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CICLO XXXI

Epidemiology of herbicide resistance evolution in rice weeds and variability in *Echinochloa* spp.

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Coordinatore: Ch.mo Prof. Giuseppe Zanin

Supervisore: Maurizio Sattin

Co-Supervisore: Prof. Roberta Masin

Dottorando : Elisa Mascanzoni

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November 29th 2018 Elisa Mascanzoni

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Alice ed Irene

Look wide,
and even when you think you are looking wide,
look wider still.

Robert Stephenson Smyth Baden Powell, Lord of Gilwell.

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Chapter I

INTRODUCTION

1.1 Man vs weed

Multiple studies with the aim to objectively clarify "what makes a weed, a weed" (Sutherland, 2004) have been published but the concept of weed remains mostly human centered and perceptive.

The simplest definition is that a weed is "a plant growing in the wrong place at the wrong time" (Sattin *et al.,* 1995). Ross & Lembi (2009) defined weeds as "plants that interfere with the growth of desirable plants and are unusually persistent. They damage cropping systems, natural systems and human activities and are such undesirable". Weeds possess – by definition - specific biological characteristics, such as the ability to live in a variety of environments and produce an abundant quantity of seeds potentially affecting yield and quality of crops they infest (Baker, 1965; Holzner, 1982).

Humankind has struggled against the negative impact of weeds on crops since the dawning of agriculture (Hay, 1974): weeds economic impact on farm profitability is heavier than any other pest or disease causing both direct and indirect losses: e.g. yield reduction due to weeds competition with crops and the reduction of yield pricing or quality (Oerke, 1994, 2006).

1.2 Herbicide brief history

Weeds control technology has evolved during centuries, but the biggest step forward was done after the end of World War II with the development and introduction in the market of 2,4-D (2,4 – dichlorophenoxyacetic acid) and MCPA [(4-chloro-2-methylphenoxy)acetic acid]. They were the first selective herbicides, initially developed for military purposes, which were made available to farmers.

Chemical weed control has significant advantages in comparison with hand weeding and other mechanical techniques: it is in fact cheaper and more reliable and has led to a more abundant and constant food production worldwide (Oerke, 2006; Powles & Shaner, 2001).

220 herbicides belonging to 60 chemical families assigned to 26 Sites of Action (SoA) were commercialized in the following years and are now included in the 10th edition of the Herbicide Handbook of Weed Science of America (Shaner, 2014).

Glyphosate was introduced in the '70s, bringing an outstanding control on both perennial grasses and broadleaved weeds. In the '80s two other classes of highly selective and very active herbicides were commercialized: the graminicides acetyl coenzyme A carboxylase (ACCase) inhibitors and the acetolactate synthase (ALS) inhibitors, which set a new standard with the commercialization of sulfonylureas and imidazolinones, due to the high efficacy at very low doses as well as to their good toxicological and environmental profiles.

Since mid-1980's, the discovery of herbicides with new SoA has drastically slowed down and none have been commercialized (Duke, 2012). This is the consequence of the very high costs of development for a single new molecule (Rüegg *et al.* 2007) and the introduction, in 1996, of genetically engineered crops. These crops, first created to tolerate glyphosate and then other active ingredients (synthetic auxines, 2,4-D and dicamba), guaranteed high profits to farmers, thanks mostly to their flexibility and ease of management.

In the last decades there has been an ever increasing concern related to the use of herbicides, not only because of their impact on both the environment and human health, but also because the over-reliance on chemicals, coupled with the simplification of cropping systems, has led to the appearance of herbicide resistant (HR) weed populations.

1.3 Herbicide Classification

Several classification methods for herbicides exist, the most used of which is the one proposed by the Herbicide Resistance Action Committee (HRAC available at www.hracglobal.com), where herbicides are classified according to their Mode of Action (MoA). MoA is defined as the sequence of events between herbicide absorption and the final effect caused by the molecule. A total of 18 MoA is included in the HRAC classification (Fig. 1).

Some MoA are divided in sub-groups, dependently from the Site of Action (SoA) of the herbicide: e.g. in the photosynthesis inhibitors group C, there are three subgroups C_1, C_2 and C_3 indicating a different binding behavior with protein D_1. SoA is the specific process that the herbicide disrupts, interfering with plant growth and development. Consequently herbicides are divided into 26 Sites of Action (www.weedscience.org; Heap, 2018). This classification method is

important at technical level for the prevention and management of resistance evolution, which includes, among other practices, the rotation of herbicides with different SoA.

Until a few years ago it was more common to use Mode of Actions (MoA) to describe the different classes of herbicides instead of SoA while today it is considered more correct to use Site of Action relatively to resistance issues.

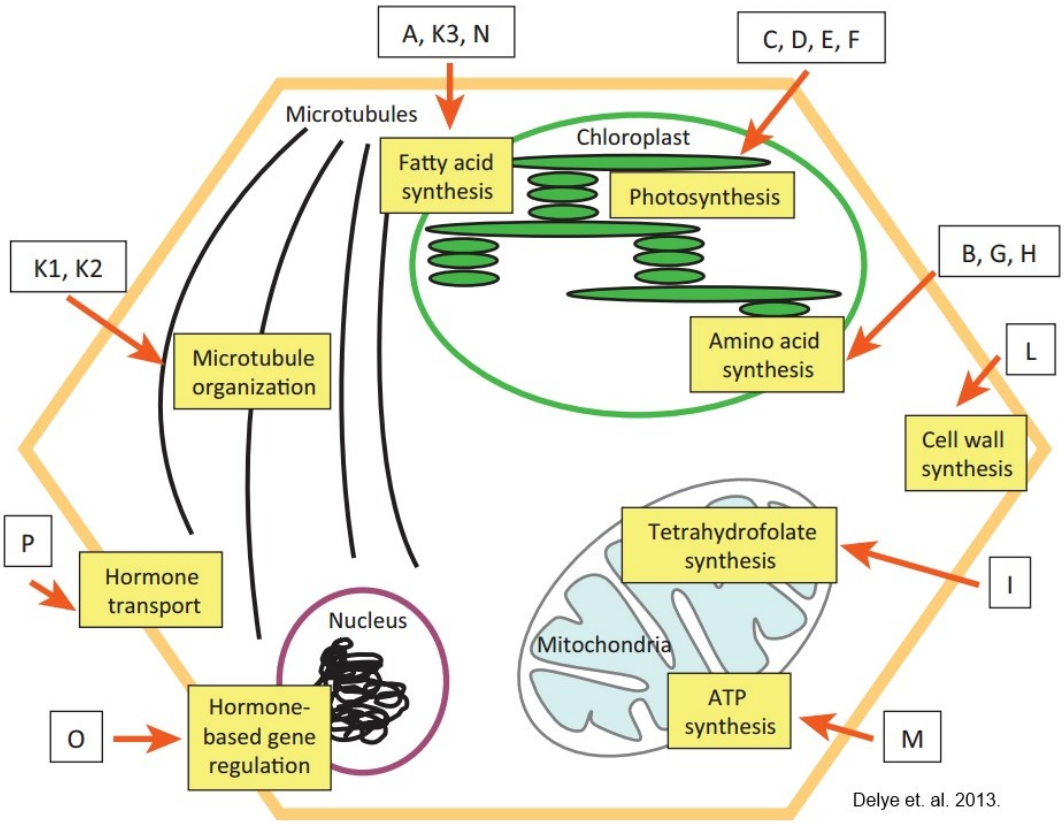


Fig. 1: Herbicide Resistance Action Committee (HRAC) herbicide classification, displaying cellular targets of each MoA (Délye *et al.* , 2013)

The most widely used herbicide SoA are group A acetyl coenzyme A caboxylase (ACCase) inhibitors, group B acetolactate synthase inhibitors (ALS) and group G 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase inhibitors. ACCase inhibitors include three chemical families: aryloxyphenoxypropionate (FOP), cyclohexanedione (DIM) and phenylpyrazoline (DEN). They inhibit the carboxyltransferase activity of the ACCase enzyme, which catalysis the first step in fatty acid biosynthesis eventually resulting in death of the plant (Burton *et al.*, 1991). FOP and DIM bind

to the homomeric ACCase CT domain in competitive way causing changes in the structure (Zhang *et al.*, 2004).

ALS inhibitors are large spectrum herbicides, targeting both grasses and broadleaved weeds. Five families are included in this group: sulfonylureas (SU), imidazolinones (IMI), triazolopyrimidines (TP), pyrimidinyl(thio) benzoates (PTB) and sulfonylamino-carbonyl-triazolinones (SCT). These herbicides targeting ALS (or AHAS) - an enzyme involved in the biosynthesis of branched aminoacids leucine, valine and isoleucine – bind in a channel that leads to the active site.

Another important class of herbicides is the synthetic auxins (group O and P of the HRAC), which induce effects similar to those of indole-3-acetic acid, a natural plant hormone. Thanks to their higher stability in comparison to indole-3-acetic acid inside plants, symptoms induced by auxin herbicides, such as tissue swelling and root growth inhibition, are severe and can cause plant death. Herbicides in this group belong to multiple chemical classes such as phenoxycarboxylic acids, benzoic acids, pyridinecarboxylic acids, aromatic carboxymethyl derivatives and quinolinecarboxylic acids (Grossmann, 2010).

1.4 Herbicide resistance

1.4.1 Definitions of herbicide resistance

Resistance is – literally – nature's answer to human attempt of eradication of an organism. Affecting both agriculture and human health, it is now creating major concerns and threatening the sustainability of important cropping systems and – consequently - food production worldwide while increasing management costs. (Evans *et al.*, 2015; Mortensen *et al.* 2012).

Resistance is an evolutionary process, well synthetized by Caroline Ash (2018): "Whenever mutating or recombining organisms are faced with extirpation, those individuals with variations that avert death will survive and reproduce to take over the population. This can happen rapidly among organisms that reproduce fast and outpace our efforts to combat them."

The Herbicide Resistance Action Committee (HRAC) defines Herbicide Resistance as "the naturally occurring inheritable ability of some weed biotypes within a given weed population to survive an herbicide treatment that would, under normal use conditions, effectively control that

weed population". The European and Mediterranean Plant Protection Organization (EPPO) describe it 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" ("PP 1/213 (4) Resistance risk analysis," 2015).

In the "Resistance risk analysis" - EPPO guideline PP 1/213 (4) (2015) - is also introduced the concept of practical resistance, which discriminated between resistance observed on field and the one selected in laboratory: "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 susceptibility". A weed population is considered affected by practical resistance when at least 20% of the plants, derived 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 (Panozzo *et al.*, 2015b).

Herbicide resistance is often confused with herbicide tolerance, defined by Holt & Lebaron, (1990) as "the normal variability of response to herbicides present among plant species, not involving selection of mutations that made the population tolerant".

1.4.2 Factors influencing resistance evolution

How does herbicide resistance evolve? In a certain population there are already rare individuals that are naturally resistant to a specific active ingredient and survive to an herbicide dose that would normally control all other plants in that population: their frequency varies depending on the weed species, herbicide SoA and resistance mechanism involved. Repeated treatments with herbicides with the same Site of Action remove susceptible individuals, consequently leaving space to the resistant ones to reproduce and disperse, leading – often in a relatively short time – to the evolution of a resistant biotype (Fig. 2).

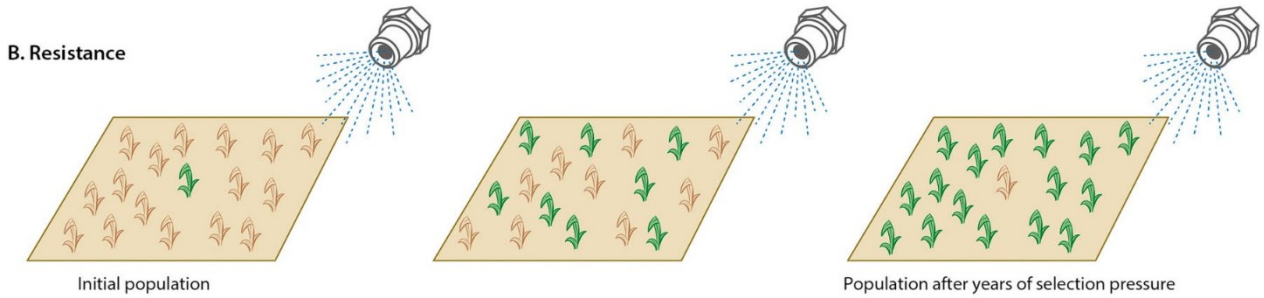


Fig. 2: herbicide resistance's evolution: in a certain mixed population there are already a few resistant individuals (left); the repeated use of herbicides with few or also the same SoA select these plants, that are able to reproduce and disperse (centre) finally taking over the whole population (right)

Resistance evolutionary process is influenced by many factors, the main are described by Onofri & Covarelli (2001):

(1) Initial frequency of the resistant trait in an unselected population: genetic variation for resistance is required in a susceptible population for the selection of a resistant population and the most important source of this variation is likely to be a gene mutation that spontaneously occurs. The initial frequency of resistant plants roughly varies between 10^{-5} and 10^{-6} (Diggle *et al.*, 2003; Jasieniuk *et al.*, 1996) depending on the target gene of the herbicide.

(2) Resistance genetic bases: e.g. number of involved alleles

(3) Selection pressure due to the repeated application of herbicides having the same SoA: this is considered the main factor influencing weed resistance evolution (Maxwell *et al.*, 1990).

(4) Relative fitness of resistant and susceptible genotypes: fitness is defined as the success in producing offspring contributing to the next generation by a particular genotype in comparison to another in a specific environment (Primack & Kang, 1989). If fitness of the resistant individuals is lower than fitness of the susceptible ones the population will evolve resistance slower than others, or not evolve it at all.

(5) Soil seed bank: seed bank dynamics in soil play an important role in resistance evolution: the more concentrated are the emergence of seedlings, the less persistent is the soil seed bank and the faster will be herbicide resistance selection. In other terms, soil seed bank acts as a buffer.

(6) Seed production of resistant weeds.

(7) Residual activity of the herbicide.

1.4.3 Resistance Mechanisms

Resistance mechanisms involve multiple plant biological metabolic pathways and they can be divided into two categories “Target Site” resistance (TSR) and “Non Target Site” resistance (NTSR).

TSR is a well-known mechanism due to mutation(s) in the gene encoding the herbicide target protein causing structural changes at the herbicide binding site(s). NTRS includes all mechanisms that determine a reduction in the amount of herbicide reaching their target-site (Heap, 2014a), i.e. reduced herbicide uptake, decreased rates of herbicide translocation and increased detoxification or herbicide sequestration (Délye, 2013).

Because of weed’s genomic plasticity there can be countless NTSR mechanisms. The most common and established involve an increased expression of cytochrome P450 monooxygenase, glycosyl transferase and glutathione-S-transferase that can metabolize herbicides (Yu & Powles, 2014). These enzymes belong to major enzyme superfamilies and some of them are involved in the detoxification of xenobiotics. Another mechanism is called gene amplification and involves the over-expression of the enzyme target of the herbicide: as a consequence a higher dose of herbicide is needed to reach the target and site and inhibit the enzyme causing plant death (Gaines *et al.*, 2010; Yuan *et al.* 2007).

Other less common mechanisms are sequestration and reduced absorption/translocation. (Heap, 2014a).

NTSR are mainly reported in grasses although their importance in broadleaves might be underestimated (Délye, 2013; Délye *et al.*, 2011; Yuan *et al.*, 2007).

NTSR is more complex to demonstrate respect to TSR. However, the detection of NTSR is crucial to modulate agricultural practices because it can confer resistance to herbicides with different SoA leading to the appearance of unexpected multi-herbicide resistances (Preston, 2004; Yuan *et al.*, 2007).

When a resistance mechanism confers resistance to different herbicides having the same SoA, the resistance is called “cross-resistance”. Instead, “multiple resistance” affects herbicides with different SoA and is due to multiple resistance mechanisms which coexist in the same plant. This

is the result of a either concurrent or sequential selection caused by more herbicides with different sites of action.

Cross resistance can be both TS and NTS depending on species and SoA: e.g. cross resistance to bispyribac-sodium and bensulfuron-methyl, two ALS-inhibiting herbicides, is related to cyt P450 monooxygenases in *Echinochloa phyllopogon*, while is target-site mediated in *Cyperus difformis* (Osuna *et al.*, 2002).

1.4.4. Herbicide resistance: current situation worldwide and in Italy

The first herbicide resistant populations were collected in 1968: two populations of common groundsel (*Senecio vulgaris* L.) that had evolved resistance to the PSII inhibitors simazine and atrazine (Ryan, 1970). Since then, the number of reported cases has steadily increased, with about 11 new cases confirmed every year. There are now 495 unique biotypes of herbicide resistant weeds globally.

By 2018, 255 weed species (148 dicots and 107 monocots) have evolved resistance to 23 of the 26 available SoA involving 163 herbicide molecules. (Heap, 2018). Resistance first heavily affected PSII inhibitors and then other to two SoA: i.e. ALS and ACCase inhibitors (Fig. 3). ALS inhibitors resistance is mostly due to a reduced susceptibility of the target ALS enzyme (Heap, 2014; Saari *et al.*, 2018), a second mechanism involved is the enhanced metabolism resulting in the rapid detoxification of the herbicide (Yu *et al.*, 2009). This class of herbicides is the most "prone" to select resistance, because of their high efficacy and very specific target site (Saari *et al.*, 2018).

For ACCase inhibitors the wide use of FOP and DIM has led to the evolution of resistant populations. To date, 57 weeds are involved (Heap, 2018), with both target site and non-target site resistance mechanisms.

More recently, resistance to glyphosate (EPSPs inhibitor) has increased significantly, mainly due to the cultivation of glyphosate-tolerant crop variety in Americas and consequent widespread use of this SoA (Heap & Duke, 2018). In Europe glyphosate resistance is less diffused and mainly affects perennial crops (Collavo & Sattin, 2012), although a few resistant populations of *Lolium* spp. infesting arable crops have been reported (Collavo & Sattin, 2014).

Considering synthetic auxins, resistance to this SoA is not widespread and has little economic impact, as synthetic auxins remain one of the least prone herbicides to resistance's selection. (Heap, 2013).

Herbicide resistant weeds have been reported in 92 crops from 70 countries: the most affected countries are USA with 161 unique cases of resistance confirmed, followed by Australia (90), Canada (88), Brazil (48) and China (44) (Fig. 4).

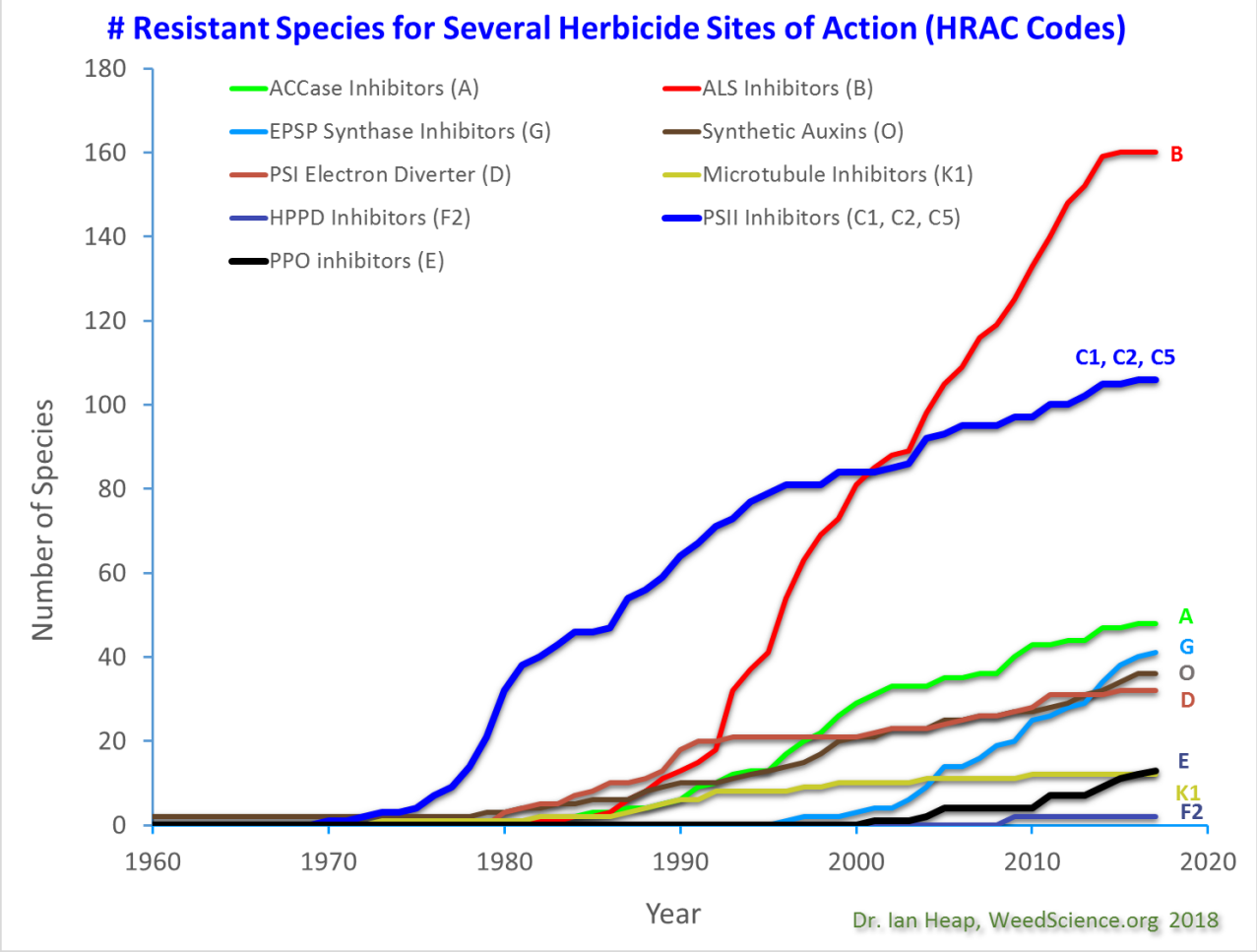


Fig. 3: Cumulated resistant species worldwide, sorted by herbicide SoA (Heap, 2018).

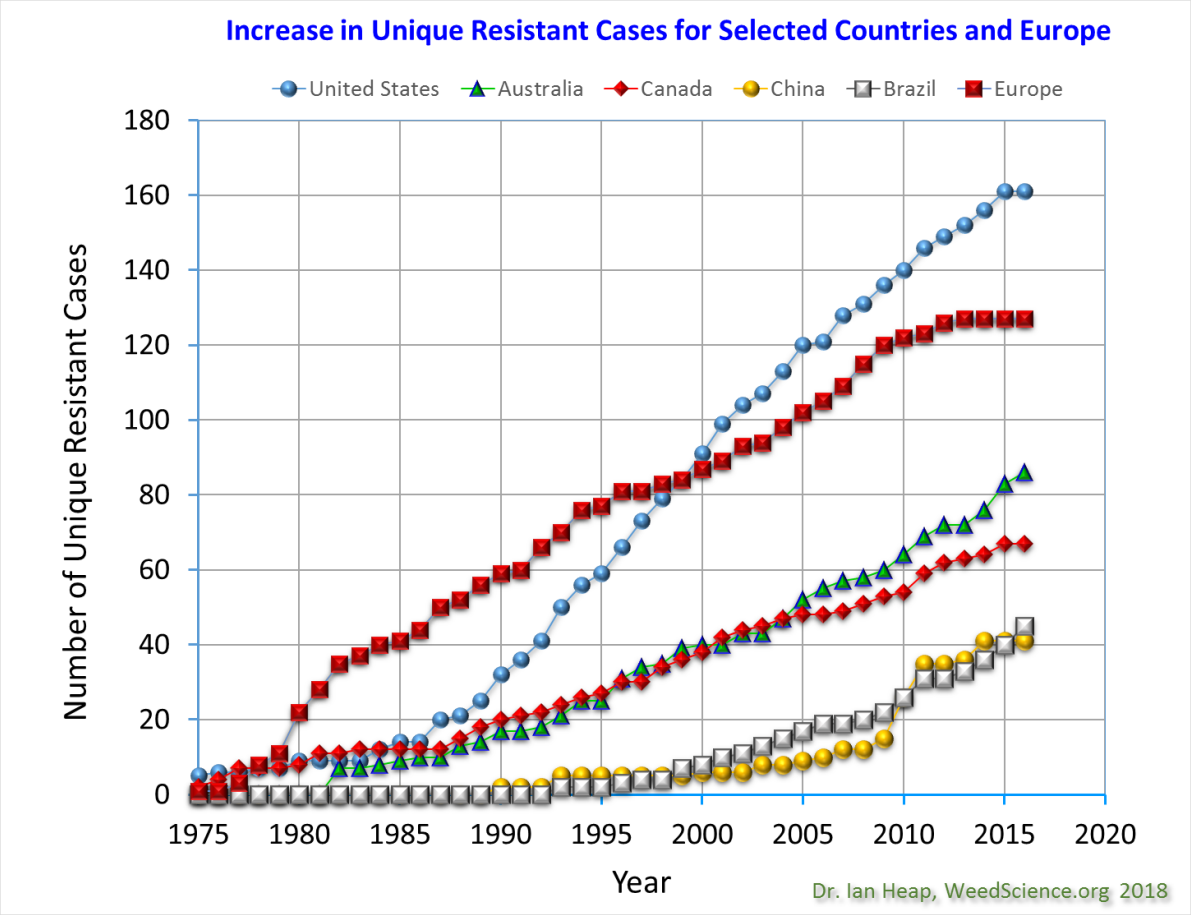


Fig. 4: Cumulated unique resistant cases sorted by country (Heap, 2018)

In Italy, herbicide resistance was first reported in 1978 and remained marginal until early 90's (Cantele *et al.*, 1985; Porceddu *et al.*, 1997). It involved only atrazine resistant populations of *Solanum nigrum* L., *Amaranthus* spp. and *Chenopodium album* L. found between 1978 and 1982 in maize crops. The situation rapidly worsened in the mid '90s after the introduction of ALS and ACCase inhibitors. The first ALS resistant populations of *Alisma plantago-aquatica* L. and *Schoenoplectus mucronatus* L. Palla were found in rice in 1994 and 1995, while the first population of ALS-resistant *Papaver rhoeas* was found in 1998 in durum wheat in Puglia. ACCase inhibitors resistance first appeared in *Poaceae*: *Avena sterilis* L. in 1992, *Lolium* spp. in 1995 and *Phalaris paradoxa* L. in 1998. Only a few populations of *Papaver rhoeas* L. resistant to synthetic auxins have been reported in durum wheat fields.

By 2018, 19 weeds (9 dicots and 10 monocots) have evolved resistance to the most important herbicides SoA used in 16 out of 20 Italian regions.

The Italian situation is constantly monitored by the Italian Herbicide Resistance working Group (Gruppo Italiano Resistenza Erbicidi, GIRE) since 1997. Basing its activity on farmers and farmers' advisors complaints of poor herbicide efficacy, GIRE collects and tests putative herbicide resistant populations nationwide and publish results, including maps of diffusion as well as guidelines for resistance management for all resistant biotypes on its website (www.resistenzaerbicidi.it).

1.4.5 Resistance Management

Herbicide resistance management, which includes preventive and curative measures, should be based on Integrated weed management (IWM) (Barzman *et al.*, 2015; Berti *et al.*, 2001). IWM aims at increasing diversity in cropping systems by reducing the standardization of cropping practices. To do so it is necessary to integrate a range of weed control tools including chemical, physical and agronomic techniques in order to prevent/slow down the appearance of resistant populations, without excessive reliance on one method only (Powles & Matthews, 1992).

The main tools are:

(1). Crop rotation: it is the key tool for increasing diversity in the cropping system by diversifying the types of disturbance.

(2). Other agonomic techniques: they reduce herbicide input and their selection pressure. Available options include the use of different types of tillage, cover crops, exploitation of allelopathy, hand weeding, mowing, stale seedbed and grazing.

(3). Chemical control: rotating and mixing herbicides with different SoA, but active on the same target weed can delay resistance evolution. SoA rotation is complementary to crop rotation: the second is poorly effective without the first. HRAC herbicide classification is a handy tool allowing to choose and mix herbicides from different groups, with different SoA in order to minimize the selection of resistant biotypes.

For the Italian situation, specific guidelines for resistance management are reported in the GIRE website.

The agronomic and economic impact of resistance and consequent losses for farmers have been widely described in many publications and many others describe how to prevent, slow down or manage it (e.g.: Délye, 2013; Juraimi *et al.*, 2013; Norsworthy *et al.*, 2012; Orson, 1999) converging on a series of best management practices such as herbicide strategies diversification, monoculture reduction, promotion of the correct use of herbicides and deepening the knowledge of weed biology and other agronomic techniques (Beckie & Harker, 2017; Norsworthy *et al.*, 2012; Evans *et al.*, 2015) reaching a sustainable intensification of agriculture: i.e. where production, profitability and sustainability meet a possible compromise (Jordan & Davis, 2015).

Although resistance is a well-known topic counting over 3000 publications since 1980, at field technical level resistance is very often treated as a temporary problem that will be solved with new SoA commercialization. Instead, resistance should be considered – at all levels – as a "wicked problem" (Gould *et al.*, 2018), the result of multiple economic, social and biological variables interacting in complex and unexpected ways.

