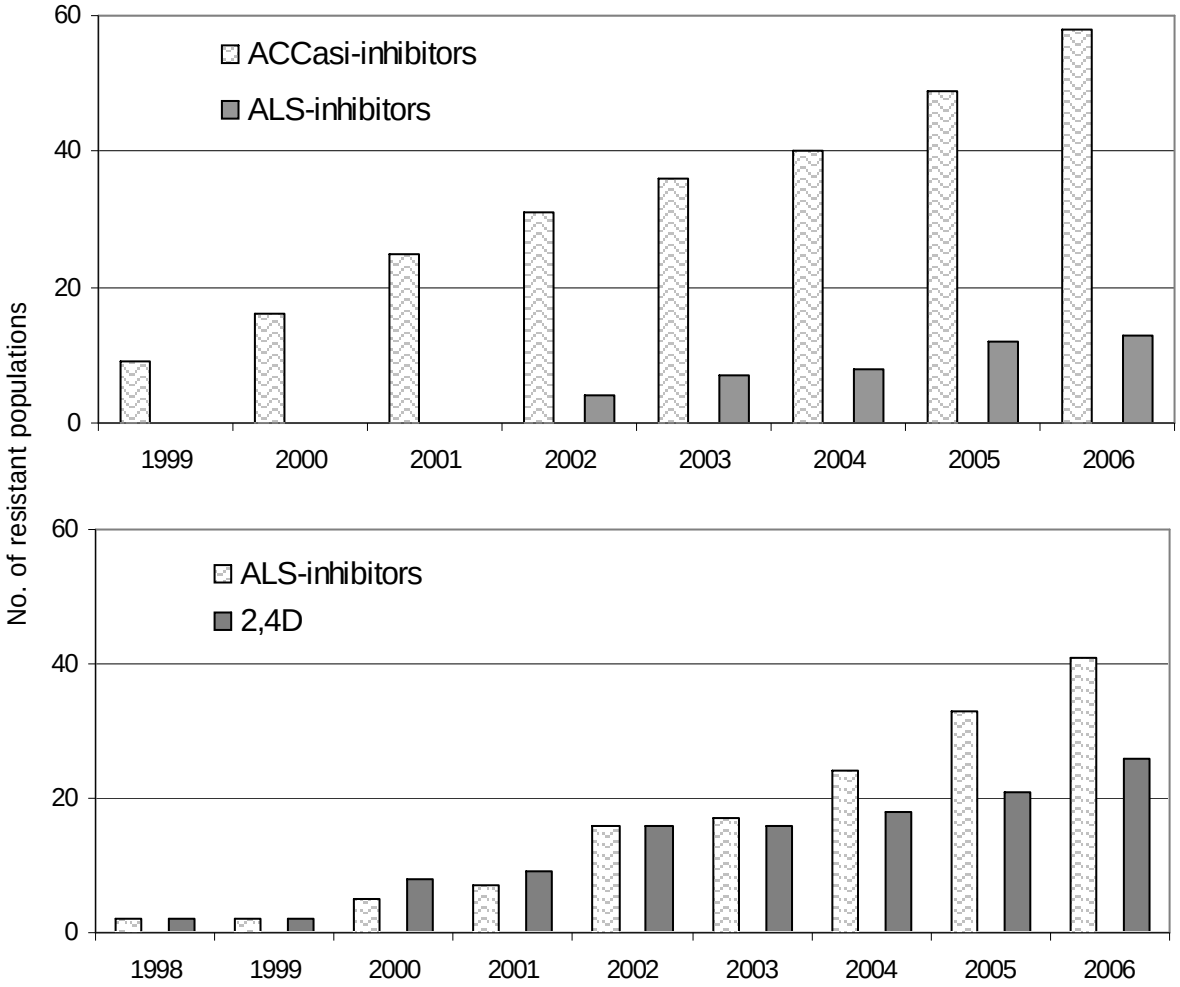


Figure 5. Top: *Lolium* spp.: cumulative trend of ACCasi- and ALS-inhibitors resistance (60% resistant at least to 1 ACCase-inhibitor, 36% cross-resistant among ACCase-inhibitors and 25% resistant at least to 1 ALS-inhibitor). Bottom: *Papaver rhoeas*: cumulative trend of ALS-inhibitors and 2,4D resistance (59% resistant at least to 1 ALS-inhibitor, 38% resistant to 2,4D and 23% multiple resistant).

1.2 Acetyl-Coenzyme A Carboxylase (ACCase)

Acetyl-Coenzyme A carboxylase (ACCase; EC 6.4.1.2) is a key enzyme involved in the first step of fatty acid biosynthesis. Plant lipids have different functions: they are the central components of cellular membranes and founded in chloroplast thylakoids; acyl lipids, in form of oils and fats store energy - it is well known their importance as edible sources and raw substrate for industry; many plant lipids and their metabolites have acute biologic activity; lipids are constituents of cell membranes and cuticle layers as waxes, cutin and suberin (Harwood, 1996). In plants, there are two isoforms of ACCase: the plastidic ACCase is essential in biosynthesis of primary fatty acids while the cytosolic ACCase is involved in biosynthesis of long chain fatty acids. All ACCase isoforms contain three catalytic domains: the biotin carboxyl carrier (BCCP), the biotin carboxylase (BC), and the carboxyl transferase (CT) domains (Nikolau et al., 2003).

The formation of fatty acids in plants starts by de novo saturated long chain fatty acids through the combined activity of acetyl-CoA carboxylase (ACCase) and fatty acid synthase. Acetyl-CoA carboxylase carries out the first committed step in fatty acid biosynthesis and is the target-site of three classes of graminicides: aryloxyphenoxypropionates, cyclohexadiones and phenylpyrazoline (including only pinoxaden herbicide), used to control grass weeds (not only in broad-leaved crops). ACCase catalyses its reaction in two main steps on two physically and kinetically distinct catalytic sites. In the first ATP is used to carboxylate the biotin prosthetic group of biotin carboxyl carrier protein (BCCP) and in the second step at level of carboxiltransferase (CT) the carboxyl group is transferred to acetyl-CoA to yield malonyl-CoA (Harwood, 1996).

1.2.1 Acetyl-Coenzyme A Carboxylase inhibitors

ACCase-inhibitors consist of three herbicide classes of with specific graminicides activity: aryloxyphenoxypropionates (commonly referred to as APPs or FOPs), cyclohexanediones (CHDs or DIMs) and the new class phenylpyrazolin (DEN) (Muehlebach, 2007). These herbicides inhibit the plastidic form of ACCase which catalyzes the first dedicated step in acyl lipid biosynthesis (Harwood, 1999). Studies using diclofop (an APP class molecule) indicated that the herbicide was inhibiting *de novo* fatty acid biosynthesis

(Walker et al., 1989), and other studies indicated that also CHDs were inhibiting the same pathway (Burton et al., 1991; Kobek et al., 1988; for a review see Harwood, 1996). Molecular and biochemical studies have clearly established that the CT domain of the plastidic homomeric ACCase is the primary target-site for APP and CHD herbicides, and two regions of the CT domain of the plastidic ACCase are critical for sensitivity to these herbicides (Zhang et al., 2004; for review see Délye, 2005).

The homomeric ACCase in the cytosol of nearly all plant species and the heteromeric ACCase in the chloroplasts of dicotyledonous are insensitive to APP and CHD herbicides. In contrast, the plastidic homomeric ACCase in nearly all grass species is herbicide sensitive, and this is the basis for selective control of grass weeds by ACCase herbicides. As APPs and CHDs show competitive binding characteristics, with overlapping binding sites (Rendina et al., 1989) it has to be expected that resistance can be acquired to both classes of graminicides and to the new class phenylpyrazoline (i.e. pinoxaden) which bind the same target (Hofer, 2006). Studies on pinoxaden revealed that this molecule binds both plastidic and cytosolic ACCase enzyme (Campagna and Rueegg, 2006).

Translocation of CHD and APP is very important in their mode of action since these herbicides act in the meristematic regions of the plant. Surprising is that very little (1 to 5%) is translocated out of the treated leaf, this suggests that the active ingredients of these herbicide are very active in the plant meristematic regions (Burton, 1997).

1.2.2 A new ACCase inhibitor (pinoxaden)

Pinoxaden is a herbicide which belongs to a new chemical class (phenylpyrazoline - DEN), compared to the current standard ACCase herbicides it has a greater activity to a broad spectrum of grass weeds infesting cereals worldwide. The target species on which pinoxaden field dose has been calibrated are *Lolium* spp., *Avena* spp., *Alopecurus myosuroides*, *Phalaris* spp., *Apera spica-venti* and *Setaria spp.* (Muehlebach et al., 2007). The main difference compared to other ACCase-inhibitors, which selectively target the plastidic form of the enzyme, is that pinoxaden targets both the plastidic and cytosolic forms of the enzyme in monocotyledons (Campagna and Rueegg, 2006).

The adjuvant (Adigor®), specifically developed for this herbicide, is based on methylated oilseed and non-ionic tensioactives and ensures an optimal and quick uptake

not affected by a rainy event just after 30 minutes. The rapid leaf penetration permits translocation to the site of action in the meristematic growing tissue. Pinoxaden is taken up primarily through leaves of treated grasses and then basipetally and acropetally translocated. It has many favourable features compared to other members of ACCase-inhibitors, such as a more flexibility in application timing - even if the correct plant stage is still related to herbicide efficacy (Campagna et al., 2006). Together with the adjuvant, pinoxaden can be also sprayed in non optimal conditions (i.e. low air humidity). Recommended application rates are low and vary from 30 to 60 g a.i. ha^{-1} depending on the target grass species and location. It can be applied in autumn or spring from the 2-leaf stage up to the pre-boot stage of crops; applications show no significant carry-over, i.e. no rotational crop limitations the following growing season (Hofer et al., 2006); it has also a very favourable toxicological and ecotoxicological profile (EPA, 2005).

Selectivity towards the main cereal crops (wheat and barley) is excellent compared to the current cereal herbicide standards. Tolerance is obtained by adding the safener cloquintocet-mexyl to the herbicide formulation, which induces the synthesis of herbicide degrading enzymes in cereal crops (Hofer et al., 2006), but do not interfere with grass weeds metabolism.

1.2.3 Resistance to ACCase herbicides and other graminicides

Up to date, 73 grass weeds over 290,000 fields have evolved resistance to graminicides (Heap, 2008). Resistance to ACCase inhibitors concern 35 species in 51 states (large countries as Australia, Canada and USA account with the states or provinces where resistance has been reported in Weedscience.org, the International Survey of Herbicide Resistant Weeds - Heap, 2008). The reported cases are 124 and this is clearly just an underestimation of the worldwide resistance problem. Multiple resistance, up to 7 modes of action, has been reported in 27 cases. In Italy the three major grass weeds which evolved resistance to aryloxyphenoxypropionates and cyclohexanediones are *A. sterilis*, *Lolium* spp. (*L. multiflorum*, *L. rigidum* and hybrids) and *P. paradoxa* (Sattin, 2006). Some populations are not even controlled by the newly marketed phenylpyrazolin (Hofer et al., 2006) class herbicide pinoxaden (Collavo et al., 2007; Hochberg et al., 2007).

Resistance can be due to target-site or not-target-site mechanisms. The most studied is the target-site resistance mechanism because of the relatively simple techniques to detect mutations available in biomolecular laboratories. While non-target-site mechanisms need biochemical laboratory facilities and know-how which are not so common.

Several mutations in the ACCase gene endowing target-site based herbicide resistance have been identified in some ACCase herbicide-resistant grass weeds populations (for a review see Délye, 2005). In the present study new mutations causing resistance in *Lolium* spp. and *P. paradoxa* have been identified. Thus far, seven distinct amino acid substitutions in the CT domain of the plastidic ACCase gene, that individually endow resistance to certain ACCase-inhibitors, have been characterised, at the beginning mainly in *Alopecurus myosuroides* and later in other grass weed species, as reviewed by Délye et al. (2005) and updated in Table II.

Many resistant populations can also have non-target-site resistance mechanisms such as enhanced rates of ACCase herbicide metabolism (Matthews et al., 1990; Holtum et al., 1991; Tardif et al., 1993, 1996). Those species which are allogamous can easily accumulate different resistance mechanisms (Hall et al., 1994; Preston and Powles, 1998; Vila-Aiub et al., 2005a), while completely or almost completely autogamous species are disadvantaged in accumulating.

Specific graminicides are the herbicides belonging to ACCase-inhibitors. Among those molecules used also as graminicides in wheat there are members of the sulfonylureas class, which inhibit the Acetolactate Synthase (ALS). This stops the production of the branched amino acids isoleucine, leucine and valine, finally causing plant death of susceptible plants. The first sulfonylurea to be used against grass weeds was chlorsulfuron, which was commercialised in 1979. In 1999 a new sulfonylurea, iodosulfuron, has been available for controlling broad leaved weeds and some grasses (at low dosages of 10 g a.i. ha^{-1}) in winter, spring and durum wheat, triticale and rye (Hacker et al., 1999). Two years later mesosulfuron was introduced (Hacker et al., 2001). Mesosulfuron and iodosulfuron show a complementary efficacy, in fact they are marketed in mixtures only. These products have been addressed to be used in situation where grasses have been found resistant to other mode of action. This strategy has to be valuated very carefully, since ALS-inhibitors are very prone to rapidly select for resistance (Moss, 2007).

Nowadays, the group of the ALS inhibitors is the most widely used. These are very valuable molecules, especially sulfonylureas, primarily due to their high efficacy and selectivity in a large number of crops, low application rates, low mammalian toxicity, and high environmental safety (Saari et al., 1994). Unfortunately this wide use and the high efficacy has led to the development of the major number of resistant cases worldwide (Figure 2). Until now 95 species have been selected for resistance using ALS-inhibitors, 273 resistant cases have been reported in Weedscience.org (Heap, 2008).

Table II. Amino acid substitutions within plastidic, homomeric ACC and associated cross-resistance patterns observed at the whole plant level (modified from Délye, 2005).

Amino acid residue[b]		Weed species	Resistance[a]												
			APPs[c]						CHDs[d]					DEN[e]	Reference[f]
Wild-type	Resistant		Cd	Cy	Dc	Fx	Fz	Hx	Ct	Cx	Sx	Tk	Te	Pxd	
Ile_{1781}	Leu	*Alopecurus myosuroides*	S/R	R	R	R	R	S/R	S	R	R	R	R		1; 2; 5; 12
	Leu	*Avena fatua*			R						R				3
	Leu	*Avena sterilis* ssp. *Lud.*	R								R				10
	Leu	*Setaria viridis*			R	R					R	R			6; 9
	Leu	*Lolium* spp.	S/R		R	R	R	R	S/R	R	R	R		R	4; 5; 12; 13; 14
	Val	*Phalaris paradoxa*	R		R	R	R	R	R	R	S	R		R	4
Trp_{1999}	Cys	*Avena sterilis* ssp. *lud.*	S			R		S			S				10
Trp_{2027}	Cys	*Alopecurus myosuroides*	R			R		R	S	S					8
	Cys	*Avena sterilis* ssp. *lud.*	R			R		R			S				10
Ile_{2041}	Asn	*Alopecurus myosuroides*	R			R		R	S	S					7
	Asn	*Lolium* spp.	R		R			R		S					7, 15
	Val	*Lolium* spp	S					R		S					7
	Asn	*Avena sterilis* ssp. *lud.*	R			R		R			S				10
Asp_{2078}	Gly	*Alopecurus myosuroides*	R			R		R	R	R					8
	Gly	*Avena sterilis* ssp. *lud.*	R			R		R			R				10
	Gly	*Phalaris paradoxa*	R		R	R	R	R	R	R	R	R		R	4
	Gly	*Lolium* spp.	R		R				S/R		R			R	4; 13
Cys_{2088}	Arg	*Lolium* spp.	R		R		R	R	R		R	R		R	13
Gly_{2096}	Ala	*Alopecurus myosuroides*	R			R		R	S	S					8

[a] S and R respectively indicate that plants containing at least one copy of the ACC mutant allele are sensitive or resistant to the corresponding herbicide either in the field or in bioassays. S/R indicates different results in different papers using different herbicide doses or different bioassays.
[b] Amino acid number is standardized to A. myosuroides plastidic, homomeric ACC (EMBL accession AJ310767).
[c] Cd, clodinafop;Cy, cyhalofop; Dc, diclofop; Fx, fenoxaprop; Fz, fluazifop; Hx, haloxyfop.
[d] Ct, clethodim; Cx, cycloxydim; Sx; sethoxydim; Tk, tralkoxydim; Te, tepraloxydim.
[e] Pxd, pinoxaden.

[f] References:

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1.3 Rapid tests to detect herbicide resistance

Testing for resistance is fundamental to manage herbicide resistance. Many techniques have been developed to detect herbicide resistance at plant, seed, pollen or enzyme levels.

Greenhouse pot assays experiments are usually reliable, but take about 5 to 7 weeks to produce results (Moss, 1995; Beckie et al., 2000). Many other techniques are based on pollen detection of resistance (Richter and Powles, 1993; Letouzé and Gasquez, 2000) or enzyme bioassay. Often these latter tests are useful and precise in discriminating the mechanism responsible for resistance, but this could be a limitation as more mechanisms could be involved (Moss, 1995). Most of these tests are costly in terms of labour, time, space, reagents (i.e. enzyme assay) or require greenhouse facilities. A different diagnostic test was developed by Boutsalis (2001) for grass weeds surviving a herbicide treatment and tillers from survivors were transplanted into pots to regenerate new leaves and treated with different herbicide.

The main features that a diagnostic test should have are rapidity, accuracy, reliability and possibly inexpensiveness.

Seedling assays have been developed for different species and herbicide groups (Beckie et al., 2000). They vary in methodology: in many quick tests pre-germinated seeds and herbicide aqueous solutions are used and shoot and/or root length is assessed (Burke, 2005; Letouzé et al., 1997; Letouzé and Gasquez 1999; Retrum and Forcella, 2002). Other tests consider fresh weight (Kim et al., 2000) or germination rate (Cirujeda et al., 2000; White et al., 2005) and dry weight (Kuk et al., 2003). Non pre-germinated seeds are also used (Burgeois et al., 1997; Kotoula-Syka et al., 2000; Li et al., 2002; Murray et al., 1996; Tal et al., 2000).

Rapid bioassays have demonstrated to be an important tool to screen high numbers of putative weed resistant populations. The information should then be promptly given to stakeholders asking for herbicide resistance testing and shared among farmers to be advised for herbicide options.

1.4 Resistance management

Farmers in high-input cropping systems, usually are worldwide known to be not much proactive but reactive to herbicide resistance appearing (Powles et al., 2001). Even after many years of resistance problems, it has been seen that growers tend to wait until the problem is evident in the field. This approach is obviously wrong and relay on short-term economic return and show the inability to assess the risk associated to herbicide resistance (Beckie, 2006). Weeds are relatively immobile and it is therefore it is primarily in the farmers' hands preventing and managing herbicide resistance (Moss et al., 2007). Another aspect is also 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.

Herbicides can be thought as a not renewable resource (Beckie, 2007) and studies on the economics of herbicide resistance management have been focusing on bio-economic modelling of herbicide resistance, e.g. annual ryegrass (*L. rigidum*) resistance to post-emergence selective herbicides. In these studies a set number of effective herbicide applications are assumed to be available before resistance develops (Weersink et al., 2005), and results show that there is often no economic advantage from taking pre-emptive action to delay the onset of herbicide resistance (Powles et al., 2001). In environments where crop rotation is not possible or limited, it is hard to believe in a long term prevention strategy that does not have a positive impact.

Not all farmers act in this way, as reported by Beckie (2006) in Canada in 1998 around 50% of farmers were practising herbicide group rotation and by 2003 70 to 90% of them claimed to rotate herbicide by site of action. Any weed management option that reduces herbicide-imposed selection pressure will reduce the rate of appearance of herbicide resistance.

It must be stressed again that agricultural chemical industry regularly reminds that there will be no new herbicide chemistries in the near future.

1.4.1 Integrated Weed Management (IWM)

The research efforts in the last decades have also been devoted to address long-term strategies for resistance management (Berti et al., 2001). The main goal is to reduce the selection pressure through the implementation of Integrated Weed Management (IWM - Shaw, 1982), and more in general of integrated crop management (ICM). 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 tactics is to practise a continuum of different disturbances to the infesting flora such as crop rotation, cultural techniques and chemical control.

Crop rotation. Rotations of crops with different time of sowing and cultural cycle interfere with weed seedling emergence and often imply the use of different herbicide modes of action.

Cultural techniques. Type of tillage, i.e. no or minimum tillage vs. ploughing, interferes with the dynamics of the seed-bank. Stale seedbed preparation will encourage weed emergence and a subsequent disturbance (mechanical or chemical) will kill the emerged seedlings. The choice of more vigorous cultivars will help in contrasting weeds to reach maturity and produce seeds.

Chemical control. Use of herbicides with different mode of action whenever possible, careful choice of herbicide doses.

The general strategy should be to keep **selection pressure** as low as possible. The tactics depend on the genetics of the species and more precisely the gene(s) system(s) involved. When a major gene is involved in resistance a high selection pressure will select those genotypes which carry mutation(s) endowing herbicide resistance. Reducing selection pressure will slow down the selection. This can be achieved in different ways: reducing the dosage of the selecting herbicide(s); using herbicides with different MoA in mixture, since the frequency of mutants for two different targets is less likely to occur; rotation of herbicide MoA, rotation of chemical control with not-chemical control. Lowering of the selection pressure may delay the evolution of resistance in monogenic systems, whereas if polygenic resistance is present the lowering of the selection pressure (i.e. lower herbicide doses) has the opposite effect. Genetic recombination of minor genes, each contributing in a minor way, in a genotype will be favoured in a low selection pressure context while a higher pressure would control those genotypes with a small enhancement

of resistance (Gressel, 1995).

The country that currently has the world's most severe herbicide resistance problems is Australia. Here *Lolium* spp. was planted as pasture for sheep over huge areas. With the collapse of wool prices pasture have been converted into intensive agriculture, basic no till crop fields (mainly wheat) with intense herbicide selection. Australian farms are quite big (thousand hectares) and highly mechanised, usually minimum or no tillage is adopted to reduce soil erosion risk and minimise costs. There is a strong relay on herbicide, fields are characterised by low rainfall and poor soils: this situation reflects the yields which are obviously poor. Typically Australian farmers cut herbicide doses endowing enhanced herbicide metabolism, if not the selection pressure of field doses select for resistance mutations in a species like *Lolium* spp. highly prolific and completely out-crossing. In such a scenario there is a strong lack in diversity which stands for high herbicide reliance. In 12 million hectares of Western Australia wheat-belt which in 1970 had all the populations' herbicide susceptible, has been reported that in 2004 50% of crop fields had high frequencies of multiple herbicide resistant *Lolium* spp. (Powles, personal communication). To manage the Australian situation in the early 90s the Western Australia Department of Agriculture started education campaigns on resistance selection, workshops to communicate with the farmers have been supported along with developing of computer models (i.e. Ryegrass Integrated Management by Pannell et al., 1999). By necessity, many Australian farmers adopted diverse combinations of weed control measures, consistent with the concept of "integrated weed management" (IWM). To reduce the *Lolium* spp. infestation the seeds are removed (seed catching) or destroyed and crop stubble burned. This allows reducing the charge of seeds which reflects in reducing the probability to select for resistance. Comparing the Italian situation, where different crops can be rotated, farms are quite small, farmers are rather individualist and agriculture has been not so extensive (except rice) it is possible to recognise why herbicide resistance had not been a problem until the last decade. Therefore, the best resistance management strategy is to apply IWM.

1.4.2 European legislation

European Union regulates the marketing of plant protection products through the EU Council Directive 91/414/EEC. The directive takes into account the resistance risk which has to be considered for registering or re-registering products. Risk analysis has to be conducted at local level, related to the specific condition of use of the product for each country.

To support and harmonise the implementation of resistance risk analysis, the European and Mediterranean Plant Protection Organisation (EPPO, http://www.eppo.org/) has developed specific guidelines (EPPO standard PP 1/213(2); Anonymous, 2006) indicating what applicants and registration authorities are expected to do. In the EPPO standard, resistance risk analysis consists of two parts: resistance risk assessment and, if necessary, resistance risk management where strategies to avoid or delay resistance are identified. The management of resistance is a continuous process, it starts assessing resistance risk during product development, and continues with the selection of appropriate measures before the start of sales, and with the implementation of the measures throughout the commercial use of the active substance.

The application of directive is yet a sophisticated, complex and costly registration system and has caused a significant loss of existing active ingredient on the market due to the high cost of producing the data required for re-registration. This particularly affects minor crops (e.g. fruit and vegetables) because many products have low value in relation to the registration cost. Member states can only authorise marketing and use of plant protection products where the a.i. are listed in Annex I, except where transitional arrangements apply. A negative consequence of this is in managing resistance risks since there are less opportunities for rotating different MoA.

On the other side now there is a high safety level for users, consumers and the environment, establishing of a positive list (Annex I) of a.i. which have proved to be without unacceptable risk to humans or the environment and there is a greater harmonisation between EU countries policies.

1.5 Environmental fate of herbicide

Pesticides in the environment are affected by many processes such transfer, adsorption, breakdown and degradation. The transfer of the herbicide away from the target can occur through volatilisation, spray drift, runoff, leaching, absorption and crop removal.

Volatilisation is the process of solids or liquids converting into a gas, which can move away from the initial application site (vapour drift) and could damage nearby crops. Hot, dry or windy weather and small spray drops increase volatilisation. Incorporating herbicide into soil can reduce volatilisation.

Spray Drift is the airborne movement of spray droplets away from a treatment site during application. It is affected by herbicide spray droplet size (the smaller the droplets, the more likely they will drift), wind (the stronger the wind, the more pesticide spray will drift), distance between nozzle and target plant or ground (the greater the distance, the more the wind can affect the spray). Drift can damage nearby sensitive crops or can contaminate crops ready to harvest and can be harmful to animals. It could contaminate water basins causing hazards to animals and plants distant from the herbicide application.

Runoff is the movement of pesticides in water when encountering a sloping surface. The pesticides are either mixed in the water or bound to eroding soil. Runoff can also occur when water is added to a field faster than it can be absorbed into the soil. Pesticides may move with runoff as compounds dissolved in the water or attached to soil particles. Runoff from areas treated with pesticides can pollute streams, ponds, lakes, and wells. Pesticide residues in surface water can harm plants and animals and contaminate groundwater. Water contamination can affect livestock and crops downstream. Pesticide runoff can be reduced by using minimum tillage techniques to reduce soil erosion, grading surface to reduce slopes, leaving border vegetation and plant cover to contain runoff. Weather forecast should be taken into account before spraying herbicide treatments.

Leaching is the movement of pesticides in water through the soil. Leaching occurs downward, upward, or sideways. The factors influencing whether pesticides will be leached into groundwater include characteristics of the soil (i.e. sand content, texture) and pesticide (solubility, adsorption), and their interaction with water from a rain-event such as irrigation or rainfall. These factors are summarised in the table below.

Absorption is the uptake of pesticides and other chemicals into plants or microrganisms. Most pesticides break down once they are absorbed. Pesticide residues may be broken down or remain inside the plant or animal and be released back into the environment when the animal dies or as the plant decays.

Crop Removal through harvest or grazing may remove pesticide residues.

Adsorption is the binding of pesticides to soil particles and it is dependent on the type of pesticide, soil, moisture, soil pH and soil texture. Usually a herbicide is better adsorbed by soil with a high content in clay or organic matter.

Degradation is the process of breakdown of herbicide after application. The breakdown of chemicals could produce molecules which are biological inactive or not. The process can be biologic through microbiological breakdown (by bacteria or fungi) or chemical by reactions in the soil. Photodegradation is the breakdown due to sun light and it is dependent on intensity, spectrum and exposition to the light.

1.6 Wheat crops and weeds

Wheat is a cereal that is cultivated worldwide, it has the largest total production and total area and it is the first food grain consumed by humans. *Triticum aestivum* L. and *T. durum* Desf. are the two most cultivated species. They are cool season crops produced between latitudes 30 to 60 °N and 27 to 40 °S. wheat can be growth where annual precipitations are as low as 250-175 mm, but most areas have a precipitation range from 375 to 875 mm annual rainfall (Powles and Shaner, 2001). FAO reports that in 2006 over 216 million ha of wheat was harvested worldwide with a production about 606 million t.

Among cereals wheat represents the major fraction of cultivated area (32%) with a production fraction of 27%, which is at the third place after maize (31%) and rice (29%) (Figure 6). Global distribution and production for 2007 were about 99 million ha and 272 million tons in Asia; 56.1 mil. ha and 191 mil. t in Europe; 39.7 mil. ha and 107 mil. t in America; 10.2 mil. ha and 25.1 mil. t in Africa and 11.2 mil. ha and 10.1 mil. t in Oceania. The amount of wheat produced in developed countries was 47.3% (FAO, 2008).

The worldwide distribution of resistant weeds depends on the biology of the species

involved, crops rotations, cultural practices, chemicals availability and socio-economical factors. It is not surprising that the worst cases of resistant weeds are from developed and industrialised country (Powles and Shaner, 2001). Grasses comprise about 25% of weedy species and they account for 40% of resistant biotypes (Beckie, 2007). ACCase- and ALS-inhibiting herbicides account for more than 75% of the reported cases of resistance in wheat (Heap, 2008). ACCase-inhibitors were introduced in the late '70s and the first reported case of resistance is in an Australian wheat field in 1980 (Heap and Knight, 1982). ALS-inhibitors were introduced in wheat in 1982 and the first resistant biotype in wheat field has been reported in USA in 1987 (Mallory-Smith et al., 1990).

The main cereal grown in Italy is wheat, *T. aestivum* is cultivated mainly in northern Italy while *T. durum* in the centre and in the south. The total area cultivated with wheat crops in 2006 was nearly 2 million ha and the production around 7 million tons (Table III). The country is characterised by a highly diverse environment. A survey conducted by Viggiani (2005) revealed that Italian wheat crops reflect this diversity with the presence of 186 species belonging to 33 different families. Monocotyledonous account for 23% of the total, 73% for dicotyledonous and 4% for other families. The most common dicotyledonous is *Papaver* spp. (mainly *P. rhoeas*) representing 15% of the average infestation. Among grasses the most important species is *A. sterilis*, representing (10%). Others major weeds are *Lolium* spp. (7%), *Phalaris* spp. (6%) and *A. myosuroides* (5%). *Avena* spp. and *Lolium* spp. are frequent and abundant throughout the country, while *A. myosuroides* and *Phalaris* spp. are spread mainly in the north and in the centre-south, respectively. On the basis of the species distribution it is possible to identify six homogeneous areas in terms of weed flora: northern, Adriatic-central, Tyrrhenian-central, Southern-peninsular, Sicily and Sardinia.

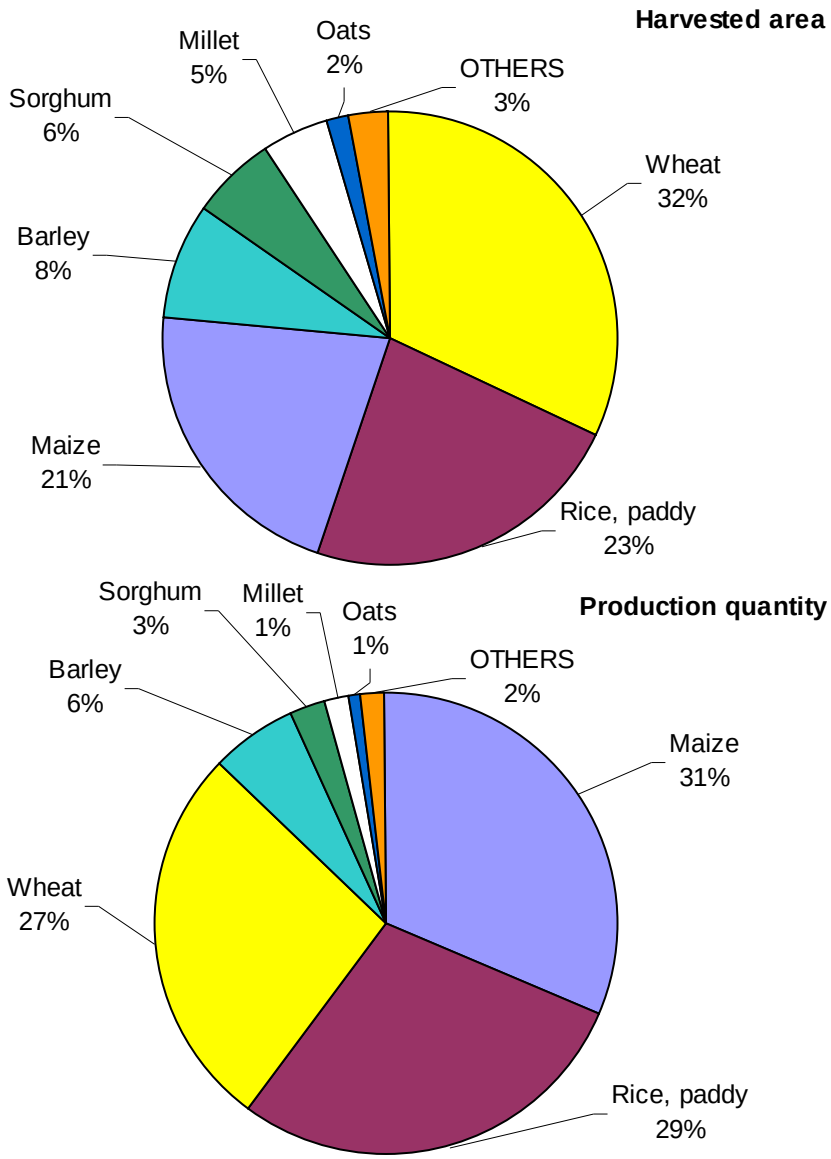

Figure 6. Percentage of worldwide cereals harvested area (tot. 674 mil. ha) and production quantity (tot. 2221 mil. t) in 2006.

Cultural practices influence the composition of weed flora, in recent years there has been a slow but steady increase of minimum and no-tillage. These practices favour mainly perennial and species with weak seed dormancy. The shift is more evident where chemical control is poor.

Areas with wheat monoculture (usually central and southern Italy) or rotated with crops having the same cultural cycle (e.g. autumn sown sugarbeet) suffered a high selection pressure because herbicides with the same MoA were used.

Table III. Wheat production (million tons) and area harvested (million ha) in Europe in 2006. Production, yield and area are sorted in order of importance (FAO, 2008).

	State	Production quantity (Mil t)	State	Area harvested (Mil ha)
1	France	35.4	France	5.25
2	Germany	22.4	Germany	3.11
3	United Kingdom	14.7	Poland	2.18
4	**Italy**	**7.09**	Romania	1.99
5	Poland	7.06	Spain	1.96
6	Spain	5.58	**Italy**	**1.93**
7	Romania	5.53	United Kingdom	1.83
8	Denmark	4.8	Hungary	1.08
9	Hungary	4.38	Bulgaria	0.970
10	Czech Republic	3.51	Czech Republic	0.782
11	Bulgaria	3.3	Denmark	0.686
12	Sweden	2.00	Greece	0.610
13	Belgium	1.58	Sweden	0.366
14	Austria	1.4	Slovakia	0.349
15	Greece	1.38	Lithuania	0.344
16	Slovakia	1.34	Austria	0.285
17	Netherlands	1.21	Latvia	0.215
18	Lithuania	0.810	Belgium	0.198
19	Ireland	0.768	Finland	0.192
20	Finland	0.684	Netherlands	0.141
21	Latvia	0.598	Portugal	0.112
22	Portugal	0.260	Estonia	0.091
23	Estonia	0.220	Ireland	0.088
24	Slovenia	0.134	Slovenia	0.032
25	Luxembourg	0.076	Luxembourg	0.013
26	Malta	0.010	Cyprus	0.007
27	Cyprus	0.007	Malta	0.002

1.7 Objective of the research: case studies of *Lolium* spp. and *Phalaris paradoxa*

1.7.1 Genus *Lolium*

The taxonomic classification within *Lolium* is not clearly established (Warpeha et al., 1998). Terrell (1968) recognised eight species and two compatibility groups that segregated the allogamous from the autogamous species. Fertile hybrids occur between *Lolium* and the genus *Festuca* (e.g., *Festulolium* Aschers. and Graebn). According to Stammers and colleagues (1995), some species of *Festuca*, particularly *F. pratensis* might be classified as a *Lolium*. Dinelli et al. (2002) tried to classify *Lolium* populations. Electrophoretic patterns revealed a significant number (40–60%) of hybrid individuals in all Italian populations collected from Central Italy. It was postulated they resulted from both intrageneric hybridisation among different *Lolium* species and intergeneric hybridisation between *Lolium* and *Festuca*. In each population some plants looked similar to *L. multiflorum* whereas others to *L. rigidum*, generally plants coming from southern Italy were identified as *L. rigidum* while the ones collected in central Italy showed intermediated characteristics between *L. multiflorum* and *L. rigidum*. Therefore, many population could consist of a mix of hybrids. None of the populations tested has been ascribed as *L. perenne*.

Lolium spp. are native to the Mediterranean basin, and nowadays have become important weeds in many other regions with a Mediterranean climate (Martin, 1996). Species belonging to this genus produce highly competitive infestations (Lemerle et al., 1996; Cousens and Mokhtari, 1998; Lemerle et al., 2001), have a weak dormancy (Baskin and Baskin, 1998; Steadman et al., 2003 and Steadman, 2004). Seed production can be as high as 1000 seeds per plant, seed production up to 45,000 seeds m^{-2} has been reported in irrigated wheat crop (Rerkasem et al., 1980) *Lolium* has a high genetic variability (Gill et al., 1996) and plasticity.

This species is considered the weed with the worst resistance problems worldwide. A recent study conducted in Australia demonstrated that pollen carrying resistant traits could move and fecund plants up to 3 km away (Busi, personal communication).

Since *Lolium rigidum* seed bank life is of 3-4 years with a decline of 80% per year (Matthews, 1994), IWM strategies can relay on this aspect to reduce resistant *Lolium* spp. populations.

1.7.2 ACCase-inhibitors resistant *Lolium* spp.

Lolium spp. is the weed with the highest level of resistance to many mode of action and with broad cross-resistance and multiple resistance patterns (Heap, 2008). The biological characteristics of the species - high seed production, high genetic diversity, out-crossing and low seed dormancy - are directly related to the occurring and diffusion of resistance.

The objectives of this part of the research were to infer mechanism of resistance in Australian population resistant to clethodim and Italian population resistant to pinoxaden as from preliminary work it was suspected that the resistance to these herbicides was due to common mutations of the ACCase enzyme.

In Australia *Lolium* spp. was planted by colons as pasture for sheep. It is not surprising that Australia suffers the worst resistant problems with this species since when converting from pasture to wheat crop the selection for resistance started with an abnormal infestation of *L. rigidum* (Powles, personal communication). The biochemical basis of ACCase herbicide resistance has been revealed in several populations to involve resistant ACCase (Matthews et al., 1990; Holtum et al., 1991; Tardif et al., 1993, 1996). Many resistant populations can also have a non-target-site based resistance mechanism of enhanced rates of ACCase herbicide metabolism (Tardif and Powles, 1994; Preston et al., 1996; Preston and Powles, 1998). *L. rigidum* is an obligate cross-pollinated plant and it is emphasized that individual plants and populations can accumulate resistance mechanisms. Specific ACCase mutations confer peculiar cross-resistance patterns to ACCase herbicides (for review, see Délye, 2005). The Ile1781Leu mutation is associated with resistance to APP and some CHD herbicides (not including clethodim). The Trp2027Cys, Ile2041Asn, or Gly2096Ala mutations confer resistance only to APP herbicides. The Asp2078Gly mutation confers resistance to many APP and CHD herbicides including clethodim. The Trp1999Cys mutation confers resistance only to the APP herbicide fenoxaprop (Liu et al., 2007). Despite widespread resistance to certain ACCase herbicides, in 1998 a survey across 300

western Australian crop fields confirmed that the CHD herbicide clethodim was still effective on many otherwise ACCase herbicide-resistant *L. rigidum* populations (Llewellyn and Powles, 2001). Five years later, however, a random survey of 452 ryegrass populations from the same region revealed clethodim resistance to be present in 8% of these populations (Owen et al., 2007). Thus far, the Asp2078Gly mutation in the plastidic ACCase enzyme is the only known mutation endowing clethodim resistance (Délye et al., 2005). As *L. rigidum* is a highly genetically variable species, all possible herbicide resistance endowing mechanisms can be present and enriched in large populations of this species under herbicide selection (Powles and Matthews, 1992). Thus, a number of different mutations endowing ACCase herbicide resistance is expected, and could be enriched both within and between different resistant populations. The hypothesis tested here is that field evolved ACCase herbicide-resistant *L. rigidum* populations would be comprised of individuals carrying a diverse range of resistance-endowing mutations and that individuals would be heterozygous or homozygous for one or any two possible combinations of different mutations. To examine this in depth 12 clethodim-resistant Australian *L. rigidum* populations, together with two resistant Italian *Lolium* populations have been selected. In addition, (derived) cleaved amplified polymorphic sequence ([d]CAPS) markers for the 2041, 2078, and 2088 mutations to enable rapid detection of these mutations in the *Lolium* populations have been developed.

The activity done in Italy concentrated on three pinoxaden resistant populations: 05281, 05256 and 06302. All these populations have been selected with ACCase-inhibitors. Two mutation have been known to cause resistance to pinoxaden, Ile1781Leu and Asp2078Gly (Collavo et al., 2007a). Plants of the selected populations have been grown in agar medium with pinoxaden [0.1 µM]. The survivors have been analysed with CAPS for the mutation 2041 and (d)CAPS for the mutations 1781 and 2078. The uncertain results have been confirmed by sequence.

1.7.3 Genus *Phalaris*

The genus *Phalaris* had a very complicated taxonomic and nomenclatural history. A recent review (Baldini, 1993) based on the centres of differentiation, recognised two groups: the first include 13 “old world” species and the second 8 “new-world” species.

Worldwide the genus occurs in areas with a Mediterranean climate, except *P. arundinacea* which has a wider range of diffusion being found in wet habitats (Baldini, 1995).

From the ecological point of view, the species produces a bulk of useful pasture forage but it is a major weed of winter cropping systems, particularly on heavy soils (Viggiani and Angelini, 1993), it emerges mainly during autumn-early winter period: Gasparetto et al. (2003) showed that more than 95% of *P. paradoxa* emerged within two months after seedbed preparation. It is a medium to highly competitive species in winter crops, especially durum wheat. It is able to set seed before most crops are ready for harvest. The species is mainly self-pollinated with a rate of out-crossing estimated around 8%, but unfortunately specific studies are not available in the literature.

In Italian wheat crops *P. paradoxa* is mainly distributed in the centre and south (Viggiani, 2005).

Newly harvested *P. paradoxa* seeds exhibit at least 50% germinability. *Phalaris* spp. form a persistent soil seed bank, emerging from shallow depths (<3 cm depth), and the annual germination and establishment of seedlings has been recorded to be between 8 and 11% of the seed bank.

1.7.4 ACCase-inhibitors resistant *Phalaris paradoxa*

Worldwide target-site resistant *P. paradoxa* biotypes have been reported, in Mexico (~400-4000 ha), Australia (~30-150 ha), Italy (~2000 ha) and Israel (~200-400 ha). No ALS resistant *P. paradoxa* biotypes are reported so far (Heap, 2008). Among *Phalaris* species the world situation concern largely *P. minor* Retz. (littleseed canary grass) resistant to isoproturon, especially in India (~400000-800000 ha; Heap, 2008), *P. minor* and *P. paradoxa* against diclofop (Mexico) (Malik et al., 1996) and triazine resistant *P. minor* biotypes have been identified in Israel (Yaacoby et al., 1985), South Africa multiple resistant.

The objective of the research on this species was to characterise populations resistant to ACCase-inhibitors through dose response experiments and infer the resistance mechanism to the new marketed herbicide pinoxaden. From the observation of the screening result of 85 *P. paradoxa* Italian populations suspected to be resistant, assessed from 1998 to 2007, the main difficulties in controlling were from diclofop-methyl (Illoxan),

clodinafop-propargyl (Topik) and tralkoxydim (Grasp). Seventeen populations result to be resistant to APPs, 11 to CHDs and 4 to the DEN. This study investigates the resistance status of seven Italian *P. paradoxa* biotypes characterised with two outdoor dose-response experiments. The biotypes were collected from southern and central Italian fields where inadequate levels of control of the species was observed in the 2000 and 2003 growing seasons following an extensive history of repeated ACCase application. The aims of the research were to understand patterns and level of resistance to ACCase inhibitors and particularly if any mutation in the ACCase enzyme was causing resistance to pinoxaden. The populations inadequately controlled by pinoxaden have been sequenced to detect if any amino acid substitution was present.

1.8 Aims of the research

In this research ACCase-inhibitors resistant *Lolium* spp. and *P. paradoxa* were characterised.

The aims of the research were:

1. to monitor of the resistant situation throughout the country coming from complaint monitoring;
2. to develop a cheap, fast and reliable seed bioassay for detecting ACCase-inhibitors resistance to be used as routine screening;
3. to characterise at molecular and physiological level resistance to ACCase-inhibitors (with emphasis to clethodim and pinoxaden) in *Lolium* spp., developing molecular marker for rapid detection of mutations affecting ACCase;
4. to characterise at whole plant level ACCase-inhibitors resistant P. paradoxa and at molecular level resistance to pinoxaden.

2. MATERIALS AND METHODS

Plant material analysed belongs to *Lolium* spp. - it consists of populations of *L. rigidum*, *L. multiflorum* and hybrids - and *Phalaris* spp. - which are mainly *P. paradoxa* and a population of *P. brachystachys*. The word biotype is referred as a group of organism having the same genotype or more precisely is a group of plants within a species that has biological traits that are not common to the population as a whole. Population consist of all the organisms that constitute a specific group or occur in a specified habitat. In this research group samples coming from a specific field are referred as population, which usually consist of a group of individuals shifting from resistant to susceptible genotypes. The field has a own history derived from the strategies that have been adopted (i.e. his treatment, crop rotation) and the provenience from a farm is the criteria to identify a population. When fields of a same farm have different histories the samples are ascribed to different populations.

2.1 Screening

Seed samples (41) of *Lolium* spp. and *P. paradoxa* (17) were collected during three years (2004-2006) from fields, mainly cultivated with durum wheat crops, where poor control of the three weeds was reported to agrochemical industry. Seeds were collected from plants that had survived a herbicide treatment. Available information on historical records of herbicide use and other agronomic techniques used in the sampled fields were collected from the farmers.

All experiments have been done at Legnaro (PD, 45° 21' N, 11° 58' E) in the greenhouse of the Institute of Agro-environmental and Forest Biology (IBAF – CNR). Samples of each population were cleaned from chaff and preserved in double paper bags at room temperature in a low humidity environment (Beckie et al., 2000). The susceptible *Lolium* spp. population used was 04204L, which is the reference which has been used in the last six years. It was not possible to find a susceptible population of *P. paradoxa* from untreated areas so a susceptible check from Herbiseed (No. 9527 in the 1999-2000 catalogue, CNR-IBAF code 0041) was purchased and included in the experiments. It was necessary to reproduce (0441L) the original stock (0041) because of reduced vitality of

seeds in 2004. To break dormancy, seeds of *Lolium* spp. and *P. paradoxa* were vernalised in a fridge at 4 °C in petri dishes on wet filter paper in dark conditions for 3 and for 7 days, respectively. They were then placed in other petri dishes on 0.6 % (wt/V) agar with the addition of 0.2 % KNO_3 in the case of *P. paradoxa* to improve germination and placed in a germination cabinet [temperature (day/night) 25/15 °C, 12 hour photoperiod with neon tubes providing a Photosynthetic Photon Flux Density (PPFD) of 15-30 µmol m^{-2} s^{-1}]. Germinated seedlings of similar growth stage were transplanted into plastic trays (325 x 265 x 95 mm) filled with a standard potting mix (60 % silty loam soil, 15 % sand, 15 % perlite, 10 % peat). Thirty *Lolium* spp. and 24 *P. paradoxa* seedlings were transplanted into each tray at uniform density. Plants were treated at two-three leaves, corresponding to growth stage 12-13 of the Extended BBCH Scale (Hess et al., 1997).

The experimental layout was a completely randomised design with two replicates of one tray for each population.

Upon reaching the 2 to 3 leaf stage, herbicides were applied as commercial formulations in a precision bench sprayer delivering 300 L/ha, at a pressure of 215 kPa, and a speed of 0.75 m/s, with a boom equipped with three flat-fan (extended range) hydraulic nozzles (TeeJet, 11002) with recommended surfactants. Populations were screened at the recommended field dose (1x) and three times that (3x) with the following herbicides: clodinafop-propargyl, pinoxaden, sethoxydim and a mixture of mesosulfuron and iodosulfuron (5:1), see table IV for details. Sethoxydim when used in a greenhouse is more effective than outside (Beckie et al., 2000), therefore the herbicide dose was defined through specific greenhouse dose-response experiments on susceptible populations which allowed the determination of the dose discriminating between S and R populations (Sattin et al., unpublished data).

Table IV. Herbicides, dose of active ingredient used (1x as field dose) and adjuvant.

Herbicides	**1x dose (g a.i. ha^{-1})**	**Adjuvant**
clodinafop-propargyl 22.2% (240 g L^{-1}; 0.25 L ha^{-1})	60	-
sethoxydim 20% (185 g L^{-1}; 0.8 L ha^{-1}) used with *P. paradoxa**	148	Trend 0.5%
sethoxydim 20% (185 g L^{-1}; 0.4 L ha^{-1}) used with *Lolium* spp.*	74	Trend 0.5%
pinoxaden (100 g L^{-1}; 0.3 L ha^{-1})	30	A12127R 0,5%
mesosulfuron:iodosulfuron (5:1) (15 g ha^{-1} p.a.; 500 g ha^{-1})	10	Biopower (1 L/ha)

* Sethoxydim dose is not the field dose, see explanation in the text.

Survival and visual estimate of biomass (VEB) in relation to the untreated check were recorded around 21 days after ACCase-inhibitors herbicide treatment and around 28 days after when an ALS-inhibitor was used. 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. Standard error was calculated per each mean value.

Most screening tests were done during autumn/winter/spring, so light was supplemented using 400 W metal-halide lamps, which provided a Photosynthetic Photon Flux Density (PPFD) of about 150 µmol m^{-2} s^{-1} and a 14-hour photoperiod. The temperature varied between 8 and 20 °C and 20 to 35 °C night/day, respectively.

2.2 Petri dish seed quick-test

2.2.1 Plant material

Four resistant *Lolium* spp. populations (05157, 04256, 04258 and 04259) as well as two Australian resistant references (AUS93 and AUS97) were chosen to set up a rapid test in agar for discriminating between resistant and not resistant populations (Table V). A susceptible check was also included: population 04 204L, which is the same used in traditional pot screenings. Among the resistant populations, target-site resistance involved the following substitutions: Ile1781Leu (population 05157), Asp2078Gly (04256) and Cys2088Arg (04259). A metabolic resistant population was included (AUS97, Kaundun, personal communication). Ten populations collected in 2006 and survived at field treatments in wheat crops were used to validate the quick test (Table V). Experiments comparing technical grades with commercial products have also been conducted with this species.

Two target-site resistant and a slightly resistant *P. paradoxa* populations were included in the set up of the quick test (Table VI). The lack of seeds and low and prolonged germination in almost all the populations forced us to use few populations to validate the test. Susceptible check was the population 0441L which is the same used in traditional pot screenings. Two resistant target site populations have a point mutation either in position 1781 and 2078. It is worth of note that population 0478L has a substitution in position

1781 of a Ile to a Val instead of the most common Ile to Leu and population 0025 has a substitution at 2078 is of a Cys to an Arg.

To break seed dormancy all *Lolium* spp. seeds were vernalised in Petri dishes with filter paper saturated with distilled water and placed in a fridge at 4 °C for three days. A 0.2% KNO_3 water solution was used for vernalising *P. paradoxa* and the seeds were left in the fridge for 7 days.

2.2.2 Seedling bioassay

The test has been developed using clodinafop, pinoxaden, clethodim and sethoxydim for *Lolium* spp., clodinafop, pinoxaden and clethodim for *P. paradoxa* (Table VII). Quick-test experiments were conducted using petri dishes 90 mm diameter. Herbicides were first dissolved in water to obtain a stock solution 10,000 µM, hence diluted to other desired concentration to be mixed in agar (0.6%). Care was taken to operate at a temperature around 42 C. Higher temperatures would denature the herbicides while lower temperatures would solidify the agar solution.

Two replicates of 50 seeds for each herbicide and concentration were placed in petri dishes and incubated for 7 days in an incubator with 12 hours photoperiod at 25 C and a 12 hours dark period at 15 C. Firstly two commercial products containing clodinafop or pinoxaden were compared with the technical grades to assess if differences in mortality or shoot length were detectable between the two herbicide forms. Secondly, using only commercial products, a broad range of herbicide concentrations (0, 0.01, 0.1, 1, 10 and 100 µM) was explored to roughly determine the range of herbicide concentrations where to focalise attention. Thirdly a narrower range of herbicide concentrations was used to determine the most suitable concentration to discriminate between susceptible and resistant biotypes. Finally, the fourth step was to identify a quick-test herbicide concentration comparable to the recommended field dose used in pot screenings. Populations chosen for the set up of the quick-test have also been screened in pots, at field dose and three times that using two replicates. Pot screening experiment has been repeated twice. To validate the quick test, other populations - as well as one susceptible and three resistant references - have been tested in agar with herbicide at the chosen "comparing" concentration, using three replicates of 50 seeds each and experiment has

been repeated twice. Results have been compared with pot screenings, at recommended field dose, two replicates and experiment has been repeated twice. Unfortunately in the case of *P. paradoxa*, the lack of seeds, allowed the validation step using just three more populations plus the populations included in the set up.

Experiments were completed during the winter season in greenhouse. Growth stage at spraying was uniform as plants in other conditions then at 2-3 leaf stage they were removed from trays.

Table V. Code, site of origin, resistant status of the populations included in quick-test set up and validation experiments.

Population	Site of origin	Resistant status from previous pot experiments	Set-up	Validation
04204L	Legnaro (PD)	Susceptible check	x	x
04256	Trinitapoli (FG)	Resistant to clodinafop, tralkoxydim, sethoxydim 1x and iodosulfuron, substitutions Ile1781Leu, Ile2041Asn/Val, Asp2078Gly and Gly2096Ala	x	x
04258	Torre San Severo (TR)	Resistant to clodinafop and sethoxydim 1x	x	
04259	S.Caterina Cortona (AR)	Highly resistant to clodinafop, sethoxydim, pinoxaden and resistant to iodosulfuron 1x, substitution Cys2088Arg	x	
05157	Alberese (GR)	Highly resistant to clodinafop, tralkoxydim, pinoxaden and sethoxydim, substitution Ile1781Leu	x	x
AUS93	Australia	Target site resistance to ACCase inhibitors, substitution Ile1781Leu	x	
AUS97	Australia	Metabolic resistance to ACCase and ALS inhibitors	x	x
06289	S.Martino in Pensilis (CB)	Highly resistant to clodinafop, pinoxaden and sethoxydim, resistant to clethodim and cycloxydim		x
06290	Offagna (AN)	Highly resistant to clodinafop, resistant to sethoxydim, slightly resistant to pinoxaden		x
06298	Cerveteri (RM)	Slightly resistant to clodinafop		x
06299	Cerveteri (RM)	Highly resistant to all ACCase inhibitors tested		x
06302	Grosseto (GR)	Highly resistant to all ACCase inhibitors tested except to clethodim, substitution Ile1781Leu		x
06303	Grosseto (GR)	Slightly resistant to clodinafop		x
06305	Fauglia (PI)	Highly resistant to clodinafop, pinoxaden, cycloxydim and sethoxydim		x
06309	Montalcino Torrenieri (SI)	Highly resistant to clodinafop, pinoxaden, cycloxydim and sethoxydim, slightly resistant to clethodim		x
06313	S.Maria di Galeria (RM)	Highly resistant to clodinafop, resistant to cycloxydim and slightly resistant to pinoxaden and sethoxydim		x
06242chk	Grosseto (GR)	Resistant to clodinafop		x

Table VI. Code, site of origin, resistant status of the populations included in quick-test set up and validation experiments.

Population	Site of origin	Resistant status from pot experiments	Set-up	Validation
0441L	Legnaro (PD)	Susceptible check	x	x
0025	Manfredonia (FG)	Highly resistant to clodinafop, tralkoxydim, pinoxaden and sethoxydim; substitution Asp2078Gly	x	x
0460test	Maccarese (RM)	Slightly resistant to clodinafop 1x		x
0470	Rocchetta S Antonio (FG)	Resistant to clodinafop 1x		x
0478L	Zapponeta (FG)	Resistant to clodinafop, tralkoxydim, pinoxaden, sethoxydim 1x and iodosulfuron 1x; substitution Ile1781Val	x	x
0482	Belvedere Ostrense (AN)	Slightly resistant to clodinafop 1x		x
0692	Montemilone (PZ)	Slightly resistant to sethoxydim 1x	x	x

Table VII. Tested herbicides.

Active ingredient	Commercial formulation	Concentration a.i. (g L^{-1})	Molecular weight
clodinafop	Topik	240	349.8
sethoxydim	Fervinal	185	327.5
pinoxaden	Axial	100	400.5
clethodim	Select	240	359.9

2.3 Characterisation of *Lolium* spp.

Most of the work was done during a collaboration (between June 2006 and January 2007), with Western Australian Herbicide Resistance Initiative (WAHRI), at University of Western Australia, under the supervision of Professor S B. Powles and Dr. Qin Yu. The research has result with the publication: “Diversity of Acetyl-Coenzyme A Carboxylase Mutations in Resistant *Lolium* Populations: Evaluation Using Clethodim” - Qin Yu, Alberto Collavo, Ming-Qi Zheng, Mechelle Owen, Maurizio Sattin and Stephen B. Powles. *Plant Physiology*, October 2007, Vol. 145, pp. 547–558.

The research done at University of Western Australia, which was focused on clethodim resistance, here is supported by the research done at University of Padova and focused on pinoxaden resistance in the Italian populations.

2.3.1 "Australian experiments"

2.3.1.1 Plant material

Several *L. rigidum* populations resistant to clethodim were identified during herbicide screening in a large random survey across the Western Australian wheat belt (Owen et al., 2007). Seedlings of these field populations were sprayed with clethodim at the commercial rate of 60 g ha^{-1} using a cabinet sprayer delivering 113 L ha^{-1} water at a pressure of 200 kPa. The survivors of each population were grown to maturity and allowed to cross-pollinate only within the population. Seeds of 12 clethodim resistant populations of *L. rigidum* from Australia (H1/2, H1/10, H1/19, H1/25, H2/2, M1/23, M1/25, M2/3, M2/15, M2/19, M2/23 and M3/4, hereinafter referred to as R_1 to R_{12}, respectively) and two *Lolium* spp. populations from Italy (04259 and 05281) were used in this research. A known herbicide susceptible *L. rigidum* population (VLR, referred to as S_1) from Australia and a susceptible *Lolium* spp. population (204L, referred to as S_2) from Italy were used as controls. Seeds of resistant and susceptible populations were germinated in plastic trays containing potting soil and seedlings grown in a glasshouse at 20/15 °C day/night temperature under natural sunlight. At the two to three leaf stage, these seedlings were treated with 60 g ha^{-1} of clethodim. This rate killed all the plants in susceptible populations. Individual survivors from resistant populations were used for subsequent molecular and biochemical analysis.

2.3.1.2 ACCase mutations

2.3.1.2.1 Sequencing of the plastidic ACCase gene CT domain

Shoot material of individual survivors from resistant populations was used for DNA extraction. Bulked shoot material from two susceptible populations without herbicide treatment was used as a control. Genomic DNA was extracted from shoot tissues using a Nucleon Phytopure DNA extraction kit (Amersham Biosciences). Primers were used or designed to amplify regions in the CT domain known to be involved in sensitivity to ACCase herbicides (Délye and Michel, 2005). Plastidic ACCase sequences used for the primer design were from *L. rigidium* (GenBank accession numbers are AF359516, AY995225, AY995232, AY995233, DQ184633, DQ184640, and DQ184646), *L. multiflorum* (AY710293) and *A. myosuroides* (AJ310767). Cytosolic ACCase sequences were from *A. myosuroides* (AJ632096) and *Triticum aestivum* (U39321). Because of the high level of similarity between plastidic and cytosolic ACCase DNA sequences (about 74%), when designing primers, particular attention was given to consensus sequences of plastidic and cytosolic ACCase sequences, and each primer contained at least one specific nucleotide at the 3' end to discriminate plastidic and cytosolic sequences. The primer pair ACCF5/ACCR5 from Délye et al. (2002b) was used to amplify a 785-bp region of the plastidic ACCase gene containing codon 1781 (Table VIII). The primer pair ACCF1/ACCR1 was designed to amplify a 492-bp region containing codons 2027, 2041, 2078, 2088 and 2096 (Table VIII). The primer pair ACCF6/ACCR6 was designed to amplify a 484-bp region bridging the above mentioned two regions. The PCR was conducted in a 25 µL volume that consisted of about 300 ng of genomic DNA, 0.5 µM of each primer, and 12.5 µL of 2 x GoTaq Green Master Mix™ (Promega). The PCR was run in a Mastercycler (Eppendorf, Germany) with the following profile: 94 ^{o}C 4 min, 35 cycles of 94 ^{o}C 30 s, 62 ^{o}C 30 s, and 72 ^{o}C 30 s, followed by a final extension step of 5 min at 72 °C. The PCR product was directly purified or purified from agarose gel with Wizard™ SV Gel and PCR Clean-up System (Promega), and sequenced from both ends with the AB-Big Dye Terminator system using a commercial sequencing service. At lease six survivors from each clethodim resistant population were sequenced. All sequences were visually checked with chromatogram files, and assembled and aligned using the DNAMAN software. Heterozygous individuals were recognized by double peaks at the same position in nucleotide chromatograms of both

forward and reverse sequencing. Heterozygosity at position 2041, 2078 or 2088 was also further verified by using cleaved amplified polymorphic sequence (CAPS) or derived cleaved amplified polymorphic sequence (dCAPS) analysis (see below).

Table VIII. Primers used in the experiments. An introduced point mutation in the designed dCAPS primer is in bold; nucleotides discriminating grass plastid ACCase sequences from cytosolic sequences at the 3' end of the designed primers are underlined.

Primer	Sequence 5'- 3'	Usage	References
ACCF5	AATGGGTCGTGGGGCACTCCTATAATTCC	Gene-specific PCR	Délye et al., 2002b
ACCR5	GCTGAGCCACCTCAATATATTAGAAACACC	″	″
ACCF6	CATACAGCGTGAAGATCAGC	″	This research
ACCR6	TCCTGGATCAGCTGGGACG	″	″
ACCF1	CACAGACCATGATGCAGCTC	″	″
ACCR1	CTCCCTGGAGTTGTGCTTTC	″	″
NsiI1781f	CTGTCTGAAGAAGACTATGGCCG	dCAPS for 1781	Kaundun and Windass, 2006
NsiI1781r	AGAATACGCACTGGCAATAGCAGCACTTCCATG**C**A	″	″
EcoRV2078r	GCACTCAATGCGATCTGGATTTATCTTG**A**TA	dCAPS for 2078	This research

2.3.1.2.2 Cleaved amplified polymorphic sequence (CAPS) analysis

The nucleotide T to A mutation at codon 2041 in the plastidic ACCase gene, causing an amino acid Ile to Asn change, removes an *EcoRI* restriction site (Table IX). Sequence results showed no other single nucleotide polymorphisms (SNPs) around the restriction site. Thus, the primer pair ACCF1/ACCR1 (Table VIII) has been used to amplify a 492-bp fragment followed by *EcoRI* digestion at 37°C for 3 h (all restriction enzymes were obtained from Fermentas Life Science). Homozygous resistant plants with two mutant 2041-Asn alleles would display a single undigested band of 492 bp. In contrast, homozygous susceptible plants with two 2041Ile alleles would have two resolvable bands of 208 and 282 bp. Heterozygous plants with both 2041Asn and 2041Ile alleles would have all three bands.

The nucleotide T to C mutation at codon 2088, causing an amino acid Cys to Arg substitution, creates an *Eco47III* restriction site (Table IX). Sequence results revealed no other SNPs around this restriction site. Therefore, the same primer pair ACCF1/ACCR1 (Table VIII) was used to amplify a 492-bp fragment followed by *Eco47III* digestion. Homozygous susceptible plants with two 2088Cys alleles would display a single undigested band of 492 bp. Homozygous resistant plants with two mutant 2088Arg alleles would have two resolvable bands of 141 and 351 bp, and heterozygous plants with both wild type and mutant alleles would have all three bands.

Table 8. Restriction enzymes used in (derived) cleaved amplified polymorphic sequence (dCAPS) analysis

Enzyme	**Commercial isoschizomers**	**Restriction site**	**Experiment**	**Reference**
NsiI	*AvaIII, EcoT22I, Mph11031, Zsp2I*	5‘-ATGCA^T-3’ 3‘-T^ACGTA-5’	dCAPS (1781)	Kaundun and Windass, 2006
EcoRI	*FunII*	5‘-G^AATTC-3’ 3‘-CTTAA^G-5’	CAPS (2041)	This work
EcoRV	*Eco32I*	5‘-GAT^ATC-3’ 3‘-CTA^TAG-5’	dCAPS (2078)	This work
Eco47III	*AfeI, Aor51HI, FunI*	5‘-AGC^GCT-3’ 3‘-TCG^CGA-5’	CAPS (2088)	This work

2.3.1.2.3 Derived cleaved amplified polymorphic sequence (dCAPS) analysis

A dCAPS marker for the 2078 mutation (Asp to Gly) was developed in this research to facilitate rapid and accurate identification of mutant 2078Gly alleles. A 31-bp reversed dCAPS primer EcoRV2078r was designed (Table VIII) using the dCAPS Finder 2.0 software (Neff et al., 1998) based on highly conserved sequences around and especially towards the 3’ end of the 2078 codon of all sequenced plants. An A:G mismatch was introduced in the reverse primer to create a restriction site for *EcoRV* in the susceptible sequence (Table VIII). The primer pair ACCF1/EcoRV2078r (Table VIII) amplifies a 353-bp fragment using

the same PCR conditions as for sequencing. Following *EcoRV* digestion, individuals with homozygous resistant 2078Gly alleles would have an uncut band of 353 bp, while individuals with homozygous susceptible 2078-Asp alleles would have a digested band of 323 bp (Figure 7). Individuals with both susceptible and resistant alleles would have a combination of two resolvable bands (Figure 7).

The published dCAPS marker for the 1781 mutation (Ile to Leu) (Kaundun and Windass, 2005) was used with primer pair NsiI1781f/NsiI1781r (Table VIII) with modified PCR conditions as described for sequencing.

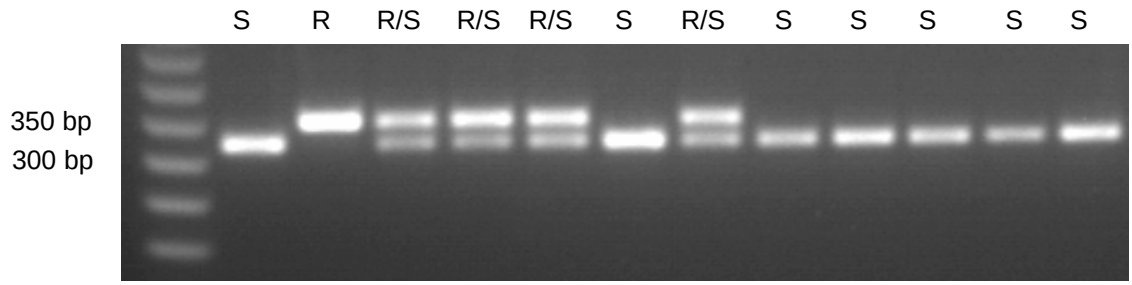

Figure 7. Derived cleaved amplified polymorphic sequence (dCAPS) analysis of individual *L. rigidum* plants homozygous susceptible 2078Asp (S), homozygous resistant 2078Gly (R) or heterozygous 2078Gly/Asp (R/S). The sizes of restriction enzyme (*EcoRV*) digested fragments are 353 bp and 323 bp, respectively.

2.3.1.3 *In vitro* inhibition of ACCase activity by ACCase herbicides

Individual clethodim survivors containing two mutant 1781, 2078, or 2088 alleles, and individual survivors containing two types of mutant alleles (1781Leu/2027Cys, or 1781Leu/ 2041Asn) were identified by marker analyses and sequencing. These plants were transplanted, fertilized and maintained in a glasshouse at 20/15°C day/night temperature. Shoot tissue of each genotype was harvested, snap-frozen in liquid nitrogen and immediately used for the enzyme assay. Herbicide susceptible plants from population S_1 or S_2 at the same developmental stage were used as controls. ACCase extraction and partial purification, and enzyme inhibition by ACCase herbicides, were performed as described

(Yu et al., 2004). Two sub-samples from each extraction were assayed, and there were at least two extractions per population per herbicide treatment.