1.4.6 Epidemiology in herbicide resistance studies

Many publications describe the economic and agronomic impact of resistance and many others suggest ways to prevent, slow down or manage it (e.g. Norsworthy *et al.* 2012; Delyè *et al.* 2013; Juraimi *et al.* 2013; Orson 1999), but very few contribute to elucidating the impact of interactions between major agronomic and environmental factors on resistance epidemiology (Evans *et al.*, 2015).

Epidemiology is a biomedic discipline concerned with distribution and determinants of evolution in defined populations. Although widely used in human health (Derks & Tomasi, 2015; Franklin & Lindberg, 2015) this approach is scarcely used in weed science and in particular in herbicide resistance studies, although it can provide relevant evidence-based information for preventing or reducing the spread of resistant populations by identifying the major risk factors.

This lack of information was highlighted in several recent studies and many authors claim for large-scale studies on resistance evolutionary processes to properly understand what occurs on field (Evans *et al.*, 2015; Gould *et al.*, 2018; Editorial, 2018; Shaw *et al.*, 2018). In 2015 Evans *et al.* published a study aimed to identify risk factors associated with resistance to glyphosate in

Amaranthus tuberculatus (Moq.) J.D. Sauer based on the field management data of a custom retailer spread on an area of about 800 km^2, while in 2018 Hicks *et al.* produced a research on drivers for the evolution of herbicide resistance using data of *Alopecurus myosuroides* Huds. resistance assessed on 138 fields belonging to 71 farms in UK.

Epidemiological studies at large scale were partially hindered by the paucity of field level data about field management, soil texture and structure, water management and other important factors that might affect herbicide resistance evolution in a certain field. This obstacle now is partially removed by the progressive publication "open-source" of these type of data and can be used in epidemiological studies at large scale also in weed science.

Weed infestations and herbicide selection pressure in rice crops vary widely in relation to many agronomic and pedo-climatic conditions, so herbicide resistance evolves in a context of a series of interacting factors. However, detailed field-by-field data on these factors are rarely available at a large scale and therefore the identification of concise, yet informative, agronomic predictors of herbicide resistance distribution/diffusion would significantly facilitate effective management.

The use of different statistical approaches, i.e. discriminant analysis, logistic regression and artificial neural network enables the quantification of the effect of pedo-climatic and management drivers on agro-ecological systems at large scale. For example, discriminant analysis was used to investigate the effect of rainfall-related variables on the occurrence of drought stress in maize (Zhang e*t al.*, 2013) and the effect of fertilizer regimes on the structure of the soil microbial community and its functions (Lazcano *et al.*, 2013). It has also been recently used to calculate weed distribution in maize fields (Vidotto *et al.*, 2016). Recently, a comparison of artificial neural networks and logistic regression was used to predict weed populations in chickpea and winter wheat (Mansourian *et al.*, 2017) and to investigate the contribution of topographic and soil-related traits, as well as land use and maximum rainfall intensity as landslide drivers in landslide susceptibility mapping (Gong *et al.,* 2018). The use of different approaches ensures a reliable depiction of the examined system as each approach relies on different assumptions and analytical solutions.

1.5 Rice, a key crop worldwide and in Italy

One of the most affected crops by resistance is rice, a key sector of the Italian agriculture and general economy.

Rice is a worldwide strategic crop: it is the second most cropped cereal and represents the staple food for more than half of the world population in Asia, Africa, Central and South America (Van Nguyen & Ferrero, 2006).

Italy is the first European producer with about 240,000 hectares, 92% of which is concentrated in an area located between Piedmont and Lombardy, in the North-West of the Po Valley. However, other rice growing areas are present in Emilia-Romagna, Veneto, Sardinia, Tuscany and Calabria (Fig. 5).

Fig. 5: distribution of rice in Italy (source: Ente Nazionale Risi)

Cultivated in Italy since the Middle Ages (Ferrero & Vidotto, 2010; Ente Risi), rice cultivation has become a key sector for the Italian and European agriculture and economy, thanks to its high profitability and specialization; it is also an environmental and landscape richness, as rice fields create a unique environment in Europe with specific and diverse flora and fauna.

While the total rice area has been substantially stable in the last decade, the number of farms has decreased: from 6,367 of 1996 to 4,265 in 2016, while the average farm surface has increased from 37 to 55 ha.

In 2016 about 234,000 ha of rice have been cropped to rice in Italy, nearly half of the surface was cropped to long A grain varieties.

Rice varieties cropped in Italy are divided in four main groups (Fig. 6):

- **Round grain** varieties: grain length is < 5.2 mm, length-width ratio of the grain is < 2, e.g. Sole, Selenio, Centauro
- **Medium grain** varieties: grain length is > 5.2 mm but < 6.0 mm, length-width ratio of the grain is < 3: e.g. Venere, Vialone Nano
- **Long grain** varieties:
    - **Long A** grain: : grain length is > 6.0 mm, length-width ratio of the grain is > 2, but < 3, e.g Luna CL, Dardo, Volano, Carnaroli
    - **Long B** grain: grain length is > 6.0 mm, length-width ratio of the grain is > 3: CL26, Gladio, Mare CL, Thaibonnet

All data are available on the web page of Ente Nazionale Risi (www. enterisi.it).

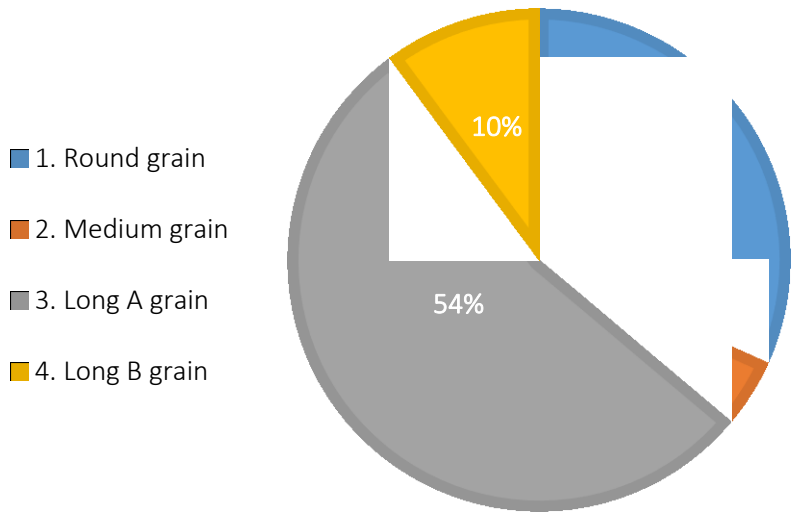


Fig. 6: Italian rice surface divided by market category: long A grain (120,056.7 ha, 51.3%), Medium grain varieties (9,727.5 ha, 4.2%), round grain (70,786.56 ha, 30.2%) and Long B grain (33,563.6 ha 14.3%).

In 2016 the most cultivated variety were Sole CL (26,000 ha), Volano (18,121 ha), Selenio (14,903 ha) and Centauro (14,807 ha).

Variety's choice is driven by multiple factors, such as price, market request and varietal productivity, but also by other farmer necessities like weed management.

BASF Clearfield Technology®, with its imidazolinone-tolerant rice (IMI-rice), was commercialized in Italy in 2006 and has now reached 80000 ha, because it offers the possibility to control weedy rice – a key weed of this crop - with a limited effort and number of treatments.

IMI rice varieties have been developed through natural selection or inducing mutations in the ALS gene (Sudianto *et al.*, 2013) and in Italy, it is imazamox (Beyond®, BASF Milan, Italy) the herbicide (ALS inhibitor) registered for Clearfield varieties (Scarabel *et al.*, 2012).

This technology was launched with strict usage rules (e.g. no more than two consecutive years of Clearfield® Technology were allowed on the same field) to prevent the development of ALS inhibitors resistant populations of red rice. Anyway the first cases of ALS inhibitors resistant red rice were recorded in 2010, four years only after the launch of this technology.

Resistance in this case can occur both through selection of already IMI-resistant mutants of red-rice or through gene-flow, i.e. cross-pollination, between IMI-rice and the wild type. (Gealy *et al.*, 2003; Scarabel *et al.*, 2012).

1.5.5 Weed flora in rice fields

Rice has evolved a specific weed infestation, which includes both aquatic and non-aquatic weeds. Major weed species can be ascribed to six groups as proposed by Ferrero & Vidotto, (2007):

(1) *Echinochloa* spp., the species that is actually causing the biggest issues in rice fields in Italy

(2) *Heteranthera* species, that were introduced in Italy in the 60's from south America (Pirola, 1962)

(3) *Alisma* species and cyperaceae weeds (sedges);

(4) *Oryza sativa* f. *spontanea* L. or weedy rice, which has always been present, but started becoming an issue in the 90's

(5) weeds typical of the dry seeded fields i.e. *Panicum dicotomiflorum* Michx, *Digitaria sanguinalis* (L.) Scop., *Poligonum* spp., *Bolboschoenus maritimus* L. Palla

(6) minor and exotic weeds who do not pose major threads and do not need specific interventions. An example is *Leptochloa fascicularis* (L.) Nees. (very common and problematic in rice fields of Extremadura (Spain) (Osca, 2013), which is now expanding in the South-West of the province of Vercelli (Benvenuti, Dinelli, & Bonetti, 2004) or *Eclipta prostrata*, found in Sardinia rice fields about 20 years ago and in Lombardy in 2000 (Viggiani & Tabacchi, 2017)

Composition and evolution of rice flora is heavily influenced by crop management techniques: the shift from hand weeding to chemical weed control with selective herbicides (propanil in 1959), the introduction of direct seeding instead of rice transplant and the adoption of short statured rice varieties (Ferrero *et al.*, 2008) has favored infestations of *Echinochloa* spp., *Alisma* spp., sedges and red rice.

In rice fields, weeds can affect final rice production, with yield losses that can reach 80-90% (Oerke, 1994; R. J. Smith, 1988).

1.5.6 *Echinochloa* spp., rice's worst weed

Weed species discrimination is an important aspect for their management, as species belonging to the same genus often respond differently to the same herbicide.

Knowing weed species and their biology (Norsworthy *et al.* 2012), is an important point to address weed control on field and manage resistance issues; according to Holm (1977) the behavior of a species can be understood – also relatively to herbicides – only when its taxonomy is clear.

The genus *Echinochloa*, class Monocotyledonous, family Poaceae, subfamily Panicoideae, tribe Panicoieae, is a widespread weed genus causing nuisance in many crops worldwide. It includes over 50 hydrophanous annual species (therophyte), whose discrimination is often difficult. It is highly competitive with crops and shows broad ecological tolerance, great ability to mimic rice and is well adapted to both temperate and tropical regions (Benvenuti *et al.*, 1997; Bouhache & Bayer, 1993; Clayton & Renvoize, 1986; Michael, 1983; Tabacchi, 2003).

Echinochloa species have a C4 photosynthetic cycle and are prevalently autogamous, although they show a certain degree of cross pollination (Maun & Barrett, 1986). Seed production is abundant and extended for a long period. In favorable conditions a single plant of *Echinochloa crus-galli* can produce up to 40,000 seeds with a germination rate of about 10-15%. Recently, it was highlighted that resistant populations of *E. crus-galli* have a lower germination capacity compared to the sensitive ones. (Serra *et al.*, 2018)

Seeds can live up to 10 years in the soil (Altop & Mennan, 2011; Barrett & Wilson, 1983; Norris, 1996).

1.5.6.1 Echinochloa spp. morphological classification

The genus *Echinochloa* shows a high degree of morphological plasticity, depending both on specie and environmental conditions: e.g. it is known that characteristics like color or awns presence and length vary depending on plant stage and environmental conditions (Ruiz-Santaella *et al.*, 2006) and it is common to find, on field, plants of *Echinochloa* that show intermediate phenotypic characteristics for which classification is impossible (Pirola, 1965).

For this genus, many classification keys have been proposed in the last century (Micheal, 1983), but none of them have been able to fulfill the task: e.g. to *Echinochloa crus-galli* (L.) P. Beauv. have been attributed over 100 names and *Echinochloa oryzicola* (Vasinger) have also been named *Echinochloa hispidula* or *E. crus-galli* by different authors (Viggiani & Tabacchi, 2017).

In Italy, and in general in the Mediterranean counties, the traditional dichotomous keys used for *Echinochloa* spp. classifications were Pignatti (1982) and Carretero (1981), which relied on different morphological markers: Pignatti used mostly to macroscopic traits such as hair presence and inflorescence bearing, Carretero used spikelet length as principal distinction trait.

Pignatti distinguished among six species: *Echinochloa phyllopogon* (Stapf), in case of presence of hairs on both stem and leaf sheath, *Echinochloa erecta* Pollacci, which presents upright panicle and stem and is hairless, *E. crus-galli, Echinochloa crus-pavonis* (Kunth) Schult.*, Echinochloa hostii* (Bieb.) Boros. and *Echinochloa colonum* L. (Fig 7).

Fig. 7: plants of *Echinochloa* spp. with hair (a) and hairless (b). The one on the left should be classified as *E. phyllopogon* on the base of Pignatti (1982) dichotomous key.

Carretero distinguished among five species: *E. colonum*, *Echinochloa oryzicola* (Vasing), *E. crus-galli*, *Echinochloa oryzoides* (Ard.) Fritsch and *E. hispidula*.

Taking one step further Carretero, Costea & Tardif (2002) proposed in 2002 an additional classification based on spikelet and caryopses characteristics where the same five species were included (Fig. 8).

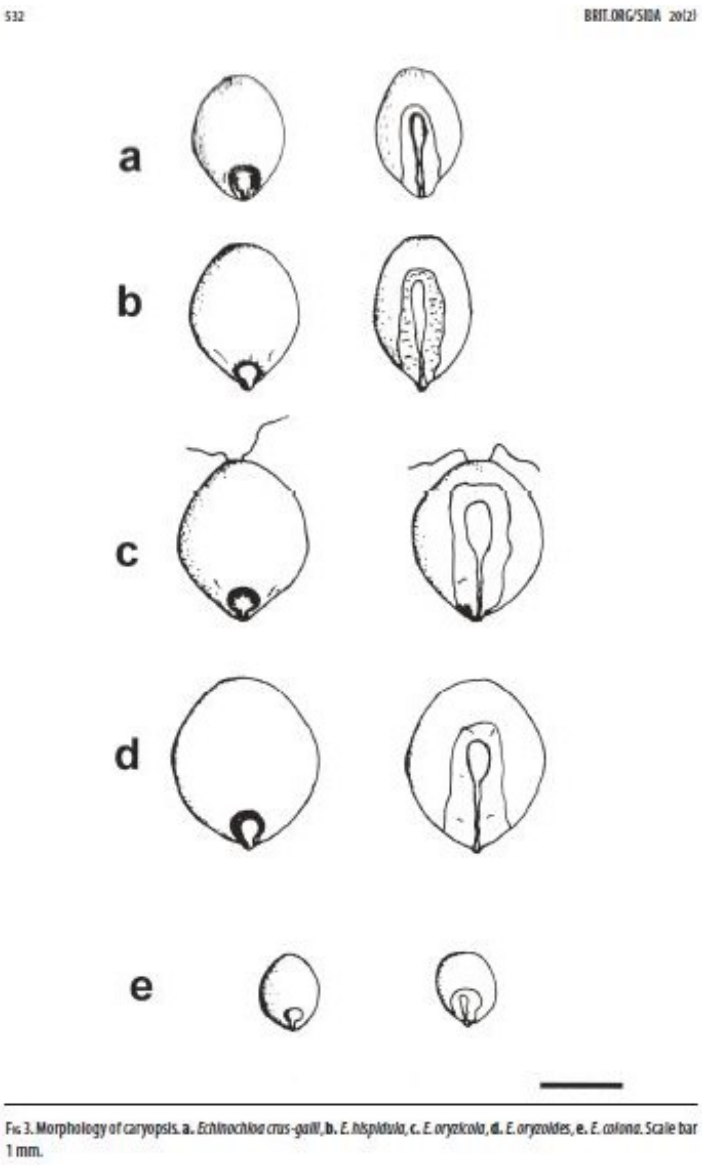


Fig. 8: Scheme of Costea&Tardif *Echinochloa* spp. classification (2002).

This classification results more complicated to be used on field as it takes into account – for the distinction of the different species – small characteristics *Echinochloa* spp. seeds, such as the embryo-caryopses ratio, the number of veins on the lower glume and the length and width of the caryopses.

Fig. 9: spikelet and caryopses of *E. crus-galli* (top) and *E. oryzicola* (bottom), according to the classification of Costea-Tardif (2002). It is visible the difference in dimension between the first and the second.

Matching morphological markers with Amplified Fragment Length Polymorphism (AFLP) markers Tabacchi proposed, in 2006, a new classification and distinguished five species: E*. colona*, *E. crus-galli*, *E. phyllopogon*, *E. oryzoides* and *E. oryzicola*.

The most recent classification was by Viggiani & Tabacchi, (2017) based on that of Costea & Tardif, (2002), also maintaining the names of the different species.

1.5.6.2 Echinochloa spp. discrimination through molecular markers

In the past classification relied on the observation of morphological characters, i.e. those used for the taxonomical approach. These markers are easy to spot (e.g. presence or absence of awns) in a certain species and less expensive in comparison with the material needed for genomic

studies, but as highlighted by Tabacchi (2003) and other authors, they also present limits that can affect their reliability for species identification (e.g. their variability in function of the plant development stage and environment influence). Also species discrimination using morphological markers requests a high number of samples and observations in order to take into account high morphological variability.

With the advent of molecular biology new scenarios appeared for plant discrimination through molecular markers. For instance it was introduced the possibility to study the polymorphisms present in the DNA and use them as markers for plant species discrimination. Techniques like AFLP, Restriction Fragment Length Polymorphism (RFLP) and Random Amplified Polymorphic DNA (RAPD) have been used in several cases for this purpose (Moser & Lee, 1994; Nissen *et al.*, 1995; Smith *et al*, 1990; Tatineni *et al.*, 1996).

Considering *Echinochloa* spp., RFLP - PCR, a technique that exploits PCR amplification and endonuclease enzymatic digestion was used for the distinction of *E. crus-galli* from E. *oryzicola* (Yasuda *et al.*, 2002), while Amplified Fragment Length Polymorphism (AFLP) was performed to investigate the phylogenetic relationship of 80 *Echinochloa* spp. accessions from Italian rice fields (Ferrero & Vidotto, 2007; Tabacchi, 2003) and to discriminate between *E. crus-galli* and *E. muricata* in Belgium (Claerhout *et al.*, 2016).

Anyway classic taxonomy remains a fundamental approach to complement and sustain classification through DNA sequences.

1.5.6.3 DNA barcoding: an innovative tool for weed species discrimination

DNA barcoding involves the sequencing of standard short sequences of DNA – between 400 and 800 base pairs (bp) - to characterize animal and plant species on the tree of life through the investigation of Single Nucleotide Polymorphism (SNP) in the DNA (CBOL Plant Working Group , 2009). Potential applications of this technique span from the discovery of species to ecological forensics and floristic surveys (Coissac *et al.*, 2016). DNA barcoding application is a two-step process: (A) the creation of a DNA barcode library of a known species followed by (B) the match of the DNA barcode sequence of an unknown sample against the DNA barcode library (Fig. 10) (Kress & Erickson, 2012).

Step (A) involves taxonomists selecting several individuals per species to build the initial library: sample sources can be both herbaria and living plants on the fields. Specimens used in this step must be appropriately labeled and vouchered, as vouchers will function as permanent records for the connection of the DNA barcode database to a certain species. After completion barcode library will be used for the comparison and identification of unknown individuals to be assigned to a certain species. To achieve a reliable comparison among plants, so using barcoding as a tool for species discrimination, suitable regions of DNA must be chosen, covering the criteria of universality, sequence quality and coverage as well as discrimination ability of the chosen sequence (CBOL working group, 2009). For animals the sequencing of standardized regions of mitochondrial gene CO1 has proved to be an efficient tool (Hebert *et al.*, 2003), but as substitution rate of mitochondrial DNA in plants is low, alternative plastid regions have been identified to become the standard for DNA barcoding: plastid genes (in particular in the chloroplast (cp)-DNA) show in fact a highly conserved gene order, low levels of nucleotide substitution and absence of recombination (Huang *et al.*, 2017).

DNA regions that best fulfilled the criteria listed above, thus chosen as a standard for plant discrimination, are genes matK+rbcL with intron psbA-trnH supporting the previous two (CBOL, 2009). In other studies discriminating power of non-coding regions trnT-L-F were investigated (Taberlet *et al*., 2006; Yamaguchi *et al.*, 2005).

Despite the potential multiple application fields of DNA barcoding it has been scarcely used in weed science, particularly for species discrimination: molecular markers for the discrimination of multiple accession of *Echinochloa* spp. have been built on non-coding regions of trnT-L-F allowing to discriminate *E. crus-galli* from *E. oryzicola* (Aoki & Yamaguchi, 2008; Yamaguchi *et al.*, 2005). While other molecular markers to the discrimination of *E. crus-galli* var. *crus-galli*, from *E. crus-galli* var. *praticola* and *E. colona* have been designed on genes psbA, psbM, psal and other intergenic regions obtaining only partial results (Zhang *et al.*, 2017).

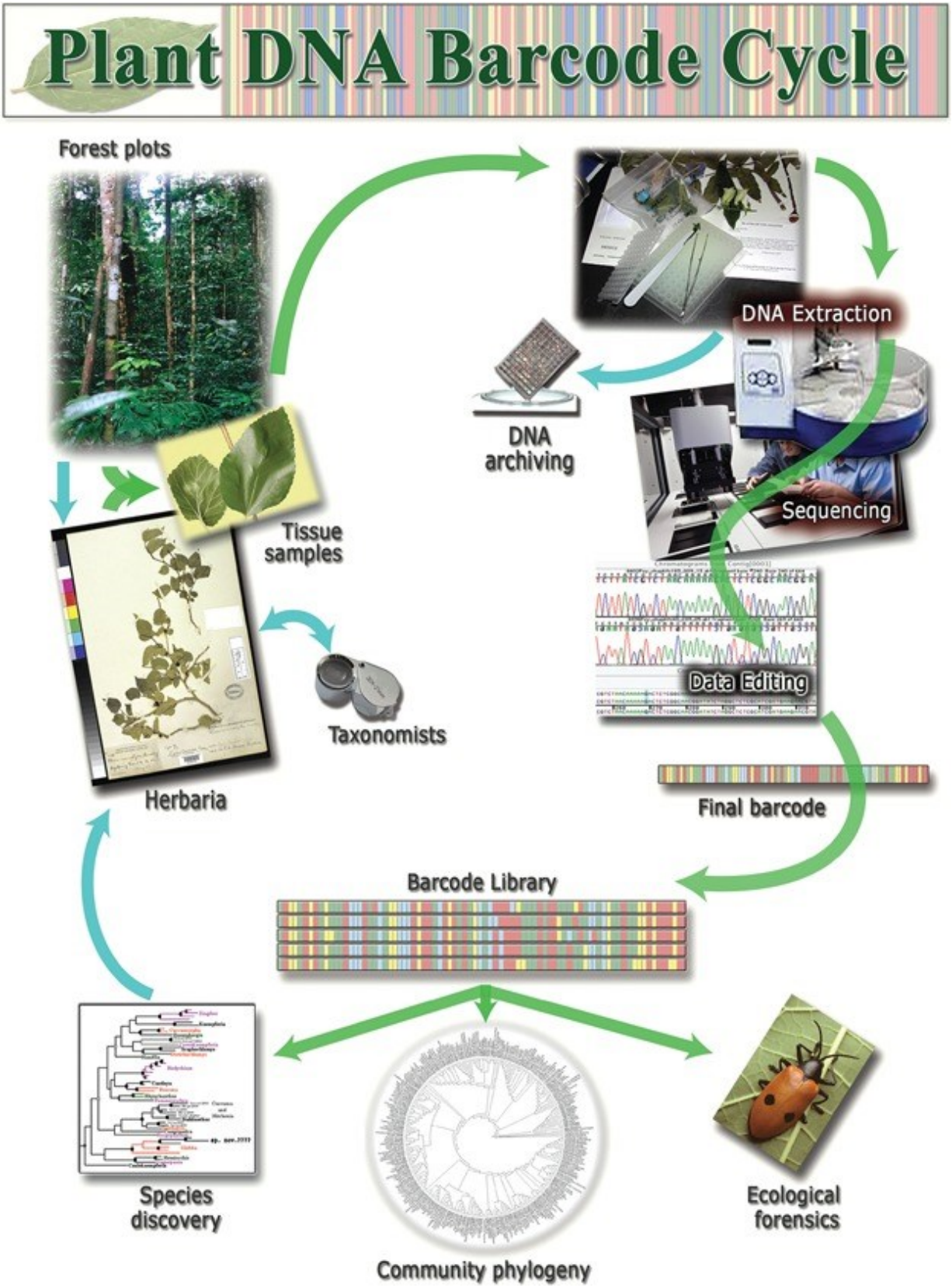


Fig. 10: steps involved Workflow in plant DNA barcoding (Kress & Erickson, 2012).

1.5.6.4 Echinochloa spp. and herbicide efficacy

The high intra and interspecific morphologic variability inside this genus often baffles farmers and sometimes leads them to make wrong correlations between *Echinochloa* spp. morphology and herbicides poor control. In rice fields there are populations with intermediate characteristics between "red" and "white" *Echinochloa* species: farmers normally refer to them as "purple" or "hybrid" type of *Echinochloa*. Although very low, the rate of cross pollination is sufficient to ensure gene exchange in *Echinochloa* spp. populations (Maun & Barrett, 1986). Anyway the existence of wild fertile or sterile "hybrid" populations of *Echinochloa* spp. has not been demonstrated in Europe or elsewhere. Nevertheless hybrids have been produced in controlled conditions, mostly using cultivated varieties of *Echinochloa* spp. (Yabuno, 1983), and in most cases, sterile F_1 were obtained (de Wet *et al.*, 1983).

The understanding of the response of the different *Echinochloa* species to herbicides having different SoA has multiple implications, such as the fact of becoming a tool for a correct weed management at field level and, whether verified, of becoming an additional trait for species discrimination. Also, discriminating different *Echinochloa* species on field knowing that they respond differently to the various herbicides could lead to a more precise weed management, ideally "ad hoc" for each field infestation.

Few studies have been done to test differences in susceptibility to herbicides of the different *Echinochloa* species (Claerhout *et al.*, 2016; Vidotto *et al.*, 2007), and great variability in results have been found depending on the history of herbicide strategies of the *Echinochloa* spp. collection site, test location, experimental conditions etc. supporting the idea that environmental factors have an important role in plant phenology.

Vidotto *et al.* (2007) investigated the response of 80 populations of *E. crus-galli*, *E. erecta* and *E. phyllopogon* to multiple herbicides in Italy demonstrating that, although all populations showed a large interspecific and intraspecific variability in herbicide susceptibility, *E. crus-galli* was generally less susceptible to quinclorac and more sensitive to azimsulfuron, cyhalfop-butyl, molinate and propanil. A difference in susceptibility was highlighted between *E. phyllopogon* and *E. erecta* as the first resulted more sensitive than the second to both molinate and quinclorac.

Studies conducted so far were performed at population level, i.e. collecting in a single field a mixed bulk of seeds, thus testing the different herbicides on a heterogeneous group of individuals sharing the same site of origin, but do not necessarily share a similar genetic background. To our knowledge only one study analysed the response of purified and classified *Echinochloa* accessions to different herbicides: Claerhout *et al.* (2016) showed that no differences in susceptibility to nicosulfuron, cycloxydim and topramezione were present between *E. crus-galli* and *E. muricata* collected from Belgian corn fields.

1.5.7 Herbicide Resistance in rice in Italy

Six weed species have evolved resistance to ALS inhibitors: *Alisma plantago-aquatica*, *Cyperus difformis*, *Oryza sativa* var. *spontanea*, *Schoenoplectus mucronatus, Echinochloa* spp. and recently *Cyperus esculentus* (GIRE 2018; Heap, 2018). *Echinochloa* spp. have also evolved multiple resistance to ALS and ACCase inhibitors. GIRE estimates that around 40% of the Italian rice area is involved in resistance issues. A total of 586 populations were tested with three herbicides and 427 proved to be resistant to at least one, mostly ALS products (Tab. I).

Weed Species	Resistant	Susceptible	Total
ALSPA	66	13	79
CYPDI	29	32	61
ECHSS	192	48	240
ORYSA	57	46	103
SCPMU	81	20	101
CYPES	2	0	2
Total	*427*	*159*	*586*

Tab. I: Number of populations collected in rice crops in Italy and tested by GIRE. Populations were considered to be resistant if at least 20% of plants survived the field dose of at least one herbicide tested. ALSPA = *Alisma plantago-aquatica*, CYPDI = *Cyperus difformis*, ECHSS = *Echinochloa* spp., ORYSA = *Oryza sativa* var. *spontanea*, SCPMU = *Schoenoplectus mucrunatus*, CYPES = *Cyperus esculentus* (Hess *et al.*, 1997).