2.3.1.4 Response of purified resistant populations to ACCase herbicides at the whole plant level

Three purified populations were obtained by bulk cross pollinating at least six plants homozygous for the mutant 1781, 2078, or 2088 alleles. Mutant 1781, 2078 or 2088 alleles were therefore purified and fixed in three sub-populations R_7P, $R_{12}P$ and 05281P, respectively. Seeds of purified populations were germinated on 0.6% agar-solidified water for 7 days. Germinated seedlings were transplanted to plastic pots (20 to 25 seedlings per pot) or trays (40 to 50 seedlings per tray) containing potting mix and were kept in naturally illuminated glasshouses at 25/15 ^{o}C day/night temperature. Seedlings in pots were treated at the two to three leaf stage with rates of clethodim (0, 0.94, 1.88, 3.75, 7.5 and 15 g ha^{-1} for the susceptible population S_1; 0, 15, 30, 60, 120, 240 and 480 g ha^{-1} for purified resistant populations) using a cabinet sprayer and each treatment contained three replicates. Seedlings in trays were sprayed with a number of other ACCase herbicides at a rate known to control susceptible plants (see Table X). Herbicides were applied as commercial formulations plus adjuvant as required, using a cabinet sprayer. Plants were returned to the glasshouse after treatment, and the mortality was recorded 21 d after herbicide application. Plants were recorded as alive if they had strongly tillered since herbicide application.

2.3.1.5 Statistical analysis

The herbicide concentration causing 50% inhibition of enzyme activity (I_{50}), or the herbicide rate causing 50% mortality (LD_{50}), was estimated by non-linear regression using the logistic model (Seefeldt et al, 1995):

$$y = C + \frac{D - C}{1 + (x / \mathrm{ED}_{50})^{b}}$$

where C = lower limit, D = upper limit, ED_{50} = dose giving 50% response and b = slope around ED_{50}. Estimates were obtained using the Sigmaplot™ software (version 8.02, SPSS Inc. 233 South Wacker Drive, Chicago, IL). A t-test (*P* = 0.05) was used to test significance of the regression parameters. Analysis of variance was performed by ANOVA and significant differences in ACCase specific activities among genotypes in the absence of inhibitor herbicides were determined by the LSD test.

Table X. Percentage survival of plants from purified homozygous resistant populations for the three ACCase mutations at herbicide application rates known to control the susceptible population S_1. Forty to 50 plants per population were treated with the respective herbicide.

Herbicides	Application rate (g ha^{-1})	Genotype (Population), % survival			
		Wild type (S_1)	1781Leu (R_7P)	2078Gly ($R_{12}P$)	2088Arg (05281P)
APP					
Diclofop	1000	1	100	100	100
Clodinafop	50	3	100	100	100
Fluazifop	100	0	100	100	100
Haloxyfop	52	0	100	100	100
CHD					
Sethoxydim	186	0	100	100	100
Tralkoxydim	800	4	100	100	100
Butroxydim	45	2	50	87	70
Phenylpyrazolin					
Pinoxaden	30	0	100	100	100

2.3.2 "Italian experiments"

2.3.2.1 Plant material

The populations investigated in Italy were 05256, 05281 and 06302. They have been found resistant to pinoxaden through the classic screening in pot during 2006 and 2007. Plants for the experiment have been selected through the quick test developed in these

thesis work (for methodology see point 2.2 above) using pinoxaden at the discriminating concentration of 0.1 µM. The selected concentration discriminates between resistant and susceptible and it is more sensitive than the field dose comparing concentration. Survivors have been transplanted in trays with soil (40% silty loam soil, 10% perlite, 50% peat).

2.3.2.2 ACCase mutations

2.3.2.2.1 Sequencing of the plastidic ACCase gene CT domain

At the stadium of 2-3 leaves samples of around 0.1 g for each plant have been collected and DNA has been extracted as reported above at point 2.3.3.

CT domain of ACCase gene has been amplified with the primers reported in Table 56. The PCR was conducted in a 25 µL volume that consisted of PureTaq™ Ready-To-Go™ PCR beads, 0.2 µL of each primer (100 µM) of each primer, 0.2 µL of genomic DNA (25 ng/µL), sterile water to final volume.

The PCR was run in a Thermocycler T1 (Biometra) with the following profile: 95 ^{o}C 5 min, 40 cycles of 95 ^{o}C 30 s, 60 ^{o}C 30 s and 72 ^{o}C 75 s, followed by a final extension step of 10 min at 72 °C. The PCR product was purified from agarose gel with Montage™ DNA Gel Extraction kit (Millipore). Sequencing has been conducted at CRIBI BMR-genomics centre at University of Padova (Italy) using the AB-BigDye Terminator™ system and sequences have been analysed through Chromas™ software. Samples of each populations have been investigated using CAPS and dCAPS described at point 2.4.2.2.2-3; those samples showing none or uncertain mutation have been sequenced using the primers reported in Table XI.

2.3.2.2.2 Cleaved amplified polymorphic sequence (CAPS) analysis

The nucleotide T to A mutation at codon 2041 in the plastidic ACCase gene, causing an amino acid Ile to Asn change, removes an *EcoRI* restriction site. Primers LOL FOR 2027/LOL REV CT (Table XI) have been used to amplify a region of 420 bp followed by a *EcoRI* (Promega, 12 U mL^{-1}) digestion at 37 °C for 1.25 hours. PCR conditions had the following profile: 95 ^{o}C 5 min, 35 cycles of 95 ^{o}C 30 s, 62 ^{o}C 30 s and 72 ^{o}C 40 s, followed

by a final extension step of 10 min at 72 °C. Homozygous resistant plants with two mutant 2041Asn alleles would display a single undigested band of 420 bp. In contrast, homozygous susceptible plants with two 2041Ile alleles would have two resolvable bands of 170 and 250 bp. Heterozygous plants with both 2041Asn and 2041Ile alleles would have all three bands (Figure 8). The program used to find the primer was *dCAPS Finder Software* (Neff et al., 1998).

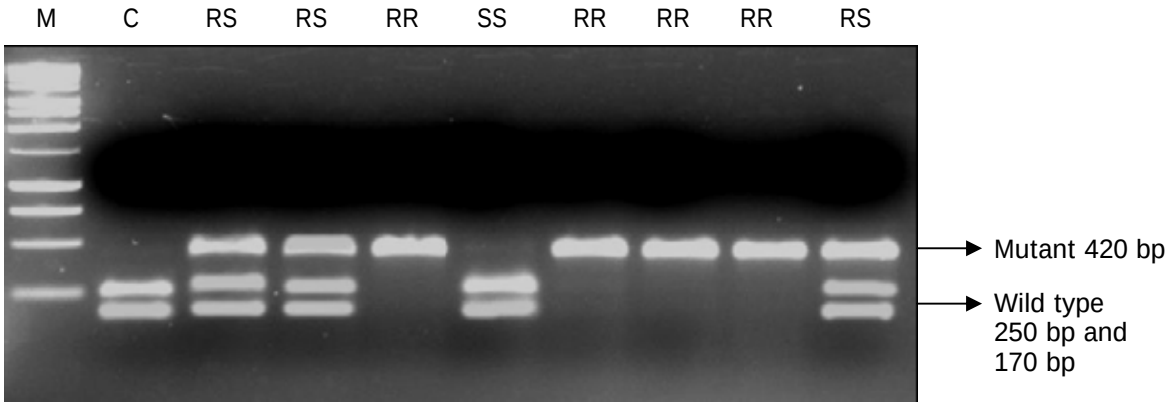

Figure 8. Cleaved amplified polymorphic sequence (CAPS) analysis of individual *Lolium* spp. plants homozygous susceptible 2041-Ile (S), homozygous resistant or heterozygous. The sizes of restriction enzyme (*EcoRI*) digested fragments (wild type alleles) are 170 bp and 250 bp, undigested (mutant alleles) 420 bp. (M, molecular marker 25 bp step; C, homozygous resistant check control; RR, resistant homozygous; RS, heterozygous resistant; SS, homozygous susceptible).

2.3.2.2.3 Derived cleaved amplified polymorphic sequence (dCAPS) analysis

The published dCAPS marker for the 1781 mutation (Ile to Leu) (Kaundun and Windass, 2005) was used with primer pair LOL FOR/REV 1781 (Table XI). Primers have been used to amplify a region of 165 bp followed by a *NsiI* (Promega, 10 U mL^{-1}) digestion at 37 °C for 1 hour. Reverse primer introduce a restriction site for *NsiI* in the wild type sequence, which is recognised by a band of 130 bp. Homozygous mutant will display an undigested band of 165 bp (Figure 9). PCR conditions had the following profile: 95 ^{o}C 5 min, 35 cycles of 95 ^{o}C 30 s, 64 ^{o}C 30 s and 72 ^{o}C 60 s, followed by a final extension step of 10 min at 72 °C.

A dCAPS marker for the 2078 mutation (Asp to Gly) was developed in this research to facilitate rapid and accurate identification of mutant 2078-Gly alleles. Primers used were MUT2 FOR/REV 2078 (Table XI) with the same PCR conditions applied for dCAPS marker for the 1781 mutation followed by a *NsiI* digestion at 37 °C for 1.25 hours. Reverse primer introduce a restriction site for *NsiI* in the mutant sequence, which is recognised by a band of 145 bp. Wild type will display an undigested band of 180 bp (Figure 10).

The program used to find the primer was *dCAPS Finder Software* (Neff et al., 1998).

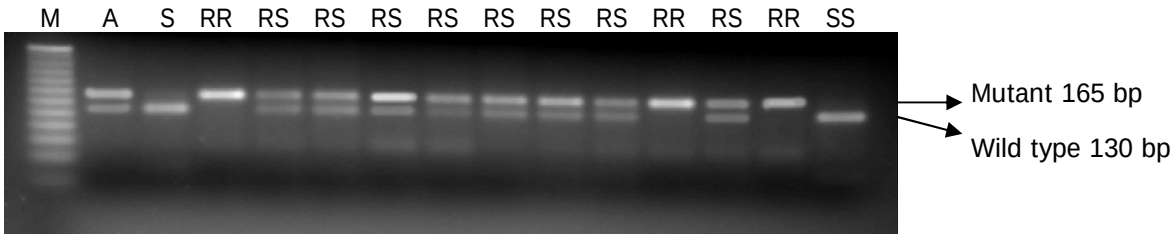

Figure 9. Derived cleaved amplified polymorphic sequence (dCAPS) analysis of individual *Lolium* spp. plants homozygous susceptible 1781-Ile (S), homozygous resistant or heterozygous. The sizes of restriction enzyme (*NsiI*) digested fragments (wild type alleles) is 130 bp and undigested fragment (mutant alleles) is 165 bp. (M, molecular marker 25 bp step; A, heterozygous check control (from population AUS93); S, susceptible check control (population 04204L); RR, resistant homozygous; RS, heterozygous resistant; SS, homozygous susceptible).

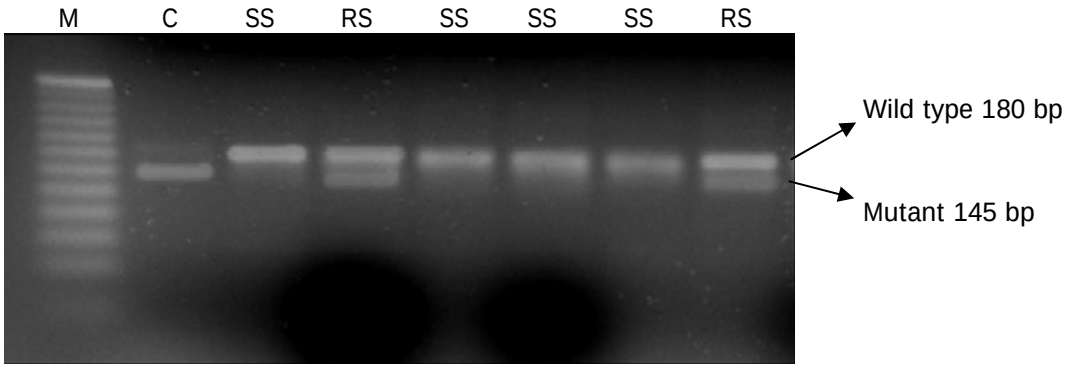

Figure 10. Derived cleaved amplified polymorphic sequence (dCAPS) analysis of individual *Lolium* spp. plants homozygous susceptible 2078 (S), homozygous resistant or heterozygous. The sizes of restriction enzyme (*NsiI*) digested fragments (mutant alleles) is 145 bp and undigested fragment (wild type alleles) is 180 bp. (M, molecular marker 25 bp step; C, homozygous resistant check control; RR, resistant homozygous; RS, heterozygous resistant; SS, homozygous susceptible).

Table XI. Primers used in the experiments.

Primer	Sequence 5'- 3'	Usage	References
LOL FOR	CTGTCTGAAGAAGACTATGGCCG	Gene-specific PCR and dCAPS for 1781	This research
LOL REV CT	ATGCATGGGTAGGCTTGATCCAG	Gene-specific PCR and CAPS for 2041	″
LOL FOR 2027	CAGCCTGATTCCCATGAGCGGTC	CAPS for 2041	″
REV 1781	AGAATACGCACTGGCAATAGCAGCACTTCCATG**C**A	dCAPS for 1781	Kaundun and Windass, 2006
MUT 2 FOR	TTCTCTGGTGGGCAAAGAGACC	dCAPS for 2078	This research
REV 2078	CATAGCACTCAATGCGATCTGGATTTATCTTG**G**TA	dCAPS for 2078	″

2.4 Characterisation of *Phalaris paradoxa*

There is little information available in the literature on patterns and levels of resistance of *P. paradoxa*. Therefore, two outdoor dose-response experiments were done followed by laboratory experiments aiming at elucidating the molecular bases of the resistance to ACCase inhibitors.

2.4.1 Plant material

The seed collections were made from surviving plants where fluoazifop-P (Fusilade), haloxyfop (Gallant), diclofop-methyl (Illoxan), clodinafop-propargyl (Topik), fenoxaprop-P (Proper Energy) had been repeatedly used (between 2 and 10 treatments). These biotypes were selected according to preliminary information on multiple and/or cross resistance and level of resistance produced by routine screenings. It was not possible to find a susceptible

population from untreated areas so were used the same susceptible checks included in pot screenings (0041 and 0441L).

Information on the past usage of herbicides was also collected from farmers (Table XII). Seed samples were cleaned, stored in double paper bags and kept in a dry environment at ambient temperature. Population 0477L and 0478L have been reproduced because of lack of enough seed after routine screening and to preserve seed for later studies.

Table XII. Origin of and herbicide history for each population (note that more than one treatment could have been done).

BIOTYPES	ORIGIN	HERBICIDES				
		diclofop-methyl	fenoxaprop-P-ethyl	clodinafop-propargyl	haloxyfop-P-methyl	fluazifop-P-butyl
0041 and 0441L*	Herbiseed - England	never treated with herbicides				
0025	Manfredonia (FG)	1		4	3	2
0034	Maccarese (RM)	3	3			
0044	Maccarese (RM)	4	3			
0047	Maccarese (RM)	6	4			
0059	S.Fetucchio (PG)	10				
0477L	S.Caterina di Cortona (AR)			2		
0478L	Zapponeta (FG)			2	1	1

* Population 0441L has been reproduced from the original stock 00 41.

2.4.2 Dose-response experiments

Seeds were vernalised in a fridge for 7 days at 4 °C to break dormancy, they were then put in Petri dishes containing 0.6% (wt/V) agar and 0.2% KNO_3 and moved to a germination cabinet [temperature (day/night), 25/15 °C, 12 hour photoperiod]. Germinated seedlings of similar growth stage were transplanted in pots of 16 cm diameter with a standard potting mix (60% silty loam soil, 15% sand, 15% perlite, 10% peat). Four

seedlings were transplanted into each pot at uniform density. Plants were grown outside with the soil maintained at or near field capacity.

Upon reaching the 2 to 3 leaf stage, herbicides were applied as commercial formulations with a precision bench sprayer delivering 300 Lha^{-1}, at a pressure of 215 kPa, and a speed of 0.75 ms^{-1}, with a boom equipped with three flat-fan (extended range) hydraulic nozzles (TeeJet, 11002) with recommended surfactants. Herbicides and doses used are reported in Table XIII.

Table XIII. Herbicides and range of doses used (1x as field dose).

Herbicides	**1x dose (g a.i. ha^{-1})**	**Range (g a.i. ha^{-1})**
clodinafop-propargyl 22.2% (240 g/L)	60	1/8x (7.5) - 32x (1920)
fenoxaprop-P-ethyl (55 g/L)	66	1/8x (8.25) - 32x (2112)
diclofop-methyl (284 g/L)	710	1/8x (88.75) - 32x (22720)
tralkoxydim 22.5% (250 g/L)	425	1/8x (53.125) - 32x (13600)
sethoxydim 20% (185 g/L)	185	1/8x (23.125) - 32x (5920)
pinoxaden (100 g/L)	30	1/32x (0.9375) - 16x (480)
iodosulfuron (50 g/Kg)	10	1/8x (1.25) - 4x (40)
chlorsulfuron (75 g/L)	15	1/4x (3.75) - 2x (30)
imazamethabenz (300 g/L)	576	1/8x (72) - 4x (2304)
isoproturon 50% (500 g/L)	1000	1/16x (62.5) - 3x (3000)

The dose range of herbicide for each population was chosen on the basis of preliminary screenings. All doses were calculated in a range using a geometric progression, with doses ranging from $^{1}/_{32}$ to 32 times the recommended field dose (indicated as 1x dose).

Given the large no. of treatments involved and the need to test new herbicides and populations, two experiments were done. In the first experiment populations 0041, 0025, 0034, 0044, 0047 and 0059 have been treated with clodinafop, diclofop, fenoxaprop, tralkoxydim, sethoxydim, iodosulfuron and chlorsulfuron. In the second experiment clodinafop, tralkoxydim, sethoxydim and iodosulfuron have been tested on populations 0441L, 0477L and 0478L, while pinoxaden and isoproturon were used on populations 0441L, 0477L, 0478L, 0025 and 0044 and imazamethabenz on populations 0441L and 0477L. Sethoxydim was included to infer the mechanism of resistance as it is not known

yet any biotype able of metabolic detoxification, consequently resistance to sethoxydim is likely to be due to an altered target-site. Isoproturon (PSII inhibitor) was chosen because of its different mode of action and it had never been used on these populations. Imazamethabenz was tested only on population 0477L because it was suspected to be resistant to ALS inhibitors.

The number of surviving plants and fresh weight was recorded 18 days after ACCase inhibitors herbicides application and 21 days after ALS- and PSII-inhibitors application. Plants were assessed to be dead if they showed no active growth: the fresh weight of all plants assessed as alive and weight of residual death plant material were then recorded.

Each experiment had three replicates with three pots per replicate. The experimental design was a complete random block design. The mean survival and fresh weight for each treatment was expressed as a percentage of the untreated control treatments. The ED_{50}, GR_{50}, for the mean percent survival and fresh weight respectively, were calculated using non linear regression analysis. Statistical analysis has been carried out using the macro BIOASSAY® developed by Onofri (2004) and running under Windows Excel™ . The macro is based on a log-logisitic equation to fit the data (Seefeldt *et al*., 1995):

$$y = C + \frac{(D - C)}{(1 + (x / \mathrm{I}_{50})^{\mathrm{b}}}$$

Y is the fresh weight or survival, *C* and *D* are the lower and upper asymptotes at higher and zero doses respectively, I_{50} is the dose resulting in a 50% reduction in plant biomass or survival, *b* is the slope.

The upper and lower asymptotes were forced through the mean of the untreated plants and zero respectively. Resistance indexes were calculated by comparing the ED_{50}, GR_{50}, of each of the biotypes to the susceptible biotype (0041 or 0441L).

2.4.3 Molecular analysis

Shoot material (0.1 g) for DNA extraction was collected 3 weeks after treatment of the plants with pinoxaden (30 g a.i. ha^{-1}). For the susceptible samples, extractions of bulks of untreated plants were performed. Genomic DNA was extracted using CTAB method (Doyle and Doyle, 1987). A portion of plant leaf of 1.5 cm long has been collected, frozen in liquid

nitrogen and grinded using an electric drill equipped with plastic pestle. Then in each tube has been added 600 µL CTAB, therefore placed in soaking bath at 60°C for 30 min. After incubation 600 µL chloroform: isoamyl alcohol (24:1 v/v) has been added and centrifuged for 15 min at 10,000 rpm at room temperature. The upper DNA containing phase has been transferred in a new tube containing 1.2 µL RNase A solution (4 mg mL^{-1}) and placed at 37 °C for 30 min. DNA has been precipitated using 2/3 of the volume of cold isopropanol, then centrifuged at 4 °C, 11,000 rpm for 20 min. After discarding the supernatant, pellet has been washed with 70% ethanol, centrifuged at 4 °C, 11,000 rpm for 5 min and discarded the supernatant again. The air-dried DNA pellets have therefore resuspended with 10 µL of sterile water. Primers (Table XIV) to amplify the region of the ACCase CT domain (Figure 11), where the known mutation conferring resistance to ACCase inhibitors have been already reported, have been designed aligning ACCase sequences of *P. minor* (GenBank accession number: AY196480 and AY196481) and *L. rigidum* (AF359516, AY995225, AY995232 and AY995233). The combination of primer Pha-For/Pha-Rev-CT was used to amplify a DNA fragment of 1160 bp encompassing the CT domain of the ACCase gene from susceptible and both resistant populations (0025 and 0478L). The amplified genomic fragments of the expected size were sequenced. The nucleotide sequences obtained from susceptible plants were compared with those of resistant plants. The PCR was run in a final volume of 25 µL containing 50 ng of genomic DNA, 0.1 µM of each primer, using a Whatman Biometra Termocycler T1 with the following profile: 94 °C 90 s, 35 cycles of 94 °C 30 s, 60 °C 20 s and 68 °C 75 s, followed by an extension step at 72 °C for 10 min. PCR product was purified from gel using Millipore Montage™ DNA Gel Extraction. Sixteen samples for each resistant population and 2 susceptible bulk samples have been sequenced from both ends. All sequences were visually checked with chromatogram files using the Chromas software.

Table XIV. Primers used for sequencing

Primer	Sequence (5'-3')
Pha-For	CTG ACG GAA GAA GAC TAT GGT CG
Pha-Rev	TGA TGC AGC TTG TCC CTG CTG AT

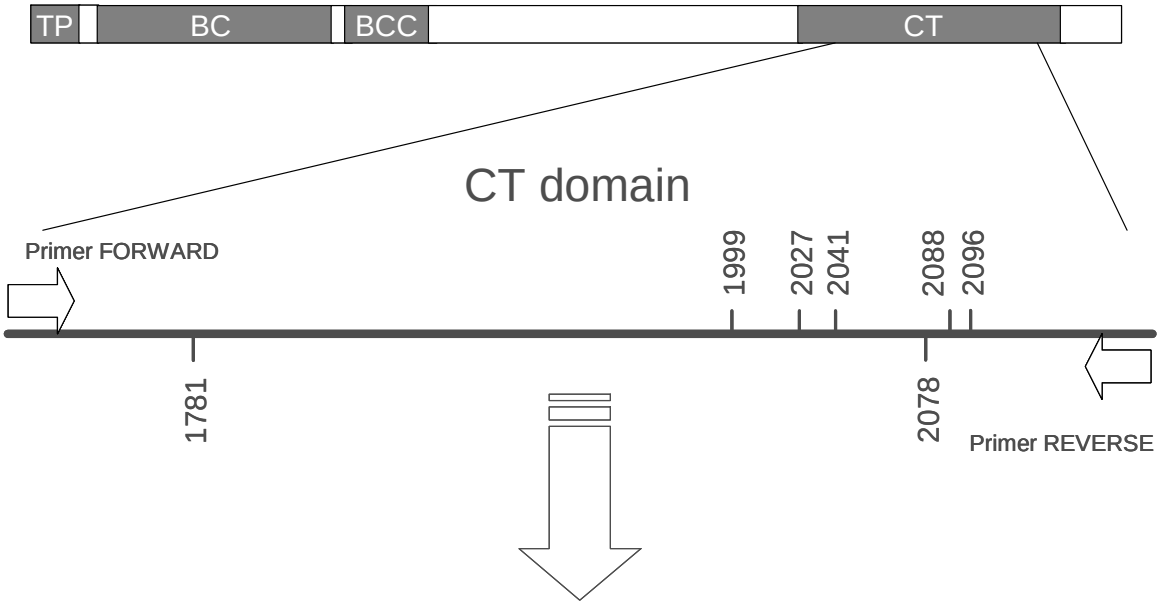

Figure 11. Representation of a plastidic homomeric acetyl coenzyme A carboxylase (ACCase) showing the three functional domains (BCC, biotin carboxyl-carrier; BC, biotin carboxylase; CT, carboxyl transferase) and the transit peptide (TP) that is absent in cytosolic ACCase. The amino acid residues critical for sensitivity to ACCase-inhibiting herbicides, displayed on CT domain, have been referenced after the sequence from *Alopecurus myosuroides* plastidic ACCase (EMBL accession AJ310767). (Modified from Délye, 2005).

3. RESULTS AND DISCUSSION

3.1 Screenings, levels and resistance patterns

3.1.1 *Lolium* spp. resistance patterns

These results do not reflect the national situation in wheat crop since the survey has not included randomly collected populations over Italy. This kind of sampling was able just to identify the worst cases where resistance was already well evolved, but probably missed most of the situations where resistance had just begun to evolve. Table XV and Figure 12 summarise the percentage of populations belonging to different resistance categories in terms of plant survival to four herbicides. Populations have been scored as S when the survivors were less than 5% of the treated plants at the field rate (1x dose), SR when the survivors were between 5 and 20% at 1x dose, R when the survivors were more than 20% at 1x dose and RR when the survivors were more than 20% survival at dose 1x and more than 10% at three time the field dose.

Clodinafop is the ACCase-inhibitor most used in Italy in wheat in the last years (commercialised since 1993) and the resistance level is always higher than other ACCase-inhibitors. The sum of clodinafop resistant populations (R+RR) is 56% of the tested populations, while pinoxaden is 37%, sethoxydim 36% but all populations are still controlled by the ALS-inhibitor although some of them showed a low level (<20% at 1x) of survival. However, considering that graminicide sulfonylureas (SUs) have been only recently introduced into the market, this may indicate that either a few plants are multiple resistant to both herbicide classes because of enhanced herbicide metabolism, regardless of these populations have been treated or not with ALS inhibitors, or the beginning of the selection process operated by SUs.

Cross-resistance among the three ACCase-inhibitors was quite high: 36.6% of the populations was resistant to all tested ACCase-inhibitors, while 9.8% was resistant only to clodinafop and sethoxydim.

Pinoxaden has been introduced in the Italian market this year (2007), the resistance already observed in some of the population is due to the selection done in the past by other ACCase-inhibitors belonging to APPs and CHDs. Some of the fields where *Lolium*

samples have been collected are rotated with wheat and autumn sown sugar-beet that is also treated with ACCase-inhibitors. Even if there is a crop rotation, crops have similar cropping season and there is no herbicide rotation so weeds are exposed to similar agricultural practises and to the herbicides with the same mode of action.

Among the tested populations (total 41) 34 samples are from the Maremma (Tuscany and northern Latium), few others from the Adriatic coast in central Italy (3), Apulia (3) and Sicily (1).

Table XV. Screenings of *Lolium* spp. populations collected during 2004-2006 with three ACCase-inhibitor and one ALS-inhibitor herbicides: percentage of populations for different resistance categories in terms of plant survival; clo, clodinafop; pin, pinoxaden; set, sethoxydim and Atl, Atlantis WG. 2004, 16 tested populations; 2005, 12 and 2006, 13.

Herbicides	**S (%)**	**SR (%)**	**R (%)**	**RR (%)**	***R+RR (%)***
2004 clo	25	31.25	12.5	31.25	*43.8*
2005 clo	16.7	8.3	0	75	*75.0*
2006 clo	7.65	38.5	7.65	46.2	*53.8*
2004 pin	87.5	6.25	6.25	0	*6.3*
2005 pin	25	8.33	33.33	33.33	*66.7*
2006 pin	46.2	7.6	23.1	23.1	*46.2*
2004 set	62.5	12.5	18.8	6.2	*25.0*
2005 set	25	0	25	50	*75.0*
2006 set	38.5	15.3	46.2	0	*46.2*
2004 Atl	93.8	6.2	0	0	*0.0*
2005 Atl	66.7	33.3	0	0	*0.0*
2006 Atl	76.9	23.1	0	0	*0.0*

S, plant survival <5% at dose 1x; SR, survival between 5 and 20% at dose 1x; R, survival >20% with 1x dose and RR, survival >20% survival at dose 1x and >10% at dose 3x.

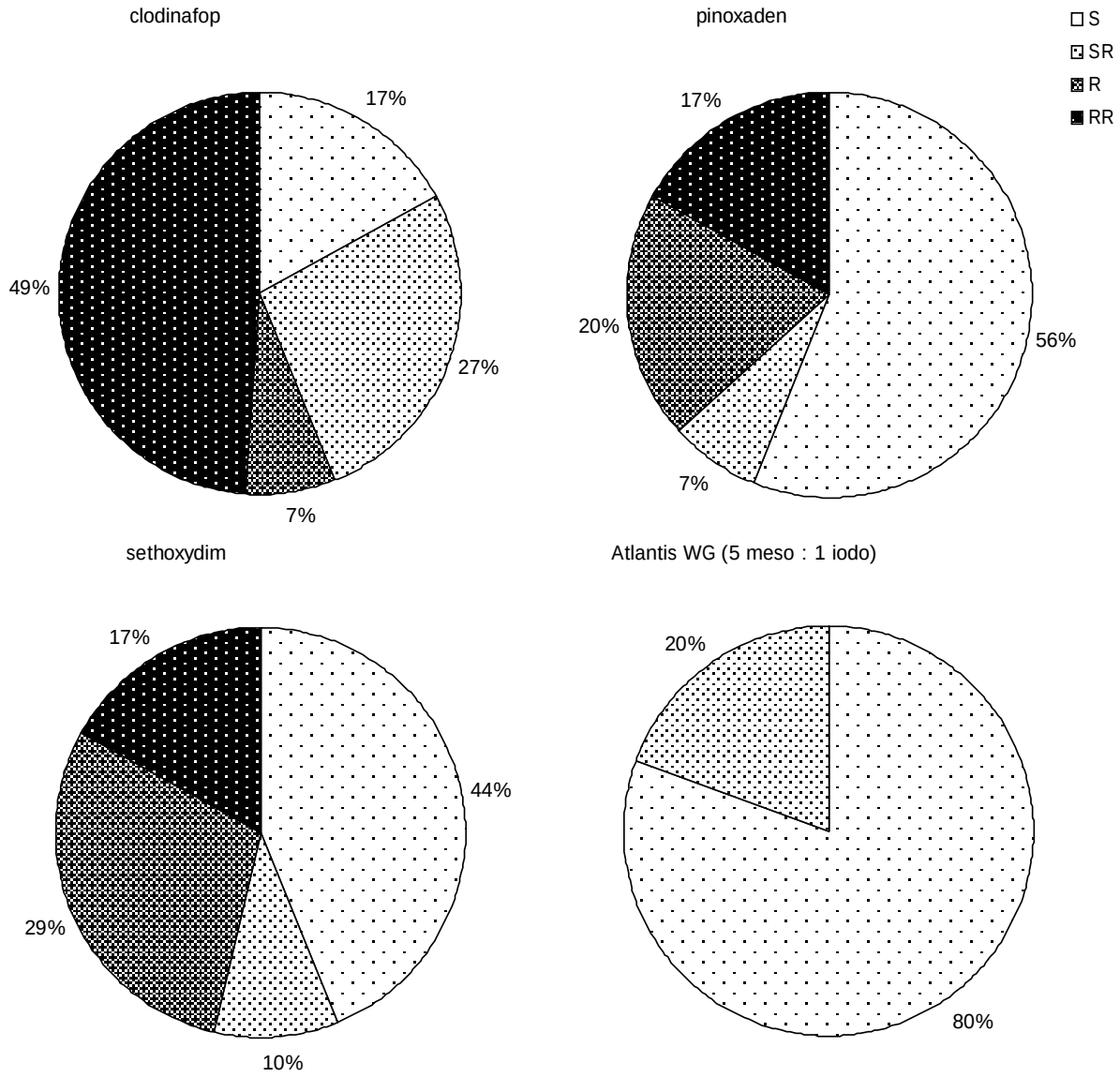

Figure 12. Screenings of all *Lolium* spp. populations collected during 2004-2006 with three ACCase-inhibitor and one ALS-inhibitor herbicides: percentage of populations showing a certain resistance level to the ACCase-inhibitors used and the ALS-inhibitors (Atlantis WG) herbicide (S, survival <5% with 1x dose; SR, survival 5-20% with 1x dose; R, survival >20% with 1x dose and RR, survival >20% survival at dose 1x and survival >10% with 3x dose).

3.1.2 *Phalaris paradoxa* resistance patterns

The number of populations tested was rather low, indicating that the impact of herbicide resistance in this species is marginal. A previous report on ACCase-inhibitors resistance (Sattin et al., 2001) showed that among 39 screened populations, during 1998-2000, just 6 were resistant with only one highly resistant.

Among the 17 populations tested over three years the level of resistance was found high in just one population collected in central Italy in 2006 (Table XVI and Figure 13). Compared to *Lolium* the resistance level is lower and the pattern of resistance is not so wide: three populations resistant to clodinafop and one of these cross-resistant to all the ACCase-inhibitor used. All the populations are controlled by the ALS-inhibitor. Pinoxaden in this species could be a valid alternative to clodinafop as just one population was being controlled, showing to be highly resistant. All the other populations were completely controlled, there are no cases reported as SR (Table XVI).

Among the tested populations (total 17) 7 samples are from the Maremma (Tuscany and northern Latium), few others from the Adriatic coast in central Italy (4), Apulia (3) and Sicily (3).

Table XVI. Screenings of *P. paradoxa* populations collected during 2004-2006 with three ACCase-inhibitor and one ALS-inhibitor herbicides: percentage of populations for different resistance categories in terms of plant survival; clo, clodinafop; pin, pinoxaden; set, sethoxydim and Atl, Atlantis WG. 2004, 8 tested populations; 2005, 2 and 2006, 7.

Herbicides	S (%)	SR (%)	R (%)	RR (%)	*R+RR (%)*
2004 clo	37.5	37.5	25.0	0	*25.0*
2005 clo	100	0	0	0	*0*
2006 clo	57.1	28.6	0.0	14.3	*14.3*
2004 pin	100	0	0	0	*0*
2005 pin	100	0	0	0	*0*
2006 pin	85.7	0	0	14.3	*14.3*
2004 set	100	0	0	0	*0*
2005 set	50.0	50.0	0	0	*0*
2006 set	0	85.7	14.3	0	*14.3*
2004 Atl	75	25	0	0	*0*
2005 Atl	50	50	0	0	*0*
2006 Atl	85.7	14.3	0	0	*0*

S, plant survival <5% at dose 1x; SR, survival between 5 and 20% at dose 1x; R, survival >20% with 1x dose and RR, survival >20% survival at dose 1x and >10% at dose 3x.

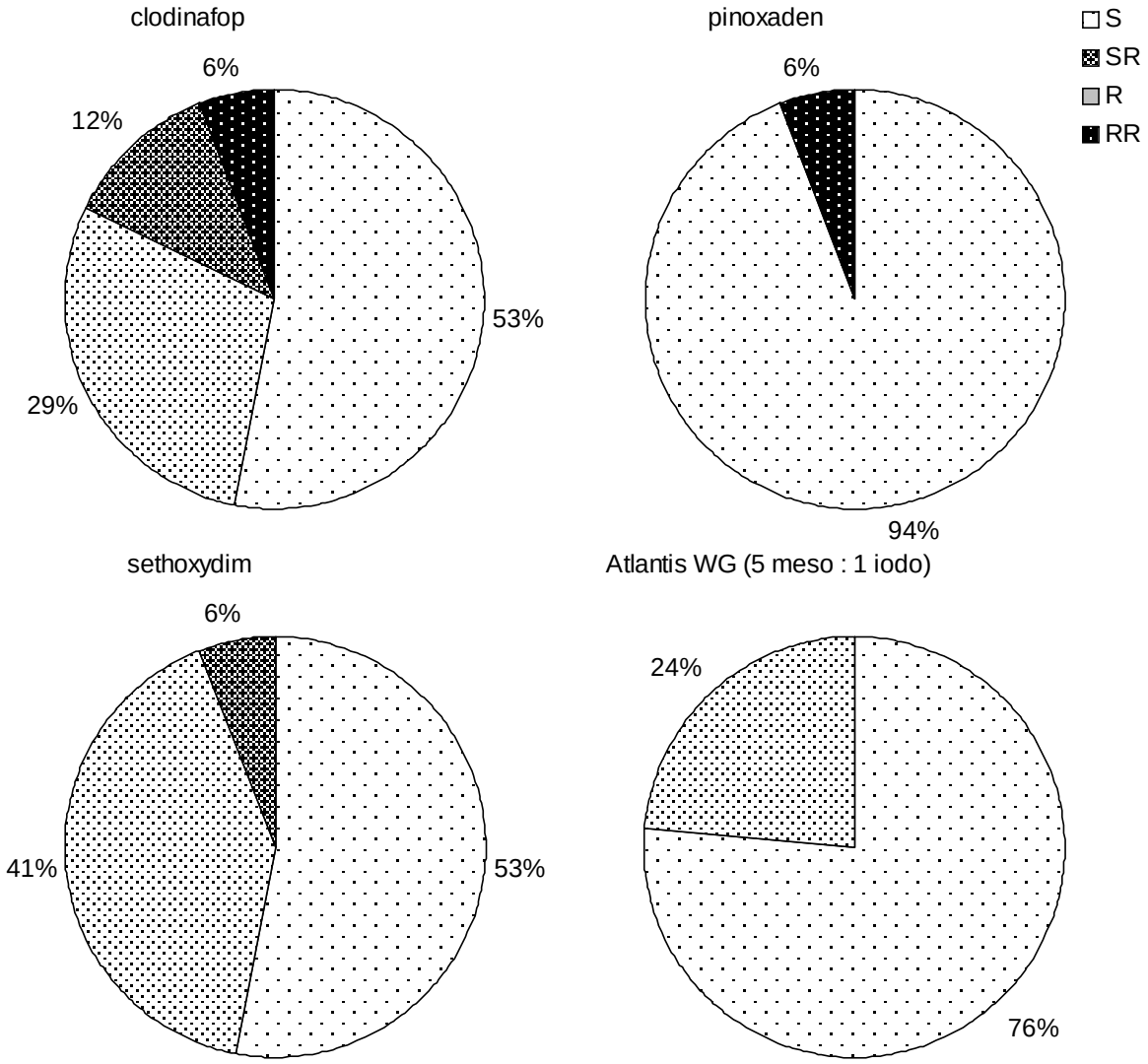

Figure 13. Screenings of all *P. paradoxa* populations collected during 2004-2006 with three ACCase-inhibitor and one ALS-inhibitor herbicides: percentage of populations showing a certain resistance level to the ACCase-inhibitors used and the ALS-inhibitors (Atlantis WG) herbicide (S, survival <5% with 1x dose; SR, survival 5-20% with 1x dose; R, survival >20% with 1x dose and RR, survival >20% survival at dose 1x and survival >10% with 3x dose).

3.2 Petri dish seed quick-test

Testing herbicide resistance has to be as fast as possible because the information should be promptly passed on to farmers and other stakeholders that have to manage the situation properly. Therefore, it is important to develop tests for practical herbicide resistance that are faster, less time consuming and cheaper than traditional greenhouse pot-experiments.

The first step in setting up the quick test was to evaluate if there was any difference between assessing shoot length and survival data. Several papers consider shoot or root length as growth parameters, rather than survival, to discriminate between resistant or susceptible populations (Beckie et al., 2000; Murray et al., 1996; Letouzé and Gasquez 1999; Retrum and Forcella 2002).

Survival is less time consuming than measuring shoots or roots, this allows increasing the number of seeds in each petri dish and therefore having a more reliable information.

3.2.1 *Lolium* spp.

A preliminary experiment was done to check whether clodinafop in the form of technical grade and as a commercial product (Topik), gave similar results in terms of shoot length and survival. Range of concentration explored was: 0, 0.01, 0.1, 1 and 10 µM. Both the shoot length and survival, which do not gave the same information from the biological point of view, can be correlated and give similar results when using the technical grade or the commercial product (Figure 14). Generally resistant plants surviving the exposition to herbicides usually show a reduced shoot biomass, for this reason the survival overestimates results of shoot length; this is not true if strong enhanced metabolism or strong mutation are expressed. One of the population used in this experiment (04258) is likely to survive because of an enhanced metabolism resistance mechanism, for this reason with short sooth the survival was high. The linear relation is not the appropriate model to correlate shoot length and survival since with null value of shoot length survival is around 28% and this is meaningless from the biological point of view. The purpose of this study is not to find out a model to correlate the two measurement methods but to justify the choice of one of the methods.

Since the technical grade is expensive and difficult to obtain, in the preliminary test the technical grades, clodinafop and pinoxaden, have been compared with the commercial formulations Topik and Axial respectively (Figures 15 and 16). Correlation between clodinafop and Topik have been investigated for both to shoot length and survival, the best estimate is obtained using survival rather than shoot because better discriminate between resistant and susceptible samples (Figure 15). Pinoxaden and Axial have been evaluated just for survival since it is a more rapid parameter to estimate resistance; the two herbicide forms are highly correlated (Figure 16).

Survival data are preferred to shoot length because it is the same parameter used for estimating resistance or susceptibility in a population in the traditional pot experiments.

These results allowed the use of the commercial products and survival assessment in subsequent experiments.

In the following discussion each herbicide will be identified with the name of the technical grade, since commercial products could change names over countries and time, but it is meant that tests have been done using the above mentioned commercial formulations.

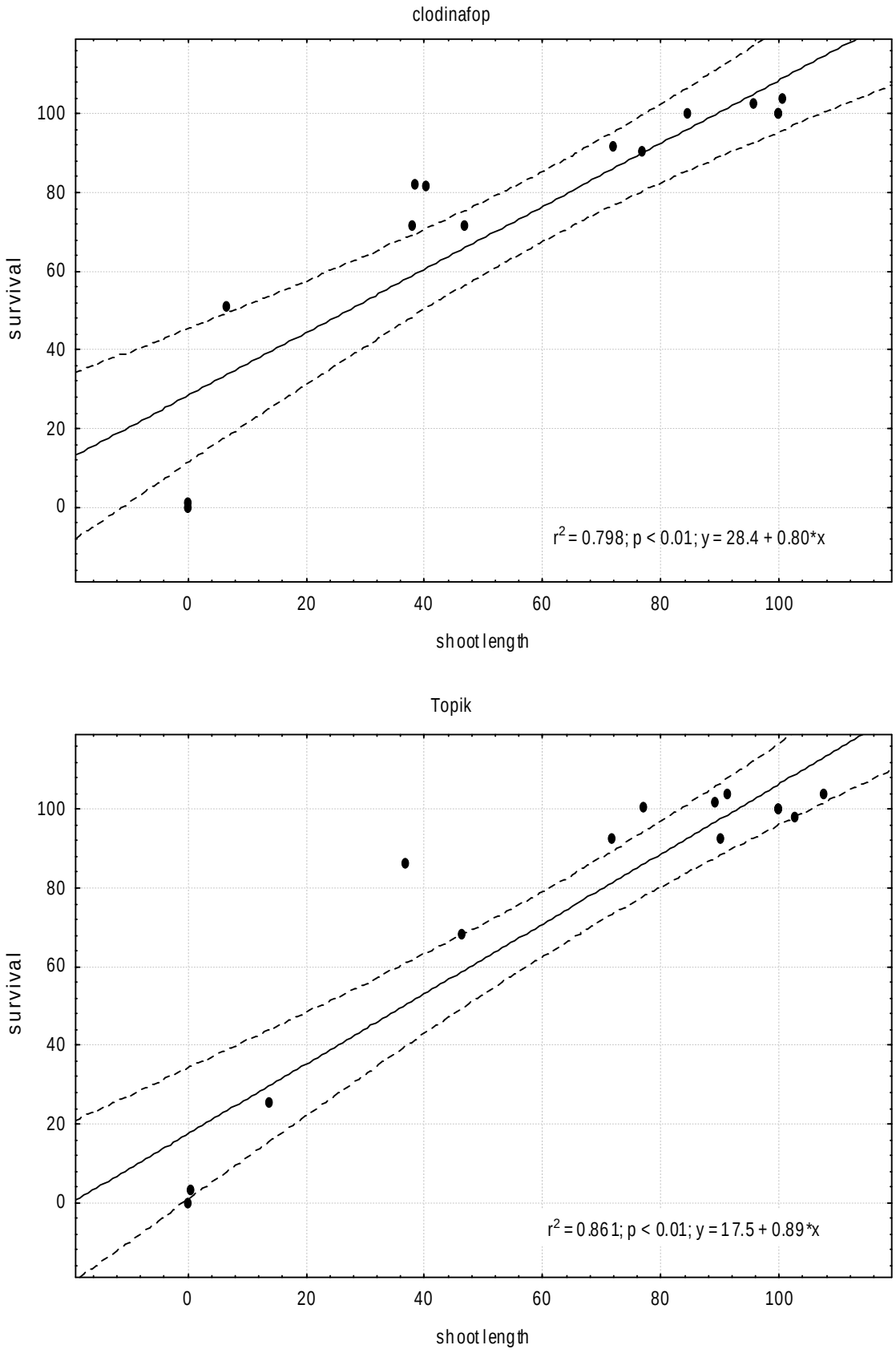

Figure 14. Correlation between shoot length and survival (percentage in relation to the untreated check) using the technical grade (a.i. clodinafop - top) and a commercial formulation Topik (bottom).

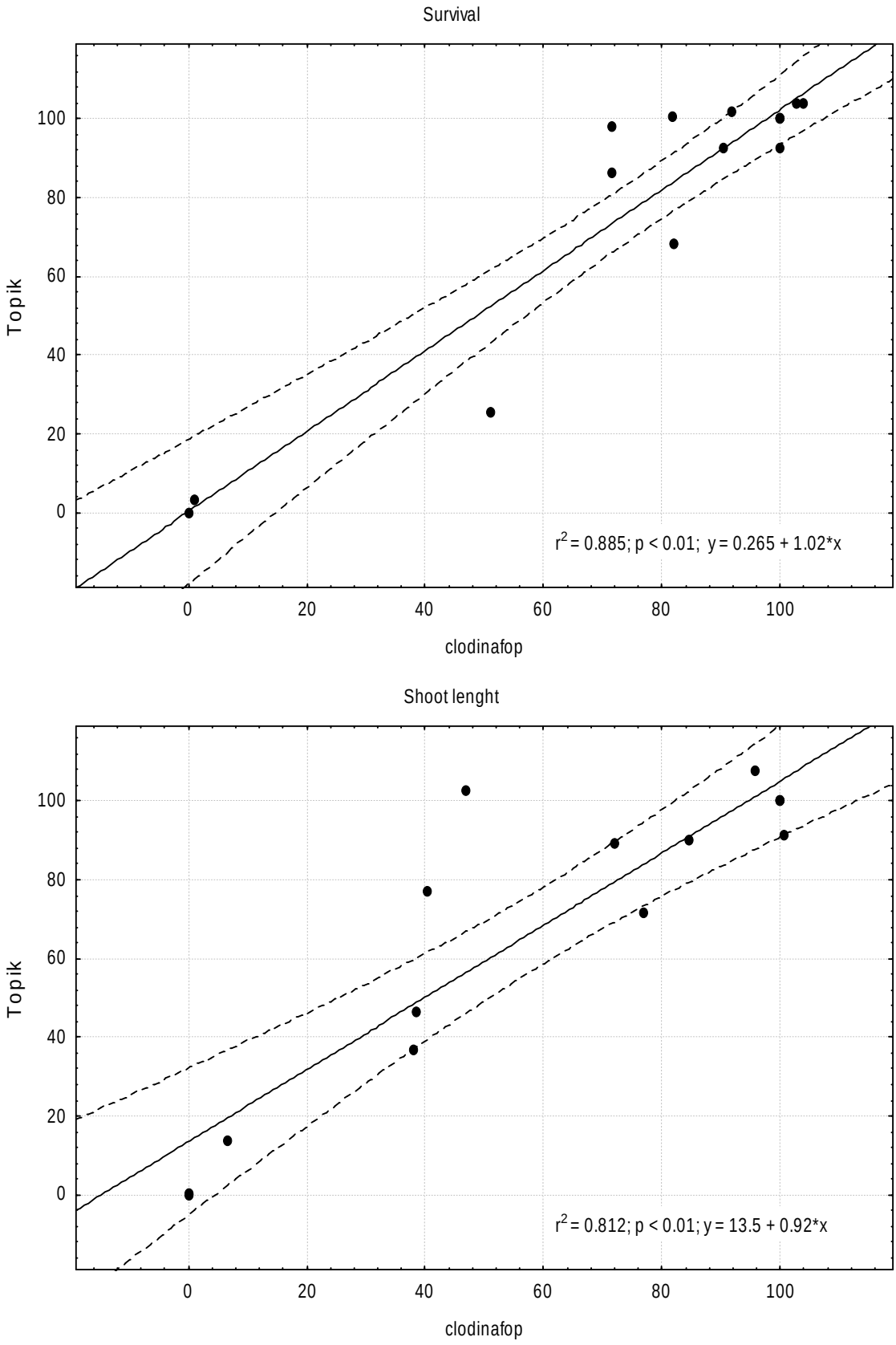

Figure 15. Correlation between the commercial formulation and the technical grade (percentage in relation to the untreated check) for shoot length (top) and survival (bottom).

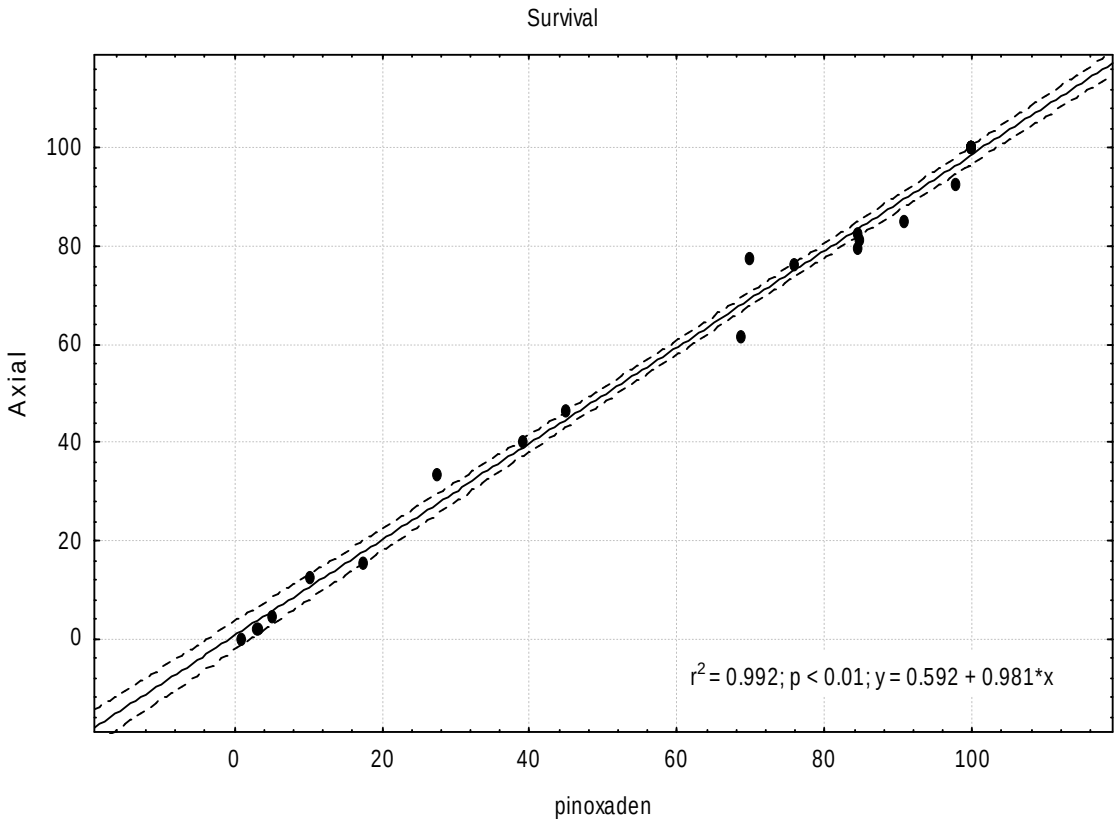

Figure 16. Relationship between the pinoxaden technical grade and the commercial formulation Axial for survival expressed as percentage of the untreated check.

The further step was to find a herbicide concentration able to discriminate between resistant and susceptible populations and that gives comparable results to the dose used in routine screenings, which usually is the field dose. There are no reports of metabolisation of the two CHDs used and they are not registered to be used in wheat or barley because they are not selective to them. Sethoxydim and clethodim are therefore used instrumentally to discriminate between target-site and non-target-site resistance. Using the field dose recommended for other crops implies to classify as susceptible plants which carry mutations endowing resistance in those crops (durum wheat) where the samples come from. For this reason CHDs included in the development of the quick test have been evaluated for the discrimination between susceptible and resistant plants.

Clodinafop (commercial formulation: Topik). The best concentration for discriminating resistant from susceptible population was 1 µM, from the graph in Figure 17

it is possible to identify that the quick test concentration of 1 µM is comparable to the field dose. From the comparison obtained from the validation experiments (Figure 18) the two groups of resistant and susceptible populations are well discriminated. In comparison with the traditional pot screening, the quick-test slightly underestimates the survival of susceptible populations and those having low to medium resistance level. A concentration of 100 µM could be used to discriminate strong from weak resistance (Figure 17). Comparing the mean of seedling survival obtained using the screening field dose and the discriminating quick test concentration a low variability has been found for most of the populations (Figure 19).

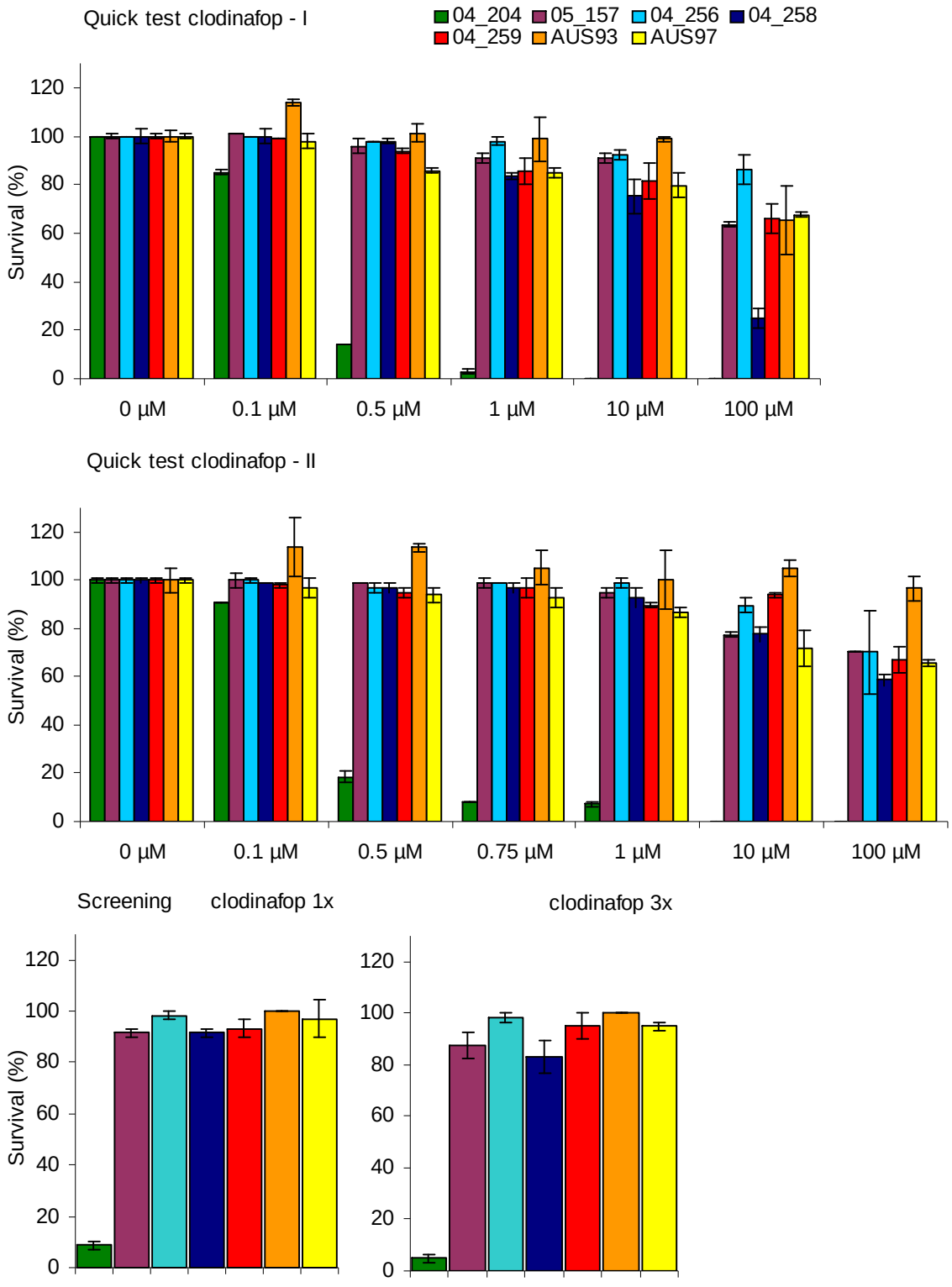

Figure 17. Petri dish test results from the set up experiments (two upper graphs). Bottom: results from routine greenhouse pot screenings using clodinafop; left: field dose (1x), right: three times the field dose (3x). All the resistant populations have a high level of resistance.

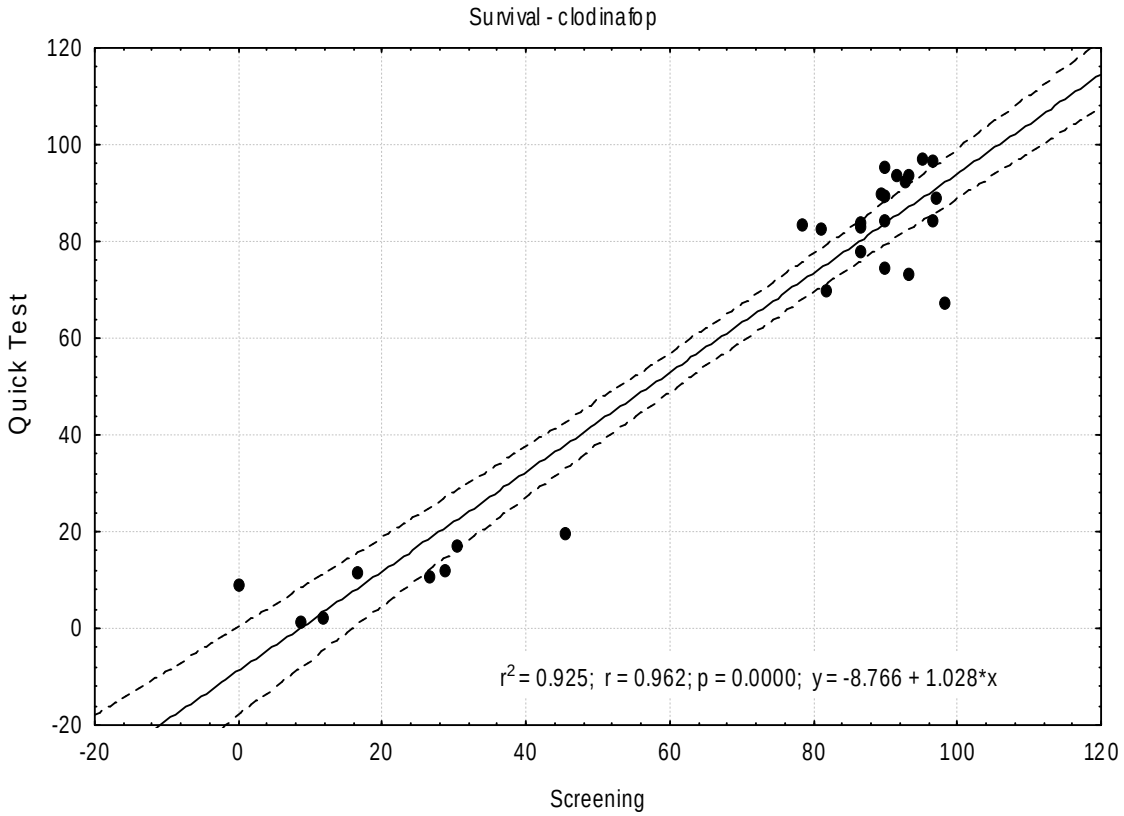

Figure 18. Correlation between traditional pot experiment screening and quick test; clodinafop: quick test dose 1 µM, screening dose 60 g a.i. ha^{-1}. The two groups of susceptible and resistant populations are well discriminated.

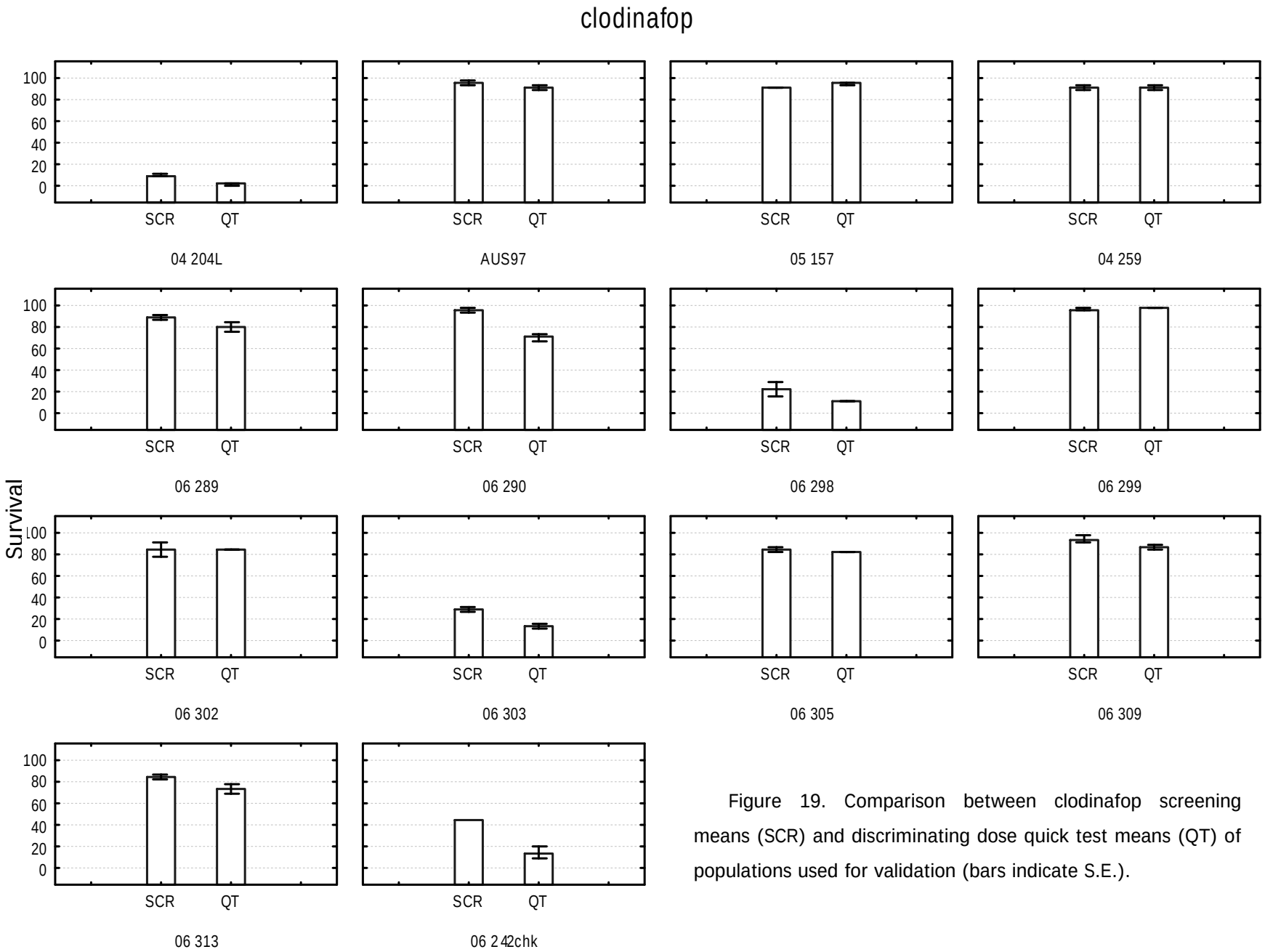

Figure 19. Comparison between clodinafop screening means (SCR) and discriminating dose quick test means (QT) of populations used for validation (bars indicate S.E.).

Pinoxaden (commercial formulation: Axial). The survival data highlighted that the concentration of 0.2 µM controls the susceptible population and select all the resistant populations. This concentration of pinoxaden discriminates susceptible from any kind of resistant population and resembles the field dose used in pot screening; concentrations higher than 0.25 µM could be used to discriminate strong from weak resistance (Figure 20). In fact the populations AUS97 (enhanced metabolism resistance mechanism) and 04258 (suspected enhanced metabolism resistance mechanism) at higher concentrations than 0.2 µM are discriminated from target site resistant populations.

Comparing the mean of survival obtained using the screening field dose and the discriminating quick test concentration (Figure 21) a low variability has been found for most of the populations (Figure 22). Pinoxaden has been the herbicide which gave the better results in comparing the classic pot screening with the rapid test developed.

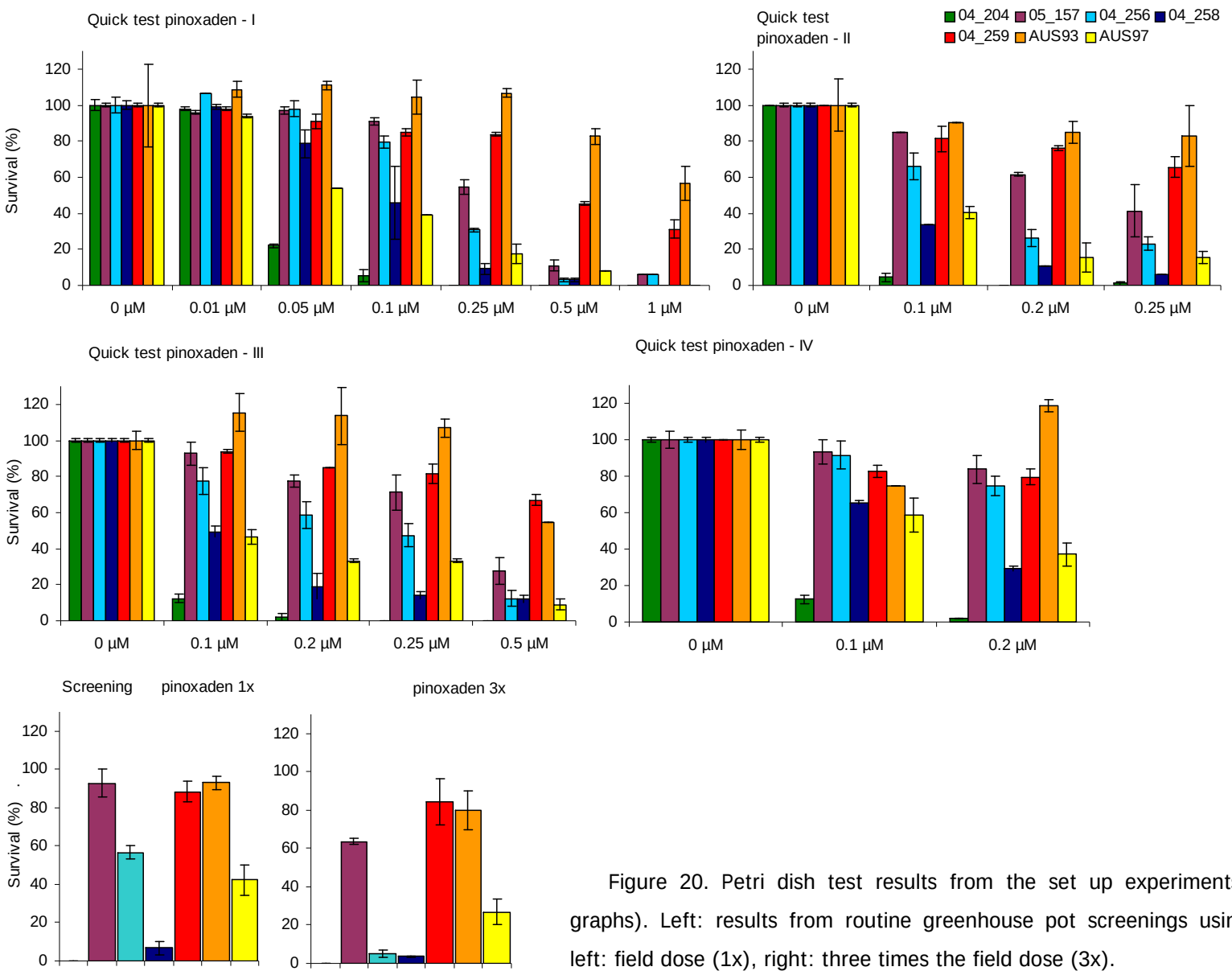

Figure 20. Petri dish test results from the set up experiments (four upper graphs). Left: results from routine greenhouse pot screenings using pinoxaden; left: field dose (1x), right: three times the field dose (3x).