The first ALS resistant populations of *A. plantago-aquatica* and *S. mucronatus* appeared in mid '90s from plants that had not been controlled by bensulfuron, only 6 years after its introduction in Italy. ALS resistant *C. difformis* was first collected in 1999 (Sattin, 2005), the same year of 3 propanil-resistant populations of *E. crus-galli* (Scarabel *et al.*, 2002).

ALS resistant *O. sativa* var *spontanea* and *C. esculentus* ALS populations were then recorded in 2012 and 2018 with populations resistant respectively to imazamox (Scarabel *et al.*, 2012) and halosulfuron (Scarabel & Miniotti, 2018) and likely cross resistant to all other ALS inhibitors.

The presence of widespread resistant populations belonging to all of the most important species of rice weeds is threatening the sustainability of rice cultivation in Italy because of the increase of weed management costs.

The biggest threat to rice worldwide are *E. crus-galli* and *E. colona,* which are considered the most widespread species and are ranked respectively third and fourth among rice worst weeds by Holm *et al.* (1977).

Resistance for this weed is spread worldwide, the first case was recorded for *E. crus-galli* in corn in the United States (1978) with populations resistant to Photosystem II inhibitors, while the first case on rice was recorded in 1986 in Greece with one *E. crus-galli* population resistant to propanil. It is now widespread in many countries and all of the principal SoAs are involved, with several cases of multiple resistant populations in Italy, USA and Brazil (Heap, 2018).

In Italy, after the discovery of the propanil resistant populations in 2000 and one population of *E. erecta* resistant to both quinclorac and propanil in 2004, the communication and confirmation of new resistant cases for this weed quickly grew up, with the intensification of ALS- and ACCase-inhibiting herbicides use in rice fields. By 2016, 10 populations have evolved resistance to ACCase inhibitors only, 105 to ALS inhibitors and 70 showed multiple resistance to ALS and ACCase inhibitors (source: GIRE).

1.5.8 Weed management in rice fields

Weed management plays a key role in rice cultivation as weed flora is often dominated by competitive and difficult-to-control species - red rice and *Echinochloa* spp. above all - and pedo-climatic conditions are favorable to their proliferation and generation of a persistent seed bank. Herbicide use is intense, with an average treatment frequency index higher than 2.5 (Ferrero & Vidotto, 2010; Scarabel *et al.*, 2013).

In the last 15-20 years the number of available herbicide SoA has significantly decreased due to the strict EU legislation, which led to the withdrawal of several effective substances and the

heavy limitation of others: e.g propanil and quinclorac, that lost the authorization in 2009 and 2013 respectively, did not receive emergency uses authorization in 2018. Oxadiazon, the only active ingredient in use for an efficacious control of *Heterantera* spp., was limited starting from 2016. Therefore farmers are forced to apply complex strategies to obtain a sufficient weed control.

The evolution of herbicide resistant populations and the loss of many previously available herbicides is making weed management more challenging as chemical control mainly relies on few SoA, mostly on ALS and ACCase: it is estimated that 95% of Italian rice paddies is treated with them every year (Sattin, personal communication).

Since the introduction, in 2006, of penoxsulam and imazamox (used in Clearfield® Technology) herbicide strategies have become simplified in comparison with the past, as these two products were able, with two or one single application, to control all of the most important weeds of rice, alone or in complement with other few SoA: e.g. cyhalofop-butyl for the control of *Panicum dichotomiflorum*, *Digitaria sanguinalis* and other weeds typical of dry seeded rice. Since the appearance of resistant populations, herbicide strategies have become more complex: involving a pre-seeding or pre-emergence application, followed by two or even three post emergence applications, containing one ALS and one ACCase inhibitor and eventually one further application to control late-born *Echinochloa* spp. For example, in water seeded rice it is common to use the "false-seeding" technique for the control of red rice and other weeds: this technique involves the submersion of the paddy before rice seeding in order to favour the germination and growth of red rice and other weeds. Paddy is subsequently drained and weeds are treated with pre-seeding products such as glyphosate, cicloxidim and propaquizafop (each one alone or in combination depending on the field weed flora composition). In post-emergence of rice and weeds one of the most common strategies is to apply cyhalofop-butyl together with profoxydim (plus adjuvant), for the early control of *Echinochloa* spp. followed by penoxsulam alone or in mixture with an hormonic active ingredient for the simultaneous control of *Echinochloa* spp. and ALS resistant ciperaceaes. Sometimes an additional treatment with cyhalofop-butyl might be necessary for the control of late-born *Echinochloa* spp. All this shows that in difficult occurrences up to nine different products might be needed to control weeds in rice paddies when resistance is present, also relying on few SoA. Beyond "false seeding", non-chemical control of weeds relies on competitive rice varieties

with certified seed, on the limitation of soil tillage, on dry seeding technique, which shifts *Echinochloa* spp. populations from the "white" species to *E. crus-galli*, generally slower in evolving resistant biotypes and other grasses, such as *D. sanguinalis* and *P. dichotomiflorum*, that did not evolve into resistant populations yet (Sparacino & Sgattoni, 1993; Tabacchi *et al*., 2006).

1.5.8.1 New molecule for weed control in rice

Florpyrauxifen-benzyl is a new pyridine-2-carboxylate (picolinate) herbicides. It is a synthetic auxin, HRAC group O (Fig. 11), developed for the control of grasses and broadleaf weeds of rice (Epp *et al.*, 2016). The commercial name of the active ingredient is RinskorTM. Mimicking the action of natural auxins, florpyrauxifen-benzyl is absorbed predominantly by plant leaves, accumulated in the meristems where it bounds with specific auxin receptors: it shows a high affinity with AFB5 and a lower one with TIR1. Consequently proteins are degraded and plants die within two weeks from the application. Florpyrauxifen-benzyl has showed a positive environmental profile, since it rapidly degrades in the environment, in soil and in the plants. It is also used at low doses (30 g a.s./ha). Florpyrauxifen-benzyl showed a very good control on most of the rice weeds, like *Alisma plantago aquatica*, *Cyperus difformis*, *Heterantera* spp. and a good control on *Echinochloa* spp. It has also shown a high selectivity on most common varieties of rice.

Its process of authorization is still ongoing in Europe.

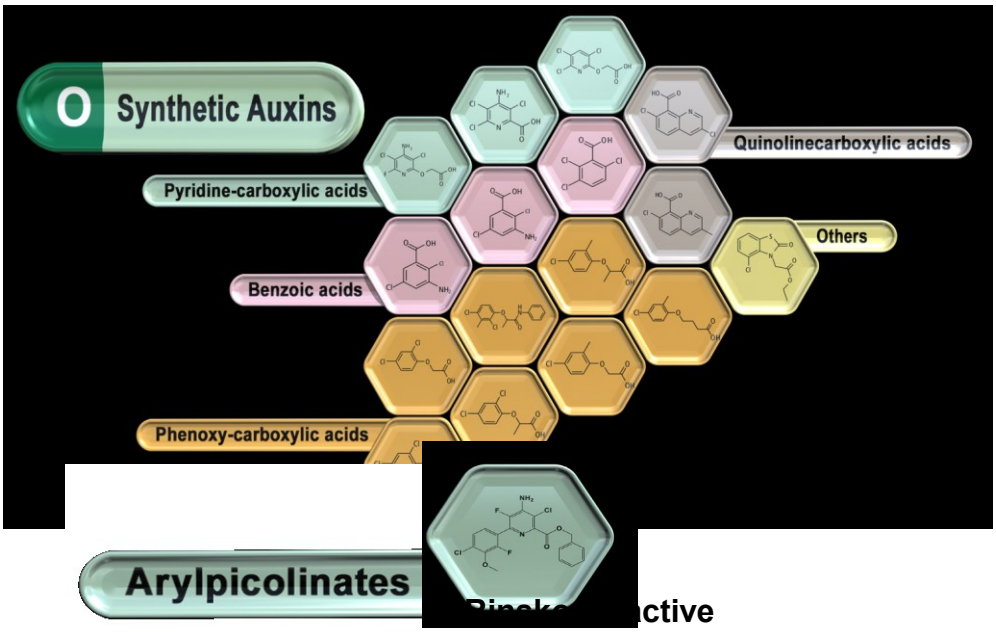


Fig. 11: RinskorTM (florpyrauxifen–benzyl) classification according to HRAC.

1.6 AIMS of the RESEARCH

This research is aimed to provide a better insight on the epidemiology of herbicide resistance into the major rice cropping areas in Italy. To our knowledge, this is the first study that determines the degree of association between herbicide resistance and a few important predictors at such a large scale. Secondly, we aimed to improve the reliability of *Echinochloa* spp. classification using innovative molecular approaches like DNA barcoding. Third we investigated on the response of different sensitive purified *Echinochloa* species to the most commonly used herbicides in rice.

For the epidemiological research the specific objectives are:

- To study the influence of agronomic and pedologic factors on the evolution of resistance through statistical analyses: i.e. discriminant analyses and logistic regression. It was decided to analyze the impact of two major agronomic techniques, rice seeding type (water- or dry-seeded) and crop rotation rate, as well as soil texture, on the diffusion of herbicide resistance on 232 municipalities, covering almost 200,000 ha;
- to estimate resistance evolution risk in these municipalities through neural network approach and create resistance risk maps.

For the *Echinochloa* spp. study the specific aims are:

- To investigate the possible match of classic dichotomous keys with discrimination provided by molecular markers found through DNA barcoding approach;
- to set up a protocol of species-specific (SS)-PCR to quickly analyze and discriminate a large number of *Echinochloa* spp. samples;
- to understand whether the most used herbicides in the Italian rice fields have different efficacy on several sensitive, purified and classified *Echinochloa* spp. accessions.

Chapter II

MATERIALS AND METHODS

2.1 Epidemiology of herbicide resistance in rice in Italy

2.1.1. Definition of the area in the study

The territory object of the study is the one where rice cultivation is deep rooted and traditional across two regions, Piedmont and Lombardy, and 6 provinces: Alessandria (AL), Biella (BL), Vercelli (VC) and Novara (NO) in Piedmont, Milan (MI) and Pavia (PV) in Lombardy. The study includes about 200,000 ha, i.e. about 92% of the total Italian rice growing area.

In the analyses only municipalities with more than 10% of UAA (Utilized Agricultural Area) cropped to rice were included.

The degree of association between resistance presence in a certain municipality and four important agronomic factors was analyzed using two different statistical tests, namely stepwise backward discriminant analyses and stepwise backward logistic regression.

Predictors chosen for the analyses are: water seeding rate per municipality (WS), rotation rate per municipality (RR), percentage of clay (PC) and sand (PS) in soil per municipality. Subsequently, neural network analyses approach was also performed to understand the ability of the selected agronomic factors to predict resistance evolution in a certain municipality and to create stochastic maps of resistance evolution risk.

This is the first study that determines the degree of association between herbicide resistance and a few important predictors at such a large scale.

An Italian municipality is a territory with autonomous administration that generally ranges in size from 15 to 40 km^2 and those included in the analyses are those where rice surface represented at least 10% of the total Utilized Agricultural Area (UAA): the municipality level was chosen because data about resistance – the benchmark of our analyses – were not available at greater detail.

2.1.2. Database building

For the statistical analyses a unique database was created, where to each municipality included in the study was assigned a single value of absence/presence of resistance – independently from

specie or herbicide SoA – the percentage of rice cropping area under crop rotation (RR), the percentage of water-seeded (WS) area, the average percentage of sand (PS) and clay in soil (PC).

These four predictors were chosen on the basis of their importance in the rice area, influence on the flora composition and data availability: e.g. herbicide strategy at municipality level was not considered among the predictors because data are scant, not homogeneous and generally too complex, although herbicide strategy is the first driver for the evolution of resistant population (Heap, 2014a; Norsworthy *et al.*, 2012).

Data on presence/absence of resistance in the rice area were extracted from the GIRE database and other resistance studies by the University of Turin, while data on RR and soil texture were obtained from Regions' environmental agencies: Regional Agency of Services for Agriculture and Forestry (Ente Regionale per i Servizi all'Agricoltura e alle Foreste, ERSAF) for Lombardy and Agriculture Registry Office (Anagrafe Agricola del Piemonte) for Piedmont. Records on WS were supplied by the National Rice Agency (Ente Nazionale Risi, ENR).

2.1.2.1 Resistance data

Since 1997 the Italian Herbicide Resistance Working Group (GIRE, www.resistenzaerbicidi.it) has been monitoring, collecting and testing putative herbicide resistant populations nationwide, based on farmers and farmers' advisors complaints of poor herbicide efficacy. All other available data on herbicide resistance in Italy was also collected and all populations that were confirmed resistant to at least one herbicide through a standardized testing procedure (Panozzo *et al.*, 2015b) were included in a national herbicide resistance database. The part of the database relative to the populations collected in the main rice producing area in Italy (approximately 200,000 ha, i.e. about 85% of the total rice growing area) was used as input to produce maps of herbicide resistance diffusion using iMAR application (Panozzo *et al.*, 2015a; GIRE, 2018). Maps are obtained by changing the color of the territory of the municipalities where at least one confirmed resistant population had been recorded. Therefore, municipalities with different numbers of resistant populations appear with the same color (Panozzo *et al.*, 2015b). This, together with the nature of the monitoring done by GIRE, which is based on (a) end users complaints about herbicide failure and (b) priority given to samples collected in municipalities where herbicide resistance had not

previously been reported, makes the output maps "qualitative" because they do not provide reliable information on the spread of resistance within each municipality. That is to say that they indicate the areas at higher risk of resistance evolution.

GIRE national rice resistance database was integrated with data provided by the University of Turin, where resistance screenings were performed in on 88 *Echinochloa* spp. populations coming from 53 municipalities in the rice area.

Six weed species have evolved resistance mainly to ALS and ACCase inhibitors: *A. plantago-aquatica* (ALSPA), *C. difformis* (CYPDI), *Echinochloa* spp. (ECHSS), *O. sativa* var *spontanea* (ORYSA), *S. mucronatus* (SCPMU) and *C. esculentus* (CYPES). The latter was excluded from the analyses because only two ALS inhibitors resistant populations were found in 2017 after the conclusion of this analyses (Scarabel & Miniotti, 2018).

A total of 584 populations were included in the initial database, 425 of which were resistant to at least one herbicide SoA (Tab. II).

Weed Species	Resistant	Susceptible	Total
ALSPA	66	13	79
CYPDI	29	32	61
ECHSS	192	48	240
ORYSA	57	46	103
SCPMU	81	20	101
Total	425	159	584

Tab. II: Number of populations susceptible or resistant to at least one herbicide for all species involved, which were tested for resistance and included in the GIRE database.

From the analyses were first excluded all the municipalities coming from outside the provinces of interest. Then data were grouped in order to have only one datum of resistance for each municipality independently from the species and SoA. Each municipality was assigned with a value of 1, when at least one case of resistance was confirmed independently from the species and the SoA and 0 when no case of resistance has ever been recorded: e.g. Bianzè municipality (VC province) counted 15 cases of confirmed resistance for *Echinochloa* spp. for multiple SoA, one for A. *plantago-aquatica* and three for *C. difformis*, so it was recorded in our database as "resistant

municipality" assigning it a value of 1. A second database was then built including only the cases of *Echinochloa* spp. resistance, being this the most important rice weed both in Italy and worldwide.

2.1.2.2. Soil data

It was decided to take into account soil texture in this study because it is a driver in the choice of seeding technique and tillage type, which are the main drivers for the composition of the weed flora in rice fields: i.e. dry seeded rice – which has a different weed flora from water seeded rice - is traditional in sandy soils, where water is not continually available.

Data about percentage of sand (PS) and clay (PC) in soil were not directly available, but both Piedmont and Lombardy region have published open access data regarding soil texture, drainage, structure and other soil characteristics in general: data for Piedmont soil are available at http://www.regione.piemonte.it/agri/area_tecnico_scientifica/suoli/suoli1_50/carta_suoli.htm, while data for Lombardy are published at www.geoportale.regionelombardia.it.

Data were downloaded as shapefiles: they were merged and transformed into easily workable Excel 2013 sheets using QGIS software version 2.14.9, to obtain a single soil database including both regions.

Per each municipality included in the analyses it was reported the number of hectares occupies by each type of soil. On the base of this data a "prevalent soil texture" was calculated making a weighted average of the different types of soil present in certain municipality, then PC and PS were calculated on the prevalent soil texture with the help of a soil triangle available at

https://www.nrcs.usda.gov/wps/portal/nrcs/detail/soils/survey/?cid=nrcs142p2_054167

Considering that the topsoil is the soil part interested by tillage and crop roots, only texture relative to this layer was taken into account for the analyses.

Given the very high correlation between the percentage of sand and silt (r=-0.96), the latter was not considered in the analyses.

2.1.2.3. Water seeding data

Water seeding data (WS) were provided by ENR already aggregated at municipality level for the three year period 2013-2015: they were expressed as hectares of water seeded and dry seeded area. An average per municipality on the three years was calculated and then expressed as percentage of the total rice area per municipality.

2.1.2.4 Rotation Rate data

Data on rotation rate (RR) was not directly available and was calculated from the "land use database" of the two regions. Data for Piedmont are available on line starting from 2013 on the site www.sistemapiemonte.it in the "Data Warehouse" page, while for Lombardy data were provided by ERSAF following an official request. Also in this case we had to focus on the three year period 2013-2015 since previous years were not available.

Land use data are detailed at "cadastral plot level": a cadastral plot is a physically continuous piece of territory – of variable dimension- located in a single municipality with a single owner, quality and culture class.

For each municipality the use of each plot in 2013, 2014 and 2015 was compared: each plot was considered rotated when at least one out of three years wasn't cropped to rice. Obviously plots never cropper to rice were totally excluded from the analyses. Hectares belonging to rotated plots were summed to obtain a single data of rotated area per municipality and transformed into percentage of the total UAA.

All information about R, PS, PC, WS and RR were then put in the final database.

2.1.3 Mapping

Starting from the GIRE national database on herbicide resistance, descriptive maps were produced as graphical support for the statistical analyses. Resistance maps were generated with iMAR application (Panozzo *et al.*, 2015a), one pooling all cases of resistance recorded from 1997 to 2015 for the five rice weeds affected by herbicide resistance (*A. plantago-aquatica*, *C. difformis*, *S. mucronatus*, *O. sativa f. spontanea* and *Echinochloa* spp.) and another for *Echinochloa* spp. only.

In both maps two areas (S and L) where resistance presence had never been recorded in 20 years of GIRE activity were evident.

Other descriptive maps were created for each of the predictors included in the study using QGIS 2.14.9 software. Data about PC, WS, PS and RR are continuous and virtually included in a 0-100% interval. To create clearly readable maps, data were clustered in 5 classes. WS and RR classes were created on a 20% interval base: 0-20%, 20-40%, 40-60%, 60-80% and >80%. For soil, classes depended on the concentration of the single element in soil: e.g. clay content ranged from 6% to a maximum of 29% so 4 intervals were created: 6-11%, 12-16%, 17-21% and 21-28%.

2.1.4 Statistical analyses

The use of different statistical approaches, namely discriminant analysis, logistic regression and artificial neural network was chosen to improve the robustness of the study.

Based on different assumptions and offering different solutions these three tests enable the quantification of the effect of pedo-climatic and management drivers on agro-ecological systems at large scale (Mascanzoni *et al.*, 2018)

Discriminant and logistic regression analyses were done in parallel. For both tests the stepwise backward selection approach of the predictors was adopted: stepwise approach is a method of selecting variables to include in the analyses by a series of F-tests. In backward stepwise selection, the analyses begins with all candidate factors and – following multiple steps - the variable whose loss implicates the least significant deterioration of the model fit is deleted at each step. This process is repeated until no further variables can be eliminated without compromising the model fit.

Neural network analysis was done after the results of the first two tests were obtained in order to understand the predictive capacity of WS, PC, PS and RR for herbicide resistance evolution.

All the three analyses were performed twice with IBM SPSS 24 software: first, resistant cases of all five weed resistant species pooled together where tested, next resistant cases of *Echinochloa* spp. only were considered. All statistical analyses were performed with the alpha set at 0.05.

2.1.4.1 Discriminant analyses

The stepwise backward discriminant analysis separates objects or observations in classes, or allocates new observations in already defined ones. It was first developed by Fisher (1936) and was used in multiple fields to separate individuals in the groups they belong to.

The aim of the analysis was to define the probability of correctly classifying a resistant (1) or non-resistant (0) municipality on the basis of WS, RR, PC and PS values.

The risk prediction model based on discriminant analysis is a process involving two steps: in the first one a large database including several training samples is needed to understand the relationship between our dependent variable "resistance" (R) and the four defined independent variables RR, WS, PC and PS. The second step is the building of the discriminant function to show this relationship.

The statistical function can be expressed as:

$$R = \beta 1(X1) + \beta 2(X2) + \beta 3(X3) + \beta 4(X4) + \cdots + C$$

Where R is the discriminant function score, β is the function coefficient, *X* is the value of the independent variable and *C* is the intercept. In our case, values of R can score only 0 in case of non-resistant municipality or 1 in case at least one case of resistance was found in that municipality.

2.1.4.2 Logistic regression

Logistic regression is a model that estimates the probability of a binary response on the basis of one or more independent variables: in other words it is used to define the relationship between a binary dependent variable and multiple independent variables (Lee, 2005).

The general equation of logistic regression is:

$$R = C0 + C1X1 + C2X2 + C3X3 + \cdots CnXn$$

where x1, x2,…, xn are independent variables and c1, c2,…, cn are the regression coefficients estimated in the analyses. R is function of the independent variables: and can take value of 1 in case of resistance presence and 0 in case of resistance absence.

2.1.4.3 Neural network approach

Previously created maps were descriptive and gave important information, but limited to the known diffusion of resistance, WS, PC, PS and RR in the municipalities. The next step was to generate stochastic maps of herbicide Resistance Evolution Risk (RER) based on the results of artificial neural network analyses (NNA).

To predict the evolution of resistance in a certain context through rigid deterministic models is rather difficult as it is an ill-defined and evolutionary phenomenon dependent on many factors. For this, an algorithm based model - such as NNA - is more suitable for this type of approach as it starts from precise information to tackle an uncertain and complex reality (Gonzalez-Andujar *et al.*, 2016)

NNA is part of a group of statistical approaches named Soft Computing Techniques or Computational Intelligence, which tolerates a certain degree of uncertainty (Das *et al.,* 2013). They are designed to mimic biological neural networks with the scope of "learning" how to perform different tasks by considering examples: e.g. they can learn to identify images of roses analyzing pictures labeled as "rose" and "no-rose", using the result to identify roses in other images with any prior knowledge of them, but automatically generating characteristics from the material they process.

The architecture of a NNA is based on a series of connected artificial neurons, which can receive a signal, process it and transmit then the signal to the other artificial neurons also called nodes. The connection is performed via coefficients or weights, being therefore numerical. For the processing of information, the processing artificial nodes have weighted inputs, transfer functions and outputs. NNA can be designed in different ways, but they all are described by the transfer function used among nodes, the training type or learning rule and by the connection formula.

In brief, a NNA consists of a number of input variables and output variables and a certain numbers of hidden layers with n nodes (Fig. 12).

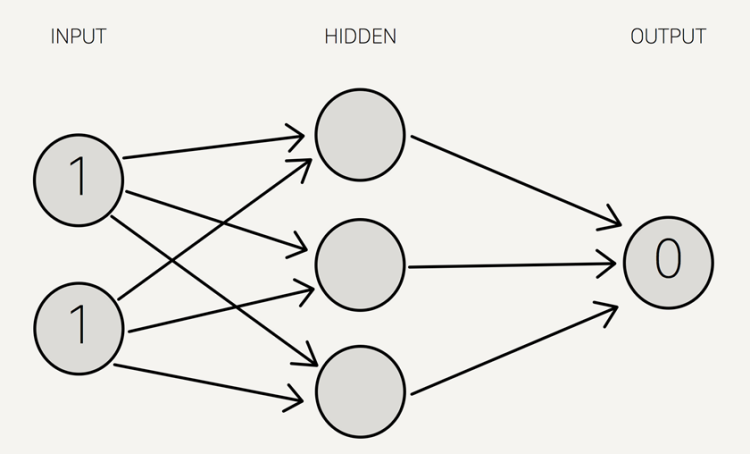


Fig. 12: Example of a simple neural network Analyses displaying the input data (1), the hidden layers were analyses id performed and the output level (0).

For the evaluation of the ability of WS, RR, PS and PC to predict resistance evolution, a feed-forward "Multi-Layer Perceptron" or MLP model was implemented in IBM SPSS 24 Software using as training method the Scaled Conjugate Gradient. MLP is suitable for complex problems as it adds more hidden layers, overcoming the drawback of the single layer perceptron. Conjugated gradient is suitable for networks with a large number of weights: i.e. big and complex databases where input data have big range of values. In particular Scaled Conjugated Gradient does not require line search at each iteration step like other conjugate training function, making this algorithm faster than others (Sharma & Venugopalan, 2014).

Both for the analyses of the database of all weeds pooled together and that of *Echinochloa* spp. alone a partition of 7 training and 3 testing was chosen: i.e. 70% of the data were used for training step and 30% for testing step.

As output two additional databases were obtained including a value of RER for each municipality. RER was expressed as a number between 0 and 1. It was then transformed into percentage: %RER were processed in QGIS software to generate the stochastic maps displaying where resistance was more likely to evolve.

All three statistical tests were performed twice: for all weeds pooled together and for *Echinochloa* spp. alone.

2.1.5 *Echinochloa* spp. random survey

To verify whether the lack of herbicide resistant weed populations observed in two areas S and L (Fig. 13) was an artifact due to the nature of resistance monitoring done by GIRE, a random sampling of 20 *Echinochloa* spp. populations was done in these areas in September 2016.

A grid of 5x5 km^2 was drawn on *Echinochloa* spp. resistance map and a seed population was collected in the rice field closest to each of the nodes of the grid where some *Echinochloa* spp. plants were present (Fig. 13).

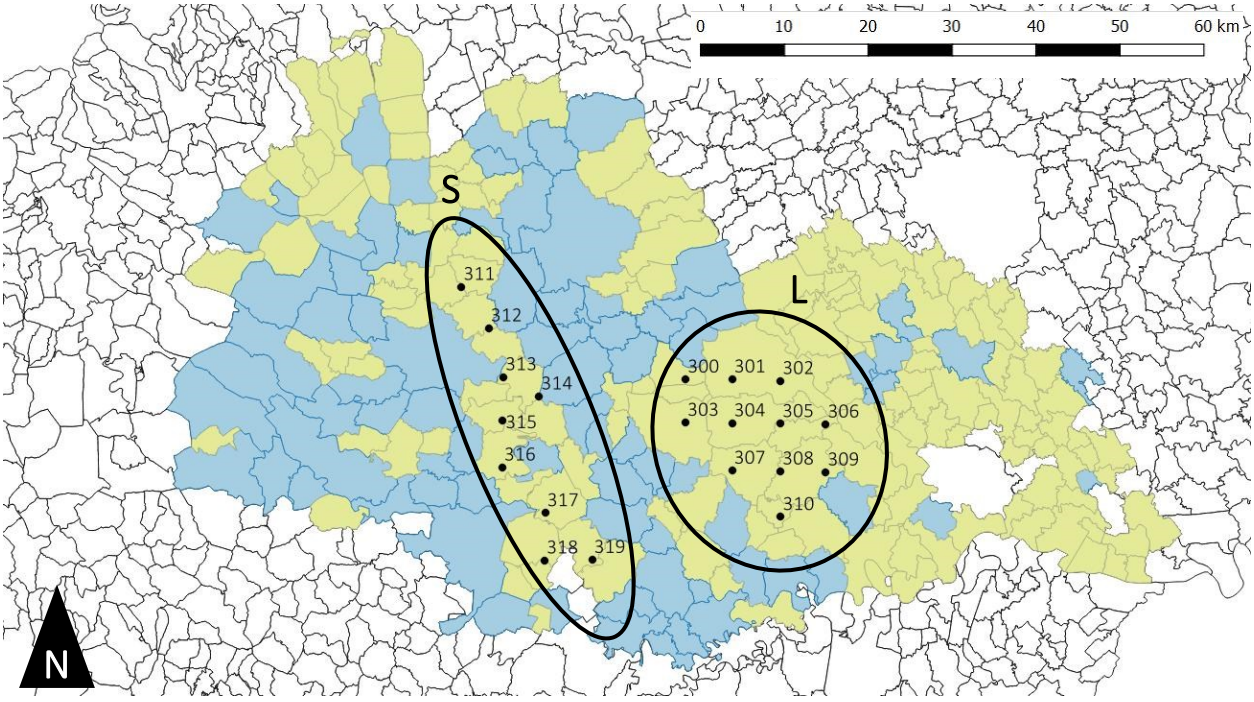


Fig. 13: map displaying the nodes of the grid for the random sampling in areas S and L. Each black dot represent a designed collection point.

Each sample harvested included seeds from at least 5 plants and harvested paddies sized about 2 ha. Samples from #300 to #310 were collected in area L while those from #311 to #319 came from area S. Sampling followed a density structured approach visually assessing the density of *Echinochloa* spp. in the field. Infestation density was divided into four categories: very low: <1 plant x 500 m^{-2}, low: about 1 plant x 100 m^{-2}, medium: about 1 plant x 10 m^{-2}, high: about or more than 1 plant x 1 m^{-2} (Tab III).

All seed samples were then cleaned and dry stored at room temperature (18-20 °C).

Region	Area	Municipality	Pop code	Latitude (N)	Longitude (E)
Lombardy	L	Cilavegna	300	45° 17' 49.00"	8° 45' 57.53"
Lombardy	L	Vigevano	301	45° 17' 48.01"	8° 49' 54.23"
Lombardy	L	Vigevano	302	45° 17' 46.66"	8° 54' 00.95"
Lombardy	L	Mortara	303	45° 15' 12.80"	8° 45' 57.32"
Lombardy	L	Gambolò	304	45° 15'11.47"	8° 49' 53.48"
Lombardy	L	Borgo San Siro	305	45° 15' 09.25"	8° 49' 00.99"
Lombardy	L	Borgo San Siro	306	45° 15' 07.29"	8° 57' 50.66"
Lombardy	L	Tromello	307	45° 12' 20.18"	8° 49' 57.79"
Lombardy	L	Garlasco	308	45° 12' 17.82"	8° 54' 01.04"
Lombardy	L	Garlasco	309	45° 12' 15.55"	8° 57' 52.47"
Lombardy	L	Alagna	310	45° 09' 35.65"	8° 53' 59.42"
Piedmont	S	Villata	311	45° 23' 21.4"	8° 27' 01.26"
Piedmont	S	Borgo Vercelli	312	45° 20' 37.36"	8° 30' 33.84"
Lombardy	S	Palestro	313	45° 17' 51.58"	8° 30' 32.49"
Lombardy	S	Rosasco	314	45° 17' 50.53"	8° 34' 10.17"
Piedmont	S	Pezzana	315	45° 15' 18.22"	8° 30' 30.94"
Piedmont	S	Caresana	316	45° 12' 30.32"	8° 30' 29.41"
Lombardy	S	Candia Lomellina	317	45° 09' 47.51"	8° 34' 07.94"
Piedmont	S	Ticineto	318	45° 07' 03.08"	8° 34' 07.12"
Lombardy	S	Breme	319	45° 16' 55.19"	8° 38' 55.19"

Tab. III: scheme of calculated latitude and longitude for the random sampling, for each population are reported region, sampling area (S for Sesia or L for Lomellina) and municipality of collection.

Two whole-plant resistance screenings were then performed in greenhouse conditions: first in autumn 2016 (A) and repeated in spring 2017 (S). Seeds were chemically scarified for twenty minutes in sulfuric acid (96%) and then carefully rinsed with cold water. They were then placed in plastic boxes containing Pot Grown H peat (Klasmann Deilmann GmbH) and stored in a germination cabinet at 26/16 °C (day/night) under neon tubes providing a Photosynthetic Photon Flux Density (PFFD) of 15-30 µmol m^{-2} s^{-1} with a 12-hour photoperiod.

At one leaf stage seedlings were transplanted into pots of 18 cm of diameter with standard potting mix (60% silty loam soil, 15% sand, 15% perlite, 10% peat) and placed in a greenhouse, with temperature ranging between 15-19 °C at night and 26-33 °C during the day. Light was supplemented using 400 W metal-halide lamps, which supplied about 450 µmol m-2 s-1 with a 16-hour photoperiod.

All populations were tested for resistance to both ALS- and ACCase-inhibiting herbicides: penoxsulam (Viper®, Dow Agrosciences, Bologna, Italy), imazamox (Beyond®, BASF, Milano, Italia), cyhalofop-butyl (Clincher One®, Dow Agrosciences Bologna, Italy) and profoxydim (Aura®, BASF, Milano, Italia). Both imazamox and profoxydim were used along with the recommended surfactant Dash HC (methyl-palmitate and methyl-oleate, BASF, Milano Italia) at 0.5% concentration and 0.9 L ha^{-1}, respectively (Tab. IV). For both experiments the experimental design was a randomized complete block with three replicates, each replicate was represented by one single pot. Each pot counted 7 plants.

Site of Action	Commercial product	Active Ingredient (g L^{-1})	Surfactant (% or L ha^{-1})	Field Dose (1x) mL ha^{-1}	Treatment timing	Rates	
						A	S
ACCase inhibitors	Aura	profoxyidim 200	Dash HC 0.9	500	2-3 leaves stage	1x, 3x	1x
	Clincher One	cyhalofop-butyl 200		1500	2-3 leaves stage	1x	1x
ALS inhibitors	Beyond	imazamox 40	Dash HC 0,5%	900	2-3 leaves stage	1x, 3x	1x, 3x
	Viper	penoxsulam 40		2000	2-3 leaves stage	1x, 3x	1x, 3x

Tab. IV: Details of herbicides treatments used in autumn (A) and spring (S) experiment. For each treatment are displayed the timing of application and the rates used: 1x indicated the field dose, 3x indicates three times that.

One known susceptible checks (07-16L) was included in the experiments. ALS-inhibiting herbicides were applied at two doses, the recommended field dose (1x) and three times that (3x). For ACCase-inhibiting herbicides it was decided to apply only the 1x recommended field dose for cyhalofop-butyl, while profoxydim was applied at 1x and 3x in the A experiment and 1x only in the S experiment. This decision was taken after the analyses of the results of the first test.

Application was performed when plants had reached 2-3 leaf stage (BBCH 12-13, Hess *et al.,* 1997), using a precision bench sprayer delivering 300 L ha^{-1}, at a pressure of 215 kPa and a speed of 0.75 m s^{-1}, with a boom equipped with three flat-fan (extended range) hydraulic nozzles (TeeJet, 11002). For each population one untreated check was included. Plant survival and shoot fresh weight were recorded 4 weeks after treatment (WAT) and expressed as percentage of the

untreated check (S). Plants were considered dead when they did not show any active growth, regardless of their color. Also completely dead plants were weighted and the real weight, even when negligible, was recorded. Standard error (SE) was calculated per each mean value.

Populations were then divided into 4 categories: S when less than 5% of plants survived the 1x dose, SR when survival at 1x dose ranged between 5% and 20%, R when survival at 1x was >20% and RR when >10% of plants survived the 3x dose and >20% the recommended field dose (Panozzo *et al.*, 2015b; Sattin, 2005).

To test whether the two experiments could be pooled Levene's test for variance homogeneity was performed.

2.2 *Echinochloa* spp. case study

2.2.1 Seed samples collection and morphological classification on original accessions.

At the end of September 2015, at *Echinochloa* seeds maturity, but before the plants scatter on the ground, two *Echinochloa* spp. seed samplings were performed in six rice fields of the Vercelli province where penoxsulam is still effective in controlling this weed. Seeds were collected from single plants to make sure that each accession included only one species of *Echinochloa*. Forty plants were sampled and a first rough morphological classification on the base of Pignatti (1982) and Carretero (1981) classification keys was performed on field, on the base of the parameters listed in the two keys.

Seeds collected from each plant were put in paper bags and coded with two ciphers: the first cipher indicating the field of origin, the second one was a progressive cipher indicating the order of the sampling: e.g. accession #34 mean field 3 plant 4.

Seeds were transported to the greenhouse of the Institute of Agro-environmental and Forest Biology (IBAF) - CNR located into the "Azienda Agricola Sperimentale L. Toniolo" in Legnaro (PD), Italy (45° 21' N, 11° 58' E) where all the experiments were carried out, let dry at room temperature (18-20 °C), cleaned and stored in paper bags in a dark room at 4 °C.

For each plant collected, five seeds were then classified according to Costea & Tardif (2002) dichotomous key. The following parameters were taken into consideration: spikelet shape, length

(SL) and width (SW), awn presence, lower glume length (GL), lower glume / spikelet length ratio (GL/SL), caryopsis shape, length (CL) and width (CW), embryo shape and stigmas presence.

Analyses were carried out using a binocular microscope supporting a camera (Leica). Photos of whole seeds and caryopsis without the glumes were taken and the different parameters were measured on the pictures using the Leica Application Suite (LAS) Software Version 4.9.0.

SL and SW were measured without considering the awn or the mucrone, which length was too variable to be considered and usually not indicative for species classification. Then awn presence and GL were recorded and GL/SL ratio was calculated.

With the aid of a tweezers, seed glumes and lemmas were eliminated from each seed in order to measured CL and CW. Caryopsis shape and embryo and scutellar region shape were visually analyzed and classified.

All measures taken were expressed in millimeters.

2.2.2 Preliminary screening

A preliminary screening on thirty seven single plants accessions collected in the fields was performed in November 2015 to test their susceptibility to penoxsulam, the main ALS inhibitor used in Italian rice fields. Three accessions were excluded as harvested seeds did not germinate or the quantity of mature seed provided by panicles was too small.

Scarification was performed by soaking 0.5 g of seeds per accession in concentrated sulphuric acid (96%) for twenty min. Acid was then removed and seeds were thoroughly rinsed under running cold water to stop the reaction and eliminate any trace of acid, which permanence on seeds might foster the appearance of molds, thus inhibiting germination.

Seeds were hence moved into plastic boxes containing Pot Grown H peat (Klasmann Deilmann GmbH) and stored in a germination cabinet at 26/16 °C (day/night) under neon tubes providing a Photosynthetic Photon Flux Density (PFFD) of 15-30 µmol m^{-2} s^{-1} with a 12-hour photoperiod.

After one week, when seedlings have reached 1 leaf stage, they were transplanted into pots with standard potting mix (60% silty loam soil, 15% sand, 15% perlite, 10% peat) and placed in greenhouse, with temperature ranging between 15-19 °C at night and 26-33 °C during the day. Light was supplemented using 400 W metal-halide lamps, which supplied about 450 µmol m^{-2} s^{-1}

with a 16-hour photoperiod. The experimental layout was a completely randomized block with four replicates of six plants each.

When plants have reached 2-3 leaves stage (BBCH 12-13, Hess *et al.*, 1997), they were sprayed with penoxsulam at the recommended field dose (1x) of 2 L ha^{-1} (Viper®, 20 g L^{-1}, Dow Agrosciences, Bologna, Italy) using a precision bench sprayer delivering 300 L ha^{-1}, at a pressure of 215 kPa and a speed of 0.75 m s^{-1}, with a boom equipped with three flat-fan (extended range) hydraulic nozzles (TeeJet, 11002). For each population, an untreated control was included.

Plant survival and the Visual Estimation of the Biomass (VEB) were recorded four weeks after treatment (WAT). The efficacy of the treatment was evaluated using a susceptible check. Plants were considered dead when they showed no active growth independently from the color or size. Plant survival was calculated as percentage of the untreated control and VEB was determined assigning a score ranging from 0 to 10 to each replicate: 0 when all plants were dead, 10 when the plant growth was comparable to the untreated control.

Accessions were then ascribed to three categories: susceptible (S) when less than 5% of plants survived the treatment, moderately resistant (SR) when 5% to 20% survived, resistant (R) when more than 20% of plants survived the treatment (Panozzo *et al.*, 2015b).

Penoxsulam screening was then repeated on these accessions in September 2016.

2.2.3 Accessions reproduction

On the base of the results of the preliminary screening and morphologic species classification (at whole plant and seed levels), in order to obtain a larger quantity of seeds for further experiments, ten accessions of *Echinochloa* spp. were chosen to be reproduced in semi-controlled conditions.

As all accessions classified as *E. crus-galli* provided small quantity of seeds or had very poor germination rates none of them was reproduced.

For each population 0.5 g of seeds were chemically scarified and put to germinate following the procedure described in section 2.2.2. When seedlings have reached one leaf stage they were transplanted into pots containing standard potting mix. For each accession 18 pots were considered, each pot containing 3-4 plants. They were placed in semi-controlled conditions in a

tunnel covered with a black thin net to provide protection against excessive solar radiation and other adverse meteorological events such as strong rain or hail, than might harm the regular plant growth during summer. An irrigation system maintained the water content of the soil substrate in optimal conditions. When plants reached tillering stage they were fertilized with ammonium nitrate (Yara, N 27.8%, nitrate 13.9%, ammonium 13.9%). Two plants per pot were eliminated five weeks after transplant. One week later, each population was covered with non-woven fabric to prevent cross-pollination, thus preserving genetic purity (Fig. 14).

Mature seeds were collected in August and placed at room temperature for one week, then placed in paper bag in a dark room at 4 °C to ensure an optimal conservation.

From one week after transplant until seed maturity regular visual assessment were performed each 10-14 days to record the morphological characteristic of each accession according to Pignatti (1981), Tabacchi *et al.* (2006) and Costea & Tardif (2002) classification keys and proceed with a more accurate morphologic classification than the first done at field level.

To distinguish the original seed stocks from the reproduced ones, new codes were assigned to the accessions formed by the year of the reproduction (i.e. 16) and the code originally assigned to the accession: e.g. 16-41.

Fig. 14: isolating cages used to prevent cross-pollination in reproduced accessions. In the back it is visible a detail of the black net used for protection of plants.

2.2.4 Collaboration with the Meise Botanic Garden (Belgium)

Objective of this cooperation was to improve our skills in species discrimination with the support of Ivan Hoste and Philipp Verloove.

For each reproduced accession 200 seeds were sent to the Meise Botanic Garden and reproduced in their greenhouse. Seeds were scarified according to the already described protocol, pre-germinated in a germination cabinet and four seedlings per accession were transplanted in pots and placed in the greenhouse in April 2018. During daytime, water was provided every two hours for 5-10 min. Temperature in greenhouse was not controlled, but in hotter days a black net was mounted over the greenhouse to provide protection against high temperatures and solar radiation.

One single evaluation on plant phenotypic characteristics was performed in August 2018, when plants had reached maturity.

2.2.4.1 Analyses of morphological data

Characteristics of plants reproduced both in Belgium and in Italy were analyzed.

It was not our intention to create a new classification key for *Echinochloa* spp., but to find a match between the morphological characterisation and molecular marker discrimination of our accessions. Qualitative parameters assessed on plants and relative scores assigned are summarized in Tab. V.

Morphological traits	1	3	5
Basal stem color	Green	Pink	Red/purple
Nodes color	Green	Pink	Red/purple
Panicle bearing	Upright	Bending	Nodding
Secondary Branching	Yes		No
Awns	Numerous	Occasional	Abstent
Max length of the awn (mm)	5	5-11	>11
Leaf Sheath Hairy	Yes	Few	No
Collar zone hairy	Yes	Few	No
Blade Hairy	Yes	Few	No
Base of blade with hairs on the border	Yes	Few	No
Different color border and midrib	Yes		No

Tab. V: list of morphological traits analyzed in plants: for each category a score of 1, 3 or 5 was given to each parameter.

For color of the base of the stems and of nodes a scale from 1 to 5 has been used: a score of 1 was given in case of green color and 5 in case of red/purple pigmentation. Panicle bearing, presence of awns and their maximum length were divided into three categories. For each characteristics recorded a score of 1, 3 or 5 was given as reported in the column headers of Tab. V. Scores of 2 or 4 were given in case of intermediate characteristics: e.g. an accession with a bending-nodding panicle bearing would receive a score equal to 4. Results were recorded in an Excel form (data not shown).

Different parameters of spikelets and caryopsis were analyzed with a binocular and measures were taken both for populations grown in Belgium and in Italy. Measures were taken in mm. Five seeds per accession were analyzed and results were then mediated within each accession. Parameters took into account were (Fig. 15):

- Upper glume length
- Fertile lemma length
- Sterile lemma length = spikelet length
- Spikelet width
- Lower Glume Length
- Spikelet/Lower Glume Ratio
- Caryiopsis length
- Caryiopsis width
- Embryo ratio considering scutellar zone
- Embryo ratio not considering the scutellar zone

In Costea & Tardif (2002) it was not described whether the embryo/caryopsis ratio was calculated considering the scutellar region or not, so this ratio was calculated twice: the first one considering the length of the embryo with the scutellar region, the second not taking it into account.

Results of morphological analyses were then compared with Costea & Tardif (2002), Pignatti, (1982) and Tabacchi et al. (2006) dichotomous keys, in order to obtain a reliable classification of our accessions.

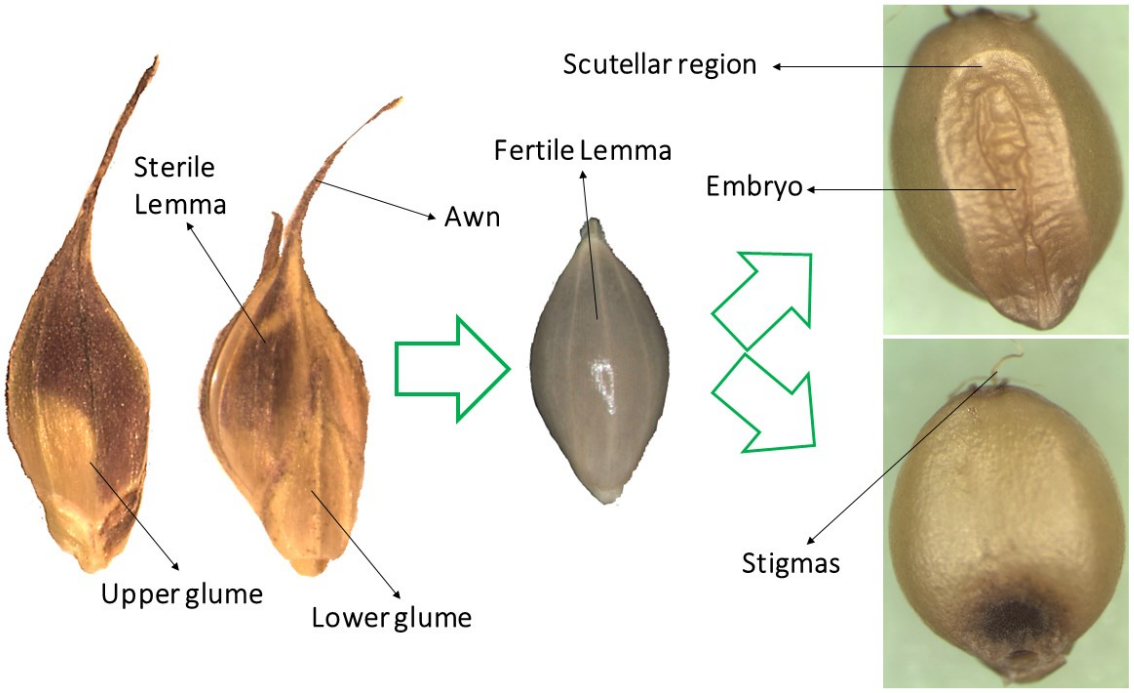


Fig. 15: *Echinochloa* spp. spikelet sections used in the morphological classification.

2.2.5 DNA barcoding

Based on the first morphological characterization (see section 3.2.1 for results) and the results of the preliminary screening, 17 susceptible accessions were chosen for the DNA barcoding.

The youngest leaf tissue was collected from one or two plants of each accession: for the nine accessions for which the different classification keys used were consistent, two leaf samples (named “a” and “b”) were collected. For the remaining accessions the different classification keys gave conflicting indications, therefore only one leaf sample was collected in order to make a secondary classification based on the DNA barcoding.

2.2.5.1 gDNA extraction

Genomic DNA (gDNA) was extracted from plants of the untreated control used in the preliminary screening following the protocol developed by Doyle & Doyle (1987) with some modifications. One leaf tissue was grinded with liquid nitrogen using a mortar and a pestle, the grinded material was transferred into 1.5 mL collection tubes, before tissues thawed and 600 µL

of CTAB buffer [20 g citrimonium bromide (CTAB), 200 mL Tris-HCl 1M (pH 7.5) final concentration 200 mM, 40 mL ethylenediaminetetraacetic acid (EDTA) pH 8 final concentration 20 mM, 81.8 g NaCl] pre-heated at 60 °C were added. Tubes were incubated for 30 min at 60 °C. 600 µL of chloroform-isoamyl alcohol (24:1 V/V) were added to the mixture, in order to separate the nucleic acids from the other tissue components, tubes were mixed by inversion and centrifuged at 10,000 rpm for 15 min at room temperature (18-20 °C).

Surfactant was collected and transferred in a new 1.5 mL tube, 1.2 µL of RNAase (A) were added and tubes were incubated at 37 °C for 30 min. DNA was then precipitated adding 400 µL of cold isopropanol and centrifuged at 10,000 rpm at 4 °C for 20 min. Surfactant was discarded and DNA pellet was washed with 200 µL of ethanol 70% and centrifuged at 10,000 rpm at 4 °C for 5 min. Surfactant was again discarded and pellet was dried at room temperature to eliminate any ethanol residue. DNA was then suspended in 30 µL of double distilled water (ddH_2O).

Concentration and quality of DNA was determined using Nano Drop spectrophotometry (Applied Biosystems) evaluating the absorbance at the wavelengths of 260 nm, 280 nm, 320nm and the different ratios. Each sample was diluted with ddH_2O to reach the final concentration of 100 ng μL^{-1} and then stored at -20 °C for following uses.

2.2.5.2 cpDNA genes amplification and sequencing

Genes used for DNA barcoding approach were chosen on the base of literature: matK, rbcL supported by the intron psbA – trnH are considered as the most reliable DNA regions for this approach (CBOL Plant Working Group, 2009; Chase *et al.*, 2007; Hebert *et al.*, 2003; Hilu & Liang, 1997) in plant science. trnL was already used in *Echinochloa* spp. to discriminate among *E. crus-galli* and *E. oryzicola* in Japan (Yamaguchi *et al.*, 2005). ITS was chosen as "complementary" to the other genes (White *et al.* , 1990).

Finally four genes (matK, rbcL, ITS and rbcL) and one introns (psbA-trnH) of the cpDNA were taken into account and analyzed on the plants of the nine accession for which the different classification keys used were consistent.

The vouchered nucleotide sequences already available for any *Echinochloa* species in the main DNA sequences databases: i.e. GenBank nucleotide (www.ncbi.nlm.nih.gov) and Barcode of Life

Data Systems (Ratnasingham & Hebert, 2007; www.boldystem.org) were downloaded and aligned in order to design some primers for the amplification of the different DNA sequences. Many sequences were available for rbcL, matK and psbA-trnH and specific primers could be designed on conserved DNA regions. For ITS and trnL, some primers were already available in the literature: White *et al.* (1990) for ITS, Yamaguchi *et al.* (2005) and Drábková *et al.* (2006) for trnL (Tab. VI).

Alignment and primer design was performed with MEGA 6 software. Primers were then analyzed for complementarity, presence of hairpin and self-dimerization using OligoAnalyzer 3.1 (http://www.idtdna.com/calc/analyzer) and synthetized by Invitrogen.

Primer name	Primer sequence (5'-3')	Amplicon size (bp)	T_a (°C)	t_e (s)	Reference
psbA-trnH_F	GTA ATG CTC ACA ACT TCC CTC TA	592	58	40	HQ600068, FJ766205, KR048637 , KR048634
psbA-trnH_R	GCT GGA TAA GGG GCG GAT GTA				
matK_F1	AAT GGT GCC GAA CCT GTG GAA A	1257	56	80	KF010243, KF010243, KR058325, KC164269
matK_R3	ATG CAA CGA TTA GGT TCC GTA				
rbcL_F1	GCA GCA TTC CGA GTA ACT CCT CA	1093	60	80	KF163507, KF163509, KT365290, KR058324
rbcL_R2	TTG GTG GAG GAA CTT TAG GAC ATC				
ITS1	TCC GTA GGT GAA CCT GCG G	800	56	60	White *et al.*, 1990
ITS2	GCT GCG TTC TTC ATC GAT GC				
trnC	CGA AAT CGG TAG ACG CTA CG	600	57	60	Yamaguchi *et al.*, 2005
trnD	GGG GAT AGA GGG ACT TGA AC				

Tab. VI: list of primers used to amplify different DNA regions, their sequences, amplicon size produced, annealing temperature (T_a) and extension time (t_e) used in the PCR amplification process. In the last column the reference where the primers are present or some GenBank and BOLDsystem identification codes (vouchers) of sequences used for primers design were reported for example.

PCR amplification was conducted using the GoTaq® G2 Hot Start Polymerase (Promega) in a 25 µL final volume mixture including 5 µL 5x Go Colorless Taq Flexi Buffer, 2.5 µL $MgCL_2$ 25 mM, 0.5 µL dNTPs mix 10 mM, 1 µL of forward (F) and reverse (R) primer, 0.2 µL GoTaq® G2 Hot Start Polymerase and 100 ng gDNA.

PCR reaction was conducted in a T1 Thermocycler (Biometra) using the following program: DNA denaturation for 2 min at 95 °C, 35 cycles with 30 s at 95 °C, 30 s at the specific annealing temperature (T_a), and 72 °C for the specific extension time (t_e), a final extension step at 72 °C for 5 min. PCR products were analyzed in 1% agarose gel.

Amplicons obtained were purified using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel), quantified using the UVI-1D software (Uvitech, Cambridge) and sequenced using both F and R primers by BMR genomics (Padova, IT). Sequences were analyzed with Finch TV 1.4.0, consensus sequences were built using SeqMan software included in the package DNASTAR® and aligned to be compared with MEGA 6® Software. UPGMA dendrograms were built to clustering data.

Following the cooperation with the Meise Botanic garden (Meise, Belgium), 14 accessions of *E. crus-galli* and *E. muricata* from Belgium maize fields were included in the study. Phenotypic classification (Tab. VII) of these accessions was performed by Ivan Hoste and Philipp Verloove according to I. Hoste dichotomous key (2004). gDNA was extracted from two plants for each accession, matK, rbcL and psbA-trnH were amplified as described above, sequenced and compared both with sequences downloaded from GenBank and Boldsystem and the Italian accessions ones.

Herbarium I. Hoste	Location	Taxon	Details
17015	Bellem (Aalter)	*E. crus-galli*	Plant multistemmed, stems ascending
17016	Zomergem	*E. muricata* var. *microstachya*	Plant multistemmed, stems straight
17017	Zomergem,	*E. crus-galli*	Plant multistemmed, stems straight
17018	Merendree (Nevele)	*E. muricata* var. *wiegandii*	Plant multistemmed, stems straight
17019	Ursel (Knesselare)	*E. muricata* var. *microstachya*	Plant multistemmed, stems straight
17020	Knesselare, Berglanden	*E. crus-galli*	Plant(s) high and multistemmed, stems straight and up to 190 cm, inflorescences upright, lower branches of the inflorescence in whorls
17021	Knesselare, Driepikkel	*E. muricata* var. *microstachya*	Inflorescences deep purple; plants usually not with multiple stems; stems straight
17022	Aalter	*E. muricata* var. *wiegandii*	Plant multistemmed, stems straight
17023	Aalter	*E. crus-galli* (cf. var. praticola?)	Plant multistemmed, stems straight; inflorescences upright and protruding well above the uppermost leaf
17024	Aalter	*E. crus-galli*	Plant multistemmed, high (up to 200 cm), green, with no tinge of purple; stems straight; inflorescences strongly curbed
17027	Nevele	*E. crus-galli*	Plant multistemmed, with straight stems and upright inflorescences; plant collected from the outer border of the maizefield; similar looking plants growing a little further away from the border of the field seem to differ only in being usually single-stemmed
17028	Nevele	*E. crus-galli*	Rather small, multistemmed plant(s) with ascending stems, inflorescences very dark and somewhat curbed
17029	Ursel (Knesselare)	*E. crus-galli*	Rather small, multistemmed plant, inflorescences rather upright
17030	Aalter	*E. crus-galli*	Often small plant(s), [the collected specimen relatively large!], with dark and strongly curbed to drooping inflorescences

Tab. VII: Morphological classification performed on the 14 accessions of *E. crus-galli* and *E. muricata* collected in Flanders (Belgium) corn fields.

2.2.6 Specie specific PCR

Sequences obtained from matK analysis highlighted the presence of SNPs able to discriminate between different "white" *Echinochloa* species also providing the best match with phenotypic classification. Therefore, it was chosen for the set-up of a Specie-Specific (SS) – PCR protocol able to discriminate among three different *Echinochloa* species: *E. oryzicola*, *E. phyllopogon* and the "unclassified SE" *Echinochloa*.

Specie-specific (SS) forward primers were designed to match the two SNPs found. Different mismatches were inserted at the 4th and 3rd position from the 3'-end to improve specificity (Taylor, 1997; You *et al.*, 2008). The reverse primer SS-R was designated to be universal for each *Echinochloa* species (Tab. VIII).

Primer	Nucleotide position	Primer sequence (5'-3')
F1_ERE	141	TAT CCA TTT AGA AAT CCT G**A**T T
F3_ERE	141	TAT CCA TTT AGA AAT CCT **T**GTT
F4_ERE	141	TAT CCA TTT AGA AAT CCT G**T**TT
F1_SE	246	GTC TTA TTA CTT CAA TGA AA**G** CC
F3_SE	246	GTC TTA TTA CTT CAA TGA A**C**T CC
F4_SE	246	GTC TTA TTA CTT CAA TGA AA**C** CC
SS_R	368	CTT CTT GCT TAC GAT TAA CAT C

Tab. VIII: SS-PCR primers sequences. In bold the mismatches inserted to improve the specificity.

The SS specific F_ERE and F_SE forward primers have been used in combination in the same PCR reaction with the common reverse primer (SS-R) to obtain an amplicon of 248 bp for the samples attributable to *E. oryzicola* species, or an amplicon of 144 bp for the samples attributable to the "unclassified SE" *Echinochloa* or no amplicons for the samples attributable to *E. phyllopogon* (Fig. 16).