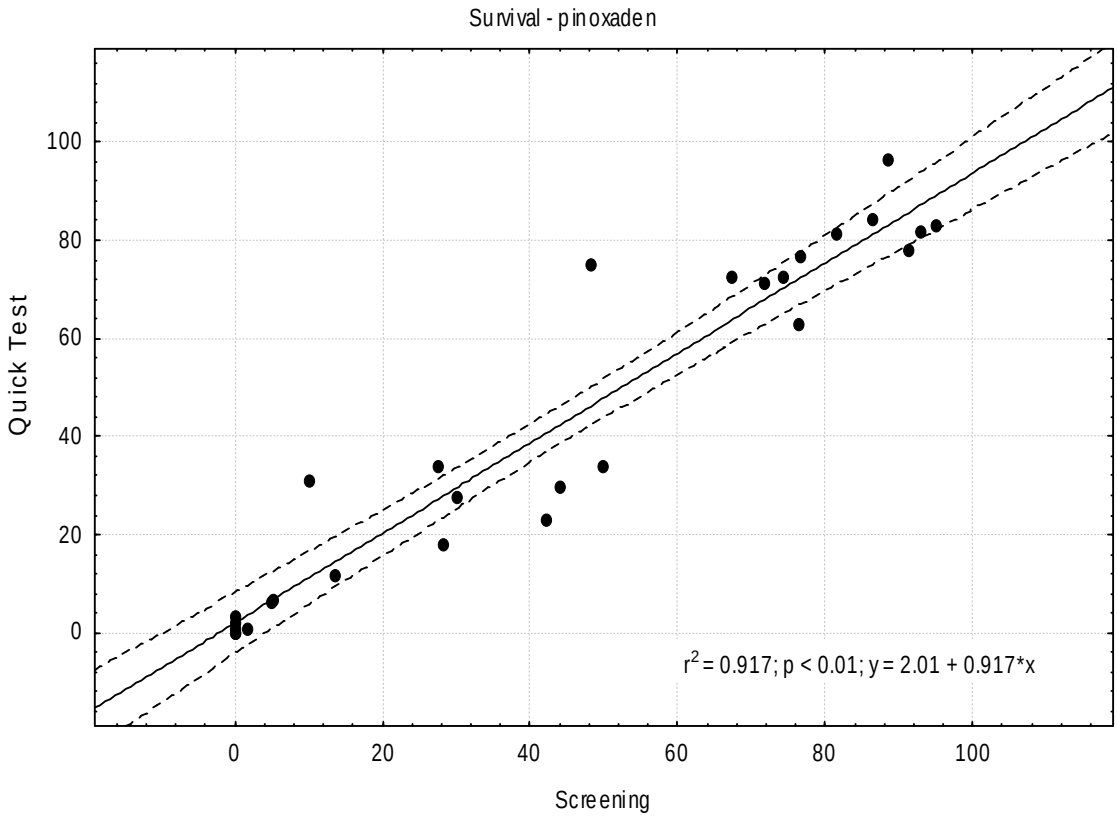

Figure 21. Correlation between traditional pot experiment screening and quick test; pinoxaden: quick test dose 0.2 µM, screening dose 30 g a.i. ha^{-1}.

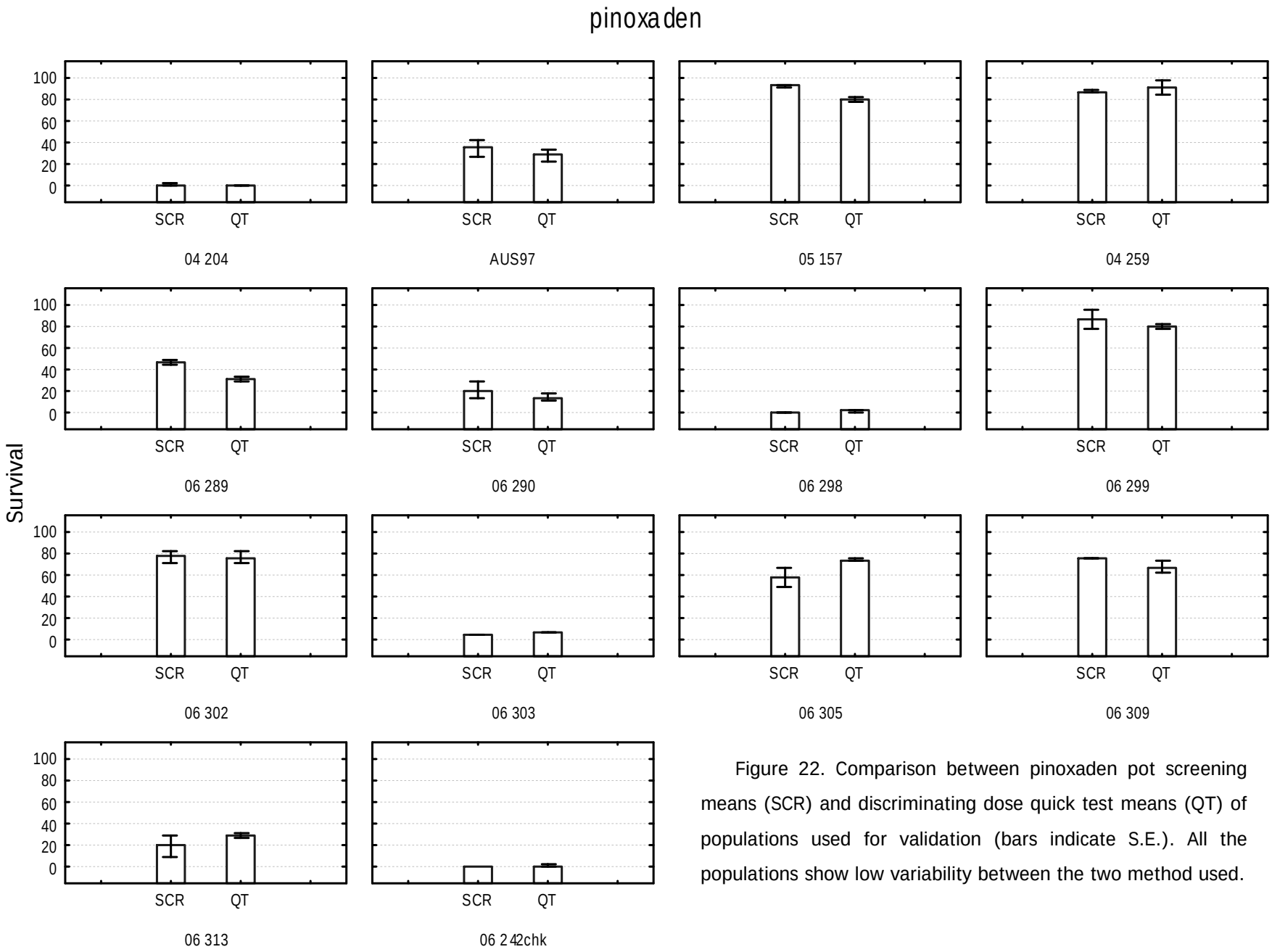

Figure 22. Comparison between pinoxaden pot screening means (SCR) and discriminating dose quick test means (QT) of populations used for validation (bars indicate S.E.). All the populations show low variability between the two method used.

Clethodim (commercial formulation: Select). The survival data indicated that the concentration to discriminate from susceptible to any kind of resistant was already 0.05 µM. The concentration 0.1 or 0.2 µM could be used to discriminate resistant populations - 0.2 µM is slightly more effective than 60g of a.i. ha^{-1} used as field dose (Figure 23). With this herbicide the greatest variability among experiments was observed. It can be stated that concentration 0.1 µM well discriminates susceptible from resistant populations. Moreover it is interesting to note that among resistant populations, a concentration of 0.1 µM discriminates also the target site resistant populations carrying mutations in position 1781 (05157 and AUS93) and 2088 (04259) (Figure 23). For most of the popularions the results of pot screening and quick test are not well correlated (Figure 24 and 25). The field dose used in Italy is 144 g a.i./ha and provides a great control among resistant populations. Australian dose is 60 g a.i./ha, provides a good control of resistant populations and allows recognizing resistant populations otherwise killed by the Italian dose. For this reason a Petri-dish concentration giving responses similar to field dose of 60 g a.i./ha has been searched (Figure 23). Probably the high effectiveness of clethodim in pot experiment is due to the location where these experiments have been conduct. In fact all pot screenings have been done in greenhouse, and CHDs herbicides are known to be more effective.

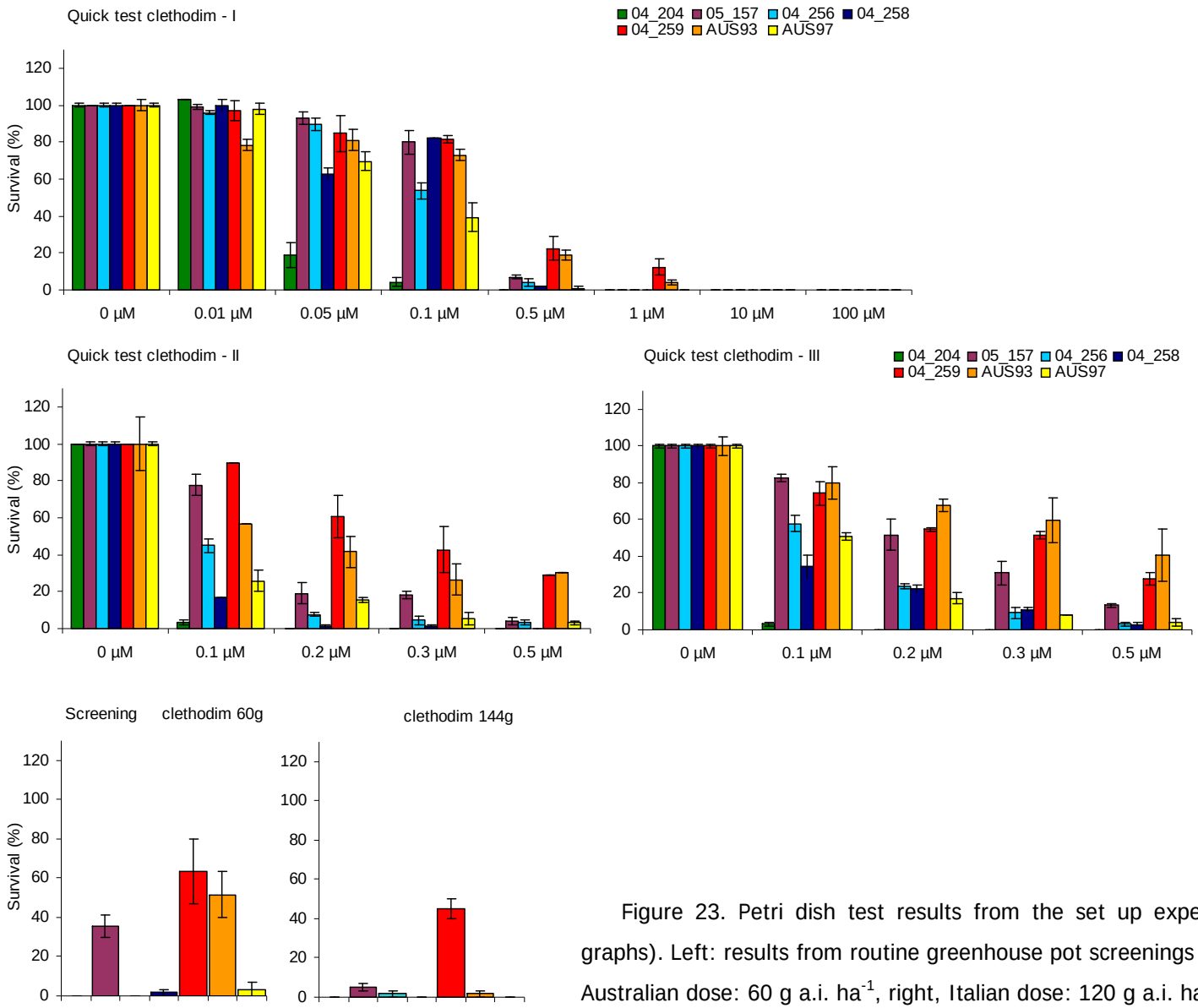

Figure 23. Petri dish test results from the set up experiments (three upper graphs). Left: results from routine greenhouse pot screenings using pinoxaden; left, Australian dose: 60 g a.i. ha^{-1}, right, Italian dose: 120 g a.i. ha^{-1}.

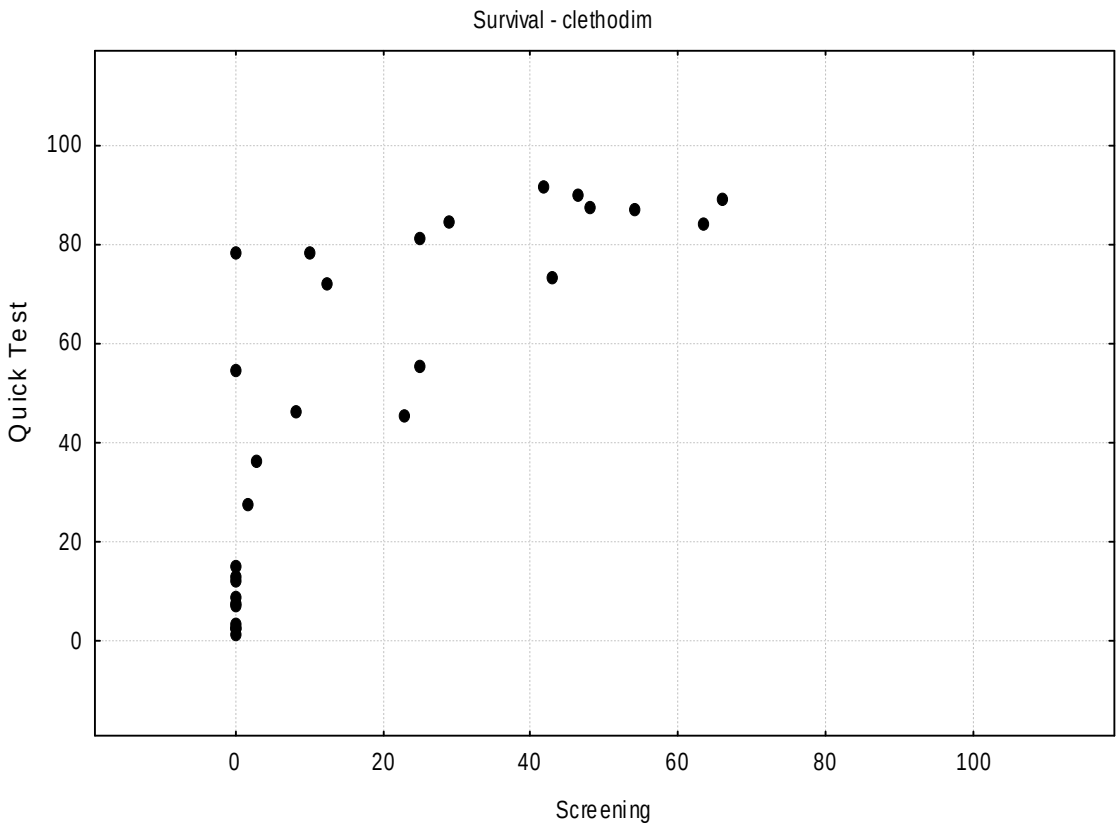

Figure 24. Correlation between traditional pot experiment screening and quick test; clethodim: quick test dose 0.1 µM, screening dose 60 g a.i. ha^{-1}.

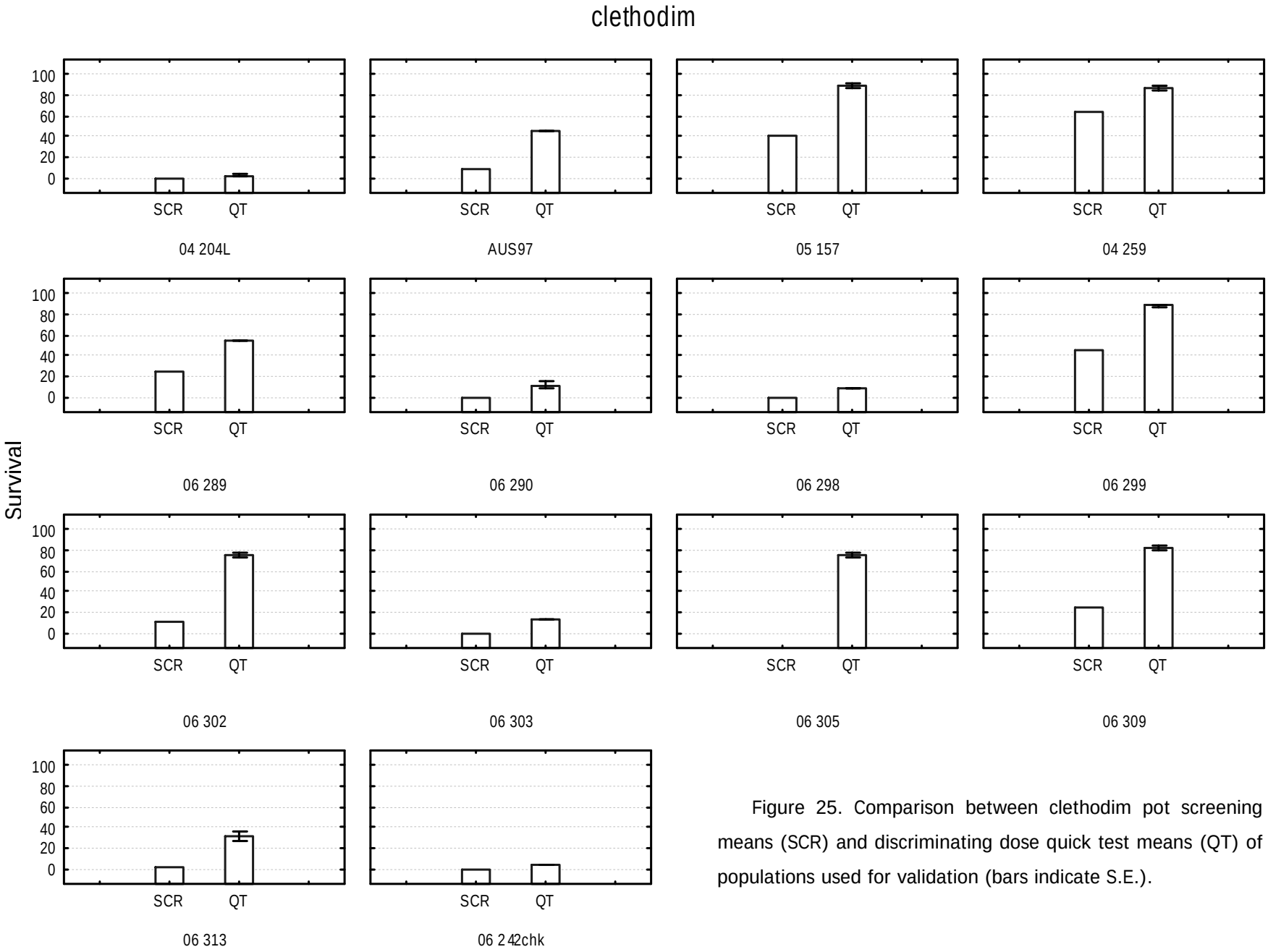

Figure 25. Comparison between clethodim pot screening means (SCR) and discriminating dose quick test means (QT) of populations used for validation (bars indicate S.E.).

Sethoxydim (commercial formulation: Fervinal). Due to a lack of seeds and because of the relatively high variability among experiments recorded in the greenhouse screenings the set up and the pot screening have been repeated just one time and validation had two repetition. The survival data indicated that the concentration of 0.1 µM discriminated well the susceptible to any kind of resistant populations (Figure 26). The concentration 1 µM could be used to discriminate strong from weak resistance: In fact populations 05157 and AUS97 (amino acid substitution Ile1781Leu) and population 05259 (Cys2088Arg) have a much greater survival at higher concentration than the discriminating concentration and at 6x the screening field dose. Population 04256, which has a pool of different mutations (Ile1781Leu, Ile2041Asn/Val, Asp2078Gly and Gly2096Ala) with the 51.6% of Ile2041Asn/Val (see point 3.4.2) of the plant selected with 0.1 µM pinoxaden had a survival at 0.5 µM of sethoxydim while other included target site populations have more than 80% survival (Figure 26).

However the difficulties of obtaining Fervinal or other commercial products containing sethoxydim alone and not in mixture in the Italian market makes not convenient using this product in routine Petri dish tests and could be substituted with clethodim which is also not-metabolised. The chosen concentration is not representative of the field dose (Figure 26), but provides a discrimination from susceptible to resistant populations. Comparing the mean of survival obtained using the screening field dose and the discriminating quick test concentration (Figure 27) sethoxydim gave results that differ more than other herbicides among repetition of experiments. Variability among populations is quite high (Figure 28).

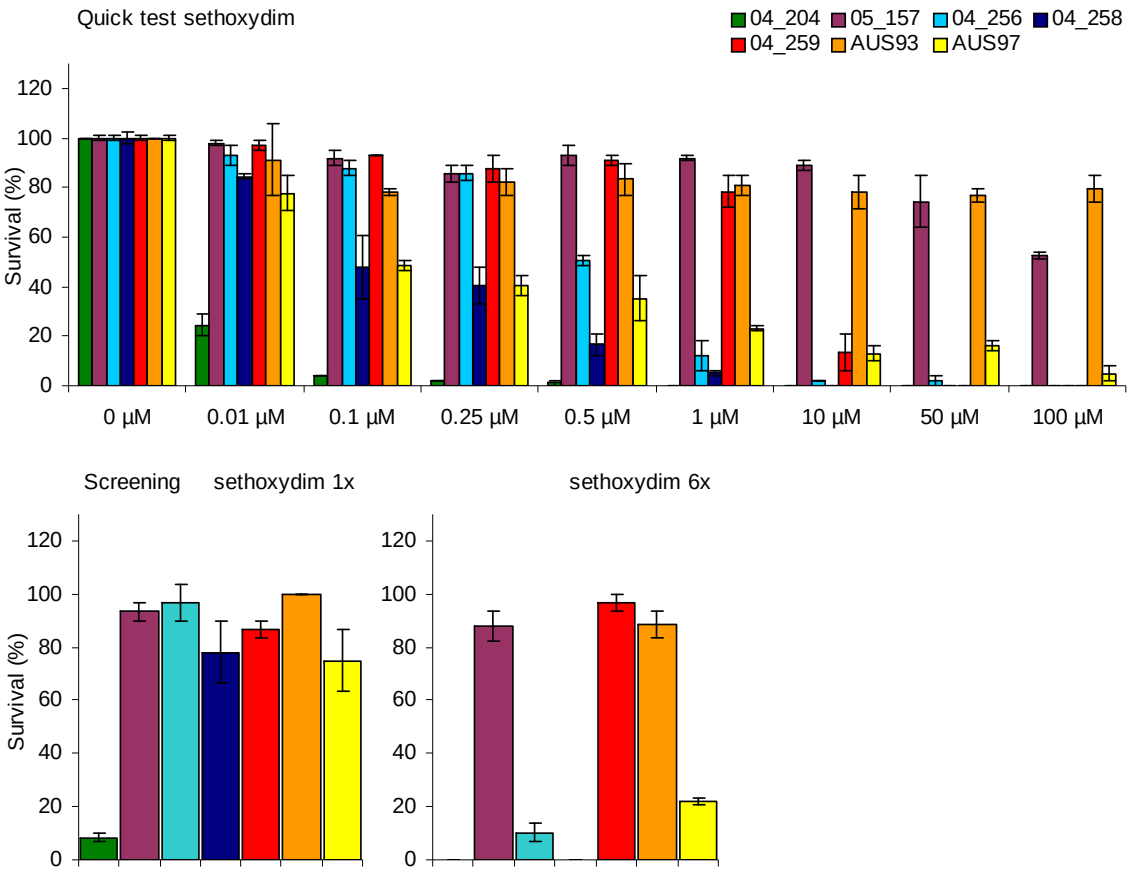

Figure 26. Petri dish test results from the set up experiments (top). Resistant populations have a high level of resistance. Bottom, left: results from routine greenhouse pot screenings using sethoxydim; left, 1x dose: 74 g a.i. ha^{-1}, right, 6x dose: 444 g a.i. ha^{-1}.

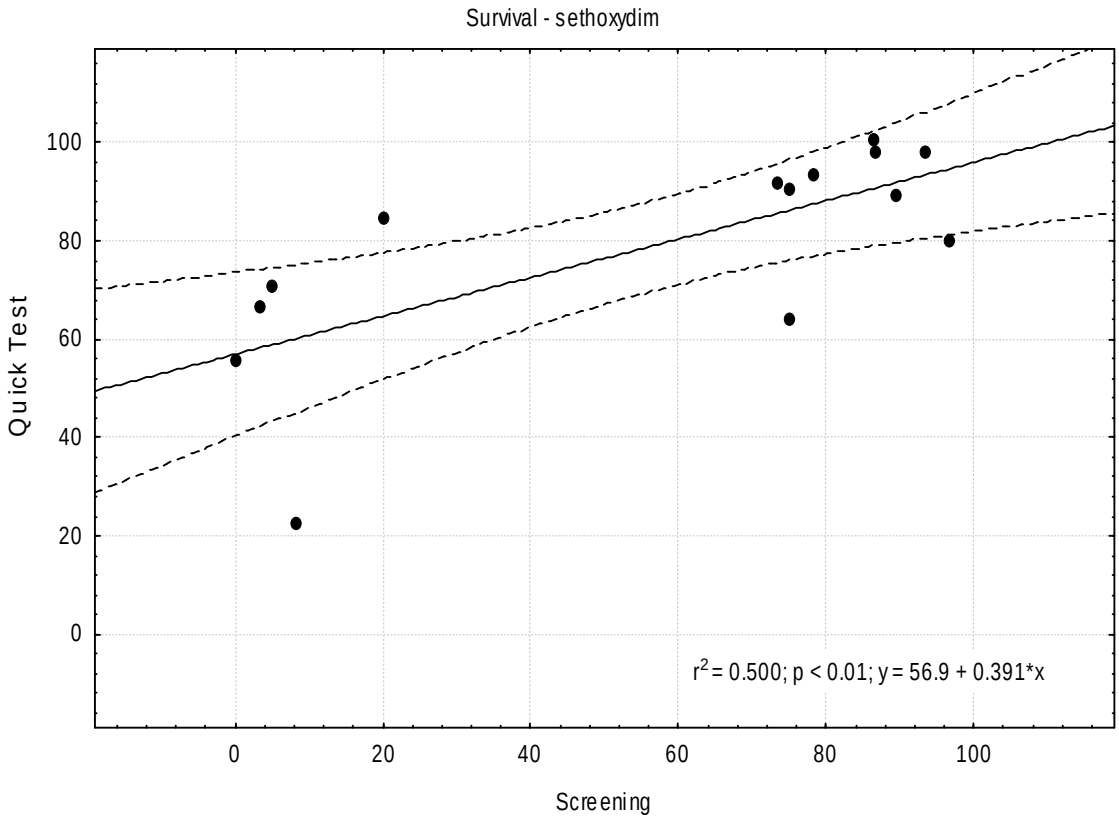

Figure 27. Correlation between traditional pot experiment screening and quick test; sethoxydim: quick test dose 0.1 µM, screening dose 74 g a.i. ha^{-1}.

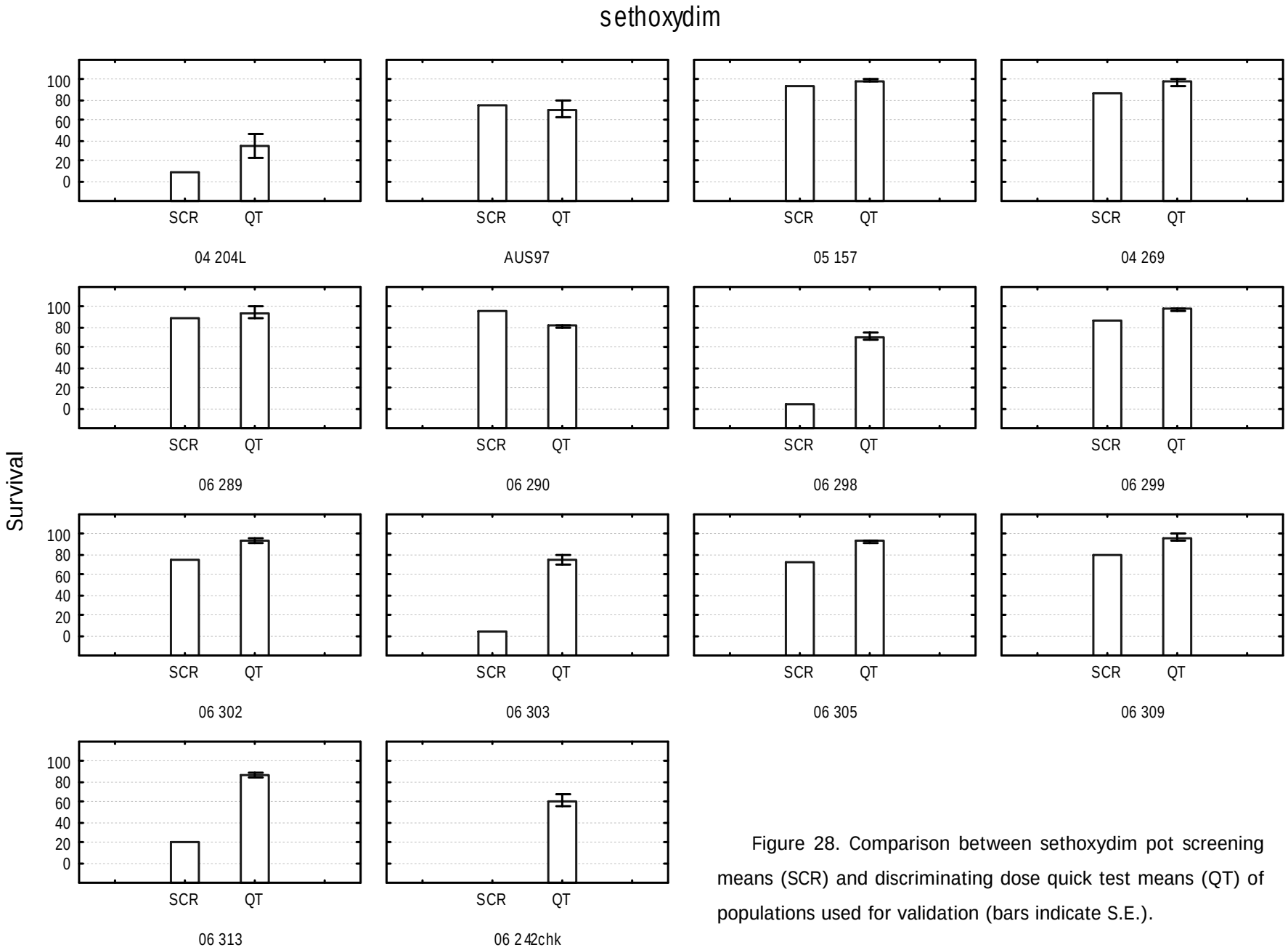

Figure 28. Comparison between sethoxydim pot screening means (SCR) and discriminating dose quick test means (QT) of populations used for validation (bars indicate S.E.).

3.2.2 *Phalaris paradoxa*

The major problem in setting up and validating a quick test for this species was the lack of seeds in the most peculiar populations. Furthermore, this species is characterised by a strong dormancy and prolonged germination. Setting up and validations have been carried out using only the commercial formulations as the comparison between the two forms of herbicides gave a good correlation using *Lolium* spp. It was possible to compare the results from quick-test and pot screening just for clodinafop and pinoxaden. Correlation between quick-test and screening survival data allows the identification of the two groups of resistant and susceptible populations (Figures 29 and 30).

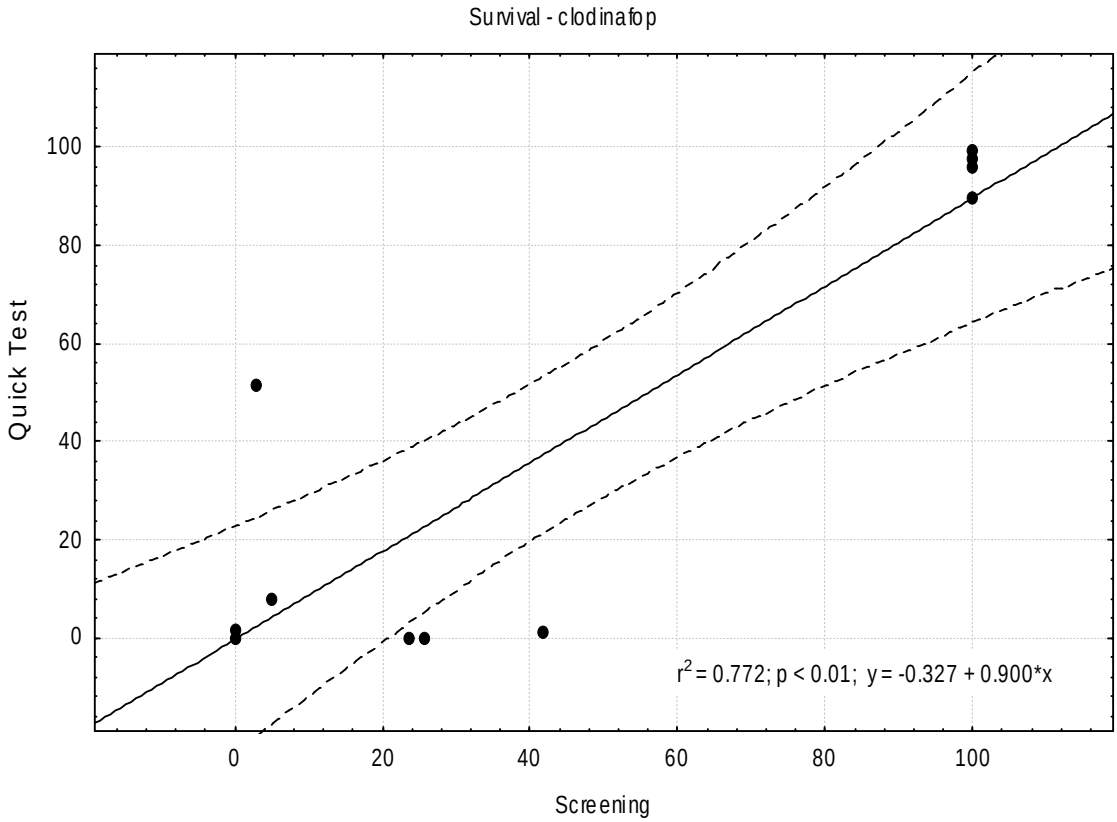

Figure 29. Plant survival: correlation between traditional pot experiment screening and quick-test using clodinafop. Clodinafop: quick test concentration (QT) 0.1 µM, screening dose (SCR) 60 g a.i. ha^{-1}.