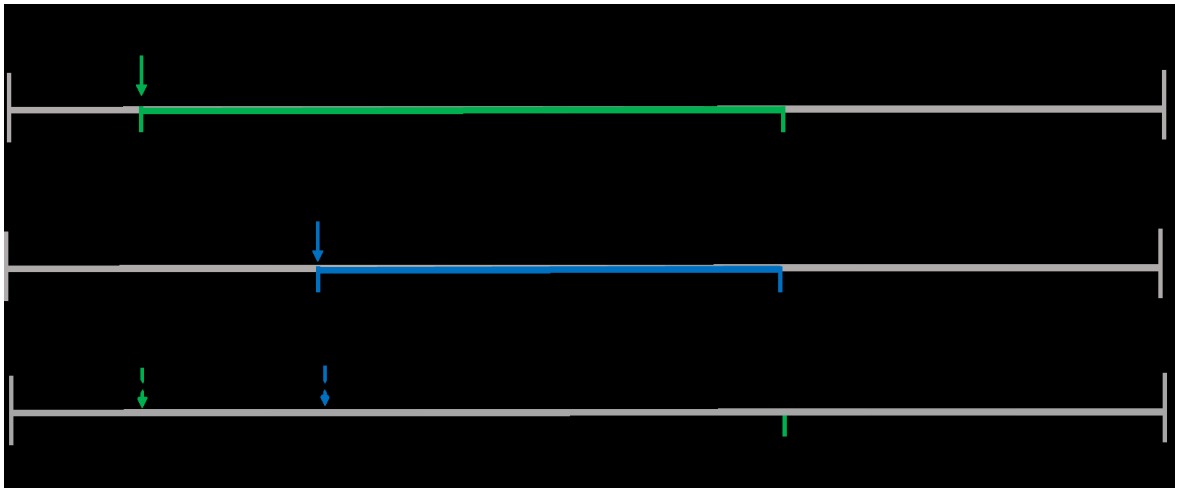


Fig. 16: Scheme of the SS-PCR showing position of the designed primer for species discrimination and the length of the amplicon expected.

PCR amplification was conducted using the Go Taq® G2 Hot Start DNA Polymerase (Promega) in a final volume of 25 µL including 5 µL of 5X Colorless Go Taq® Flexi Buffer, 1.5 µL $MgCL_2$ 25 mM, 0.5 µL of both forward primers, 1 µL of SS-R primer, 0.5 µL dNTPs 10 mM, 50 ng gDNA.

PCR reaction was performed in the T1 Thermocycler (Biometra) following the program: 2 min at 95°C for DNA denaturation, 35 cycles with 30 s at 95 °C, 30 s at 52 °C and 30 s at 72 °C, and a final extension time of 5 min at 72 °C. PCR products were analyzed in 1% agarose gel.

2.2.7 Herbicide efficacy on different *Echinochloa* species

The last step of the analyses was to verify whether different *Echinochloa* species had a different response to the most used herbicides in Italian rice fields. For this reason two dose-response (DR) pot experiments, following a greenhouse probe, were carried out in 2017 and 2018. The first was done in summer 2017 in semi-controlled conditions (outodoor), the second was done in spring 2018 in the IBAF-CNR greenhouse. The decision to perform two different studies at two different conditions, was driven by the necessity to understand the different behavior of *Echinochloa* spp. toward herbicides in different conditions.

Three herbicides with different SoA were included in the experiment: penoxsulam, cyhalofop-butyl (two of the most used herbicides in rice in Italy) and a new herbicide, with an alternative SoA, developed by Dow Agrosciencies: florpyrauxifen-benzyl (Rinskor® Active) (Tab. IX).

Commercial name	Active ingredient ($g\ L^{-1}$)	Field dose (1x) ($mL\ ha^{-1}$)	Chemical family	SoA	HRAC group
Viper®	Penoxsulam (20)	2000	Triazolopirimidines	ALS inhibitor	B
Clincher One®	cyhalofop-butyl (200)	1500	Arilossifenossipropionate	ACCase inhibitor	A
Rinskor® Active	florpyrauxifen - benzyl (25)	1200	Arilpicolinates	Synthetic Auxin	O

Tab. IX: Details of herbicides used in preliminary screening and dose-response experiments.

2.2.7.1 Greenhouse preliminary screening

A preliminary screening was conducted in greenhouse conditions in spring 2017 to understand which populations had to be included in the DR experiments and choose the correct range of doses for the three herbicides. All the 10 reproduced accessions were included in the experiment, together with *E. crus-galli* 15-12 and 15-9.

Seeds were scarified and germinated as described in section 2.2.2. Seedlings were transplanted in pots, each one containing six plants.

The experimental design was a randomized complete block with three replicates, each replicate composed by a single pot.

It was decided to try different doses for the three active ingredients to have a preliminary understand of each herbicide performance: both cyhalofop–butyl and florpyrauxifen–benzyl were used at 1/4x, 1/2x and 1x of the field dose. As penoxsulam have already been tested twice on these accessions, the performance of 1x dose (40 g a.s. ha^{-1}) was already known. This dose was then excluded from the analyses to focus on the behavior of lower doses: i.e. 1/8x, 1/4x and 1/2x.

Plants were sprayed when they have reached 2-3 leaves stage (BBCH 12-13, Hess *et al.*, 1997). For each accession in the study an untreated control was included.

Plant survival and fresh weight were recorded 4 WAT. Plants were recorded as dead when they show no active growth independently from size and color. Results of both survival and fresh weight were expressed as percentage in comparison with the untreated control.

2.2.7.2 DR experiments design

On the base of the probe experiment and of both genetic and morphologic classification, nine *Echinochloa* spp. accessions were chosen for the two dose-response experiments.

In both experiments the same herbicides were used: penoxsulam, cyhalofop-butyl and florpyrauxifen-benzyl. The experimental design was a randomized complete block with three replicates with six plants each.

Scarification, germination and herbicide treatments were performed as explained in the section 2.2.2. After germination, seedlings were placed outside in semi-controlled environment in 2017, while in greenhouse conditions in 2018 experiment. 24h before herbicide application, pots were irrigated to reach soil maximum capacity. Spraying was carried out when leaves were dry.

Herbicides were applied at eight geometrically progressive doses: 2017 experiment doses were chosen on the base of probe screening results, while those for 2018 test were chosen on the base of 2017 results (Tab. X).

Experiment	herbicide	1/32x	1/16x	1/8x	1/6x	1/4x	1/2x	1x	2x	4x
Outdoor 2017	penoxsulam	X	X	X		X	X	X	X	X
	cyhalofop butyl	X	X	X	X	X	X	X	X	
	florpyrauxifen - benzyl	X	X	X	X	X	X	X	X	
Greenhouse 2018	penoxsulam	X	X	X		X	X	X	X	X
	cyhalofop butyl	X	X	X		X	X	X	X	X
	florpyrauxifen – benzyl	X	X	X		X	X	X	X	X

Tab. X : range of doses of the herbicides used in the outdoor experiment in 2017 and greenhouse experiment in 2018.

Plant survival and fresh weight were recorded 4 WAT for cyhalofop and penoxsulam and 5 WAT for florpyrauxifen-benzyl.

2.2.7.3 Statistical analyses

Plant survival and fresh weight were calculated as percentage of the untreated control and SE were calculated as mean of the three replicate for each herbicide dose considered. It was performed a non-linear regression based on the log-logistic equation (Seefeldt *et al.*, 1995) to fit the data:

$$Y = C + \frac{(D - C)}{1 + (\frac{x}{ED50})^b}$$

Where Y is the value of survival or fresh weight, C and D are respectively the lower and upper asymptote at the highest and lower dose, ED_{50} (or GR_{50}) is the dose giving 50% of response, x is the herbicide dose and b is the slope of the curve.

The higher asymptote of the regression curve was constrained to 100, 100% of plant survival and fresh weight is in fact the value corresponding to the untreated control. The lower asymptote was left not constrained, it was constrained to 0 only when it resulted negative.

The data of both plant survival and fresh weight were first analyzed separately as single curve to estimate the parameters for each herbicide and accession. For the 2018 experiment, accession belonging the same species were regressed together first: the most complex model (i.e. the one with no common parameters) was compared with progressively simplified models that have common parameters among curves. The lack-of-fit F test was performed at each step, stopping when a significant lack of fit occurred (Onofri & Pannacci, 2011). This procedure is performed to understand whether different curves have equal ED_{50} (or GR_{50}). In the latter case, only this parameter is used to explain the response of two or more accessions to a single herbicide. This type of analysis was not performed for summer 2017 trial, due to extreme variability of the results.

All analyses were performed using the macro BIOASSAY® (Onofri, 2004) running in Windows Excel® environment.

Chapter III

RESULTS and DISCUSSION

3.1 Epidemiology of herbicide resistance in rice in Italy

3.1.1 Database analyses

The initial database includes 232 municipalities with an average area of 19 km^2.

Lombardy's territory is more fragmented: i.e. it contains more municipalities with a smaller size compared to Piedmont (16 km^2 vs 22 km^2).

The largest rice area is in Pavia province with about 80,000 ha cropped to rice (54% of the total UAA) and Vercelli with 67,000 ha (66% if the UAA) (Tab. XI).

Province	Municipalities (N)	Area (km^2)	UAA (ha)	Rice area (ha)	Rice (% of UAA)
MILANO	28	378	45,745	27,339	59.8
PAVIA	102	1,707	148,348	80,517	54.3
ALESSANDRIA	11	244	15,774	10,723	68.0
BIELLA	9	157	22,438	13,860	61.8
NOVARA	33	718	51,088	31,781	62.2
VERCELLI	49	1,132	70,852	46,948	66.3

Tab. XI: number of municipalities per province with the total area covered, the total UAA and the area cropped to rice expressed both in ha and as percentage of UAA.

Of the 232 municipalities included in the dataset, in 115 (49.6%) of them at least one resistant population was found (GIRE, 2018). Considering only *Echinochloa* spp. there were 78 municipalities (33.6%) where resistance had previously been confirmed.

While over 60% of Piedmont municipalities has developed at least one resistant population, only 40% of the Lombardy municipalities did. Though widespread it appears that resistance is not evenly distributed in the study area.

By using the dynamic mapping system available on the Italian Herbicide Resistance Working Group website (GIRE, 2018), it appeared that herbicide resistance in rice did not evolve evenly within the study area as it had not been reported in two relatively large pockets (Fig. 17, areas S and L) regardless of weed species or type of resistance. A non-homogeneous distribution of resistant populations was made clear both with all weeds (Fig. 17A) and with *Echinochloa* spp. only (Fig. 17B).

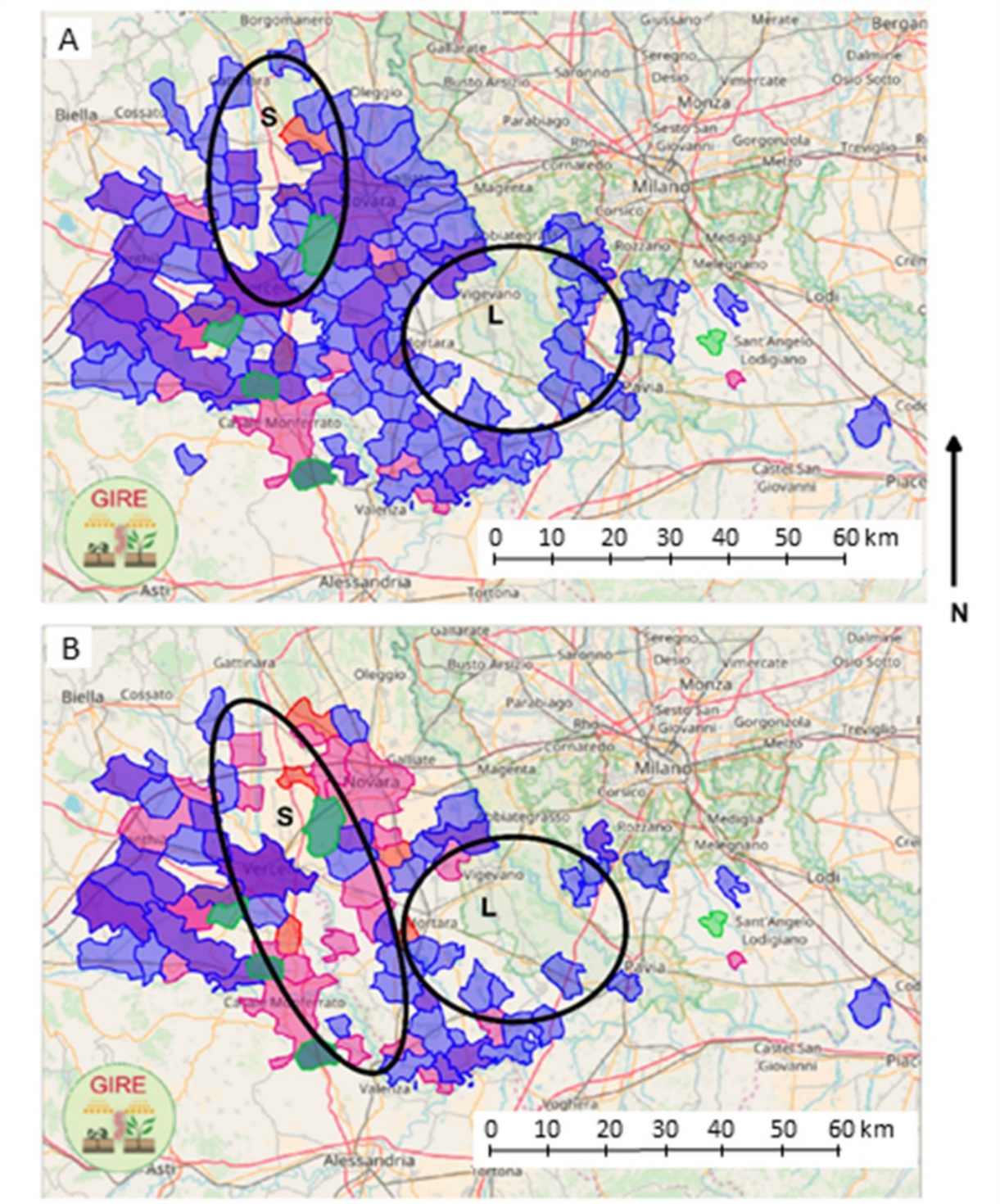


Fig. 17: Map of all resistant cases (A) and *Echinochloa* spp. only resistant cases (B) recorded in the rice area: two "resistance free" areas are evident: one (L) in Pavia province and a second (S) along the Sesia river. A municipality changes color when at least one population has been confirmed resistant in its territory. Different colors refer to resistance to herbicides with different site of action. Available online: www.resistenzaerbicidi.it (accessed on: 21st July 2018).

Area S, covers approximately 330 km^2 and is located in the North-West of the study area along the Sesia, a river Po tributary, which flows on the border between Piedmont and Lombardy. Area L is in Pavia province, specifically in the center of an area called Lomellina and covers about 420 km^2.

3.1.2 Discriminant analysis and logistic regression

Descriptive maps were produced with QGIS software, showing the distribution and frequency of percentage of clay (PC), percentage of rotation rate (RR) and percentage of water seeding (WS) in each municipality in the study, also providing graphic support to the two statistical tests – namely discriminant and logistic regression – used for the correlation analyses (Fig. 18 a, b and c).

Stepwise discriminant analysis including all weeds eliminated PS at the third step of the analysis, while for *Echinochloa* spp. only WS was retained after the first step. Discriminant analysis was able to correctly group 65.2% of "resistant" municipalities and 70.9% of "non-resistant" ones for all weeds, 64.1% and 65.6% for *Echinochloa* spp., respectively.

Stepwise backward logistic regression performed on all weeds pooled together showed that WS, RR and PC are highly correlated with resistance presence ($p<0.001$, $p=0.003$ and $p=0.009$, respectively), whereas the correlation with PS resulted as not significant. For *Echinochloa* spp. alone, only WS resulted as significant ($p<0.001$). RR and PC were negatively correlated with resistance while WS was positively correlated with it (see also Fig. 18 a, b and c).

When all weeds were analyzed together RR and PC were also significant and this is likely a consequence of using a larger dataset. It is clear that the three predictors are somehow inter-dependent, i.e. WS is less frequent in areas where PC is lower and RR is higher. The two virtually resistant-free areas, S and, especially, L (Fig. 17), display this pattern (Fig. 18a, b and c). Where WS is practiced, weed control strategies are generally based on fewer herbicide modes of action and rely more on ALS inhibitors (Ferrero *et al.*, 2008), thus increasing the herbicide selection pressure.

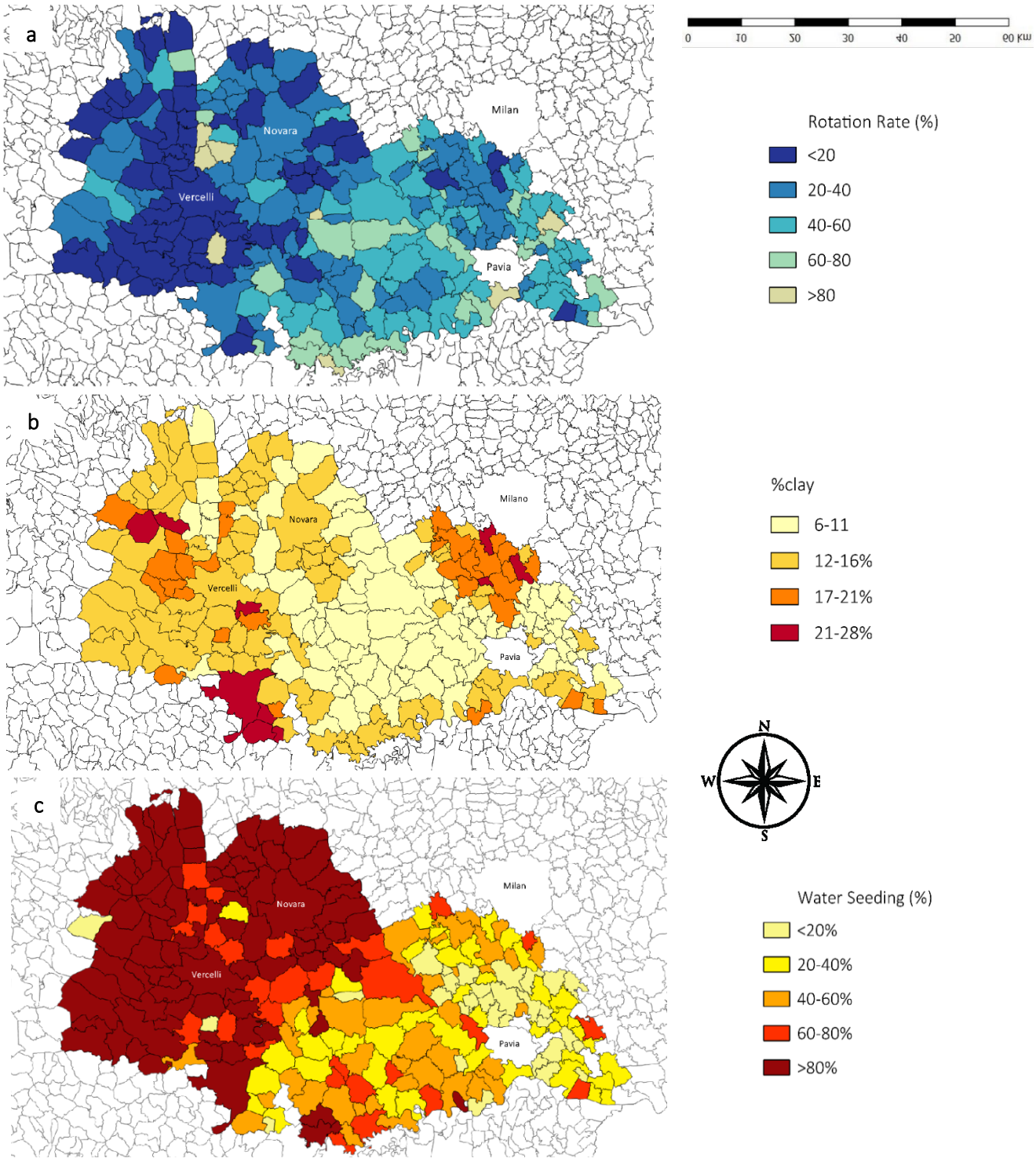


Fig. 18: Diffusion of RR (a), PS (b) and WS (c) in the area of the study. Results are expressed as classes, representing the percentage of each factor in each municipality.

Municipalities with resistant cases have a percentage of WS sensibly higher than the others (41.5% vs 29.4%) and lower rates of RR (44.3% of 64.1%) (Tab. XII).

Resistance presence	RR (%)	WS (%)	PC (%)
0	41.5	44.3	13.2
1	29.4	64.1	12.6

Tab. XII: averages of water seeding (%WS), rotation rate (%RR) and percentage of clay (%PC) in municipalities with at least one case of resistance (1) vs no resistance cases (0).

These data are also supported by the descriptive maps produced in QGIS (Fig. 18): WS and low RR are widespread in the North-West of the area where water is continuously available and rotation is difficult (Fig. 18a and 18c): e.g. in the Vercelli area soils are frequently waterlogged and the shift to other crops is almost impossible, farmers agree that they are "obliged to crop rice". On the other hand in the South East area, i.e. in Milan and Pavia province, rotation is more common: here water is not continually supplied in most cases and farmers are obliged to rotate rice with corn or soybean to deal with the lack of water.

PC map is not that informative (Fig. 18b): in the North-West are concentrated soils with higher quantity of clay, but most soils contain less than 16% of clay, so this difference can be considered negligible.

Both statistical analyses highlighted the strict relation between the presence of resistance and the more traditional system of seeding rice in flooded paddies. This is reinforced by the observation that the five weed species that evolved herbicide resistant populations are well adapted to humid and flooded conditions (Osuna *et al.*, 2002; Viggiani & Tabacchi, 2017).

Where WS is common, weed control strategies are generally based on fewer herbicide modes of action and rely more on ALS inhibitors (Ferrero *et al.*, 2008), thus increasing the herbicide selection pressure.

WS resulted the only factors significantly correlated with *Echinochloa* spp. resistance evolution. This is probably due to the shift in *Echinochloa* spp. species when seeding technique changes from water seeding to dry seeding. *Echinochloa* spp. changes from the "white" types to *Echinochloa crus-galli*, which is easier to manage because it has a less extended germination and a slower resistance evolution when compared to the other "white" species such as *E. oryzicola*.

To our knowledge, this is the first study that determines the degree of correlation between herbicide resistance and a few important predictors at such a large scale (about 200,000 ha).

3.1.3 Neural network analyses

The maps generated by the GIRE website simply give a snapshot of diffusion of resistance based on complaint monitoring. Instead, we aimed to estimate the risk of resistance evolution in the various municipalities through an innovate approach such as neural network, and generate a resistance risk map (Fig. 19a and 19b).

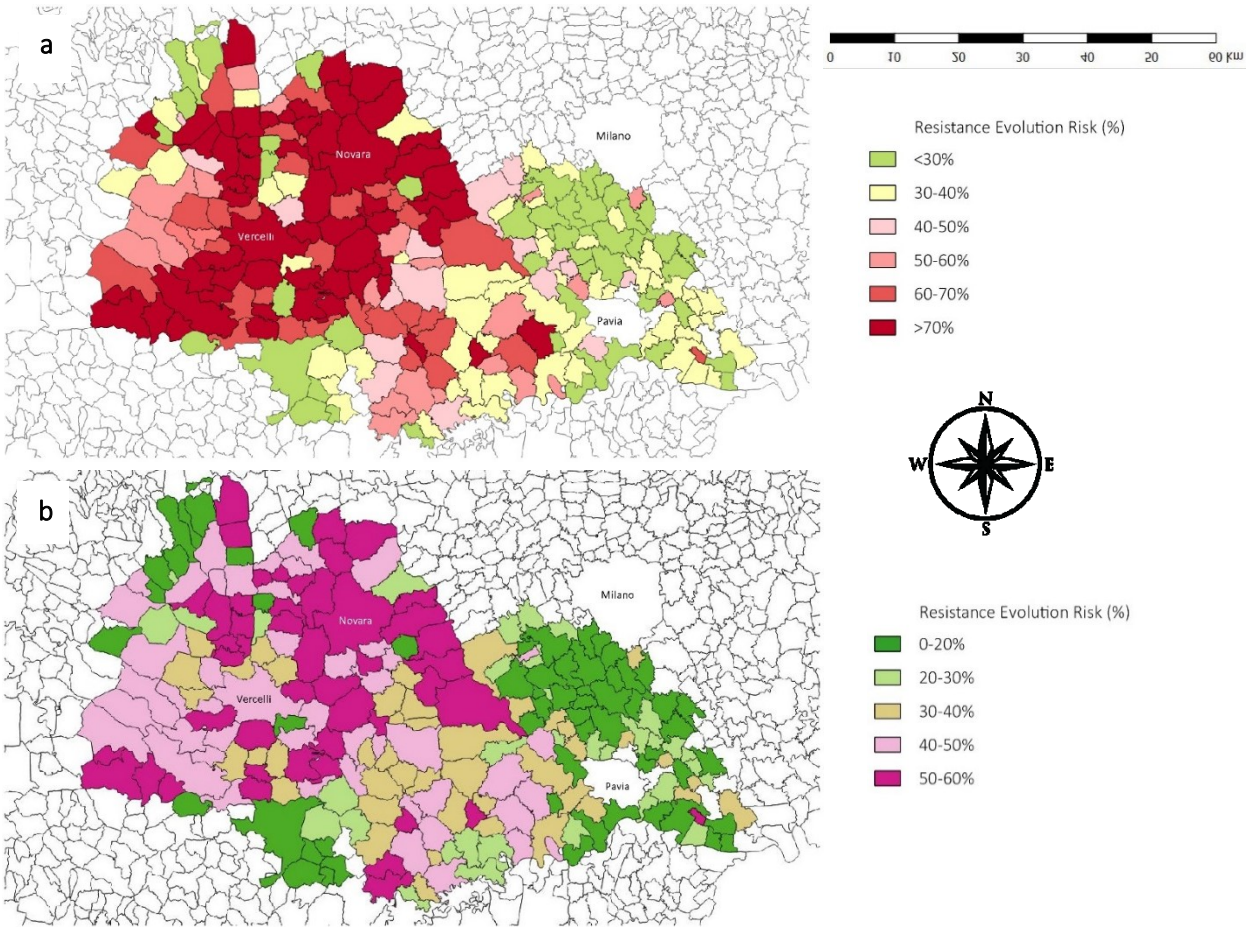


Fig. 19: RER (Resistance Evolution Risk) maps create on the results of the Neural Network Analyses for all weeds (a) and for *Echinochloa* spp. only (b). Results are expressed as % of RER split into classes.

Neural network analyses was performed twice, once for all weeds pooled together and then for *Echinochloa* spp. alone.

The neural network analysis confirmed that WS, PC and RR are good predictors of resistance, with a normalized importance of 100%, 95% and 75%, respectively. When all "resistant" weeds were considered, the analysis correctly predicted 63.8% of "resistant" municipalities and 68.4% of "non-resistant" ones during the training step, while the testing step correctly predicted 86.5% and 61.1%, respectively.

Neural network analyses performed considering only the *Echinochloa* spp. resistance cases correctly predict only about 30% of resistant cases, specifically 32.2% in the training step and 31.6% in the testing step. This suggests that when the number of resistant cases significantly decreases, this analyses loses part of its predictive ability.

Weeds are not equally distributed in the rice area, so different variables acts differently and have different importance in different analyses.

In 48% of municipalities the probability of resistance evolution is higher than 50%. Resistance risk is higher in the central-western part of the study area (Piedmont region, risk >60% in 64% of municipalities) than in the central-eastern area (Lombardy region, risk >60% in 21% of municipalities) (Tab. XIII).

RER	Municipalities (%)	Piedmont (%)	Lombardy (%)
< 0.3	19	8	26
0.3 - 0.39	26	17	32
0.4 - 0.49	7	3	10
0.5 - 0.59	9	7	11
0.6 - 0.69	13	18	9
> 0.7	26	46	12
Total	100	100	100

Tab. XIII: partition of municipalities respect to the resistance evolution risk obtained with neural network analyses, the first column displays results independently from the region, while the following two the results of municipalities split per region.

It is worth mentioning that in the 60 municipalities where the risk is higher than 70%, the average WS and RR are about 88% and 16%, respectively (Tab XIV).

RER (%)	Municipalities (%)	PC (%)	WS (%)	RR (%)
< 30	43	16.2	23.0	48.2
30 - 40	60	12.3	35.6	45.1
40 - 50	17	10.8	41.4	44.1
50 - 60	22	11.7	62.2	40.9
60 - 70	30	12.9	73.0	26.6
> 70	60	12.1	87.8	16.0

Tab. XIV: RER in comparison with the number of municipalities per interval and relative rates of PC, WS and RR.

In general resistance evolution risk is lower when *Echinochloa* spp. alone is considered: it ranges from 9 to 60%, while in the previous analyses ranged from 20 to 80% (data not shown).