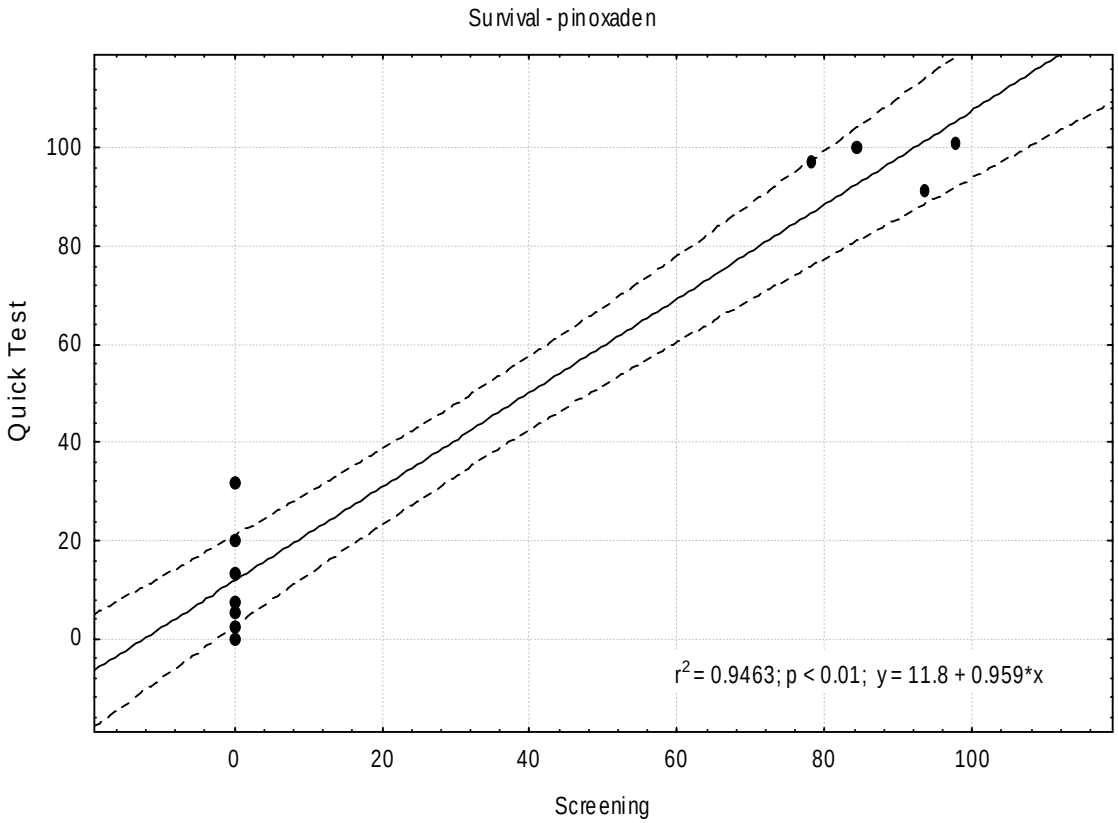

Figure 30. Plant survival: correlation between traditional pot experiment screening and quick-test using pinoxaden. Pinoxaden: QT concentration 0.05 µM, SCR dose 30 g a.i. ha^{-1}.

Clodinafop (commercial formulation: Topik). The best concentration for discriminating resistant from susceptible population was 0.1 µM, from the graph in Figure 31 it is possible to identify that the quick test concentration of 0.1 µM is comparable to the field dose. Comparing the average of survival obtained using the screening field dose and the discriminating quick test concentration, a low variability has been found for most of the populations (Figure 32). The survival data indicated that the concentration of 0.1 µM controls susceptible population while the plants of the resistant target site populations (0025 and 0478L) grew almost as the untreated seeds (Figure 31). Other populations show a wider variability (i.e. populations 0460test, 0470, 0482 and 0692).

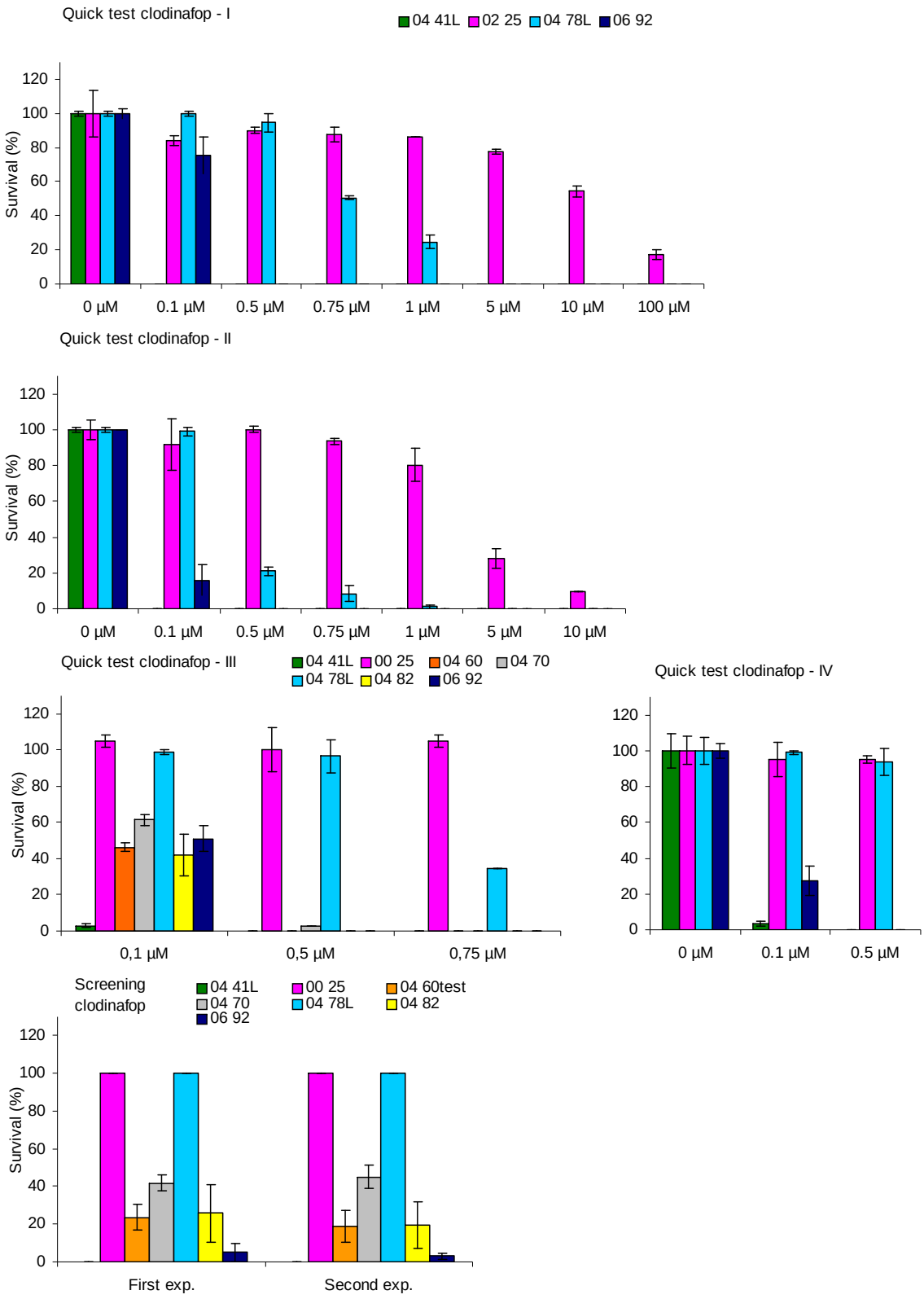

Figure 31. Petri dish clodinafop test results from the set up experiments (four graphs on top). Bottom: results from routine greenhouse pot screenings (1x dose: 60 g a.i. ha^{-1}).

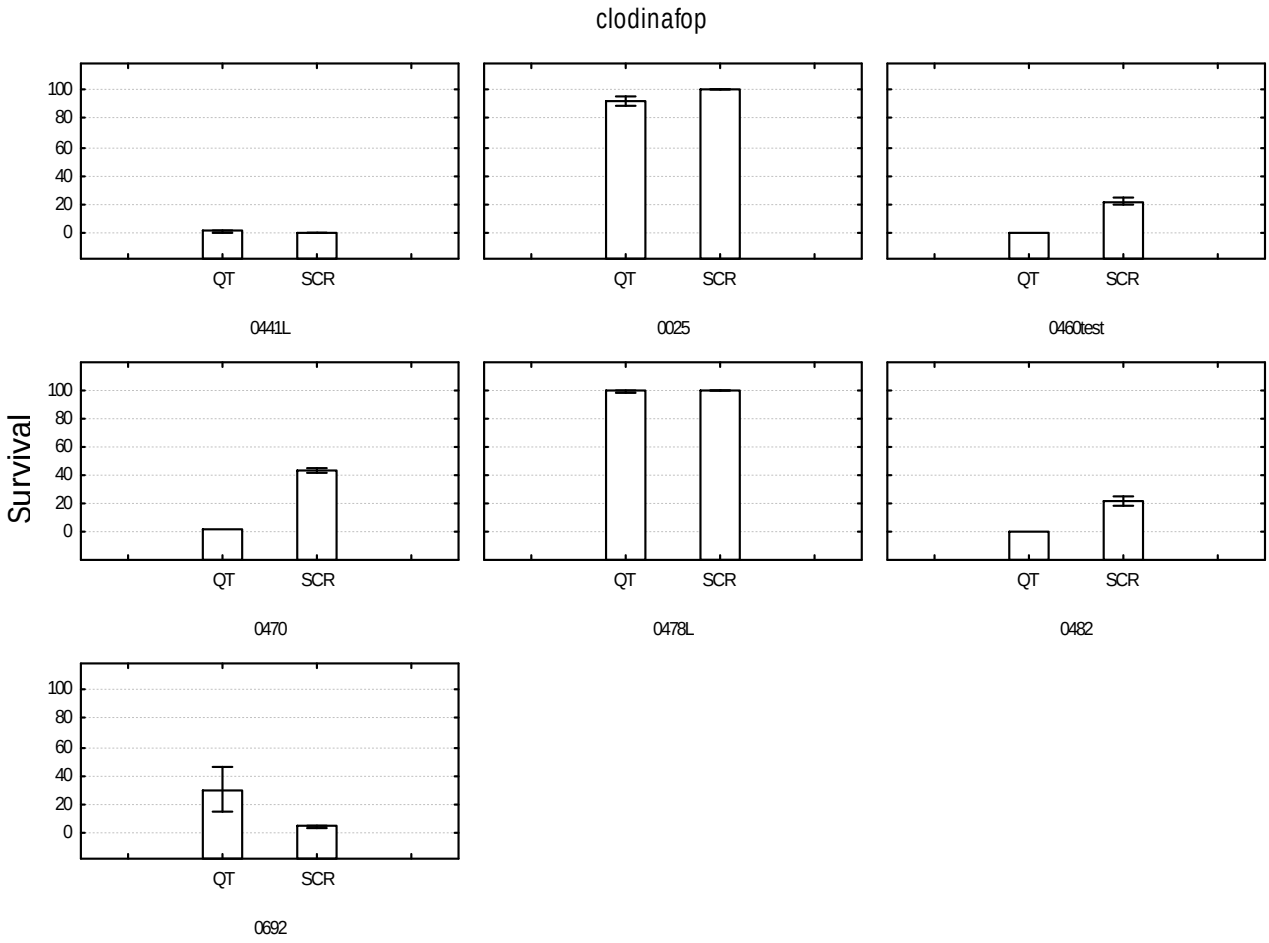

Figure 32. Comparison between clodinafop screenings dose means (SCR) and discriminating concentration quick test means (QT) of populations used for validation.

Pinoxaden (commercial formulation: Axial). As it is reported in Figure 33, the concentration of 0.05 µM controls the susceptible population (04204L) and selects the resistant populations, while a concentration of 0.1 µM more resembles the field dose used. The survival data highlighted of the two type of experiment indicates that screenings are more conservatory than quick test concentration for some populations, especially 0470 (Figure 34). The variability of the populations was not so high in complex, but unfortunately there was not the possibility to test more populations.

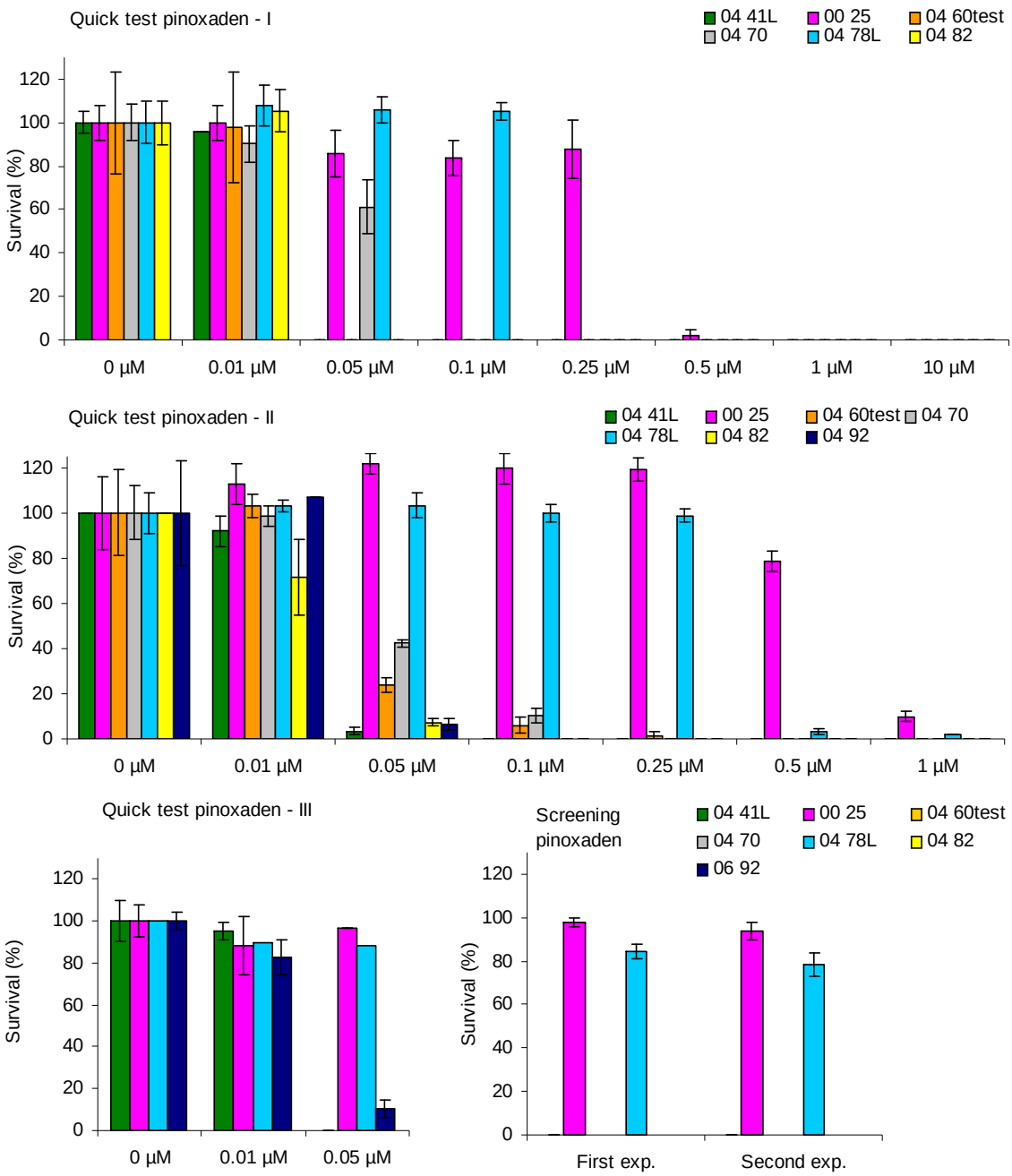

Figure 33. Pinoxaden: Petri dish test results from the set up experiments (four graphs on top). Bottom, right: results from routine greenhouse pot screenings (1x dose: 30 g a.i. ha^{-1}).

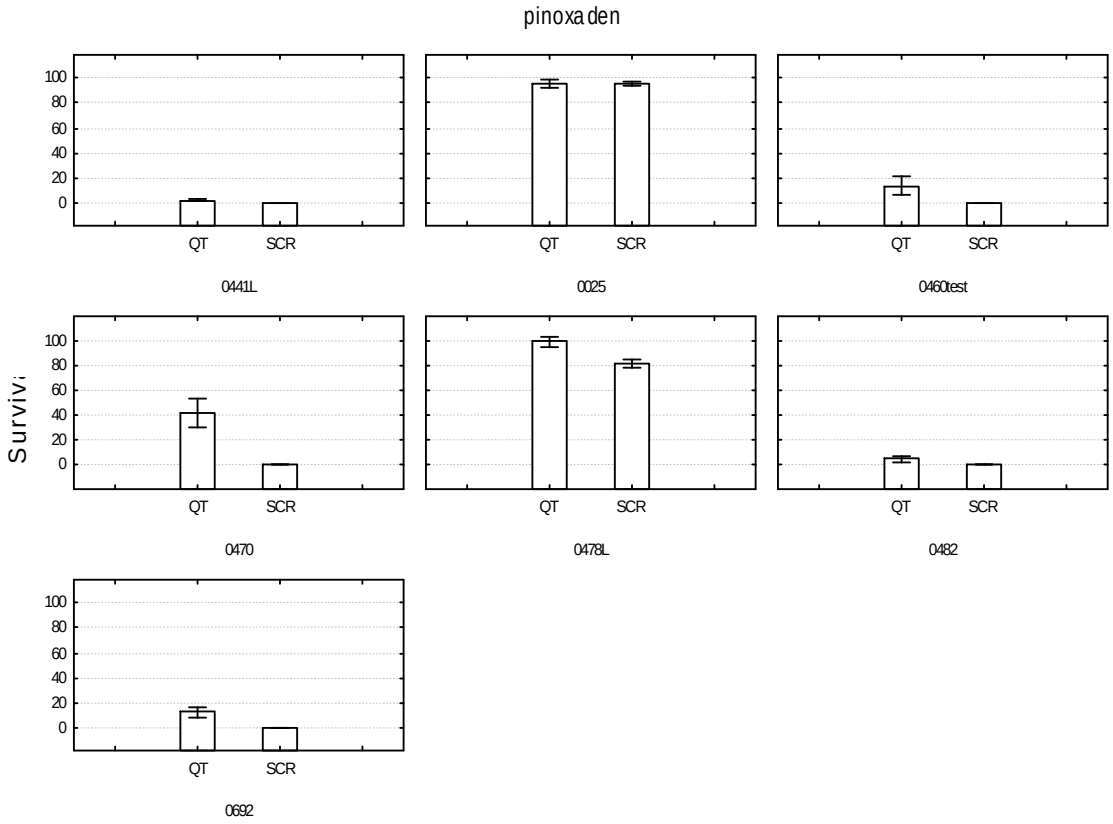

Figure 34. Comparison between pinoxaden screenings dose means (SCR) and discriminating concentration quick test means (QT) of populations used for validation.

Clethodim (commercial formulation: Select) is an herbicide that cannot be metabolized. The survival data indicate that concentrations above 0.05 µM discriminate from susceptible (0441L and 0692) and the two target site resistant (0025 and 0478L) populations (Figure 35). Unfortunately the lack of seeds precludes testing these populations in traditional pot screenings. To be more cautionary a concentration of 0.1 has been taken as discriminator between susceptible and resistant populations. The choice of a discriminating concentration between susceptible and resistant using a not metabolisable herbicide is justified to select target-site resistant populations from non-target-site resistant ones.

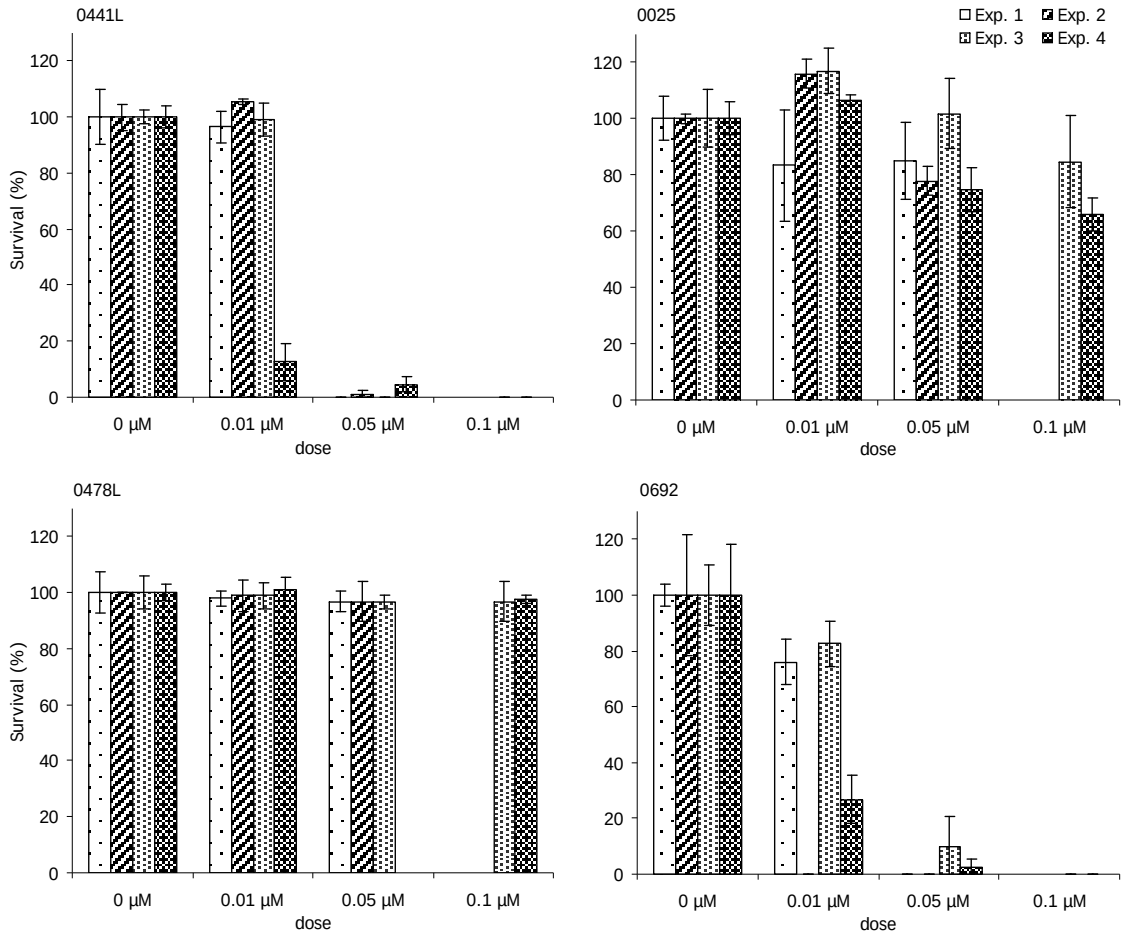

Figure 35. Clethodim quick test, 4 different experiments, at different concentrations. Populations 0025 and 0478L are well discriminated from susceptible ones with a herbicide concentration above 0.05 µM. Concentration 0.1 µM has been tested with replicates 3 and 4 only.

3.3 *Lolium* spp. characterisation

3.3.1 ACCase mutations revealed in clethodim-resistant *Lolium* populations

At least six clethodim resistant individuals from each clethodim resistant population were initially sequenced. Subsequently, a total of 124 individual plants were sequenced from 12 clethodim resistant Australian *L. rigidum* populations and two Italian *Lolium* populations. Using three overlapping primer pairs (Materials and Methods, Table VII), were amplified three regions containing all known potential ACCase mutation sites (Délye and Michel, 2005) in the CT domain (Materials and Methods, Figure 11 - amplified fragment differs between *Lolium* spp. and *P. paradoxa*) of the plastidic ACCase genes. A contig of 1513 bp was clearly identified and assembled from sequence results of all individual resistant and susceptible (bulked) plants. When compared with other ACCase gene sequences in GenBank, this assembled contig showed 99% identity with the plastidic ACCase gene from *L. rigidum* (accession No. AY995232) and *L. multiflorum* (AY710293), 95% identity with *Avena fatua* (AF231335), 93% identity with *Aleopecurus myosuroides* (AJ310767) and *P. minor* (AY196481), and 91% with *Triticum aestivum* (AF029895). However, only 77% and 76% identity was shared with cytosolic ACCase genes of *A. myosuroides* (AJ632096) and *T. aestivum* (U39321), respectively. Sequence comparison between individual resistant plants from each population and two susceptible populations revealed four mutations previously established to endow ACCase herbicide resistance (Table XVII): Ile1781Leu (referred to as 1781Leu), Trp2027Cys (2027Cys), Ile2041Asn (2041Asn), and Asp2078Gly (2078Gly). In addition, a new mutation of Cys2088Arg (2088Arg) was also detected in five populations (sequences have been deposited in GenBank with accession numbers EF538937-EF538943). Sequence alignment of 1513 bp contigs from susceptible controls and resistant plants containing the 2088 mutation revealed 18 synonymous single nucleotide polymorphisms (SNPs) and ten non synonymous SNPs. Among the ten nonsynonymous SNPs, only the SNP at 2088 differs between resistant and susceptible sequences (Table XVIII). In addition, the sequence results containing the Cys to Arg mutation were also validated by restriction analysis (cleaved amplified polymorphic sequence - CAPS). Therefore, the mutation Cys to Arg at position 2088 is very likely a newly identified mutation endowing resistance to ACCase herbicides (including clethodim).

Table XVII. Combinations of ACCase mutant alleles that were identified in individual plants that survived the field rate of clethodim treatment (60 g ha^{-1} in Australia) from 14 clethodim resistant *Lolium* populations. Twenty-one plants in population R_7, 11 plants in R_{12}, 12 plants in 04259, 15 plants in 05281 and 6 plants in each of other populations were analysed.

Group	Genotype	Population (and number of plants) where detected
1	1781-Leu/1781-Leu	R_6 (2), R_7 (21), R_8 (4), R_{10} (2)
2	1781-Leu/2027-Cys	R_2 (3)
3	1781-Leu/2041-Asn	R_8 (1), R_9 (1), R_{10} (4), R_{11} (1)
4	2078-Gly/2078-Gly	R_1 (4), R_5 (6), R_9 (1), R_{11} (4), R_{12} (11), 05281 (1)
5	2078-Gly/2078-Asp*	R_3 (2), R_4 (1), R_9 (2), 04259 (2), 05281 (2)
6	2078-Gly/1781-Leu	R_1 (3), R_4 (1), R_6 (4), R_8 (1), R_9 (3), R_{11} (1), 05281 (1)
7	2078-Gly/2041-Asn	R_9 (2)
8	2088-Arg/2088-Arg	R_2 (1), R_3 (2), R_4 (3), 04259 (10), 05281 (8)
9	2088-Arg/2088-Cys*	05281 (2)
10	2088-Arg/1781-Leu	R_2 (2)
11	2088-Arg /2041-Asn	R_4 (1)
12	2088-Arg/2078-Gly	R_2 (1), R_3 (2), 05281 (1)

* 2078-Asp and 2088-Cys are wild-type alleles.

To facilitate quick and accurate identification of mutant ACCase alleles, a published dCAPS marker for the 1781 allele has been used and (d)CAPS markers for 2041, 2078 and 2088 alleles have been developed. A published dCAPS marker for detection of mutants in position 1781 (Kaundun and Windass, 2005) was tested for suitability for genotyping *Lolium* populations under our modified PCR conditions. For a total of 84 samples of known genotypes tested, the accuracy was > 97%. This dCAPS marker was, therefore, employed to genotype the clethodim resistant population R_7 (n = 40). Remarkably, all plants in this population were homozygous for the resistant 1781Leu allele. When these genotyped homozygous plants were tested with the field rate of clethodim (at 3-4 leaf stage), they all survived, while all susceptible plants (S_1) died. Clearly, homozygous mutants for the 1781Leu allele can withstand the Australian field rate of clethodim, whereas heterozygous

mutants cannot. Therefore, the specific mutation, homozygosity versus heterozygosity and the rate of herbicide used in testing for resistance are all important in determining the level of resistance in an individual or population.

Table XVIII. Nonsynonymous single nucleotide polymorphisms (SNPs) with corresponding amino acid substitutions in the CT domain of plastidic ACCase of susceptible (S) populations S_1 and S_2 and resistant (R) populations containing a Cys to Arg mutation. Amino acid positions correspond to the full length plastid ACCase in *A. myosuroides*. Nucleotide position numbers refer to the sequenced region (1513 bp) of the plastidic ACCase gene (GenBank accessions EF538937-EF538943).

Nucleotide position	221	729	741	752	884	933	957	1154	1281	**1382**
SNP alleles	A, T	G, C	A, C	A, C	A, G	A, G	A, G	T, A	C, T	**T, C**
Amino acid position	1701	1870	1874	1878	1922	1938	1946	2012	2054	**2088**
S_1-bulk	Leu/Met	Arg/Pro	Glu/Ala	Asn/His	Ser	Lys	Glu/Asp	Met/Leu	Thr/Ile	**Cys**
S_2-bulk	Leu/Met	Arg/Pro	Glu/Ala	Asn/His	Ser/Gly	Lys/Arg	Glu/Asp	Met/Leu	Thr/Ile	**Cys**
R_2-3	Leu/Met	Arg/Pro	Glu/Ala	Asn/His	Ser	Lys	Glu/Asp	Met/Leu	Thr/Ile	**Arg**
R_3-6	Leu/Met	Arg/Pro	Glu/Ala	Asn/His	Ser	Lys	Glu/Asp	Met/Leu	Thr/Ile	**Arg**
R_4-5	Leu	Pro	Ala	His	Ser	Lys	Glu	Leu	Ile	**Arg**
04259-2	Met	Arg	Glu	Asn	Ser	Lys	Asp	Met	Thr	**Arg**
05281-6	Leu	Pro	Ala	His	Ser	Lys	Glu	Leu	Ile	**Arg**

A dCAPS marker for detection of the mutant 2078Gly allele (see Materials and Methods, Figure 7) has been developed in *Lolium* populations. The robustness and accuracy of this marker was tested with a total of 120 samples of known genotypes from across 14 resistant *Lolium* populations, and the results obtained matched sequencing results by > 98%. This dCAPS marker was therefore used to genotype the clethodim resistant population R_{12} (n = 45). Genotype frequencies were found to be 0.60 for homozygous resistant 2078Gly individuals, 0.02 for homozygous susceptible 2078Asp

individuals, and 0.38 for heterozygous individuals. The heterozygous individuals were further analysed by the 1781 dCAPS marker, and it was found that all heterozygous individuals for the resistant 2078 allele also contained one resistant 1781 allele. Therefore, at the commercial herbicide use rate, 98% of the population was found to be clethodim resistant.

A CAPS marker for the 2088 mutation was designed and tested against known genotypes. However, this marker is not ideal for large scale genotyping due to the cost limitation of an expensive restriction enzyme. Marker analysis for the 2027 allele was not developed in this study due to limited numbers of clethodim survivors carrying the mutation.

Among 14 clethodim resistant populations tested, the mutant 2078Gly allele(s) was found in clethodim survivors from 12 populations and 1781Leu from 10 populations, while mutant 2088Arg or 2041Asn alleles were identified in 5 populations, and 2027Cys only in one population (Table XVII). Clearly, clethodim resistance can be related to one or more of several specific mutant alleles.

At least two types of mutant ACCase alleles were present in most populations except for some populations (R_5, R_7, and R_{12}) in which only one type of mutant allele was detected (Table XVII). Moreover, different mutant alleles can be present in the same *Lolium* individual, as has already been observed in cross-pollinated *A. myosuroides* (Délye et al., 2005). For example, individual resistant *Lolium* plants could possess one 2078Gly allele together with a 2088Arg, 1781Leu or Asn2041 allele. It is emphasised that diploid *L. rigidum* is obligate cross-pollinated, and therefore resistant individuals easily hybridise in the field and therefore, there is enrichment of all possible resistance alleles in the progeny. A given individual *L. rigidum* plant can contain, at most, two distinct mutant ACCase alleles. As summarized in Table XVII, twelve combinations of mutant alleles (genotypes) in individual plants were found in 14 resistant populations. Most individuals surviving clethodim treatment usually had two mutant ACCase alleles (either a single type or two types), although a few surviving plants were heterozygous for the mutant 2078Gly or 2088Arg allele (Table XVII, Group 5 and 9).

Substitution of amino acid ***Asp2078Gly****.* Until now, the Asp2078Gly substitution has been the only ACCase mutation known to endow clethodim resistance and only reported in

A. myosuroides (Délye et al., 2005). Here, the Asp2078Gly mutation was identified in individuals within 12 of 14 (86%) clethodim resistant *Lolium* populations examined (Table XVII), indicating that this mutation is relatively commonly associated with clethodim resistance. The Asp2078Gly substitution has been confirmed that results in an ACCase enzyme resistant to clethodim and the other ACCase herbicides tested (Figure 37, Table XIX). The level of resistance conferred by the Asp2078Gly mutation at the enzyme level in *Lolium* (Table XIX) was found to be similar to the level of resistance confirmed by this mutation in *A. myosuroides* (Délye et al., 2005). The purified population ($R_{12}P$) consisting of individuals homozygous for the mutant 2078Gly allele was 24-fold more resistant to clethodim than the susceptible population (Figure 38), and found to be cross resistant to all the APP and CHD herbicides tested, as well as the phenylpyrazolin herbicide pinoxaden (Table XXI). Therefore, it can be concluded that the Asp2078Gly substitution endows resistance in *Lolium* to clethodim and the other ACCase herbicides tested.

*Substitution of amino acid **Cys2088Arg** endowing resistance to clethodim and other ACCase herbicides in Lolium populations.* In this study a new ACCase mutation, a Cys to Arg substitution at position 2088, has been identified and characterized in five resistant *Lolium* populations (Table XVII and XVIII). This mutation confers an ACCase herbicide resistance profile (determined at the enzyme and whole plant level) similar to the Asp2078Gly mutation (Tables XIX and XXI, Figures 37 and 38). The Cys2088Arg mutation can confer resistance to clethodim and other ACCase herbicides. In fact, the amino acid residue at position 2088 was largely conserved as Cys among 28 grass species putatively susceptible to ACCase herbicides, with only a few species displaying Phe at this position (Figure 36). Using ACCase three-dimensional models derived from the structure of the yeast CT-APP complex, Délye et al. (2005) assessed the consequences of various amino acid substitutions identified in A. *myosuroides*, and predicted that a region including amino acid 2027 to 2096 may contain more unknown amino acid residues involved in sensitivity to ACCase herbicides. Our finding supports this hypothesis. Plants homozygous for the mutant 2088 allele survived the field or higher rate of clethodim (60-120g ha^{-1}) (Figure 38). However, only two individuals (from population 05281) heterozygous for this mutation survived the commercial field rate (60 g ha^{-1}) of clethodim (Table XVII, Group 9). This indicates the strong interaction between the specific resistance endowing mutation, homozygosity versus heterozygosity of this mutation, and the rate of herbicide use. These

results show that homozygous and heterozygous plants have different levels of resistance, and that the resistant 2088 allele is incompletely dominant above the field rate of clethodim. The same 2078Gly and 2088Arg mutations were reported in *A. fatua* by Christoffers et al. (2000) in their preliminary studies, and proposed as being responsible for low level clethodim resistance (below the field rate of 140g ha^{-1} in U.S) (Christoffers et al., 2005).

```
                                      2078      2088           2096
                      consensus      GGAWVVIDSKINPDRIECYAERTAKGNVLEPQGLIEIKFR
Lolium rigidum        (AY995232)     ....................T.................
Lolium multiflorun    (AY710293)     ....................T.................
Avena fatua           (AF231335)     ....................T.................
Briza media           (AJ966444)     ....................T.................
Lolium sp.            (AJ966457)     ....................T.................
Festuca arundinacea   (AJ966454)     ....................T.................
Melica ciliata        (AJ966458)     ....................T.................
Dactylis glomerata    (AJ966450)     ......V.............T.................
Setaria viridis       (AJ966464)     ......V.............................
Digitaria ischaemum   (AJ966452)     ......V.............................
Echinochloa crus-galli (AJ966453)    ......V.............................
Zea mexicana          (AJ966468)     ......V.............................
Panicum capillare     (AJ966459)     ......V.............................
Panicum miliaceum     (AJ966460)     ......V.............................
Zea mays              (U19183)       ......V.............................
Setaria italica       (AF294805)     ......V.............................
Alopecurus myosuroides (AJ310767)    ....................................
Phalaris minor        (AY196481)     ....................................
Apera spica-venti     (AJ966442)     ....................................
Poa supina            (AJ966463)     ....................................
Poa infirma           (AJ966462)     ....................................
Bromus arvensis       (AJ966445)     ....................................
Triticum aestivum     (AF029895)     ..................F.................
Hordeum vulgare       (AJ966456)     ..................F.................
Bromus diandrus       (AJ966446)     ..................F.................
Tragus racemosus      (AJ966465)     ..................F.................
Dasypyrum villosum    (AJ966451)     ..................F.................
Aegilops cylindrica   (AJ966440)     ..................F.................
```

Figure 36. Alignment of partial amino acid sequences of chloroplastic homomeric ACCases from 28 grass species which are putatively susceptible to ACCase herbicides. Numbers above the sequences indicate amino acid positions within the *A. myosuroides* full ACCase sequence (GenBank accession AJ310767). Amino acid residues 2078, 2088 and 2096 are in bold and conserved amino acids are indicated by dots. The 2088 residue was conserved as a Cys (C) among most grass species except for a few species as a Phe (F).