Results of neural network analyses were analyzed for areas S and L in comparison with the data of Lombardy, Piedmont and the whole area in the study (Tab. XV).

Areas	RR (%)	WS (%)	PC (%)	RER (%)
Lomellina (L)	57.2	44.5	10.4	42.9
Sesia (S)	38.1	66.9	13.5	52.2
Piedmont	22.3	72.8	14.2	59.5
Lombardy	45.8	39.4	11.9	42.9
Whole study area	34.1	56.1	13.1	51.2

Tab. XV: RER in comparison with the values of %RR, %WS and %PC in the different areas of the study: areas S and L, Piedmont, Lombardy and the whole area included in this research

The two regions show very different values of water seeding and rotation rate.

In area L rotation rate is higher than those of the two regions and equal to 52.2%. In area S this value is equal to 38.1%, higher than that of Piedmont, lower than that of Lombardy.

Water seeding rate in area L is lower than the general average and rotation rate is higher. In area S rotation rate is higher than that of Piedmont, water seeding rate is higher than the average of the whole area in the study, but lower than Piedmont.

Comparison between the maps of WS, RR and PC and the resistance evolution risk maps (Fig. 19) highlights again that the traditional rice cropping systems based on water-seeding and lack of rotation (Ferrero *et al.*, 2008) are at higher risk. Therefore, contrary to what was presented in a recent article on a different cropping system (Hicks *et al.*, 2018), we demonstrate that in areas

where a combination of management strategies increasing system diversity are used, the evolution of resistance is slower.

3.1.4 *Echinochloa* spp. resistance screening

Levene's test performed on A (autumn) and S (spring) tests confirmed homogeneity of the variances both for Fresh Weight (FW) and Survival (S) with p values equal to 0.22 and 0.74 respectively. Data from the two experiments were therefore pooled and analyzed together.

Data were not pooled for profoxydim dose 3x: this dose was in fact used only in experiment A and already at 1x provided complete control for all populations with a negligible final FW (data not shown). For this reason dose 3x was eliminated in the following experiment.

The results of the screenings done on *Echinochloa* spp. populations sampled randomly in areas L and S (Fig. 20) disprove the initial hypothesis of lack of resistance in those areas. Only four accessions (300, 303, 307 and 310) resulted as still being susceptible to all four herbicides, all of them coming from area L.

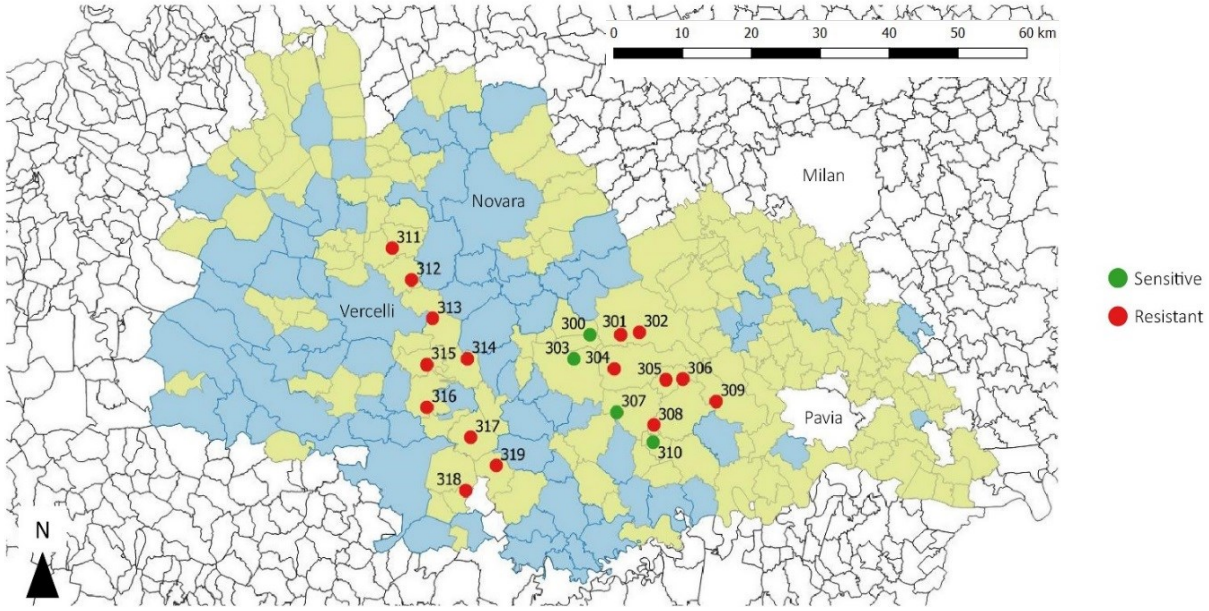


Fig. 20: map of screening results: 4 populations resulted sensitive to both ALS- and ACCase-inhibiting herbicides used in the analyses, 16 resulted resistant.

In particular only #300 and #310 resulted S for all herbicides, while #303 and #307 showed a partial resistance to ALS inhibitors when applied at field dose (1x). Pop #303 showed a plant

survival equal to 19.4% and 16.7% for imazamox and penoxsulam respectively and pop #307 showed 15.7% of plant survival to imazamox only. SE in all of the three cases were high in comparison with the result. On the other hand FW was very low, suggesting that although alive, these plants would not be competitive on fields. For this both populations #303 and #307 were considered S and not SR.

Results of the screening are reported in Tab. XVI and XVII.

Pop. code	Infestation density	cyhalofop-butyl		profoxydim	
		% plant survival (SE)	% fresh weight (SE)	% plant survival (SE)	% fresh weight (SE)
07-16L	-	2.4 (2.38)	1.9 (0.74)	0 (0)	1.6 (0.6)
300	Low	0 (0)	1.4 (0.64)	0 (0)	1.2 (0.5)
301	Low	0 (0)	3.7 (18)	0 (0)	2.2 (0.3)
302	Low	0 (0)	4 (0.94)	0 (0)	2.1 (0.4)
303	High	7.9 (3.56)	8.9 (3.44)	0 (0)	0.9 (0.1)
304	Medium	5.2 (3.27)	3.9 (2.11)	0 (0)	1 (0.2)
305	High	2.8 (2.78)	6.1 (1.88)	0 (0)	2.4 (0.8)
306	High	2.8 (2.78)	2.2 (0.83)	0 (0)	0.7 (0.1)
307	Medium	12 (11.9)	5.2 (1.24)	0 (0)	1.2 (0.3)
308	Medium	11 (5.3)	14 (3.1)	4.8 (4.8)	1.9 (0.3)
309	Medium	0 (0)	1.4 (0.49)	0 (0)	0.4 (0.1)
310	Low	5.6 (3.51)	3.4 (1.16)	0 (0)	2.1 (0.6)
311	Low	9.4 (4.25)	2.8 (1.35)	0 (0)	0.5 (0.2)
312	Low	3.3 (3.33)	3.6 (38)	0 (0)	0.5 (0.1)
313	Low	35 (15.8)	34 (15.6)	0 (0)	1.9 (0.7)
314	Medium	6.1 (3.89)	9.9 (1.69)	0 (0)	2.2 (0.7)
315	Low	44 (9.5)	54 (14)	0 (0)	3.8 (0.7)
316	Low	3.3 (3.33)	3.9 (3.21)	0 (0)	0.7 (0.2)
317	Low	5.6 (5.56)	4.2 (3.67)	5.6 (5.6)	2.7 (2.1)
318	Very Low	0 (0)	1.6 (0.32)	0 (0)	0.6 (0.2)
319	Low	0 (0)	3.6 (0.78)	0 (0)	1.9 (0.2)

Tab. XVI: Plant survival and fresh weight calculated as percentage of the untreated control for the recommended field dose (1x) of the most used ACCase inhibitors in rice cyhalofop-butyl and profoxydim. The data are means of autumn and spring experiments; standard error (SE) is given in brackets

Tab. XVII: Plant survival and fresh weight calculated as percentage of the untreated control for the recommended field dose (1x) and three times that (3x) of the most used ALS inhibitors in rice imazamox and penoxulam. The data are means of A and S experiments; standard error (SE) is given in brackets.

Pop. Code	Infestation density	Imazamox				penoxsulam			
		1x dose		3x dose		1x dose		3x dose	
		% plant survival (SE)	% fresh weight (SE)	% plant survival (SE)	% fresh weight (SE)	% plant survival (SE)	% fresh weight (SE)	% plant survival (SE)	% fresh weight (SE)
07-16L	-	0 (0)	2.2 (0.5)	0 (0)	2.2 (0.6)	0 (0)	2.6 (0.7)	0 (0)	2.4 (1.1)
300	Low	9 (6.6)	4.9 (2)	0 (0)	1.7 (0.4)	0 (0)	2.7 (1)	0 (0)	2.1 (0.6)
301	Low	100 (0)	94.7 (4.7)	100 (0)	90.9 (5.8)	100 (0)	91.3 (5.5)	97.1 (2.9)	76.8 (14.2)
302	Low	97.6 (2.2)	88.2 (7.1)	97.2 (2.8)	82.5 (8.9)	94.8 (3.3)	67.5 (9.9)	76.7 (19.4)	44.8 (2.1)
303	High	19.4 (10.10)	5.9 (3.2)	0 (0)	2.9 (1.5)	16.7 (13.6)	5.6 (1.4)	0 (0)	1.9 (0.3)
304	Medium	97.2 (2.2)	100 (0)	100 (0)	100 (0)	100 (0)	98.4 (1.6)	100 (0)	98 (2)
305	High	82.1 (9.9)	78.4 (7.3)	55.2 (10.5)	72.7 (12.8)	91 (4.1)	82.4 (6)	67.1 (7.7)	63.8 (11.6)
306	High	100 (0)	94 (2.9)	88.7 (5.6)	90.5 (5.8)	97.6 (2.4)	96.7 (2.7)	84.8 (4.6)	83.6 (10.5)
307	Medium	15.2 (6.6)	7.3 (1.3)	0 (0)	2.5 (0.7)	0 (0)	2.8 (0.6)	0 (0)	3.1 (0.8)
308	Medium	32.5 (10.10)	11.5 (3.9)	0 (0)	2.3 (0.2)	76.8 (8.1)	60.3 (11.4)	55.2 (12.7)	20.8 (5.6)
309	Medium	100 (0)	75.2 (12.7)	100 (0)	87.1 (10.4)	100 (0)	94.2 (5.8)	97.1 (2.9)	91 (9)
310	Low	9.9 (7.7)	6.3 (1.5)	0 (0)	3.8 (0.8)	2.8 (2.8)	4.1 (1)	0 (0)	2.9 (0.7)
311	Low	52.9 (2.2)	27.8 (5.8)	36.3 (4.5)	14 (4.4)	54.9 (3.6)	20.2 (2.3)	37.3 (8.9)	13.2 (4.6)
312	Low	100 (0)	67.6 (13.1)	100 (0)	32.5 (2.6)	100 (0)	39 (5.1)	80 (20)	22 (6.3)
313	Low	100 (0)	86.3 (10)	71.2 (6.5)	56.4 (5.4)	100 (0)	79.1 (8)	95 (5)	70.6 (9.4)
314	Medium	100 (0)	100 (0)	100 (0)	95 (3.1)	100 (0)	97.1 (1.9)	96.7 (3.3)	87 (5.2)
315	Low	74.8 (5.5)	81.9 (8.1)	59.2 (7.2)	65.5 (11.8)	71.1 (9.5)	61.4 (11.3)	73.3 (13.5)	80.9 (10.3)
316	Low	71.1 (9.9)	72.1 (8.7)	66.7 (8.3)	43.7 (4.6)	68.4 (12.9)	37.5 (13.5)	66.5 (10.5)	24.9 (8.1)
317	Low	36 (10.10)	27.5 (10.3)	26.9 (9.9)	42.8 (11.6)	38.3 (5.1)	50.2 (13.8)	35 (10.5)	40.9 (14.9)
318	Very Low	100 (0)	81.3 (6.1)	100 (0)	45.3 (5.9)	100 (0)	75.2 (11)	75 (19.4)	25.9 (8.3)
319	Low	96.7 (3.3)	71.5 (11.8)	83.3 (16.7)	74.1 (14.8)	94.4 (5.6)	91.2 (5.4)	96.7 (3.3)	74.2 (12.9)

Sixteen populations proved to be resistant to at least one herbicide. Two accessions (313 and 315) were multiple resistant to both ALS and ACCase inhibitors: multiple resistance was first reported in Italy in 2012 and - although less common than ALS resistance only - its frequency has increased since then (source: GIRE). The efficacy of penoxsulam was similar to that recorded for imazamox, while the efficacy of profoxydim was higher than cyhalofop-butyl. ACCase inhibitors resistance was weaker than resistance to ALS inhibitors as only two populations were resistant to this SoA. The dose effect for ALS-inhibiting herbicides was low, indicating that a target-site related resistance mechanism may be involved (Powles & Yu, 2010). The results of the screening on randomly sampled populations proved that resistance is frequently present even in the two areas where it had not previously been recorded through complaint monitoring.

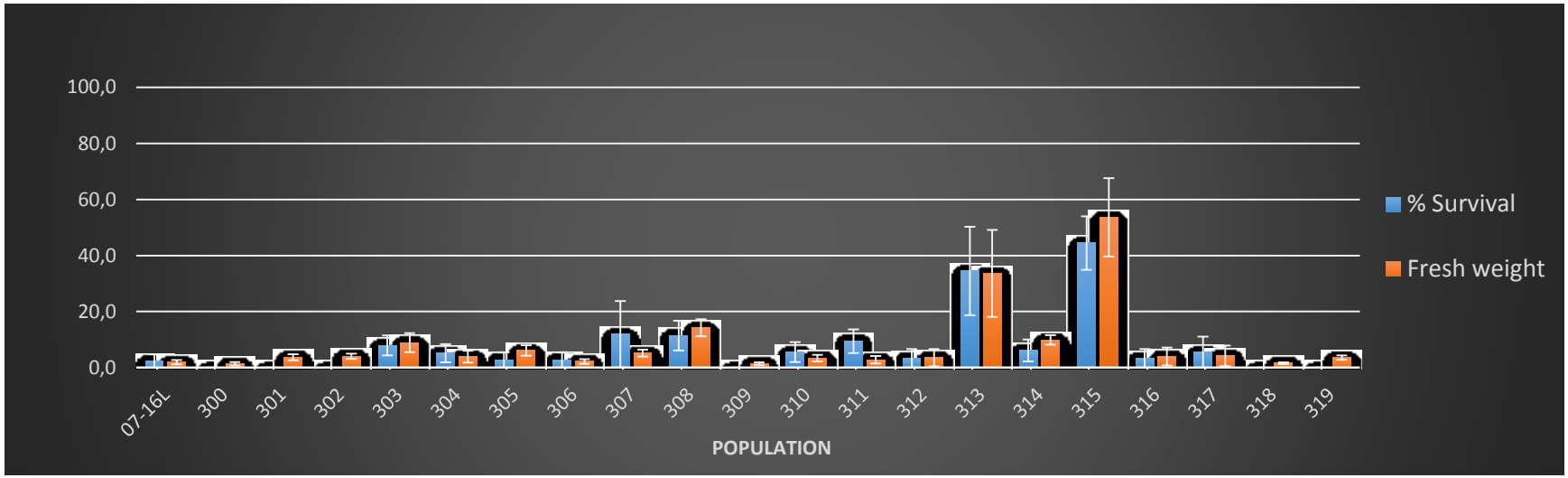
While profoxydim controlled all populations already at 1x dose, two populations resulted R to cyhalofop-butyl at the recommended field dose: #313 and #315. This difference in ACCase inhibitors susceptibility suggests the presence of a target-site resistance mechanism against FOP and not DIM, which is also the normally predominant type of ACCase inhibitors resistance (Devine & Shukla, 2000).

Analyzing the whole response of populations to cyhalofop-butyl, many populations showed S and FW values bigger than 5% with high standard errors, suggesting that there might be an initial herbicide resistance effect. For these accessions the variability is considered too high and the hypothesis of a partial herbicide resistance (SR) is refused: e.g. population #308 showed 11% of plant survival and 14% of biomass in comparison with the untreated check, but as SE for plant survival was 5.3 it was considered sensitive anyway (Fig. 21).

Sixteen populations showed very high levels of ALS inhibitors cross-resistance (Fig. 22). This pattern was expected: penoxsulam and imazamox have been widely used in rice fields since their launch around 2005, so their selective pressure on weeds is very high and this type of cross-resistance is common (Panozzo *et al.*, 2012).

The results of the screening disprove the initial hypothesis of lack of resistance in the S and L areas. Conversely, it is more frequent than what we initially thought. This results confirms those obtained by GIRE in the Italian rice fields, with ALS inhibitors resistance in *Echinochloa* spp. recording the biggest number of confirmed cases.

Fig. 21: results of screening test for ACCase inhibitors used in the experiment: cyhalofop-butyl (top) and profoxydim (bottom) at recommended field dose for susceptible check (07-16L) and randomly collected populations. Both plant survival and fresh weight are displayed. SE is expressed as vertical bars.



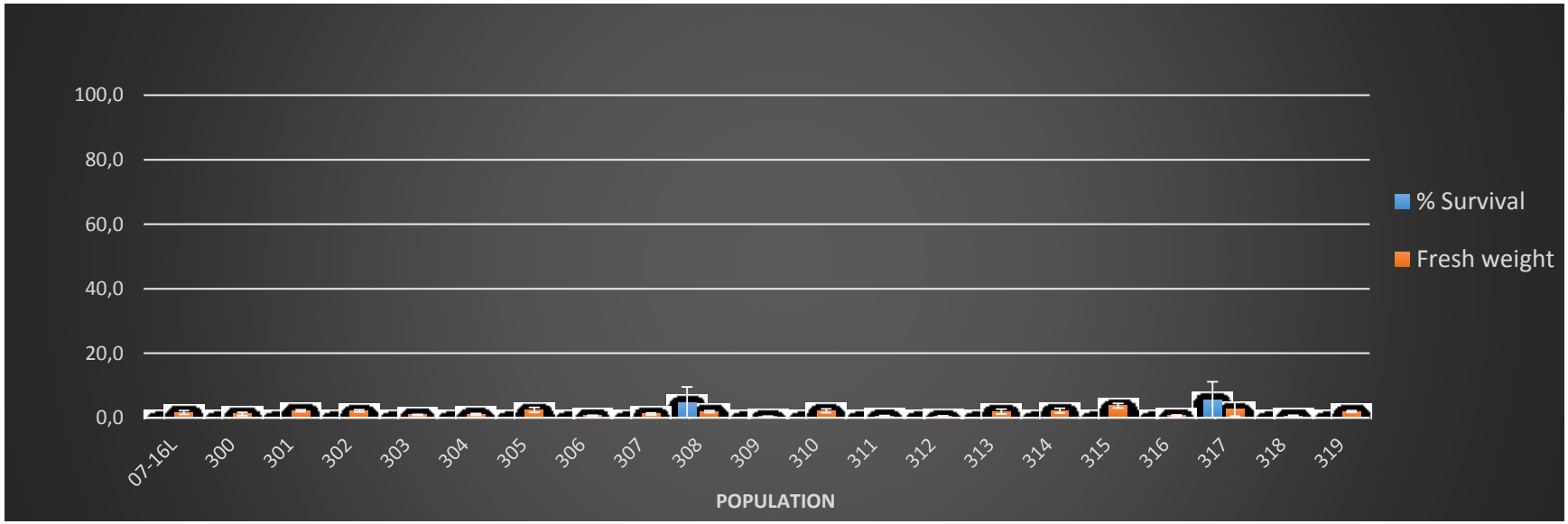
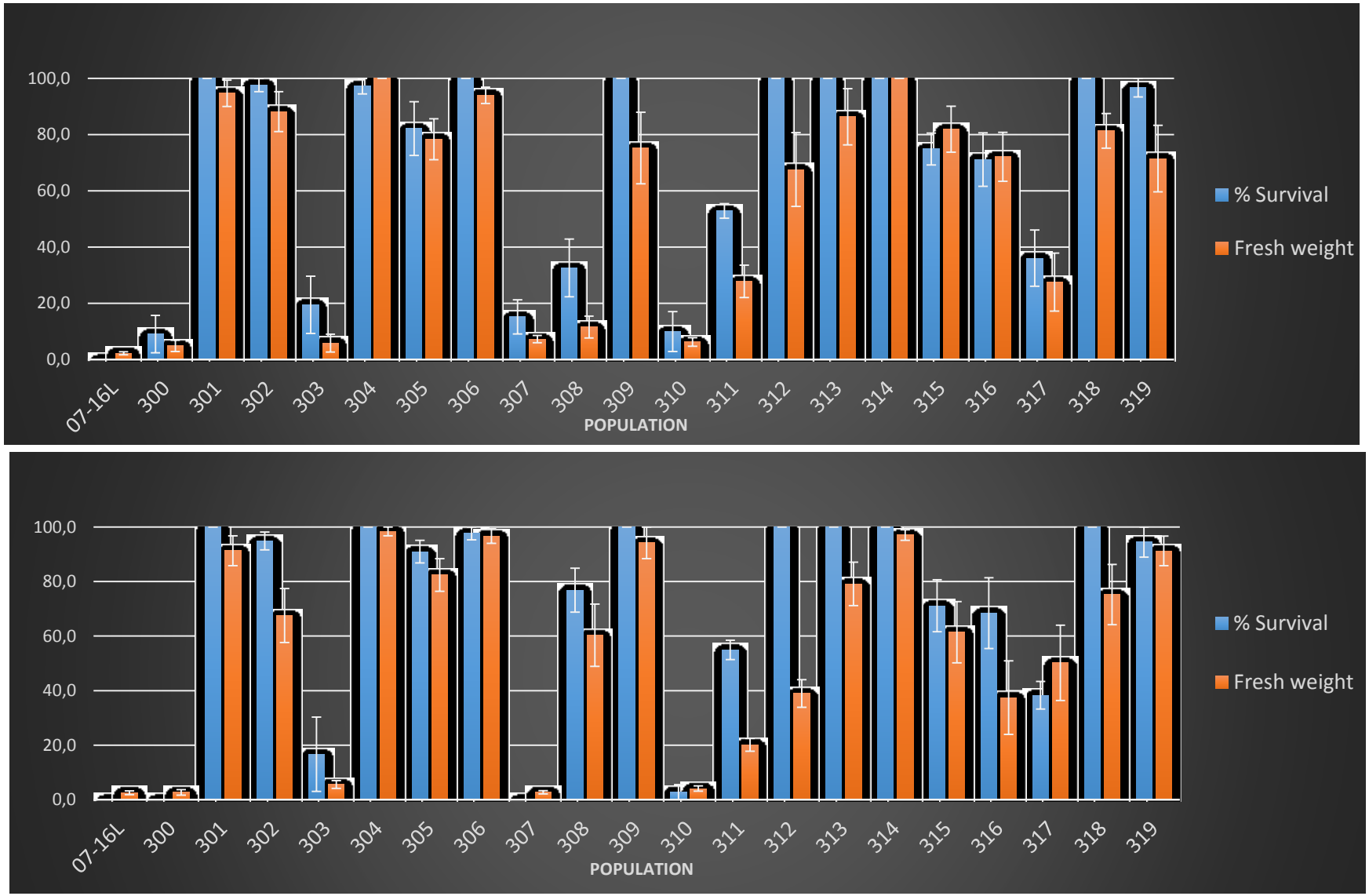


Fig. 22: results of screening test for ALS inhibitors used in the experiment: imazamox (top) and penoxsulam (bottom) at recommended field dose for susceptible check (07-16L) and randomly collected populations. Both plant survival and fresh weight are displayed. SE is expressed as vertical bars.



3.1.5 Association between resistance in collected populations and initial infestation density

Most of the infestation densities recorded during sampling were medium to low (between about 1 plant x 10 m^{-2} and about 1 plant x 100 m^{-2}), especially in area S. Only three infestation densities recorded during sampling are high (>1 plant x square meter) located in fields in the municipalities of Mortara (pop. #303) and Borgo San Siro (pop. #305 and #306) (area L). 16 infestation densities were medium to low (between about 1 plant x 10 m^{-2} and about 1 plant x 100 m^{-2}) and located especially in area S. One case only of very low infestation was recorded in the municipality of Ticineto (pop. 318) (Tab. XVIII).

Region	Area	Municipality	Pop name	Infestation density	Resistance level	
					ACCase	ALS
Lombardy	L	Cilavegna	300	Low	S	S
Lombardy	L	Vigevano	301	Low	S	RR
Lombardy	L	Vigevano	302	Low	S	RR
Lombardy	L	Mortara	303	High	S	S
Lombardy	L	Gambolò	304	Medium	S	RR
Lombardy	L	Borgo San Siro	305	High	S	RR
Lombardy	L	Borgo San Siro	306	High	S	RR
Lombardy	L	Tromello	307	Medium	S	S
Lombardy	L	Garlasco	308	Medium	S	RR
Lombardy	L	Garlasco	309	Medium	S	RR
Lombardy	L	Alagna	310	Low	S	S
Piedmont	S	Villata	311	Low	S	RR
Piedmont	S	Borgo Vercelli	312	Low	S	RR
Lombardy	S	Palestro	313	Low	R	RR
Lombardy	S	Rosasco	314	Medium	S	RR
Piedmont	S	Pezzana	315	Low	R	RR
Piedmont	S	Caresana	316	Low	S	RR
Lombardy	S	Candia Lomellina	317	Low	S	RR
Piedmont	S	Ticineto	318	Very Low	S	RR
Lombardy	S	Breme	319	Low	S	RR

Tab. XVIII: table showing, for each population sampled, infestation density visually assessed on field and resistance level assessed during screening.

The level of resistance recorded seems not to be connected with the initial infestation level. Most fields have low infestation level and very high levels of resistance. Two out of four sensitive populations, namely #303 and #307, come from fields which infestation was respectively "High" and "Medium" (Fig. 23).

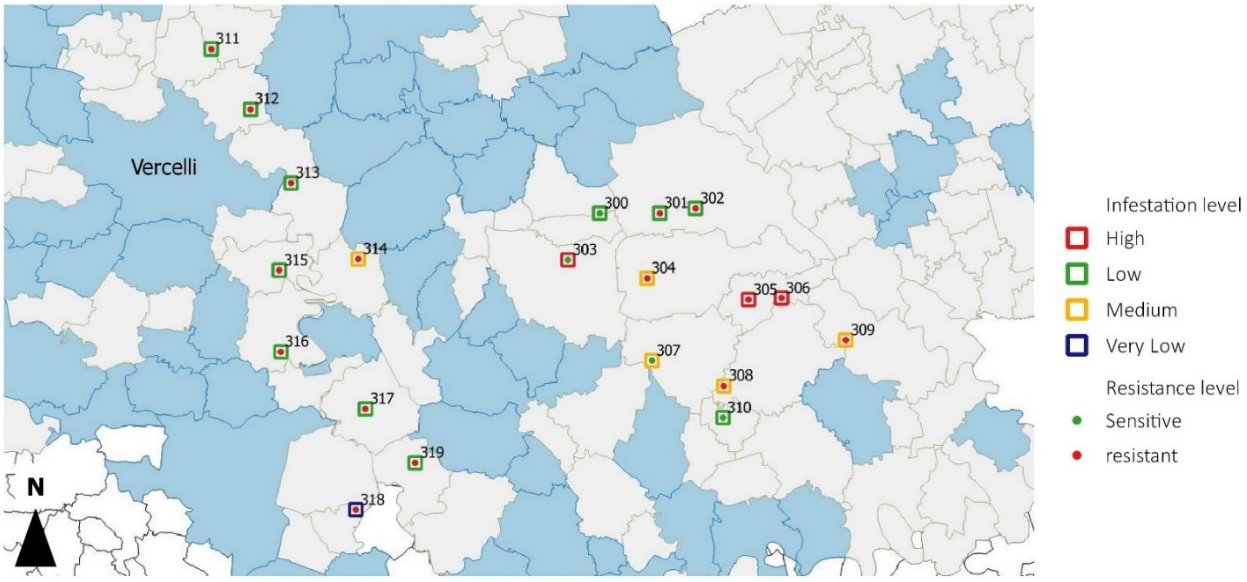


Fig. 23: results of screening test in comparison with the initial infestation recorded on field. Municipalities in light blue are those where GIRE has recorded at least one resistant case, those in grey have never registered a resistant case.

This confutes the results obtained by Hicks *et al.* (2018), i.e. that there is a positive correlation between herbicide resistance and weed density. For Italy this might be not the case.

This also suggests that the low level of infestation may not alarm farmers, so they do not complain about/report poor herbicide control. The low infestation density probably does not affect crop yield nor entails any economic loss. The generally low infestation levels are likely related to the higher level of diversity in the cropping systems (Norsworthy *et al.*, 2012; Renton *et al.,* 2014) practiced in these areas, especially in area L.; here in fact crop rotation and dry seeding are more frequent, mainly leading to the selection of different weed species (Juraimi *et al.*, 2013).

3.1.6 Specific conclusions

We present a large dataset that meets the need of both epidemiological studies at a large scale to better understand how resistance evolves and the definition of agronomic factors driving herbicide resistance evolution in the field. Although the impact of agronomic practices and environmental factors on resistance evolution is well known, this is the first time that these interactions are analyzed at such a large scale.