*Combination of two mutant **1781** alleles.* In addition to mutant 2078 and 2088 alleles, the mutant 1781Leu allele was found in many individuals within most (71%) of the clethodim resistant populations, usually in combination with another mutant allele of the same or different type (Table XVII). Plants homozygous for the mutant 1781Leu allele were able to survive clethodim at the field rate, whereas heterozygous plants could not survive this rate. The homozygous resistant genotype (1781Leu/1781Leu) was detected in four populations (Table XVII) and its resistance to clethodim was confirmed by an ACCase in vitro assay in which a moderate level of resistance (17-fold) was observed (Table XX). This genotype was found to be equally resistant to clethodim at the whole plant level, as compared to plants homozygous for the mutant 2078 or 2088 alleles (Figure 38). In addition, this genotype was found to be cross resistant to APP herbicides clodinafop, diclofop, fluazifop and haloxyfop, CHD herbicides sethoxydim and tralkoxydim, and the phenylpyrazolin herbicide pinoxaden (Table XXI). Therefore, resistance at field clethodim rates requires homozygosity of plants for the mutant 1781 alleles. Remarkably, one field evolved clethodim resistant population (R_7) was found to be 100% homozygous for the 1781 mutant alleles.

*Combination of mutant **1781/2027** or **1781/2041***. In this study with field evolved resistant *Lolium* populations, 12 patterns of mutant ACCase allele combinations endowing ACCase herbicide resistance have been revealed (Table XVII). This is to be expected in this highly genetically diverse, obligate cross-pollinated *Lolium*. Within a large herbicide treated field, *Lolium* individuals homo/heterozygous for different specific mutations of ACCase survive herbicide treatment, and in the absence of (killed) susceptible individuals, cross-pollination occurs among resistant survivors. What emerges are resistant populations comprised of individuals containing diverse ACCase mutations (a maximum of two). As expected, the genotype groups 4 to 12 would confer clethodim resistance (Table XVII). What is interesting is that the 2027Cys or 2041Asn allele is known to be mainly associated with APP herbicide resistance (Délye et al., 2005); however, combinations of 1781Leu/2027Cys alleles or 1781Leu/2041Asn alleles were found to confer clethodim resistance in *Lolium* at the field rate (Table XVII). This was also confirmed at the ACCase enzyme level with an I_{50} R/S ratio of 7 and 13 (Figure 37, Table XIX) for the mutant allele combinations of 1781/2027 and 1781/2041, respectively.

In studies with resistant *A. myosuroides* and *L. rigidum* populations from France, neither heterozygous nor homozygous mutants of 1781Leu were found to be resistant to clethodim, haloxyfop or clodinafop, and the genotype 1781Leu/2041Asn was not found to be resistant to clethodim in a seed germination assay (Délye et al., 2002b, Délye et al., 2005). Conversely, in a recent study in *A. sterilis*, 2027Cys and 2041Asn mutations were found to be associated with resistance to the CHD herbicides tralkoxydim and sethoxydim, respectively (Liu et al., 2007). Similarly, the 2041Asn mutation in *P. paradoxa* was found to confer resistance to most CHD herbicides with a lower level resistance to clethodim (Hochberg et al., 2007). These discrepancies in the cross resistance pattern endowed by a specific ACCase mutation are likely due to difference in plant species, methods of testing herbicide sensitivity, and/or especially herbicide rates used to discriminate between resistant and susceptible individuals. For example, in seed germination assay, germinating seedlings are exposed continuously to the herbicide, which is quite different from the field-simulating herbicide spray treatment used in our research. Therefore, it is possible that *A. myosuroides* plants containing 2027 or 2041 mutant alleles could survive field rates of CHD herbicides (Délye, 2005).

3.3.2 *In vitro* inhibition of ACCase activity by ACCase herbicides

ACCase assays were conducted to confirm that different mutations/combinations of mutant alleles displayed resistant ACCase. Thus, ACCase was partially purified from plants homozygous for the 1781Leu, 2078Gly or 2088Arg alleles and from plants with two different mutant alleles (1781Leu/2027Cys, or 1781Leu/2041Asn alleles). ACCase activity was evaluated in the presence of clethodim or other ACCase herbicides. Figure 37 shows that, as expected, ACCase from plants with mutant alleles was significantly less inhibited by APP herbicides (diclofop and haloxyfop acid) or CHD herbicides (clethodim or tralkoxydim). The herbicide concentration causing 50% inhibition of ACCase activity (I_{50}) was determined for each herbicide and each genotype, to give an R/S ratio (Table XIX). High level resistance to ACCase herbicides was found for ACCase from homozygous 2078 or 2088 mutants (with the R/S ratio ranging from 32 to 75). Clear but lower level resistance was found for ACCase from homozygous 1781 mutants (R/S ratio from 6 to 17).

A 7 to 13-fold resistance to clethodim was also observed for ACCase from mutant genotypes of 1781Leu/2027Cys and 1781Leu/2041Asn (Figure 37, Table XIX). Clearly, different ACCase mutations/combinations can endow different levels and patterns of ACCase herbicide resistance.

Specific ACCase activity (in the absence of herbicides) has been consistently observed to be lower in extracts from plants homozygous for the 2078 or 2088 mutant allele (three resistant populations), compared to that from plants homozygous for the wild-type allele (Table XX). Conversely, ACCase activity in extracts from plants homozygous for the 1781 mutant allele or plants of other genotypes (1781/2027, or 1781/2041) was not significantly different from that of susceptible controls (Table XX). These results were obtained by carefully conducted experiments in which protein concentration in the assay mixture was normalized for each genotype, and by using two susceptible controls.

Table XIX. Parameter estimates for logistic analysis of *in vitro* inhibition of ACCase enzyme activity by various ACCase inhibiting herbicides for the susceptible population S_1 and resistant (R) populations. Standard errors are in parentheses. Data are pooled from two extractions per population per herbicide and each assayed in duplicate.

Herbicide	Genotype (Population)	C	D	b	I_{50} (µM)	*P* value for I_{50}	R/S ratio for I_{50}
Diclofop acid	1781/1781[a] (R_7)	0.40 (1.06)	100 (0.85)	0.94 (0.04)	7.67 (0.41)	<0.01	6
	2078/2078[a] (R_{12})	1.57 (1.91)	102 (1.17)	1.52 (0.16)	40.9 (2.52)	<0.01	32
	2088/2088[a] (05281)	2.56 (1.62)	101 (0.93)	1.75 (0.16)	48.2 (2.02)	<0.01	38
	wild type (S_1)	3.22 (3.87)	98 (2.26)	0.84 (0.13)	1.27 (0.24)	<0.05	
Haloxyfop acid	1781/1781 (R_7)	3.37 (2.36)	99 (1.59)	1.11 (0.09)	23.4 (2.53)	0.01	14
	2078/2078 (R_{12})	3.57 (1.63)	99 (0.72)	1.30 (0.10)	75 (3.06)	<0.01	44
	2088/2088 (05281)	7.63 (3.86)	102 (1.92)	1.51 (0.31)	73.5 (6.92)	<0.01	43
	wild type (S_1)	3.96 (3.80)	98 (2.01)	0.89 (0.13)	1.70 (0.31)	<0.05	
Tralkoxydim	1781/1781 (R_7)	3.56 (4.60)	98 (1.71)	0.77 (0.10)	3.53 (0.72)	<0.05	17
	2078/2078 (R_{12})	0.53 (6.22)	102 (1.16)	0.80 (0.10)	11.2 (2.31)	<0.05	53
	2088/2088 (05281)	9.48 (7.45)	102 (1.03)	1.02 (0.19)	15.8 (3.65)	<0.05	75
	wild type (S_1)	7.41 (1.33)	100 (1.35)	0.86 (0.06)	0.21 (0.019)	<0.01	
Clethodim	1781/2027[b] (R_2)	6.4 (1.36)	100 (0.70)	0.79 (0.04)	1.62 (0.11)	<0.01	7
	1781/2041[c] (R_{10})	2.42 (3.92)	100 (1.21)	0.58 (0.05)	3.20 (0.56)	<0.05	13
	1781/1781 (R_7)	3.56 (1.4)	99 (0.64)	1.04 (0.04)	4.26 (0.26)	<0.01	18
	2078/2078 (R_{12})	5.20 (3.71)	98 (1.04)	1.07 (0.14)	9.76 (1.11)	<0.05	41
	2088/2088 (05281)	10.46 (4.64)	98 (1.05)	1.07(0.17)	11.57 (1.70)	<0.05	48
	2088/2088 (R_3)	4.89 (4.87)	99 (0.98)	0.93 (0.11)	11.75 (1.85)	<0.05	49
	wild type (S_1)	5.91 (3.51)	99 (3.26)	0.68 (0.10)	0.24 (0.06)	0.05	

[a] Plants homozygous for the mutant alleles of 1781Leu, 2078Gly or 2088Arg.
[b] Plants containing two types of mutant alleles (1781Leu /2027Cys).
[c] Plants containing two types of mutant alleles (1781Leu /2041Asn).

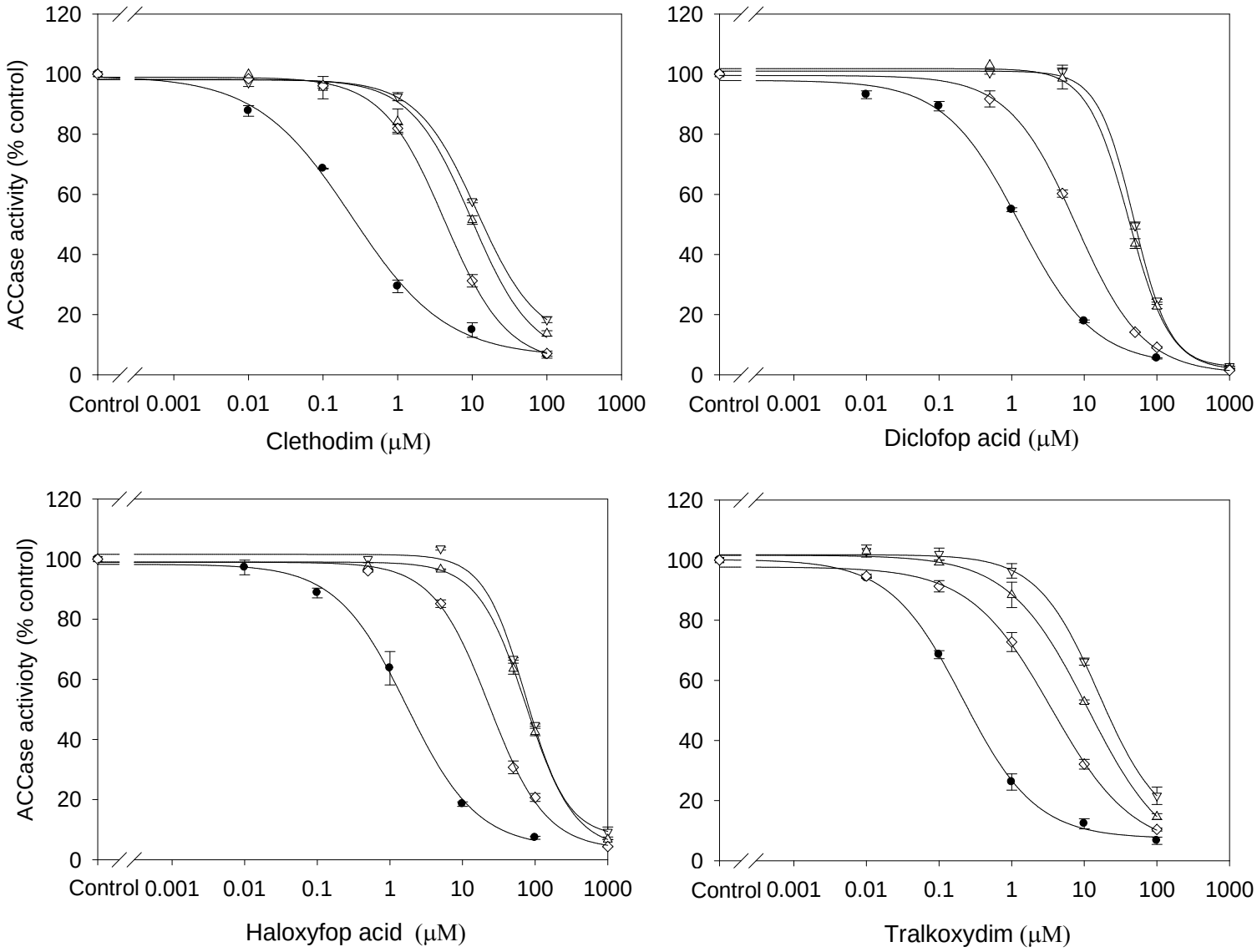

Figure 37. *In vitro* inhibition of ACCase activity by ACCase herbicides for susceptible plants (S_1, ●), resistant plants homozygous for 1781-Leu, (◇), 2078-Gly, (△) and 2088-Arg, (▽) from population R_7, R_{12} and R_{14}, respectively. Data are pooled from two extractions per population per herbicide and each was assayed in duplicate.

Table XX. ACCase activities in the absence of ACCase herbicides in the shoots of susceptible (S) and resistant (R) populations with known ACCase mutations.

Genotype used	Population	ACCase activity (nmol HCO_3^- mg protein^{-1} min^{-1})	R/S* ratio
1781Leu/1781Leu	R_7	9.10 ± 0.26 a	1.12
1781Leu/2027Cys	R_2	9.97 ± 0.25 a	1.23
1781Leu/2041Asn	R_{10}	7.88 ± 2.27 ab	0.97
2078Gly/2078Gly	R_{12}	5.86 ± 0.32 bc	0.72
2088Arg/2088Arg	R_3	4.38 ± 0.55 c	0.54
2088Arg/2088Arg	04259	4.46 ± 0.97 c	0.55
2088Arg/2088Arg	05281	4.45 ± 0.42 c	0.55
Wild type	S_1	7.38 ± 0.06 ab	
Wild type	S_2	8.85 ± 0.22 a	

Data are means ± se of two to six enzyme extractions per population and each assayed in duplicate. Means followed by different letters are significantly different at the 5% level by the LSD test.

*ACCase activities from two S populations (S_1 and S_2) were averaged for calculation of R/S ratios.

ACCase activity associated with specific ACCase mutations/genotypes. It was found in this study that plants homozygous for the resistant allele 2088 or 2078, showed lower ACCase specific activity (Table XX). Low ACCase activity was also observed in *A. myosuroides* for resistant alleles 2027Cys and 2078Gly (Délye et al., 2005). Low ACCase activity indicates that these residues are important for CT catalytic activity and these amino acid substitutions, although conferring herbicide resistance, may reduce enzyme catalytic activity and impose a fitness penalty at the whole plant level. From the three-dimensional models of *A. myosuroides* CT-herbicide complexes reconstructed from yeast, it was indicated that 2027 and 2078 mutations did not directly interfere with herbicide binding; instead, they may change the three-dimensional shape of the cavity of the CT active site by inducing a number of small allosteric changes (Délye et al., 2005). The low enzyme activity associated with the 2078 or 2088 mutation (Table XX) will be matter for

future investigation in relation to the fitness of resistant plants. For resistance to acetolactate synthase (ALS)-inhibiting herbicides certain ALS gene mutations cause fitness penalties (Roux et al., 2004; Tardif et al., 2006). Table 6 indicates this may also be the case for particular resistant mutant ACCase alleles. This work has demonstrated that the 1781 mutation does not result in lower ACCase activity (Table XX) and our fitness studies in one *Lolium* population containing the 1781 mutation showed no, or negligible, resistance fitness cost (Vila-Aiub et al., 2005a, b). However, a fitness penalty was detected in *A. myosuroides* in association with the 2078 mutation (Délye et al., 2007). On the basis of the lower ACCase activity in *Lolium* plants with the 2088 or 2078 mutation (Table XX) it can be speculated that plants carrying these mutations may suffer a fitness penalty.

3.3.3 Dose response pot experiment

Purified populations with plants homozygous for the mutant resistant 1781, 2078 or 2088 alleles were used to determine their clethodim resistance levels. As shown in Figure 38, the susceptible population (S_1) was killed at 7.5 g clethodim ha^{-1} or higher. In contrast, homozygous resistant plants were markedly less affected by clethodim, requiring a high rate (240 g ha^{-1}) for substantial mortality. The clethodim rate causing 50% mortality (LD_{50}) for the susceptible population S_1 was 4.4 ± 0.43 g ha^{-1} versus 98 ± 1.68, 105 ± 0.23 and 115 ± 0.45 for the homozygous resistant populations containing the mutant resistant 1781, 2078 or 2088 alleles, respectively. Therefore, based on the R/S LD_{50} ratio, the homozygous resistant populations are more than 20-fold resistant to clethodim at the whole plant level. Clearly, plants homozygous for the mutant resistant 1781, 2078 or 2088 alleles are all resistant to clethodim at the commercial Australian field rate. Cross resistance pattern to other ACCase herbicides was also determined. As shown in Table XXI, at field or higher rates, plants homozygous for the mutant resistant 1781, 2078 or 2088 alleles were resistant to APP herbicides clodinafop, diclofop, fluazifop and haloxyfop, CHD herbicides sethoxydim and tralkoxydim, and the phenylpyrazolin herbicide pinoxaden,. About 50%, 70% and 87% of plants homozygous for the mutant resistant 1781, 2088 or 2078 alleles, respectively, were cross resistant to the CHD herbicide butroxydim (Table XXI).

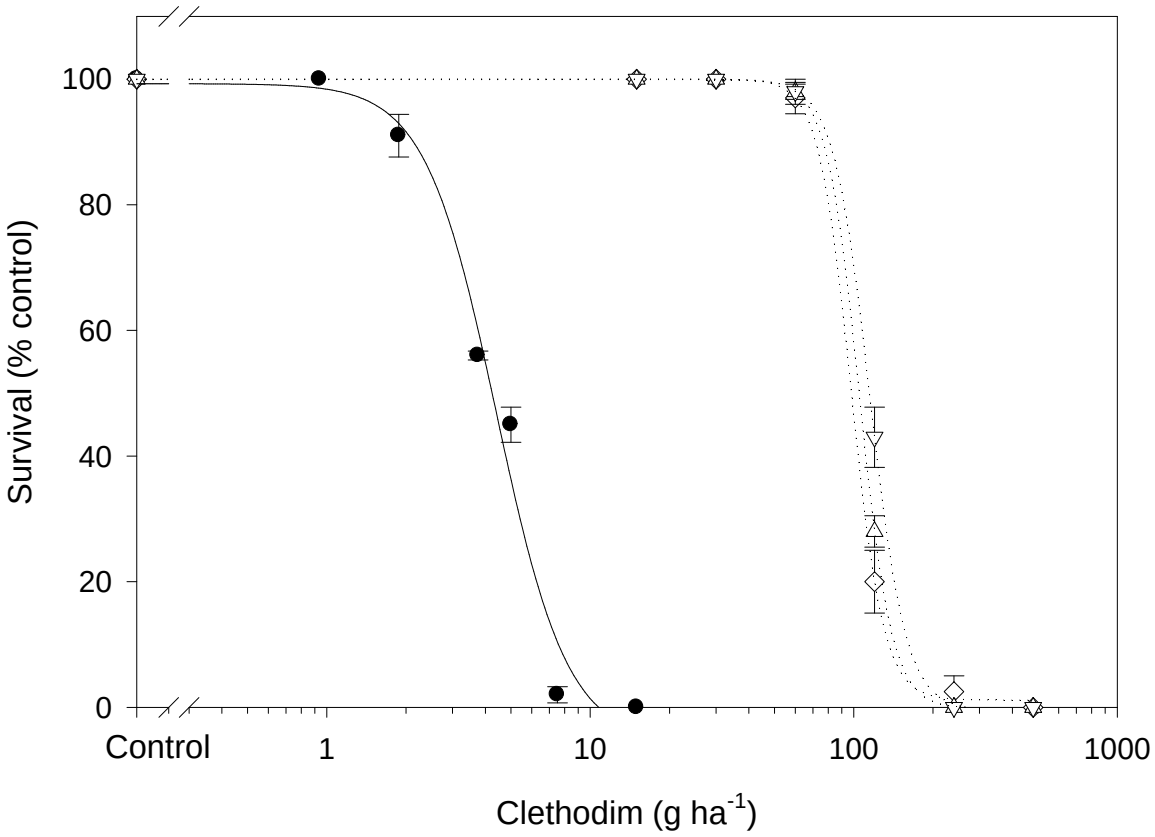

Figure 38. Clethodim dose response of the known susceptible population VLR1 (wild type,●) and purified homozygous resistant populations R_7P (1781Leu, ◇), $R_{12}P$ (2078Gly, △) and 05281P (2088Arg, ▽). Data for the susceptible population VLR1 were pooled from two experiments and data for the purified populations was each from a single experiment.

Table XXI. Percentage survival of plants from purified homozygous resistant populations for the three ACCase mutations at herbicide application rates known to control the susceptible population S_1. Forty to 50 plants per population were treated with the respective herbicide.

Herbicides	Application rate (g ha^{-1})	Genotype (Population), % survival			
		Wild type (S_1)	1781Leu (R_7P)	2078Gly ($R_{12}P$)	2088Arg (05281P)
APP					
diclofop	1000	1	100	100	100
clodinafop	50	3	100	100	100
fluazifop	100	0	100	100	100
haloxyfop	52	0	100	100	100
CHD					
sethoxydim	186	0	100	100	100
tralkoxydim	800	4	100	100	100
butroxydim	45	2	50	87	70
Phenylpyrazolin					
pinoxaden	30	0	100	100	100

3.3.4 ACCase mutations revealed in pinoxaden-resistant *Lolium* populations

All the three populations analysed carried mutations in the CT domain conferring resistance to ACCase inhibitors. Populations 05281, as reported in preliminary screenings (data not shown), had a survival between 70 and 90% at the field dose and 3 times the field dose using clodinafop and pinoxaden. The survival to sethoxydim at the field dose and 6 times the field dose was between 80 and 95%. To investigate the mutations present in this population 95 plants have been analysed through dCAPS methodology for mutation in position 1781. Wild types are visually discriminated from mutants in the electrophoretic gel because of the cut fragment after digestion with the NsiI enzyme (Materials and Methods, Figure 9). In the tested plants 21 samples did not carry the 1781Leu mutation

and in 5 samples the result was uncertain so the entire CT domain has been sequenced. Among the tested samples 69 were carrying the mutation in position 1781 (Ile to Leu, 35 heterozygous and 34 homozygous), 25 the mutation in position 2088 (13 heterozygous and 12 homozygous) and one sample apparently did not carry any mutation (Table XXII).

Table XXII. Molecular analysis for 95 plants of the population 05281 investigated for mutations in the CT domain causing resistance to ACCase-inhibitors.

Mutation		**1781Leu**	**2088Arg**	**Not detected**
N. plants	Heterozygous	35	13	-
	Homozygous	34	12	-
	Total	69	25	1
% mutant		72.6	26.3	1.1

Population 05256, as reported in preliminary screenings (data not shown), had a 100% survival at the field dose and 3 times the field dose using clodinafop. The survival to pinoxaden at the field dose was of 60% ant sethoxydim at the field dose was of 95%. Both these herbicide at the maximum dose had a survival comprised between 5 and 10%. Plants selected with pinoxaden 0.1 µM using the quick test were 101. All have been tested through dCAPS for the mutation in position 1781. Samples classified as susceptible for the mutation in position 1781 have been investigated using the dCAPS developed for the mutation 2078. Those samples which did not give a clear result for the substitution in position 2078 have been investigated using the CAPS developed for the mutation 2041. All the samples which did not give a clear result using CAPS and dCAPS have been sequenced. From the results of the dCAPS method and sequencing four different mutations have been detected. Among the tested samples 41 were carrying the mutation in position 1781 (31 heterozygous and 10 homozygous), 52 were carrying a mutation in position 2041 (26 heterozygous and 26 homozygous), 5 heterozygous for the mutation in position 2078, 1 heterozygous for the mutation in position 2096 and two samples apparently did not carry any mutation (Table XXIII). This population showed a great variability in terms of different mutation present among the tested plants.

Table XXIII. Molecular analysis for 101 plants of the population 05256 investigated for mutations in the CT domain causing resistance to ACCase-inhibitors.

Mutation		**1781Leu**	**2041Val/Asn**	**2078Gly**	**2096Ala**	**Not detected**
N. plants	Heterozygous	31	26	5	1	-
	Homozygous	10	26	-	-	-
	Total	41	52	5	1	2
% mutant		40.7	51.6	4.7	0.9	2.1

Population 06302, as reported in preliminary screenings (data not shown), had a survival between 60 and 80% at the field dose and 3 times the field dose using clodinafop and pinoxaden. The survival to sethoxydim at the field dose and 6 times the field dose was between 80%. Plants selected with pinoxaden 0.1 µM using the quick test were 86. All have been tested through dCAPS for the mutation in position 1781. Four samples did not give a clear result using dCAPS and have been sequenced. This population showed a great homogeneity displaying the mutation in position 1781 in 82 samples (58 heterozygous and 24 homozygous), the four sequence samples did not showed any mutation (Table XXIV).

Table XXIV. Molecular analysis for 82 plants of the population 06302 investigated for mutations in the CT domain causing resistance to ACCase-inhibitors.

Mutation		**1781Leu**	**Not detected**
N. plants	Heterozygous	58	-
	Homozygous	24	-
	Total	82	4
% mutant		95.3	4.7

3.4 *Phalaris paradoxa* characterisation

3.4.1 Dose-response experiments

Most the stand errors were below 10% indicating that log-logistic equation accurately fitted the data (Tables XXV and XXVI). Doses were chosen appropriately except for what concerns tralkoxydim and sethoxydim in population 0478L and pinoxaden in population 0477L that were overestimated in resistance (Table XXVI). In Figures 39-44 are reported the dose-response curves for the most relevant treatments. All resistant index (R.I.) of population 0047 are close to one, confirming a behaviour similar to the Herbiseed susceptible population (0041).

Of the seven biotypes resistant to ACCase-inhibitors, only population 0025 displayed cross-resistant to all ACCase herbicides with high levels of R.I. also for sethoxydim. This may suggest a target site mechanism of resistance involved as is not reported in literature an enhanced mechanism due to sethoxydim. Population 0478L is cross-resistant to clodinafop, pinoxaden and to tralkoxydim when survival R.I. is included (Survival R.I. to tralkoxydim is 11.3; Table XXVI, Figure 43), but not to sethoxydim. From literature, a similar behaviour of major resistance to tralkoxydim compared with sethoxydim, has been observed in *L. rigidum* (Burnet *et al*., 1994) and *Alopecurus myosuroides* (Menendez and De Prado, 1996). Population 0044 is resistant to diclofop-methyl and slightly (R.I. of 2) to tralkoxydim. Four of the five population investigated in the first experiment are resistant to diclofop-methyl but with a significant lower level than the one observed in 0025 (Figure 39 and Table XXV), this can be explained as the massive use reported in Table XII (Materials and Methods), diclofop has been also the first ACCase inhibitor introduced in the Italian market in the early 80s. Generally fresh weight R.I. was greater than survival R.I. in all populations (Table XXV and XXVI). Comparing the behaviour of the populations tested with clodinafop (Figure 40 and 41) and pinoxaden (Figure 44) it is evident how greater the level of resistance of pop. 0025 is respect to other populations. This population is also highly cross-resistant to fenoxaprop, tralkoxydim, sethoxydim and - with a lower RI - also to diclofop (Table XXV). Pop. 0478L has also a good resistance to clodinafop, tralkoxidim and pinoxaden but at two times the field dose of sethoxydim killed every plant.

All biotypes are well controlled by ASL-inhibiting products, these lead a useful tool in contrasting the rising of resistance when used according to integrated weed management strategies.

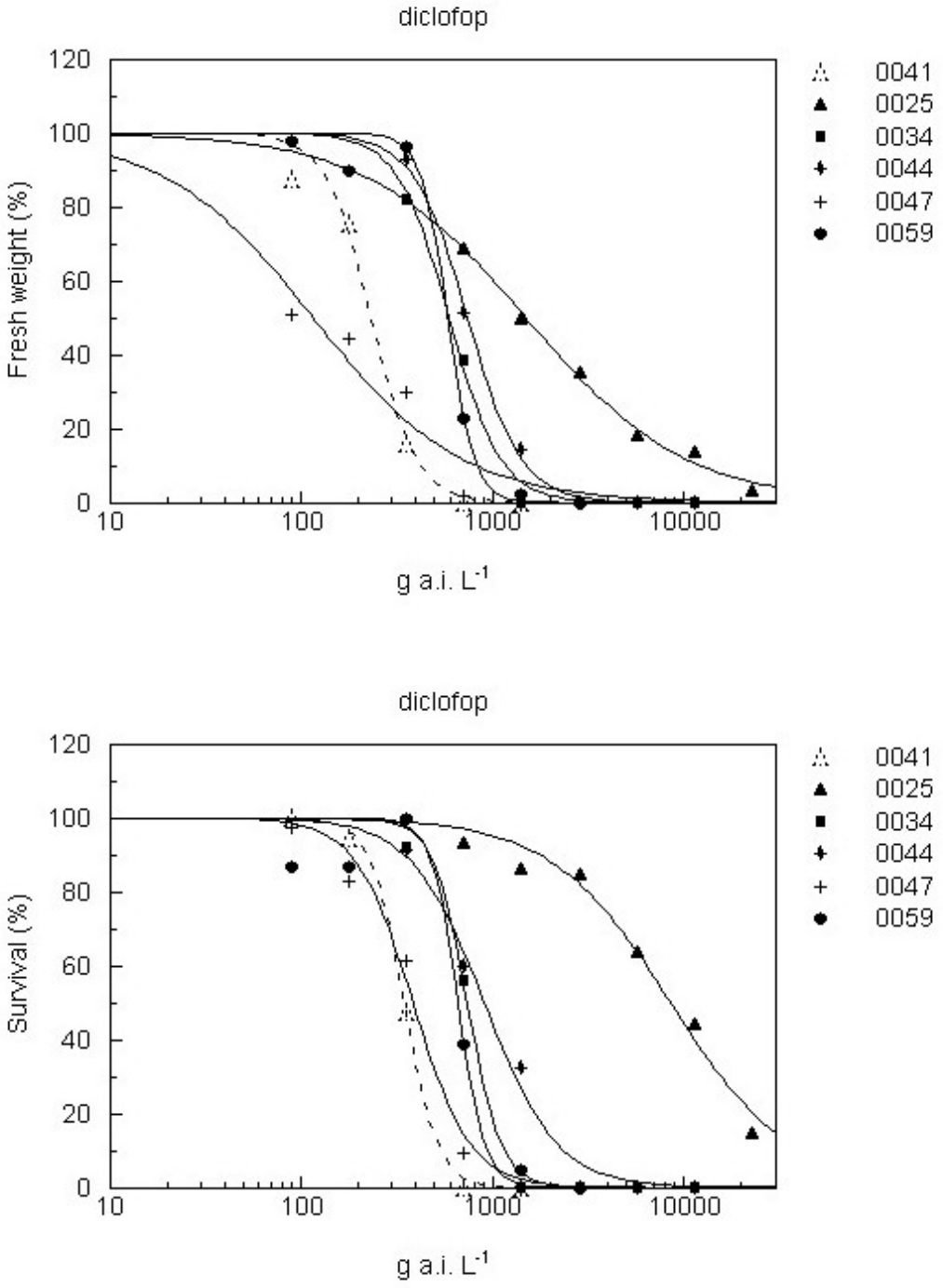

Figure 1. First experiment, effect of diclofop applied post-emergence. Fresh weight, upper graph and survival, lower graph.