By analyzing the evolution of herbicide resistance on 200,000 ha of Italian rice fields we have first of all described the variability and complexity of this phenomenon and how its evolution is driven by the interaction and combination of multiple factors.

We demonstrate that herbicide resistance is strongly correlated with traditional management practices such as seeding type and crop rotation, as well as soil clay content. Dry seeding and crop rotation rate are negatively correlated with resistance presence. Soil texture has also an impact, even if at a lesser extent; its contribution is important anyway, because, until a few years ago, dry seeding was typical of sandy soils, but now it's widely used for its technical advantages and as a resistance management tool.

Through the integration of complaint monitoring, mapping and neural network analyses we prove that a high risk of resistance evolution is associated with traditional rice cropping systems where diversity in space and time is low, it's so proved that the increase of diversity in the cultivation methods is an effective tool to slow down resistance evolution, over and above rotation and combination of herbicides with alternative SoA.

Random sampling revealed that resistance is present even in the areas where previous monitoring based on farmers' complaints had not confirmed any resistance case and that resistance is not correlated with weed density on field. The density of resistant populations is medium-low, likely does not alarm rice farmers as they can manage the problem with practices that keep resistance at an acceptable level, such as different seeding techniques and mostly crop rotation, proving that resistance is not only a technical problem but a perceptive one also.

This situation is confirmed by the resistance risk map, which shows that some risk is present also in areas S and L, even if at a lesser extent in comparison with the surroundings. GIRE data are

based on complaint monitoring, this means that the cases reported on GIRE maps are those that farmers and stakeholders decides to communicate to the organization and that GIRE confirms by the greenhouse screening tests. It is patent that many cases are not reported: it therefore appears that, although very useful for stakeholders, GIRE maps underestimate resistance as proved by the random sampling.

The identification of concise, yet informative, agronomic predictors of diffusion of herbicide resistance can significantly facilitate effective management and improve sustainability of an important sector as rice is for the Italian economy and agriculture.

3.2 Echinochlos spp. case study

3.2.1 Discrimination of Italian accession performed in 2015

In Tab. XIX are reported the results of classification performed during plant sampling in September 2015.

Pop	Municipality	Pignatti	Carretero
15-1	Costanzana	white	white
15-2		white	white
15-4		white	white
15-6		*E. oryzicola*	*E. oryzicola*
15-7		*E. crus-galli*	*E. crus-galli*
15-20	Vercelli	Unclassified SE	Unclassified SE
15-22		*E. erecta*	*E. erecta*
15-24		Unclassified SE	Unclassified SE
15-25		Unclassified SE	Unclassified SE
15-26		*E. oryzicola*	*E. oryzicola*
15-30	Villanova Monferrato	*E. erecta*	*E. oryzicola*
15-31		*E. erecta*	*E. oryzicola*
15-34		*E. crus-galli*	*E. crus-galli*
15-41	Trino	*E. phyllopogon*	*E. oryzicola*
15-42		white	*E. oryzicola*
15-43		*E. erecta*	*E. hispidula*
15-44		*E. crus-galli*	*E. crus-galli*
15-45		*E. phyllopogon*	*E. hispidula*
15-46		*E. phyllopogon*	*E. hispidula*
15-47		*E. crus-galli*	*E. crus-galli*
15-48		*E. crus-galli*	*E. crus-galli*
15-51	Trino	Unclassified SE	Unclassified SE
15-52		*E. phyllopogon*	*E. oryzicola*
15-53		*E. phyllopogon*	*E. oryzicola*
15-54		*E. phyllopogon*	*E. hispidula*
15-55		*E. erecta*	*E. hispidula*
15-56		*E. crus-galli*	*E. crus-galli*
15-58		*E. erecta*	*E. oryzicola*
15-59		*E. phyllopogon*	*E. oryzicola*
15-62	Ronsecco	Unclassified SE	Unclassified SE
15-63		*E. erecta*	*E. hispidula*
15-64		*E. crus-galli*	*E. crus-galli*
15-65		*E. phyllopogon*	*E. oryzicola*
15-66		*E. erecta*	*E. oryzicola*
15-67		*E. phyllopogon*	*E. hispidula*
15-68		*E. erecta*	*E. oryzicola*
15-69		*E. phyllopogon*	*E. hispidula*

Tab. XIX: initial discrimination performed in 2015 during sampling, according to Pignatti and Carretero dichotomous keys. Plants with intermediate characteristics were classified as "White", plants named "Unclassified SE" are those with peculiar coloration. In table are not reported those accessions that provided a too small quantity of seeds or poor germination rate and were therefore excluded from every analyses.

As expected, only in case of *E. crus-galli* the two classifications matched.

In some cases classification was not possible for the coexistence, in the same plant, of traits typical of different species: e.g. accessions 20, 24, 25, 51 and 62 showed peculiar morphological traits, like presence of thick hair tufts at leaf sheath, typical of *E. phyllopgon* together with a purple pigmentation at base and nodes, not assessed in any species before (Fig. 24).

These plants were thus coded as "unclassified SE", although Tabacchi & Viggiani (2017) consider it a "purple" variation of *E. oryzicola.*

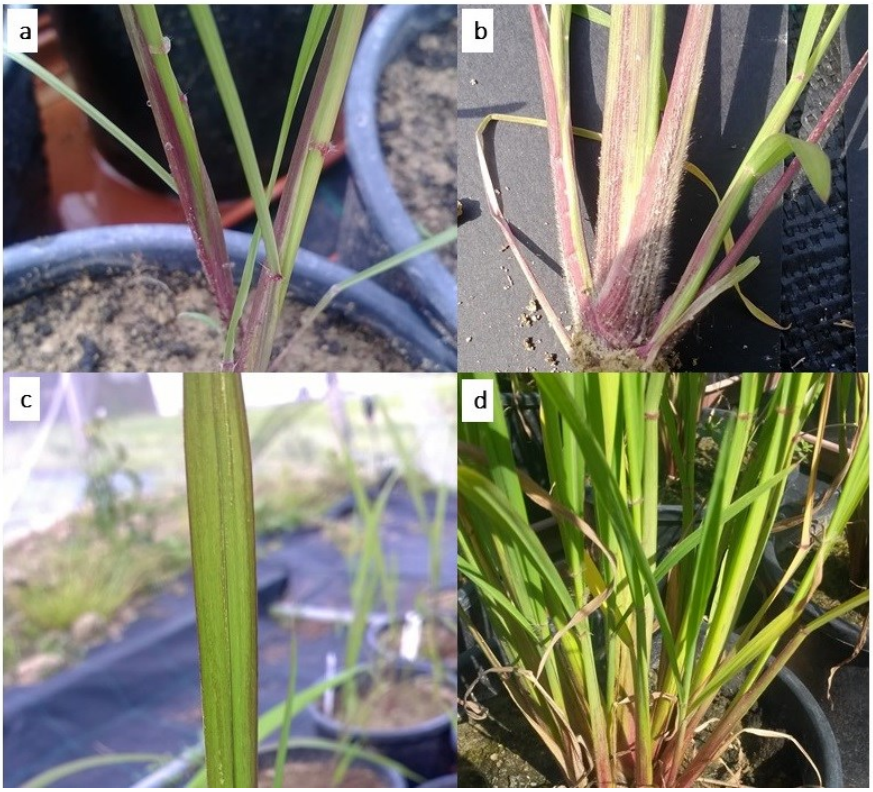


Fig. 24: accessions, 20, 24, 25, 51 and 62 of *Echinochloa* spp. showed unusual purple pigmentation at nodes (a, d), base (b) and leaves (c, d).

Three accessions had a very poor germination or provided a very scarce quantity of mature seed, for this reason they were immediately excluded from all of the analyses.

The thirty-seven accessions which provided a sufficient quantity of seed with good germination rate were then classified according to Costea & Tardif (2002): measures taken are reported in Tab. XX with standard errors in brackets.

CODE	SL (mm)	SW (mm)	CL (mm)	CW (mm)	GL/SL ratio	Awns	Costea-Tardif (2002)
15-1	4.0 (0.1)	2.2 (0.1)	2.4 (0.0)	1.9 (0.0)	0.5	< 2 cm	*E. oryzicola*
15-2	3.6 (0.1)	2.0 (0.1)	2.3 (0.0)	1.9 (0.0)	0.5	< 2 cm	*E. oryzicola*
15-4	3.8 (0.1)	2.1 (0.1)	2.2 (0.0)	1.8 (0.0)	0.6	< 2 cm	*E. oryzicola*
15-6	4.1 (0.0)	2.2 (0.1)	2.6 (0.0)	2.0 (0.0)	0.5	< 2 cm	*E. oryzicola*
15-7	3.4 (0.1)	1.8 (0.0)	1.8 (0.0)	1.4 (0.0)	0.4	< 5 cm	*E. crus-galli*
15-20	3.9 (0.1)	2.0 (0.0)	2.2 (0.1)	1.6 (0.1)	0.5	< 2 cm	*E. oryzicola*
15-22	3.9 (0.0)	2.1 (0.1)	2.4 (0.1)	1.7 (0.1)	0.6	< 2 cm	*E. oryzicola*
15-24	3.9 (0.1)	2.1 (0.1)	2.2 (0.1)	1.6 (0.1)	0.5	< 2 cm	*E. oryzicola*
15-25	4.2 (0.1)	2.0 (0.1)	2.4 (0.0)	1.8 (0.1)	0.6	< 2 cm	*E. oryzicola*
15-26	4.4 (0.1)	2.3 (0.1)	2.7 (0.1)	1.9 (0.0)	0.6	< 2 cm	*E. oryzicola*
15-30	4.0 (0.1)	2.4 (0.1)	2.5 (0.1)	2.3 (0.1)	0.5	< 2 cm	*E. oryzicola - E. oryzoides*
15-31	4.0 (0.1)	2.4 (0.1)	2.6 (0.1)	2.4 (0.1)	0.5	< 2 cm	*E. oryzicola - E. oryzoides*
15-34	3.0 (0.1)	1.6 (0.1)	1.6 (0.0)	1.2 (0.1)	0.4	Awnless	*E. crus-galli*
15-41	4.0 (0.1)	2.2 (0.1)	2.3 (0.0)	1.9 (0.1)	0.5	< 2 cm	*E. oryzicola*
15-42	4.1 (0.0)	2.2 (0.0)	2.6 (0.0)	1.9 (0.0)	0.5	< 2 cm	*E. oryzicola*
15-43	4.2 (0.1)	2.2 (0.0)	2.5 (0.1)	1.8 (0.1)	0.5	Awnless	*E. oryzicola*
15-44	3.2 (0.1)	1.6 (0.0)	1.7 (0.0)	1.3 (0.0)	0.4	Awnless	*E. crus-galli*
15-45	4.1 (0.0)	2.4 (0.1)	2.6 (0.1)	2.0 (0.1)	0.5	< 2 cm	*E. oryzicola - E. oryzoides*
15-46	3.9 (0.1)	2.2 (0.0)	2.4 (0.1)	2.0 (0.0)	0.4	< 2 cm	*E. oryzicola*
15-47	3.0 (0.1)	1.6 (0.1)	1.6 (0.0)	1.2 (0.1)	0.4	< 2 cm	*E. crus-galli*
15-48	2.9 (0.1)	1.6 (0.1)	1.8 (0.0)	1.4 (0.0)	0.4	< 5 cm	*E. crus-galli*
15-51	4.2 (0.1)	2.0 (0.0)	2.5 (0.0)	1.7 (0.0)	0.5	Awnless	*E. oryzicola*
15-52	4.4 (0.1)	2.0 (0.0)	2.3 (0.1)	1.7 (0.1)	0.6	< 2 cm	*E. oryzicola*
15-53	4.3 (0.1)	2.1 (0.1)	2.5 (0.1)	1.9 (0.1)	0.5	Awnless	*E. oryzicola - E. oryzoides*
15-54	4.6 (0.2)	2.2 (0.1)	2.7 (0.1)	2.0 (0.1)	0.6	< 2 cm	*E. oryzicola*
15-55	4.4 (0.2)	2.2 (0.1)	2.6 (0.1)	2.0 (0.0)	0.5	< 2 cm	*E. oryzicola - E. oryzoides*
15-56	3.7 (0.3)	1.9 (0.1)	2.3 (0.1)	1.5 (0.1)	0.5	< 2 cm	*E. hispidula*
15-58	4.7 (0.1)	2.2 (0.1)	2.7 (0.1)	1.9 (0.1)	0.6	Awnless	*E. oryzicola*
15-59	4.6 (0.1)	2.2 (0.1)	2.7 (0.0)	2.0 (0.1)	0.6	Awnless	*E. oryzicola*
15-62	4.2 (0.1)	2.0 (0.0)	2.5 (0.0)	1.7 (0.0)	0.4	Awnless	*E. oryzicola - E. oryzoides*
15-63	4.1 (0.1)	2.4 (0.1)	2.6 (0.1)	2.0 (0,1)	0.5	Awnless	*E. oryzicola - E. oryzoides*
15-64	2.9 (0.1)	1.5 (0.0)	1.8 (0.0)	1.3 (0.1)	0.4	Awnless	*E. crus-galli*
15-65	4.1 (0.2)	2.1 (0.1)	2.5 (0.0)	2.0 (0.0)	0.5	< 2 cm	*E. oryzicola*
15-66	4.2 (0.2)	2.2 (0.1)	2.6 (0.0)	2.5 (0.1)	0.5	< 2 cm	*E. oryzicola - E. oryzoides*
15-67	4.2 (0.1)	2.1 (0.1)	2.7 (0.1)	2.3 (0.1)	0.5	Awnless	*E. oryzicola - E. oryzoides*
15-68	4.3 (0.2)	2.0 (0.1)	2.8 (0.1)	2.1 (0.0)	0.5	< 2 cm	*E. oryzicola - E. oryzoides*
15-69	4.5 (0.2)	2.0 (0.0)	2.7 (0.1)	2.1 (0.1)	0.6	Awnless	*E. oryzicola*

Tab. XX: Plants classification according to Costea-Tardif: spikelet length and width (SL, SW) caryopses length and width (CL, CW), awns presence and length, glume/spikelet length ratio (GL/SL ratio) were assessed.

Six accessions, 15-7, 15-34, 15-44, 15-47, 15-48 and 15-64, were classified as *E. crus-galli* species, matching previous field classification. According to Costea & Tardif (2002). They can easily distinguished from the other species due to their smaller dimensions of seeds.

The distinction of *E. oryzicola*. *E. oryzoides* and *E. hispidula* was more challenging, as several spikelets and caryopses showed intermediate characteristics and measures: i.e. typical of more than one species.

Finally, only one accession was ascribable to *E. hispidula* (15-56), eight accessions were instead provided with a double classification (15-30, 15-31, 15-45, 15-53, 15-55, 15-62, 15-63, 15-66, 15-67, 15-68) as they showed intermediate characteristics between *E. oryzicola* and *E. oryzoides*, while the remaining accessions were classified as *E. oryzicola* (Tab. XX). No accessions of *E. colona* were present.

The E/C ratio (embryo/caryopses ratio) resulted always equal or superior to 0.8, also in case of *E. crus-galli*, suggesting that our instruments are not precise enough to measure at this detail. For this reason, although calculated, this parameter was not used for species discrimination. This classification provided a good representation of the actual distribution of *Echinochloa* species in the Italian rice fields, where it is estimated that *E. crus-galli* and *E. oryzicola* are the most widespread, while *E. oryzoides* and *E. hispidula* are rather rare (Tabacchi, personal communication).

3.2.2 Preliminary screening

Results of the preliminary screening highlighted, in multiple accessions, medium-high rates of resistance to penoxsulam: one harvested in Costanzana (field 0, accession 7), one harvested in Trino (field 5, accession 53) and five collected in Ronsecco (field 6, accessions 62, 63, 67 and 69) (Fig. 25).

Although only fields with a proved history of susceptibility to penoxsulam were sampled, for many accessions a few plants survived the field dose of the herbicide (15-1, 15-26, 15-30, 15-31, 15-52, 15-54, 15-56, 15-66 and 15-68), suggesting that in these fields the continued use of penoxsulam is selecting a sort of ALS resistance and that the situation need to be monitored. Twenty-two accessions tested resulted to be completely controlled (Fig. 25).

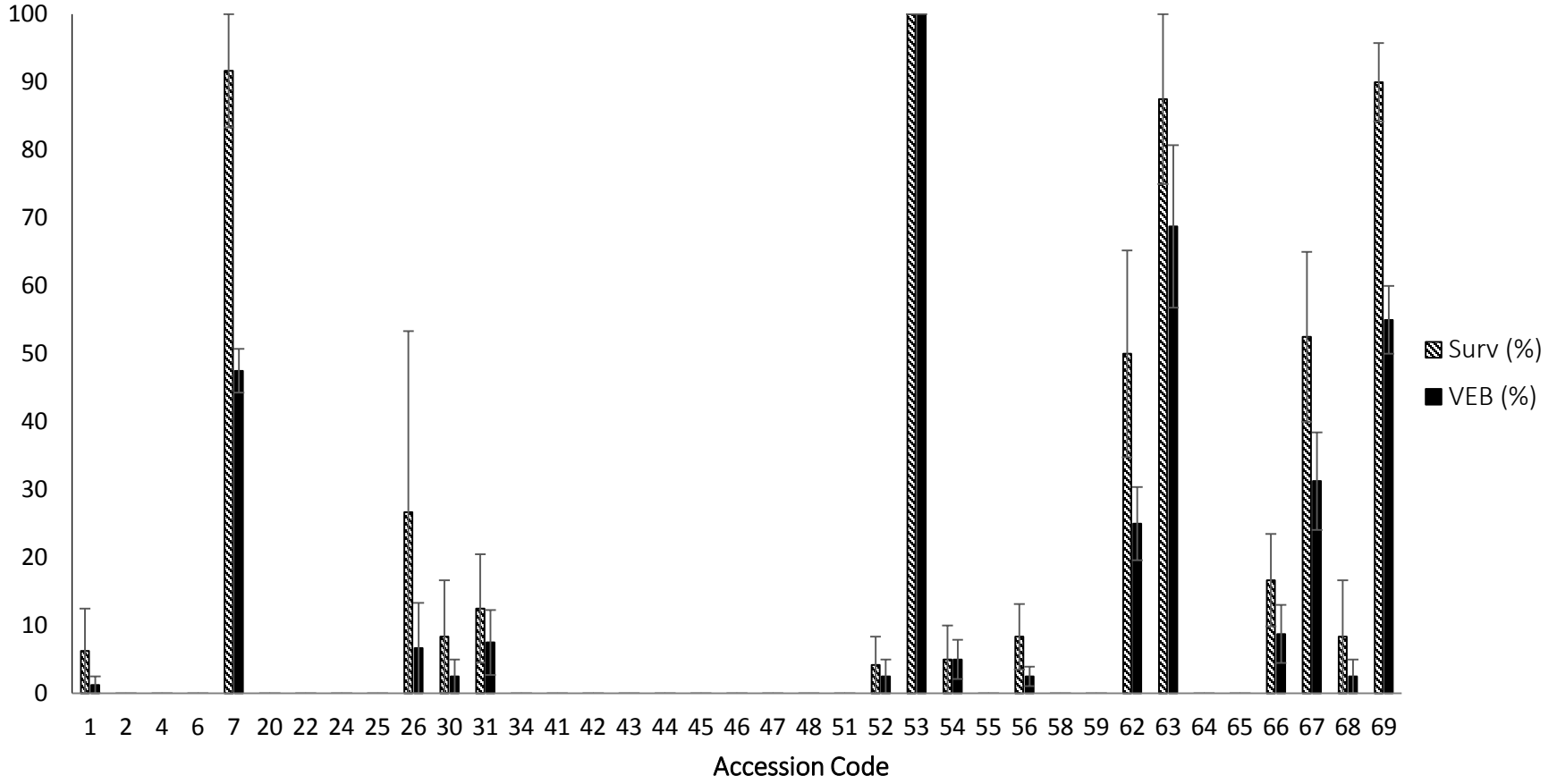


Fig. 25*:* Results of the preliminary screening performed using penoxsulam. For each accession, mean plant survival (Surv) and visual estimation of the biomass (VEB) expressed as percentage of untreated control are reported. Bars represent standard errors.

Considering that when plant survival is included between 5 and 20% (i.e. plants treated with the recommended field dose of a herbicide) (Panozzo *et al.*, 2015b), accession is still considered slightly resistant and that we would like to represent the higher *Echinochloa* species variability in Italian rice fields, both classification and results of the screening were considered when choosing accessions for molecular analyses.

Penoxsulam screening was repeated in 2016 on reproduces accessions (section 3.2.3) giving results consistent with those of 2015 (data not shown).

3.2.3 Morphologic classification of reproduced accessions

Ten accessions were reproduced and morphologically classified using four dichotomous keys: Pignatti (1982), Tabacchi *et al.* (2006), Costea & Tardif (2002), Tabacchi & Viggiani (2017).

Carretero was not used after field classification in 2015 as it was not possible to find any match between this dichotomous key and the molecular discrimination performed on both original and reproduced accessions.

Plants were grown in external conditions in Italy and in greenhouse conditions at the Botanic Garden of Meise (Belgium) to verify potential influences of environmental conditions (such as lights and temperatures) on the development of plant morphological traits.

A high degree of variability was assessed when the same plants were grown in Belgium compared to Italy: plants size, color, panicle bearing, awns presence and length were different, e.g. plants belonging to *E. crus-galli* species grew less in Belgian conditions (Fig. 26).

Nevertheless, size of the plant, color, panicle bearing and awns are all morphological characteristics that are used in classic dichotomous keys, but it is known that they show a high degree of plasticity depending on environmental conditions (Ruiz-Santaella *et al.*, 2006). For this reason these traits are used in combination with the other ones (e.g. spikelet and caryopses size) to correctly classify the species. None of the “white” accessions presented awns longer than 2 cm, suggesting that no *E. oryzoides* are included in our accessions (Costea & Tardif, 2002; Tabacchi *et al.*, 2006; Viggiani & Tabacchi, 2017).

Fig. 26: differences in grow of two accessions of *Echinochloa* spp. in Belgium greenhouse conditions. *E. crus-galli* (15-12, right) has grown smaller than *E. phyllopogon* (16-59, left), differently to what normally happen in Italian conditions.

Hair presence at base and leaf sheath did not change in Belgium compared to Italy. It was observed in our accessions that hairs at the collar regions and leaf sheath can be present at initial development of plants, disappearing later on during growth, e.g. accession 16-45 (Fig. 27). This might mislead technicians and farmers during classification on field, when plants are classified at an early stage to plan the herbicide strategy.

It is debated whether hair presence is or is not a discriminating trait for different *Echinochloa* species. Both Tabacchi *et al.* (2006) and Pignatti (1981) include it, while Carretero (1981) and Tabacchi & Viggiani (2017) do not. In our case hairs were present on leaves and at the collar region in accessions 16-41, 16-46, 16-59, 16-65, 16-24 and 16-25. This character was absent on accessions 16-42, 16-43 and 16-45.

Fig. 27: picture of accession 16-45 showing hairs at the leaf sheath at three leaves stage (left), this characteristic is lost when plant is at stem elongation stage (right).

Results of caryopses and spikelet analyses, considered by Costea & Tardif (2002) and Tabacchi & Viggiani (2017) keys, confirmed the classification performed on 2015 accessions, both on plants grown in Italy and in Belgium: i.e. accessions 15-9 and 15-12 resulted *E. crus-galli*, all of the others resulted *E. oryzicola*.

ANOVA test was performed using Statistica Software to compare the means of the different measures of spikelets and caryopses of all accessions and confirm results of visual analyses. Three types of measures - Upper Glume Length, Fertile Lemma Length and Embryo ratio without taking into account the scutellar region - were taken into account only in Belgium, for this reason a one-way ANOVA was performed on them: results did not give any relevant information that might support any morphological classification and are therefore not discussed in this analyses.

For the other parameters a factorial ANOVA was performed as both accession code and locality were inserted in the analyses as independent variables.

Factorial ANOVA results showed that parameters assessed are different in the various accessions ($p<0.0001$), while the combination of accession x locality resulted not significant, meaning that accessions behaved the same way in Italy and in Belgium and that variability is not associated to growing conditions and can be considered a trait associated with the physiology and genetic of this genus (Tab. XXI).

	Spikelet Length	Spikelet Width	Inf. Glume	Lemma-Inf. glume ratio	Cariopses Length	Cariopses Width	Ratio embryo
Loc	0.005	0.044	0.000	0.003	0.094	0.017	0.365
Accession	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Loc x Acc	0.786	0.918	0.004	0.018	0.910	0.927	0.367

Tab. XXI: p values obtained from factorial ANOVA for the spikelet and caryopses parameters measured.

ANOVA analyses highlighted statistical differences among populations, but only *E. crus-galli* accessions showed parameters that were always statistically different from the others. Both spikelets and caryopses are always smaller than those of the other species (Costea & Tardif, 2002; Viggiani & Tabacchi, 2017).

Data of morphological analyses, performed both on plants and spikelets did not provide a clear cut discrimination among species. Their use can lead to different results when different morphological keys are used and sometimes, to classify plants, some characters should not be taken into account: e.g. accessions 16-25 and 16-42 could be classified both as *E. oryzicola* and *E. oryzoides* on the base of measures of spikelet and caryopses. In these accession average GL/SL ratio is equal to 0.4. According to Costea & Tardif (2002) and Viggiani & Tabacchi (2017) they should be classified as *E. oryzoides*. Anyway they both showed very short awns and embryo almost as long as the whole caryopses. They were then classified as *E. oryzicola* (Fig. 28).

A final morphologic classification of reproduced accessions was done (Tab. XXII).

According to both Costea & Tardif (2002) and Tabacchi & Viggiani (2017) only two species are present in our bulk: *E. oryzicola* and *E. crus-galli*. According to Pignatti (1982) four species are present: *E. crus-galli*, *E. erecta*, *E. phyllopogon* and *E. hostii*. While according to Tabacchi *et al.* (2006): three species are present: *E. crus-galli*, *E. oryzicola* and *E. phyllopogon*.

The classification that is more suitable to describe our accessions is that of Tabacchi *et al.* (2006): accessions 16-24 and 16-25 should be considered variations of *E. phyllopogon,* and the purple pigmentation that characterized them was proven to be only a transitory trait linked to environmental conditions. Accessions 15-9 and 15-12 are confirmed *E. crus-galli*. Accessions 16-42, 16-43 and 16-45 are classified as *E. oryzicola*, while 16-41, 46-46, 26-54, 16-59 and 16-65 are *E. phyllopogon*.

Our intention was not to create a new classification key for *Echinochloa* spp., nor to verify the correctness of those already published, but to find the best match between morphological classification and molecular marker discrimination.

Tabacchi *et al.* (2006) was chosen as classification of reference, when discussing molecular analyses and dose response results, because it best represented the diversity of morphological characteristics found in our accessions besides providing the most suitable match with the discrimination performed with matK molecular marker.

Fig. 28: caryopsis (left) and spikelets (right) of accession 16-25 (a and b) and 16-42 (c and d). Although their measures were coherent with *E. oryzoides* according to Costea and Tardif (2002), they were classified as *E. oryzicola*.