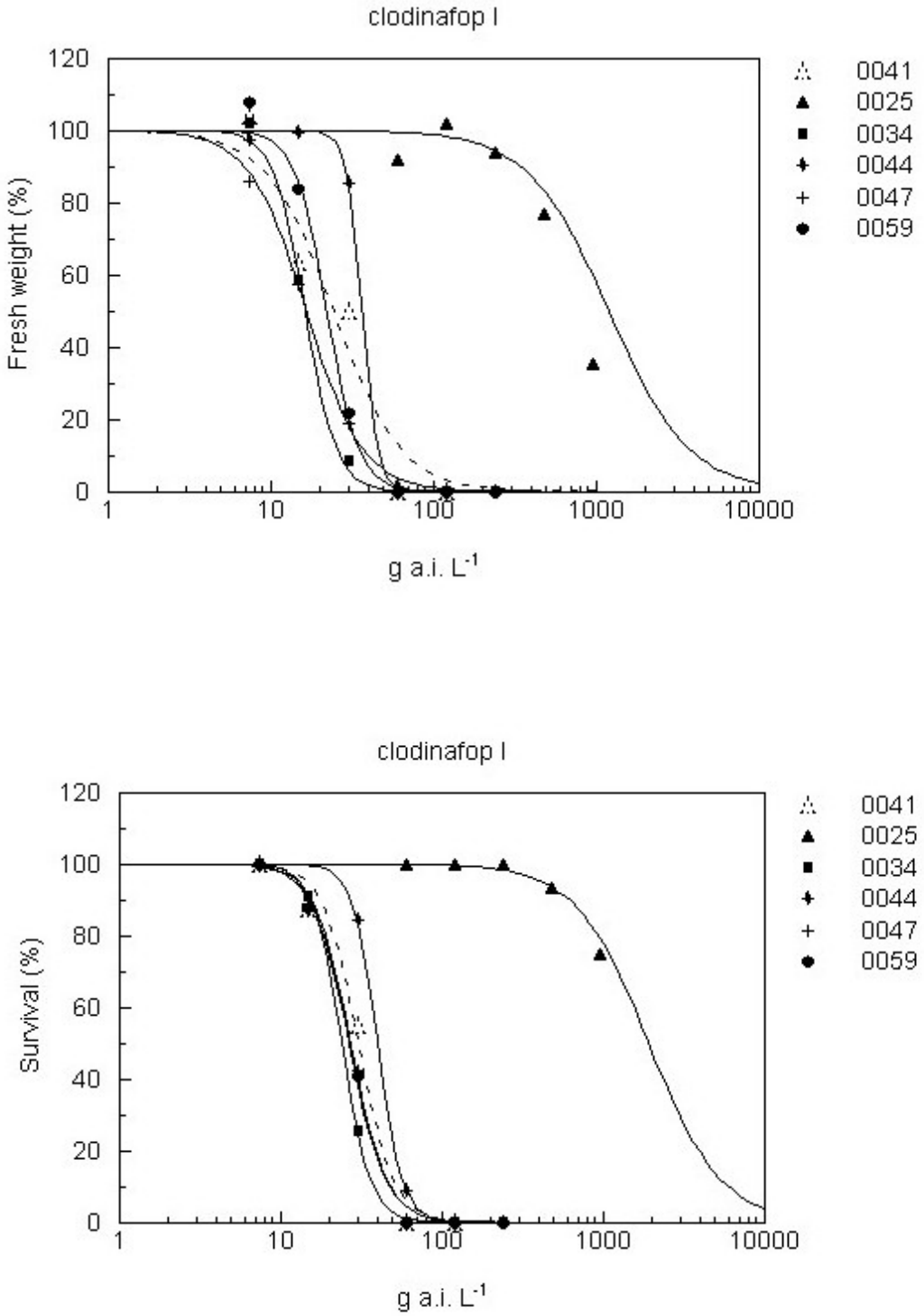

Figure 2. First experiment, effect of clodinafop applied post-emergence. Fresh weight, upper graph and survival, lower graph.

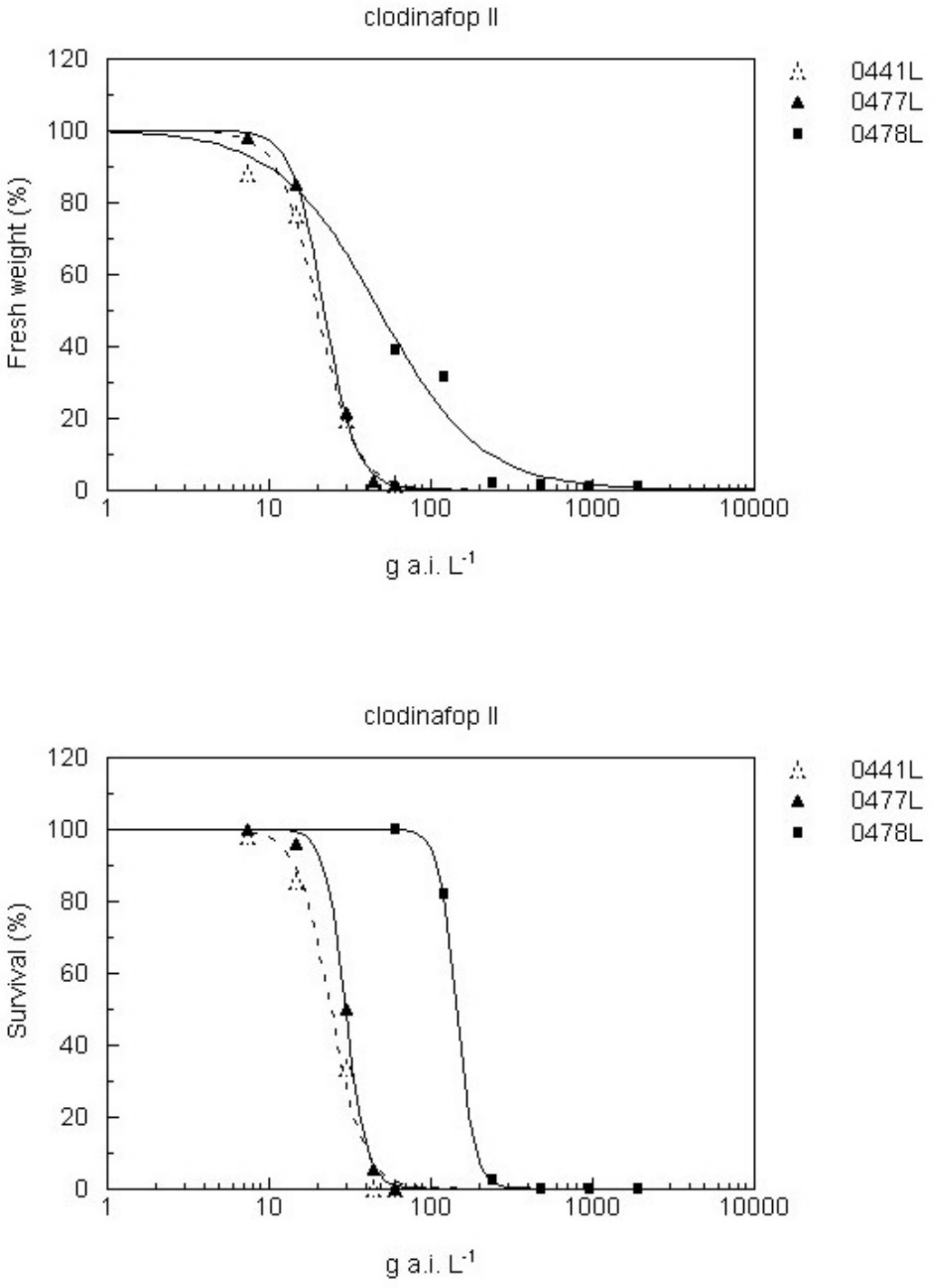

Figure 3. Second experiment, effect of clodinafop applied post-emergence. Fresh weight, upper graph and survival, lower graph.

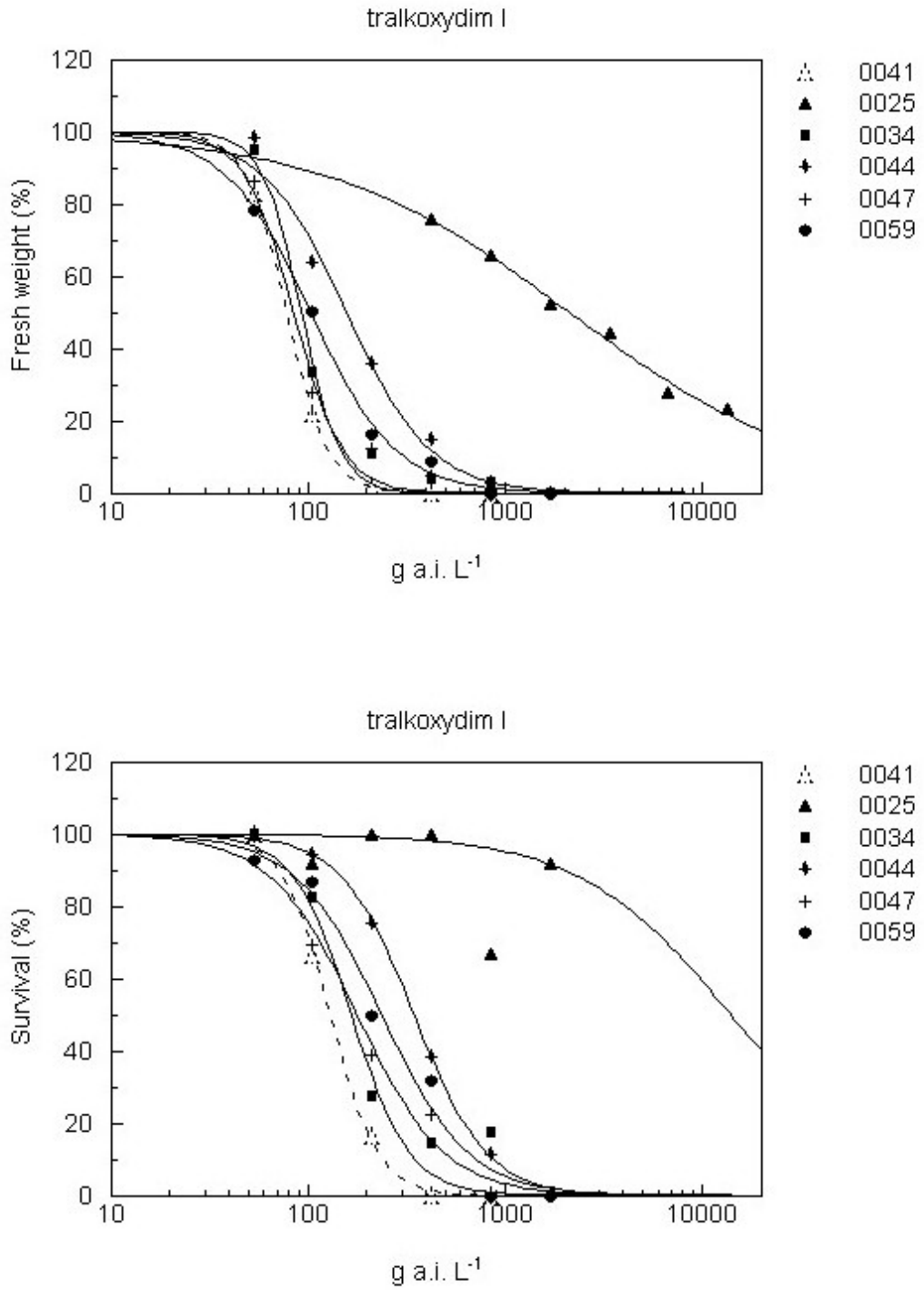

Figure 4. First experiment, effect of tralkoxydim applied post-emergence. Fresh weight, upper graph and survival, lower graph.

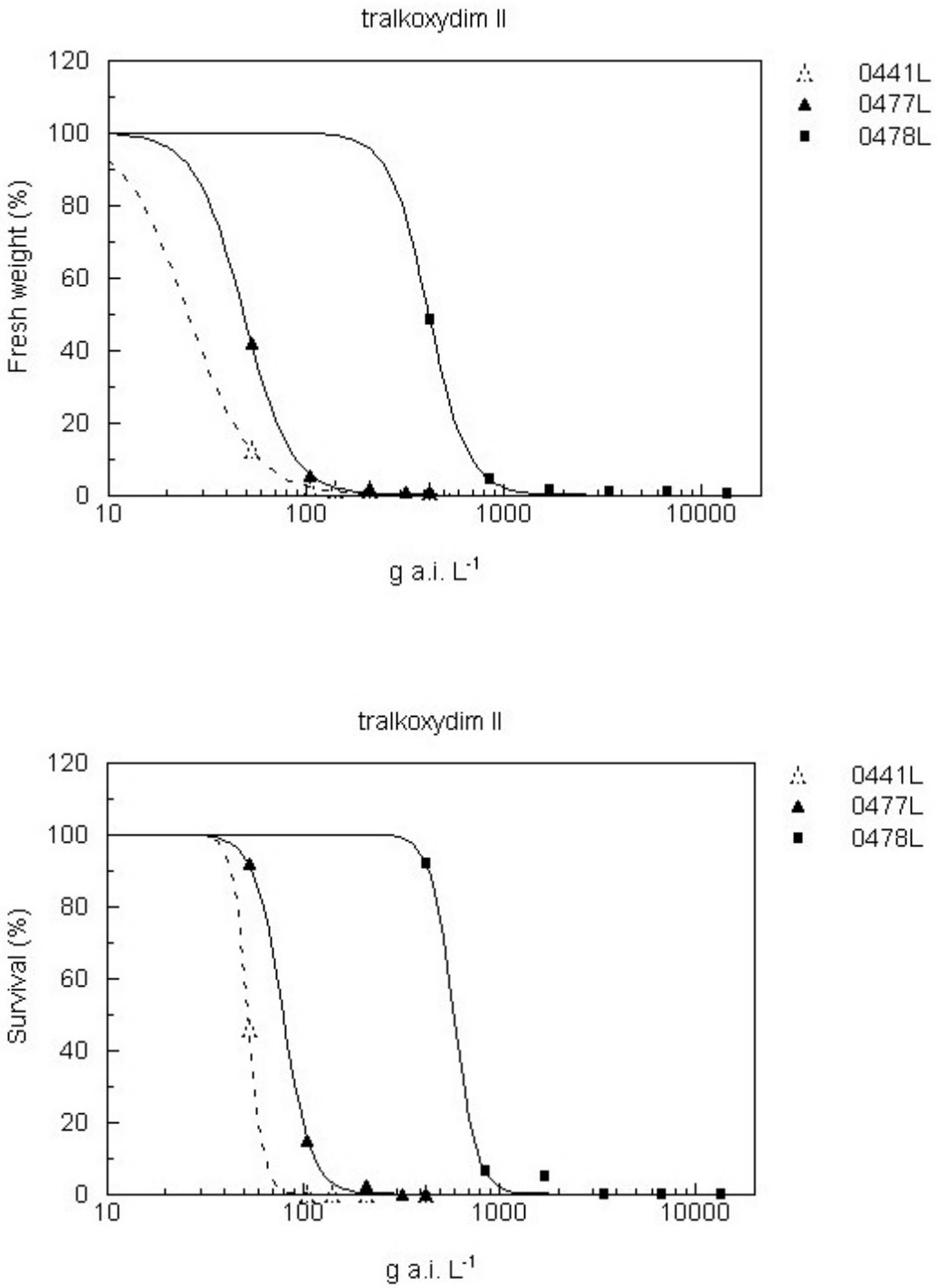

Figure 5. Second experiment, effect of tralkoxydim applied post-emergence. Fresh weight, upper graph and survival, lower graph.

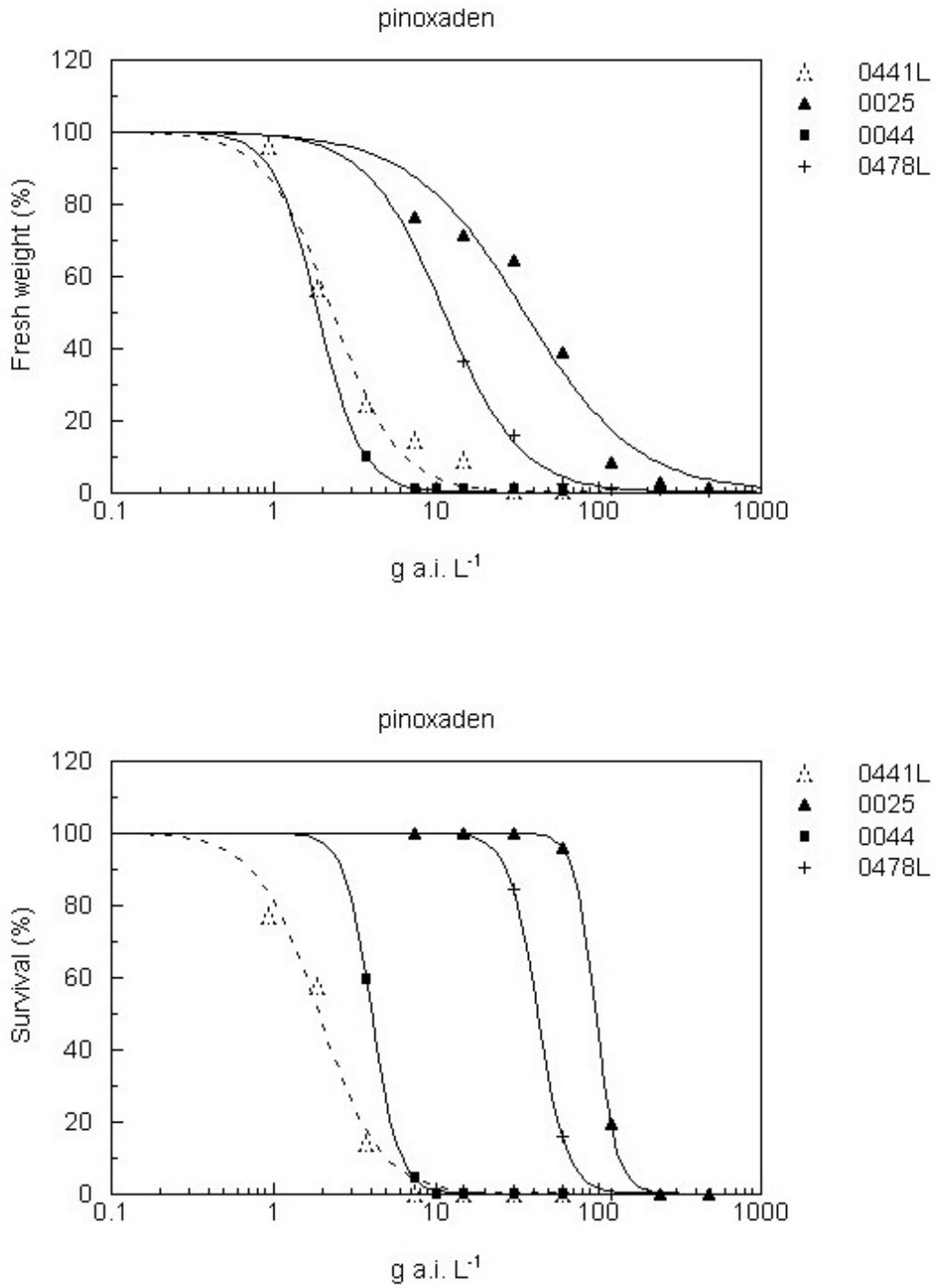

Figure 6. Second experiment, effect of pinoxaden applied post-emergence. Fresh weight, upper graph and survival, lower graph.

Table XXV. First experiment. Resistance index calculated on the basis of pop. 0041 (R.I.), herbicide doses that inhibits growth or survival by 50 percent (GR 50 or ED 50) and standard errors (S.E.)

FRESH WEIGHT

	Herbicide																	
	clodinafop-propargyl			diclofop-methyl			fenoxaprop-P-ethyl			tralkoxydim			sethoxydim			iodosulfuron			Chlorsulfuron		
Pop. code	GR 50	S.E.	R.I.	GR 50	S.E.	R.I.	GR 50	S.E.	R.I.	GR 50	S.E.	R.I.	GR 50	S.E.	R.I.	GR 50	S.E.	R.I.	GR 50	S.E.	R.I.
0041	24.3	3.66		232	13.6		35.7	0.95		78.1	0.10		53.0	1.94		2.12	0.035		2.94	0.434	
0025	1498	167.2	61.7	1491	72.4	6.4	595.6	221.22	16.7	2129	111.0	27.3	2147	110.9	40.5	1.50	0.130	0.7	3.62	0.508	1.2
0034	16.3	0.36	0.7	593	20.1	2.6	28.2	1.04	0.8	92.6	4.23	1.2	66.9	2.66	1.3	1.66	0.088	0.8	1.75	0.307	0.6
0044	36.6	1.32	1.5	739	15.0	3.2	46.8	6.60	1.3	157.0	10.19	2.0	56.8	1.45	1.1	1.74	0.085	0.8	3.95	0.427	1.3
0047	16.6	0.48	0.7	115	25.1	0.5	28.5	2.89	0.8	84.6	4.92	1.1	59.0	2.38	1.1	2.21	0.028	1.0	2.59	0.655	0.9
0059	22.2	1.58	0.9	584	35.0	2.5	45.4	1.68	1.3	103.1	3.25	1.3	60.7	2.12	1.1	1.50	0.127	0.7	3.82	0.769	1.3

SURVIVAL

	Herbicide																	
	clodinafop-propargyl			diclofop-methyl			fenoxaprop-P-ethyl			tralkoxydim			sethoxydim			iodosulfuron			Chlorsulfuron		
Pop. code	ED 50	S.E.	R.I.	ED 50	S.E.	R.I.	ED 50	S.E.	R.I.	ED 50	S.E.	R.I.	ED 50	S.E.	R.I.	ED 50	S.E.	R.I.	ED 50	S.E.	R.I.
0041	29.3	2.33		346	4.8		39.5	0.41		130	3.2		80.4	0.65		2.85	0.025		2.89	0.463	
0025	> 1920		> 65.5	8484	630.0	24.6	> 2112		> 53.5	> 13600		> 104	2350.3	11.91	29.2	2.20	0.031	0.8	3.56	0.593	1.2
0034	24.1	0.14	0.8	736	18.9	2.1	50.8	0.32	1.3	166	17.7	1.3	88.0	9.84	1.1	2.12	0.030	0.7	1.58	0.222	0.5
0044	40.1	2.70	1.4	903	55.5	2.6	65.1	1.48	1.6	345	5.5	2.6	69.4	0.07	0.9	2.56	0.004	0.9	3.92	0.459	1.4
0047	27.3	0.74	0.9	383	25.3	1.1	41.7	3.30	1.1	178	16.5	1.4	84.9	0.56	1.1	2.94	0.038	1.0	2.59	0.655	0.9
0059	26.7	0.64	0.9	661	56.2	1.9	67.4	0.64	1.7	237	19.7	1.8	86.7	0.42	1.1	2.29	0.019	0.8	3.82	0.769	1.3

Table XXVI. Second experiment. Resistance index calculated on the basis of pop. 0441L (R.I.), herbicide doses that inhibits growth or survival by 50 percent (GR 50 or ED 50) and standard errors (S.E.)

FRESH WEIGHT

	Herbicide																		
	clodinafop-propargyl			tralkoxydim			sethoxydim			pinoxaden			iodosulfuron			imazamethabenz			isoproturon		
Pop. code	GR 50	S.E.	R.I.	GR 50	S.E.	R.I.	GR 50	S.E.	R.I.	GR 50	S.E.	R.I.	GR 50	S.E.	R.I.	GR 50	S.E.	R.I.	GR 50	S.E.	R.I.
0441L	20.1	1.13		25.4	2.51		13.29	0.806		2.31	0.183		0.645	0.0723		265	10.0		527	122.9	
0477L	22.1	0.28	1.1	48.4	0.61	1.9	9.67	0.098	0.7	< 3.75			0.614	0.0486	1.0	232	6.5	0.9	259	76.3	0.5
0478L	48.0	9.75	2.4	418.8	3.09	16.5	< 370			11.19	0.459	4.8	0.906	0.0192	1.4	-	-	-	141	72.2	0.3
0025	-	-	-	-	-	-	-	-	-	34.90	5.020	15.1	-	-	-	-	-	-	332	34.4	0.6
0044	-	-	-	-	-	-	-	-	-	1.88	0.236	0.8	-	-	-	-	-	-	189	18.7	0.4

SURVIVAL

	Herbicide																		
	clodinafop-propargyl			tralkoxydim			sethoxydim			pinoxaden			iodosulfuron			imazamethabenz			isoproturon		
Pop. code	ED 50	S.E.	R.I.	ED 50	S.E.	R.I.	ED 50	S.E.	R.I.	ED 50	S.E.	R.I.	ED 50	S.E.	R.I.	ED 50	S.E.	R.I.	ED 50	S.E.	R.I.
0441L	24.3	1.16		52.3	0.05		23.3	0.06		1.9	0.11		1.85	0.055		651	9.9		1647	12	
0477L	29.9	0.30	1.2	79.2	0.86	1.5	23.2	1.74	1.0	< 3.75			2.77	0.123	1.5	557	4.0	0.9	1173	27	0.7
0478L	145.8	0.13	6.0	588.8	17.16	11.3	< 370			42.5	0.19	22.3	2.10	0.142	1.1	-	-	-	1404	105	0.9
0025	-	-	-	-	-	-	-	-	-	96.7	0.14	50.7	-	-	-	-	-	-	1001	82	0.6
0044	-	-	-	-	-	-	-	-	-	4.0	0.02	2.1	-	-	-	-	-	-	613	42	0.4

3.4.2 Molecular analysis

Alignment of the nucleotide sequences of the ACCase-amplified regions of both resistant and susceptible populations showed only one nucleotide substitution coding for an amino acid change. All 16 plants of population 0025 presented a GAT to GGT point mutation at position 2078 that determines an aspartic acid (Asp) to glycine (Gly) substitution. All the pinoxaden resistant plants analysed were homozygous at position 2078. The Asp to Gly substitution has already been reported to determine APPs and CHDs resistance in *Alopecurus myosuroides* and *Avena sterilis ssp. ludoviciana*. Consistently, the Italian population of *P. paradoxa* (0025) showed cross-resistance to both APPs and CHDs herbicides as well as to the DEN.

On the contrary, all the 11 pinoxaden selected 0478L plants showed an ATA to GTA mutation at position 1781 that changed an isoleucine (Ile) to valine (Val). All the plants analysed were homozygous at this position. It is interesting to note that mutation at position 1781 has already been reported to determine resistance in several species (Introduction, Table II), but in theses cases the isoleucine was substituted with a leucine and never with a valine. However, the new amino acid substitution identified in Italian *P. paradoxa* population determines a cross-resistance to APPs and CHDs herbicides. In comparison to the 2078 substitution observed in the other Italian resistant population, the level of resistance to both APPs and CHDs is lower.

4. CONCLUSIONS

4.1 Screenings, patterns and levels of resistance

In Italy mainly two *Lolium* species are found in durum wheat crops (*L. multiflorum* and *L. rigidum*) that interbreed easily and frequently. They are both found mainly found in central Italy where represent around 10% of the weeds found in wheat while *L. rigidum* is more typical of the southern regions (Viggiani, 2005). Where both species are present at the same time in the same infested crop we observed a continuum of hybrids between the species and most populations are made up of "intermediate" plants.

Screenings of *Lolium* (collected in 2004-2006) have shown a high percentage of resistant populations with different patterns and levels of resistance. Only a few of the tested populations were still controlled by clodinafop, while some of the resistant populations were controlled by the new herbicide pinoxaden. All resistant populations were resistant to clodinafop. Pinoxaden behaved quite similarly to the CHDs.

P. paradoxa showed a slow diffusion of resistance during the last years. Again, clodinafop proved to be the herbicide with worst resistant problems, while just one population (collected in 2004-2006) demonstrated cross-resistance to pinoxaden. From the molecular characterisation of previous 2004 populations, two samples showing target site mechanism (mutant Ile1781Leu and Asp2078Gly) are resistant to pinoxaden.

The use of pinoxaden is not recommended for populations with certain ACCase-inhibitors resistance problems. Different mutations selected by other APPs and CHDs affect this new herbicide (Collavo et al., 2007a and Hochberg et al., 2007). In these cases integrated weed management has to be adopted in the field, approaching each case individually.

The evolution of resistance appears to be very fast for *Lolium* while it is rather slow for P. paradoxa. The difference is probably due to the biological aspects of breeding characteristics and mating system of the two species: *Lolium* is a strictly cross-pollinated species while P. paradoxa is predominantly self-pollinated. An aspect to address in future work, is to determine the percentage of out-breeding in the mainly self-pollinating *P. paradoxa*.

Complain monitoring and subsequent greenhouse testing confirmed to be a reliable approach for determining the resistance status of the populations and therefore a useful tool for practical resistance management. The new herbicide pinoxaden should not be

suggested for controlling ACCase-resistance cases without knowing the molecular basis of resistance as it is emphasised in the following molecular characterisation.

ALS-inhibitors still effectively controlled ACCase resistant populations, however, quite a few of these showed a plant survival below 20%, so indicating that the selection process is ongoing. ALS-inhibitors survivals have a low biomass in comparison with the untreated control and probably would suffer the competition of the crop in field conditions. ALS-inhibitors can still be effectively used, but within a careful IWM strategy. Attention should be paid to the use of just two MoA which have a different but with very specific target (ACCase and ALS enzymes), even if rotated.

It must be stressed that there is no one herbicide able to adequately control all resistant populations.

4.2 Petri dish seed quick test

In developing the quick test for *Lolium* spp. and *P. paradoxa* priority was given to those methodologies that reduce time and costs. Agar was chosen as growth medium, the vantage of using agar was to avoid evaporation that could occur using aqueous herbicide solutions and better randomise the replicates during the incubatotion period. The discrimination between susceptible and resistant has been based on survivals identified by the presence of green tissue, active growth and roots development. Survival data give a clear cut result rather than shoot length.

The comparison between commercial formulations and technical grades gave similar results for both clodinafop and pinoxaden, therefore all the experiments for the setting up and validation of the quick test have been done using commercial formulations which are lesse expensive and readily available.

The discriminant concentration between resistant and susceptible is generally lower than the that giving results comparable to greenhouse pot screenings (hereafter called “comparing” dose), leading to an overestimation of practical resistance. For this reason a “comparing dose” was determined.

Clodinafop clearly differentiated susceptible and resistant populations, i.e. populations are either susceptile or highly resistant; while with pinoxaden the populations were distributed along the trend line because of the different resistance level to this herbicide.

The “comparing dose” has been validated using other populations not included in the set up experiments and for *Lolium* spp. they were: clodinafop 1 µM, pinoxaden 0.2 µM, clethodim 0.2 µM and sethoxydim 0.1 µM; for *P. paradoxa*, clodinafop 0.1 µM, pinoxaden 0.05 µM and clethodim 0.1 µM.

The validation phase proved that the quick test reliably discriminates susceptible form resistant populations, it could therefore be a useful tool in screening large number of samples. The results on P. paradoxa should be taken carefully because of the low number of resistant populations considered.

It was not possible to set a dose discriminating between target site and metabolic resistance: some mutations appear to be “weak”, i.e. do not confer resistance to high herbicide doses, With the characteristic resistance patterns of the populations tested, a high herbicide concentration would just discriminate between very resistant populations and less resistant ones regardless the resistance mechanism involved.

4.3 *Lolium* spp. characterisation

Herbicide rate is a potent factor in resistance evolution. Where selection occurs at a high herbicide rate, only individuals endowed with relatively strong resistance mechanisms survive. Conversely, selection at a lower herbicide rate enables survival of both groups of individuals: those with strong resistance mechanisms and individuals with weaker resistance mechanisms (Neve and Powles, 2005). At the relatively low recommended field rate of clethodim (60 g ha^{-1} used in Australia, compared with >140g/ha in North America and Europe), *Lolium* plants with certain ACCase mutations can survive. Furthermore, a herbicide dose can be lethal to heterozygous individuals, whereas homozygous individuals survive. This is evident for 1781Leu alleles in relation to clethodim resistance in *Lolium* species, which heterozygous individuals do not survive clethodim resistance at the field rate, whereas homozygous mutant individuals do. Heterozygous individuals for the mutation 1781Leu are resistant to clethodim if plants are in combination with 2027Cys or 2041Asn mutations.

A given individual plant of *L. rigidum* can contain, at most, two distinct mutant ACCase alleles.

Twelve field evolved clethodim resistant *Lolium* populations (10 Australians and two Italians) have the resistant 2078 mutation and five populations have the resistant 2088

mutation, and these two mutations endow a sufficient level of resistance to clethodim and other ACCase herbicides.

In summary, five ACCase mutations have been identified (1781Leu, 2027Cys, 2041Asn, 2078Gly and 2088Arg) and revealed 12 genotypes in 14 clethodim resistant *Lolium* populations.

This research has established that resistance to ACCase herbicides depends on the specific resistant allele(s), the homo/heterozygous status of plants for the specific resistant allele(s), and combinations of different resistant alleles plus herbicide rates are all important. To fully understand resistance, knowledge of all these factors is essential.

Any non-target-site based clethodim resistance mechanism has not been examined in these populations. However, multiple resistance mechanisms (target-site and non-target-site based) can be simultaneously expressed in individual plants of genetically diverse, cross-pollinated *L. rigidum* (Tardif and Powles, 1994; Yu et al., 2007).

Discrepancies in the cross resistance pattern endowed by a specific ACCase mutation are likely due to difference in plant species, methods of testing herbicide sensitivity, and/or especially herbicide rates used to discriminate between resistant and susceptible individuals.

Most of the mutations responsible for clethodim resistance have been found in Italian populations of *Lolium* resistant to pinoxaden. (Unfortunately we could not test the different mutant alleles at enzyme level). Using CAPS, dCAPS and sequencing have revealed the three strongest mutations, 1781Leu, 2078Gly and 2088Arg, described for clethodim resistance. Among the populations investigated in Italy, one had just the 1781 mutation which is the most common found among ACCase-resistant weeds. The molecular characterisation of the three Italian *Lolium* pop. selected with 0.1 µM pinoxaden in petri dish showed that most plants of pop. 06302 carries only one point mutation (1781Leu), pop. 05281 has a mixture of 1781Leu (73%) and 2088Arg (26%). Pop. 05256 shows a more complicated situation: 1781Leu (41%), 2041Val/Asn (52%), 2078Gly (5%) and 2096Ala (1%).

From a managerial point of view the information, collected through the molecular analysis of resistant plants, can be used to identify which mutations are affecting different ACCase-inhibitors. Rapid mutation techniques such as CAPS and dCAPS could be used to assess potential target site resistance and suggesting which chemistries have to be avoided. This lead to the use of the correct herbicide/s avoiding repeated treatments at

higher doses when inefficacy is observed in the field. This is an aspect which has to be remarked since every superfluous treatment is an environmental threat.

4.4 *Phalaris paradoxa* characterisation

Past screening results indicate that in Italy just few *P. paradoxa* populations are resistant to ACCase inhibitors. Since 1998, 85 populations have been tested, 17 have been confirmed resistant to APPs, 11 to CHDs and since the introduction of the DEN (pinoxaden) in routine screening, only 4 populations have been found resistant to the new herbicide. This characteristic does not allow a rapid gene flow from resistant to susceptible plants, mainly only the seeds of a resistant individual can carry the resistant gene(s) and this is also indicated by the patchy diffusion in infested fields.

Dose-response experiments indicate that most of the populations investigated in 2001 had some low levels resistance to with diclofop, probably due to the massive use of this herbicide in wheat crop since the early eighties, plus no rotation of MoA has been done from the field history of most of the population tested. Two of the populations, 0025 and 0478L, have also been treated with haloxyfop and fluazifop: two ACCase inhibitors that are not used in wheat crop because they are not selective.

All populations resistant to ACCase-inhibitors are controlled by ALS-inhibitors, indicating that a different MoA is still a useful tool to control Italian resistant *P. paradoxa.*

Populations 0025 and 0478L are also the only to be resistant to pinoxaden by modified target site. Two different mutations are involved, both with a similar pattern of resistance: all sequenced plants of 0025 had 2078Gly mutation and all the plants of population 0478L had 1781Val. While the first substitution has already been reported for other species, the Ile to Val substitution in position 1781 has never been reported to our knowledge. The level of resistance appears to be lower than the “traditional” mutation of an Ile to a Leu.

The overall situation of grass resistance in Italy indicates that there is no one single chemical which can solve all resistance problems. Therefore all stakeholders have to be or become aware that IWM (or better ICM) is needed to properly manage resistance in the field.

It must also be clear that IWM or ICM requires a higher technological level coupled with a deeper knowledge of all components of the cropping system.

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