Loc	Code	Base color	Nodes color	Panicle Bearing	Awns	Awn Length	Leaf Sheath hairy	Base hairy	SL	SC	G/S Ratio	CL	CW	E/C ratio	Costea-Tardif	Tabacchi-Viggiani	Pignatti	Tabacchi
BE	15-9	1	1	4	3	3	5	5	3.6	1.5	0.4	1.6	1.3	0,8	*E. crus-galli*	*E. crus-galli*	*E. crus-galli*	*E. crus-galli*
BE	15-12	1	1	3	1	5	5	5	3.2	1.6	0.4	1.6	1.3	0,8	*E. crus-galli*	*E. crus-galli*	*E. crus-galli*	*E. crus-galli*
BE	16-24	5	5	1	1	5	1	1	4.2	1.9	0.6	2.1	1.6	0,9	*E. oryzicola*	*E. oryzicola*	*E. phyllopogon*	*E. phyllopogon*
BE	16-25	5	5	3	1	3	1	1	4.5	2.0	0.5	2.4	1.8	0,9	*E. oryzicola*	*E. oryzicola*	*E. phyllopogon*	*E. phyllopogon*
BE	16-41	1	1	3	3	5	1	1	4.2	2.1	0.5	2.5	1.9	0,9	*E. oryzicola*	*E. oryzicola*	*E. phyllopogon*	*E. phyllopogon*
BE	16-42	1	1	3	1	3	5	5	4.6	2.2	0.6	2.4	2.0	1,0	*E. oryzicola*	*E. oryzicola*	*E.hostii*	*E. oryzicola*
BE	16-43	1	1	1	1	3	1	5	4.3	2.2	0.5	2.5	1.9	0,9	*E. oryzicola*	*E. oryzicola*	*E.erecta*	*E. oryzicola*
BE	16-45	1	1	1	1	3	5	5	4.1	2.1	0.5	2.3	1.8	1,0	*E. oryzicola*	*E. oryzicola*	*E.erecta*	*E. oryzicola*
BE	16-46	1	1	1	1	3	1	1	4.3	2.1	0.5	2.5	1.9	0,9	*E. oryzicola*	*E. oryzicola*	*E. phyllopogon*	*E. phyllopogon*
BE	16-54	1	1	1	1	3	1	1	4.6	2.2	0.6	2.5	1.9	0,9	*E. oryzicola*	*E. oryzicola*	*E. phyllopogon*	*E. phyllopogon*
BE	16-59	1	1	1	5	/	1	1	4.7	2.2	0.6	2.7	1.9	0,9	*E. oryzicola*	*E. oryzicola*	*E. phyllopogon*	*E. phyllopogon*
BE	16-65	1	1	3	3	3	1	1	4.7	2.2	0.6	2.5	2.1	1,0	*E. oryzicola*	*E. oryzicola*	*E. phyllopogon*	*E. phyllopogon*
IT	15-9	/	/	/	/	/	/	/	3.4	1.6	0.4	1.7	1.3	0,8	*E. crus-galli*	*E. crus-galli*	*E. crus-galli*	*E. crus-galli*
IT	15-12	/	/	/	/	/	/	/	2.9	1.5	0.4	1.7	1.3	0,8	*E. crus-galli*	*E. crus-galli*	*E. crus-galli*	*E. crus-galli*
IT	16-24	1	2	3	3	1	1	1	3.7	2.0	0.6	2.2	1.7	0,9	*E. oryzicola*	*E. oryzicola*	*E. phyllopogon*	*E. phyllopogon*
IT	16-25	1	5	1	3	1	1	1	4.2	2.1	0.4	2.5	1.8	0,9	*E. oryzicola*	*E. oryzicola*	*E. phyllopogon*	*E. phyllopogon*
IT	16-41	1	1	1	1	1	1	1	3.9	2.2	0.5	2.5	2.0	0,9	*E. oryzicola*	*E. oryzicola*	*E. phyllopogon*	*E. phyllopogon*
IT	16-42	1	1	1	1	1	5	5	4.3	2.3	0.4	2.5	2.0	1,0	*E. oryzicola*	*E. oryzicola*	*E.erecta*	*E. oryzicola*
IT	16-43	1	1	1	1	1	5	5	4.2	2.2	0.5	2.4	2.0	0,9	*E. oryzicola*	*E. oryzicola*	*E.erecta*	*E. oryzicola*
IT	16-45	1	1	1	3	1	5	5	3.9	2.1	0.5	2.3	1.9	1,0	*E. oryzicola*	*E. oryzicola*	*E.erecta*	*E. oryzicola*
IT	16-46	1	1	2	3	3	1	1	4.3	2.3	0.5	2.5	1.9	0,9	*E. oryzicola*	*E. oryzicola*	*E. phyllopogon*	*E. phyllopogon*
IT	16-54	1	1	2	3	1	1	1	4.7	2.3	0.5	2.6	2.0	0,8	*E. oryzicola*	*E. oryzicola*	*E. phyllopogon*	*E. phyllopogon*
IT	16-59	1	1	1	3	1	1	1	4.7	2.4	0.6	2.6	2.0	0,9	*E. oryzicola*	*E. oryzicola*	*E. phyllopogon*	*E. phyllopogon*
IT	16-65	1	1	3	1	3	1	1	4.8	2.3	0.6	2.6	2.1	0,9	*E. oryzicola*	*E. oryzicola*	*E. phyllopogon*	*E. phyllopogon*

Tab. XXII: classification of the Italian accessions according to the four most used classification keys: Costea&Tardif (2002), Tabacchi&Viggiani (2017), Pignatti (1981) and Tabacchi *et al.* (2006). Scores given to plants morphological traits are described in section 2.2.4.1 of Materials and Methods.

3.2.4 DNA barcoding

Seventeen of the original accessions collected in 2015 were included in the molecular analyses (Tab. XXIII). Accession 1 was added as unclassified "white" control.

Accession	Plant survival (%) (SE)	Pignatti (1982)	Tabacchi (2006)	Costea-Tardif (2002)
15-1	6.3 (6.3)	Unclassified	Unclassified	Unclassified
15-09	*NA*	*E. crus-galli*	*E. crus-galli*	*E. crus-galli*
15-12	*NA*	*E. crus-galli*	*E. crus-galli*	*E. crus-galli*
15-20	0.0 (0.0)	SE	SE	SE
15-24	0.0 (0.0)	SE	SE	SE
15-25	0.0 (0.0)	SE	SE	SE
15-34	0.0 (0.0)	*E. crus-galli*	*E. crus-galli*	*E. crus-galli*
15-41	0.0 (0.0)	*E. phyllopogon*	*E. phyllopogon*	*E. oryzicola*
15-42	0.0 (0.0)	*E. erecta*	*E. oryzicola*	*E. oryzicola*
15-43	0.0 (0.0)	*E. erecta*	*E. oryzicola*	*E. oryzicola*
15-45	0.0 (0.0)	*E. erecta*	*E. oryzicola*	*E. oryzicola-oryzoides*
15-46	0.0 (0.0)	*E. phyllopogon*	*E. phyllopogon*	*E. oryzicola*
15-47	0.0 (0.0)	*E. crus-galli*	*E. crus-galli*	*E. crus-galli*
15-51	0.0 (0.0)	SE	SE	*E. oryzicola*
15-54	5.0 (5.0)	*E. phyllopogon*	*E. phyllopogon*	*E. oryzicola*
15-59	0.0 (0.0)	*E. phyllopogon*	*E. phyllopogon*	*E. oryzicola*
15-65	0.0 (0.0)	*E. phyllopogon*	*E. phyllopogon*	*E. oryzicola*

Tab. XXIII: list of accessions chosen for the molecular analyses. It is reported the phenotypic classification according to Pignatti, Tabacchi and Costea-Tardif. Accession #1 was not classified as its phenotype was uncertain. Accessions classified as "SE" are those with intermediate characteristics between "white" and "red" types of *Echinochloa* spp. In the second column is reported the survival rate in penoxsulam screening. Standard error is reported in brackets.

Four out of five DNA regions analyzed in this study were able to discriminate among the different accessions. The only one which gave no useful distinction was ITS, therefore it is not discussed in this section.

Sequences of all genes analyzed were conserved and the BLAST analyses, which compares sequences obtained from our accessions with sequences present in the main online nucleotide

databases, gave over 99% of similarity. Also the alignments of the sequences produced for our accessions for the different genes highlighted a very few SNPs.

The entire length of regions analyzed varied from 441 bp of trnL to 936 bp of rbcL. A total of 13 single nucleotide polymorphism (SNP) was detected among all accessions: three SNP in trnL, one in rbcL, four in matK and five in the non-coding region psbA-trnH. No differences were highlighted in sequences from plants "a" and "b", for this reason in this section results of whole accessions only will be discussed.

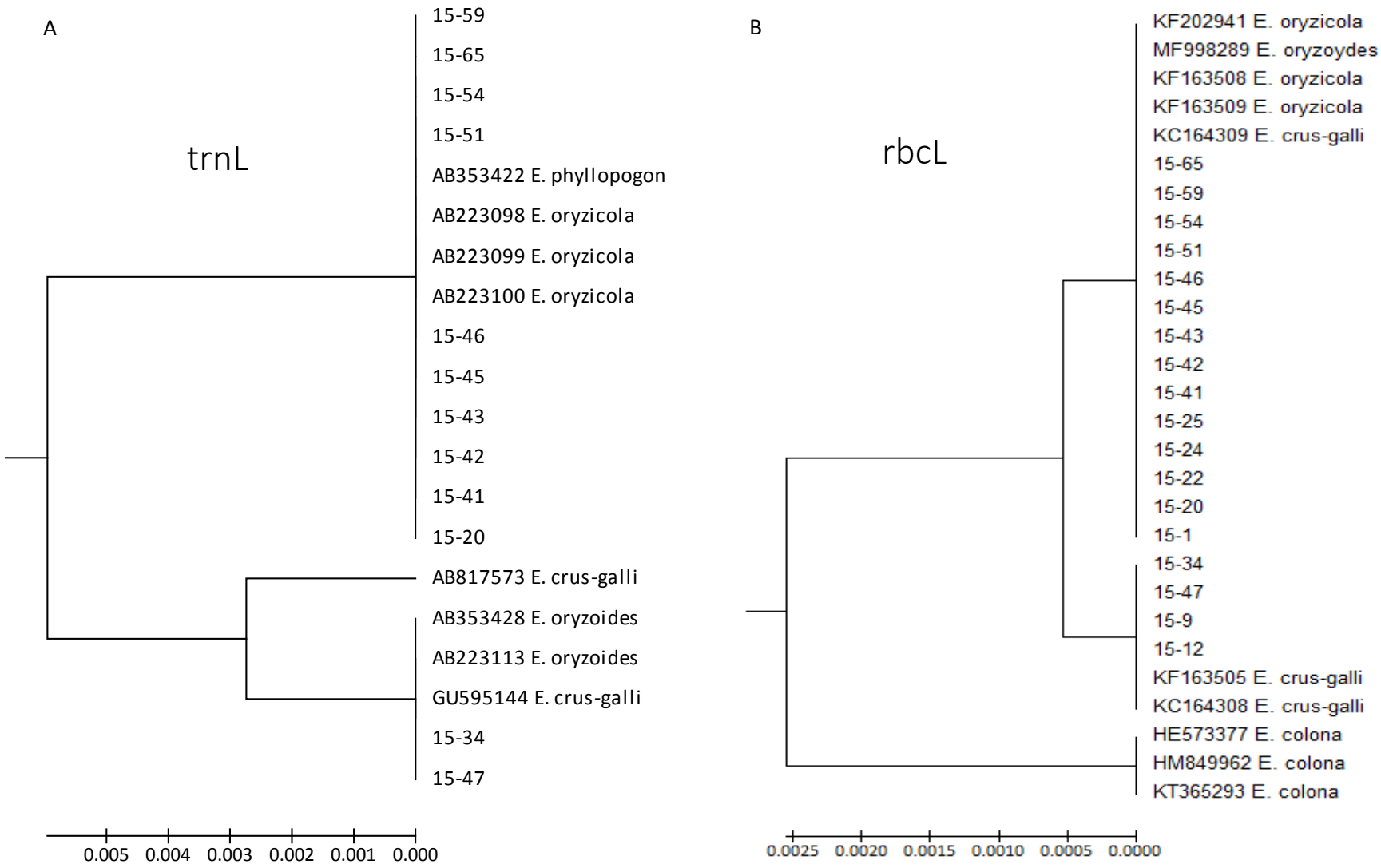
All UPGMA dendrograms built using Mega 6 software are displayed in Fig. 29.

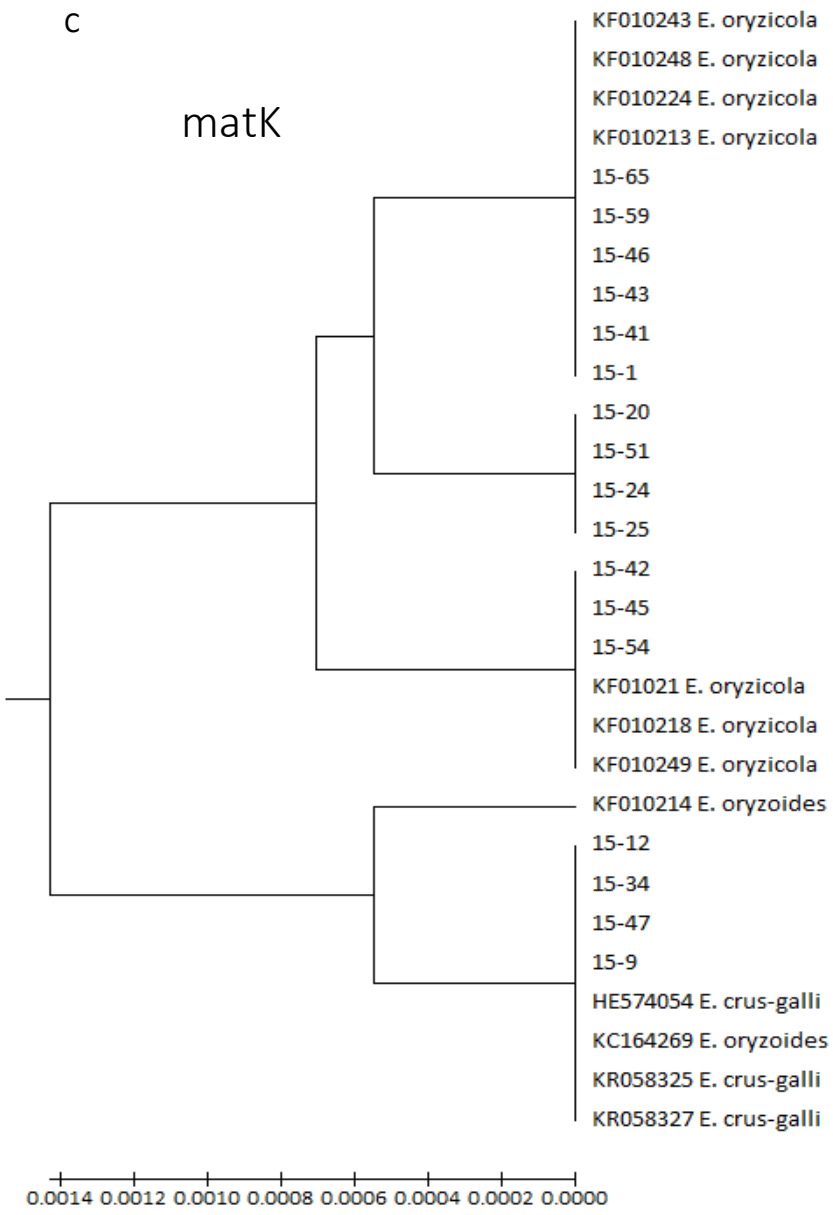
For trnL gene the sequence obtained was of 441 bp with three SNPs that led to the creation of two clusters: one containing the accessions classified as *E. crus-galli* and one with the other accessions, classified as *E. oryzicola* according to Costea & Tardif (2002). No correspondence was seen with the other dichotomous keys (Fig. 29A).

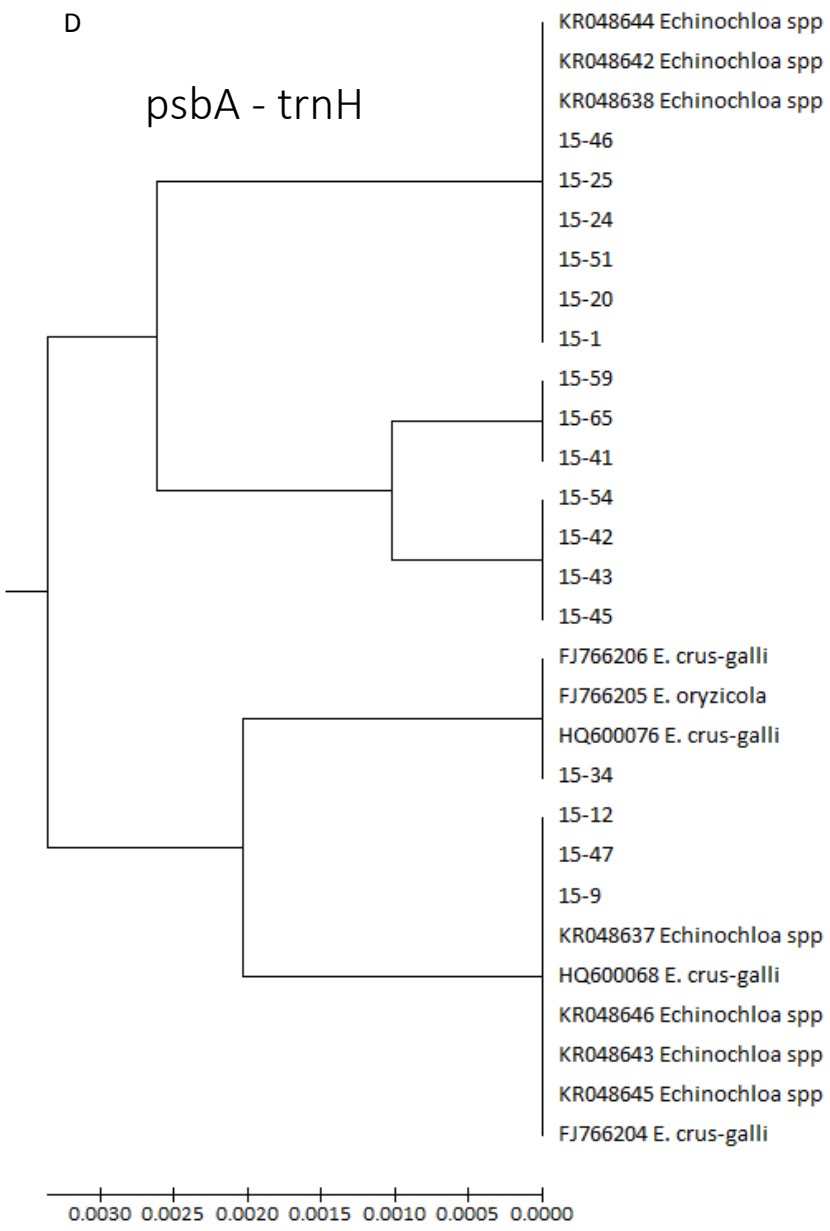
A similar result was achieved with analyses of rbcL gene (Fig. 29B). In this case a total of 936 bp was obtained highlighting only one SNP: also in this case the separation obtained was corresponding with classification of Costea & Tardif (2002). Analyses and comparison of these two dendrograms with vouchered sequences confirm the initial assumption that *E. colona* is absent. This species supposed to be rare in Italian rice fields (Pignatti, 1981): the only case was recorded in 2002, with one single population from the south-Milan area (Tabacchi *et al.*, 2006).

For matK a sequence of 917 bp with four SNPs was analyzed, leading to the creation of four clusters in this dendrograms reflecting the four SNP found (Fig. 29C). Discrimination with this molecular marker has a certain degree of correspondence with Pignatti (1981) and Tabacchi *et al.* (2006) discrimination, although there are some discrepancies. The first cluster includes the accessions classified as *E. phyllopgon*, 15-22, 15-41, 15-46, 15-54, 15-59 and 15-65, plus the unclassified white. The second includes those classified as *E. erecta* (Pignatti, 1982) or *E. oryzicola* (Tabacchi *et al.*, 2006), 15-42 and 15-45.

Fig. 29: UPGMA dendrograms of the DNA regions analyzed: trnL (A), rbcL (B), matK (C) and psbA-trnH (D) built on the alignment of sequences of the accessions. They are displayed in comparison with vouchered sequences of different classified accessions of *Echinochloa* exported from NCBI and Boldsystem.




C
matK
KF010243 E. oryzicola
KF010248 E. oryzicola
KF010224 E. oryzicola
KF010213 E. oryzicola
15-65
15-59
15-46
15-43
15-41
15-1
15-20
15-51
15-24
15-25
15-42
15-45
15-54
KF01021 E. oryzicola
KF010218 E. oryzicola
KF010249 E. oryzicola
KF010214 E. oryzoides
15-12
15-34
15-47
15-9
HE574054 E. crus-galli
KC164269 E. oryzoides
KR058325 E. crus-galli
KR058327 E. crus-galli
0.0014 0.0012 0.0010 0.0008 0.0006 0.0004 0.0002 0.0000


D
psbA - trnH
KR048644 Echinochloa spp
KR048642 Echinochloa spp
KR048638 Echinochloa spp
15-46
15-25
15-24
15-51
15-20
15-1
15-59
15-65
15-41
15-54
15-42
15-43
15-45
FJ766206 E. crus-galli
FJ766205 E. oryzicola
HQ600076 E. crus-galli
15-34
15-12
15-47
15-9
KR048637 Echinochloa spp
HQ600068 E. crus-galli
KR048646 Echinochloa spp
KR048643 Echinochloa spp
KR048645 Echinochloa spp
FJ766204 E. crus-galli
0.0030 0.0025 0.0020 0.0015 0.0010 0.0005 0.0000

The discrepancy is due by accessions 15-43 and 15-54. The first is morphologically similar to *E. erecta-oryzicola* (Pignatti, 1982; Tabacchi *et al.*, 2006), but is included in the *E. phyllopogon* cluster. The second is morphologically similar to *E. phyllopogon*, but is included in the *E. erecta-oryzicola* cluster. The peculiarity of matK tree is that the "unclassified SE" accessions 15-24 and 15-25 form one single cluster: none of the vouchered sequences used in this study contained the same mutations, suggesting that these two accessions might belong to a different species of this genus. matK is one of the most used genes in plant systematic (Hilu & Liang, 1997) but this is one of the first times it is use for the discrimination among multiple *Echinochloa* species.

PsbA-trnH provided a sequence of 493 bp with five SNPs. The cluster analyses partially matched with Pignatti (1982) and Tabacchi *et al.* (2006) classification (Fig. 29D), but it's less clear than that of matK. Here five clusters are present: *E. crus-galli* accessions are divided into two clusters. One cluster includes the "Unclassified SE" accessions together with 15-1 and 15-46, the latter classified as *E. phyllopogon*. Another one includes 15-41, 15-59 and 15-65 - *E. phyllopogon* - closer to the cluster including 15-42, 15-43, 15-45 and 15-54 classified as both *E. phyllopogon* and *E. erecta-oryzicola* (Pignatti, 1982; Tabacchi *et al.*, 2006).

For all genes accessions 15-9, 15-12, 15-34 and 15-47 groups separately from the other accessions and together with other vouchered sequences of *E. crus-galli*. This results confirms our initial classification for all dichotomous keys. Previous studies have demonstrated, through molecular and morphological tool, the clear separation of *E. crus-galli* from the other species of this genus (Yamaguchi *et al.*; 2005; Tabacchi *et al.,* 2006; Claerhout *et al.,* 2016).

MatK and psbA-trnH were able to discriminate some clusters into the "white" accessions of *Echinochloa* spp., i.e. those classified as *E. oryzicola*, *E. phyllopogon*, *E. erecta*.

In bibliography psbA-trnH is indicated as reliable for species discrimination, only when used as a support of matK and rbcL (CBOL Plant Working group, 2009), because it shows a high degree of variability both in length and presence of insertion-deletion, complicating sequence alignment (Chase *et al.*, 2007). In our case vouchered psbA-trnH sequences of *E. crus-galli* FJ766206 and HQ600076 showed SNPs that did always not match with those of our sequences of barnyardgrass.

Comparison of our sequences with the vouchered ones confirm the absence of *E. colona* from our bulk. *E. oryzoides* might also be absent: in case of trnL and matK it clusterizes with *E. crus-galli*, while for rbcL it clusterizes with *E. oryzicola*.

Comparison of our psbA-trnH sequences with those already published did not help the classification either. Most of the sequences for this intron downloaded from the NCBI website (https://www.ncbi.nlm.nih.gov) and BOLDsystem (http://www.boldsystems.org/) were not classified: i.e. they were named *Echinochloa* spp.

No match was found between molecular discrimination and Carretero (1981) classification from the beginning of the experiments, therefore this classification was not used after the first sampling in 2015.

In our accessions, plants classification according to Pignatti (1981) matched with the one of Tabacchi *et al.* (2006), both genetically and morphologically. The latter was chosen for the further part of the study as it's more recent and was also created using molecular markers as benchmark.

3.2.4.1 Molecular characterization of accessions from Belgium

Phenotypic discrimination highlighted the presence of nine accessions of *E. crus-galli*, two accessions of *E. muricata* var. *wiegandii* and three of *E. muricata* var *mycrostachia*.(De Cauwer *et al.*, 2012)

In this case, rbcL provided the best discriminating ability, while no differences were present in matK and psbA-trnH sequences of the 14 accessions (data not shown).

One single A/T substitution was found in position 421 from the 5' end. This SNP was common to all of the three accessions of *E. muricata* var. *microstachya*, two out of three *E. muricata* var. *wiegandii* and in one accession of *E. crus-galli*.

Comparison with published sequences of *E. muricata* was not possible, as published sequences of this species were too short to include this SNP. Therefore, rbcL sequences of accessions from Belgium were aligned with some rbcL sequences of Italian reproduced accessions and some rbcL vouchered sequences of other *Echinochloa* species. The resulted dendrogram (Fig. 30) showed that Italian sequences of *E. phyllopogon* (16-41), *E. erecta* (16-42) and "Unclassified SE" *Echinochloa* (16-25) clustered with Belgian *E. crus-galli* accessions as well as all other sequences

of "white" species included in the analyses, while all *E. muricata* varieties – but #24 – form a separate cluster (Fig. 30).

Although incomplete this result confirms the previous taxonomic and molecular studies performed on these two species (Hoste, 2004; Claerhout *et al.*, 2016).

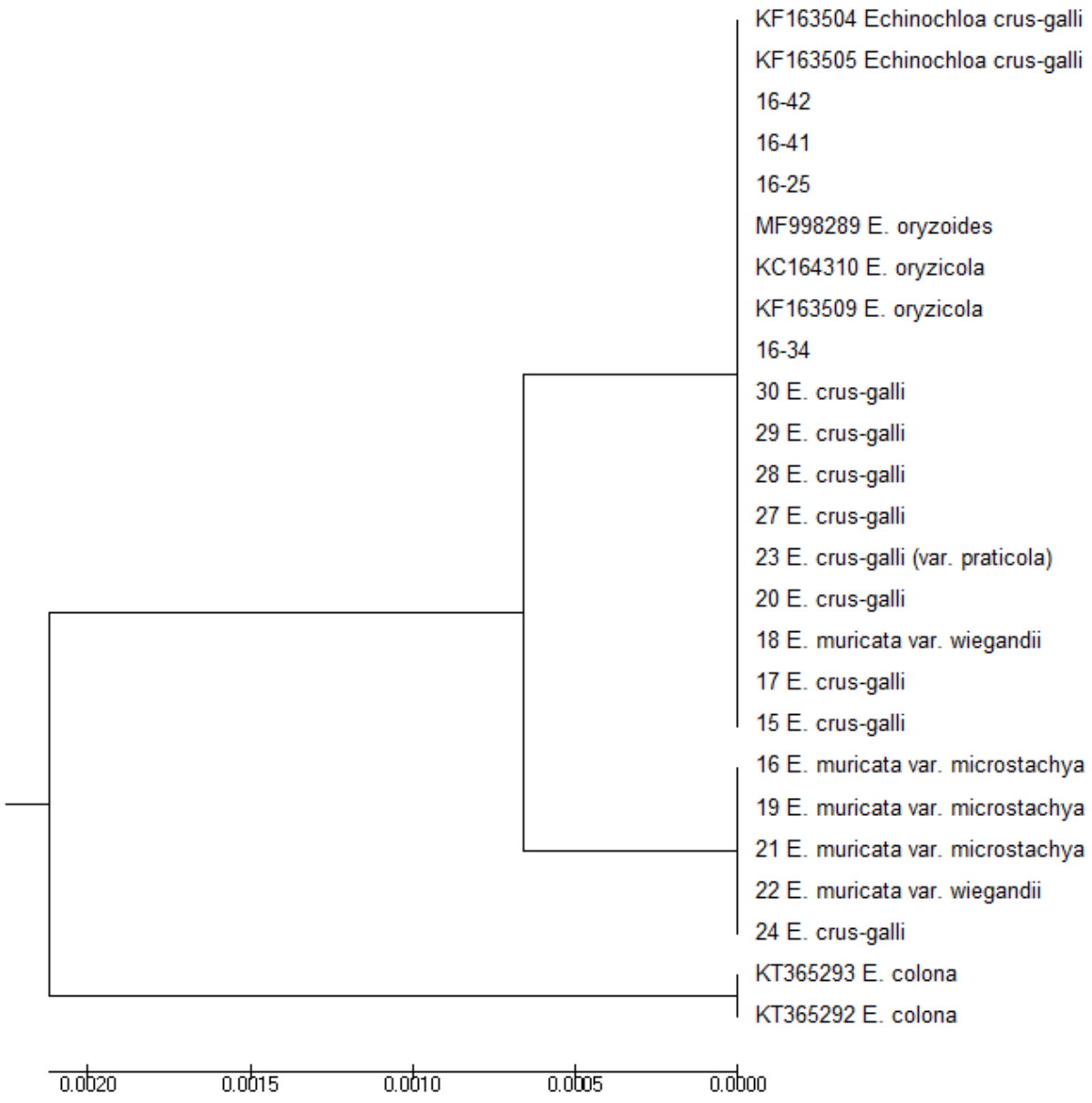


Fig. 30: UPGMA tree built on rbcL sequences of the Flanders accessions. These sequences are compared with vouchered ones belonging to other species and three Italian classified accessions: 16-25 (unclassifed *Echinochloa*), 16-41 (*E. phyllopogon*) and 16-42 (*E.oryzicola*).

3.2.5 Matching phenotype classification with molecular markers discrimination

Molecular discrimination was repeated on reproduced accessions and used as benchmark for match with the dichotomous keys. Results of molecular analyses are consistent with those made on original plants.

The high quantity of seed and plants with intermediate traits (e.g. those with traits that could lead to multiple classifications) encountered during each morphological classification suggests that when trying to classify *Echinochloa* spp. a higher number of spikelets and plants must be analyzed.

Also in this case rbcL was able to discriminate among two species: *E. oryzicola* and *E. crus-galli* matching Costea & Tardif (2002) and Tabacchi & Viggiani (2017) classification (Fig. 31).

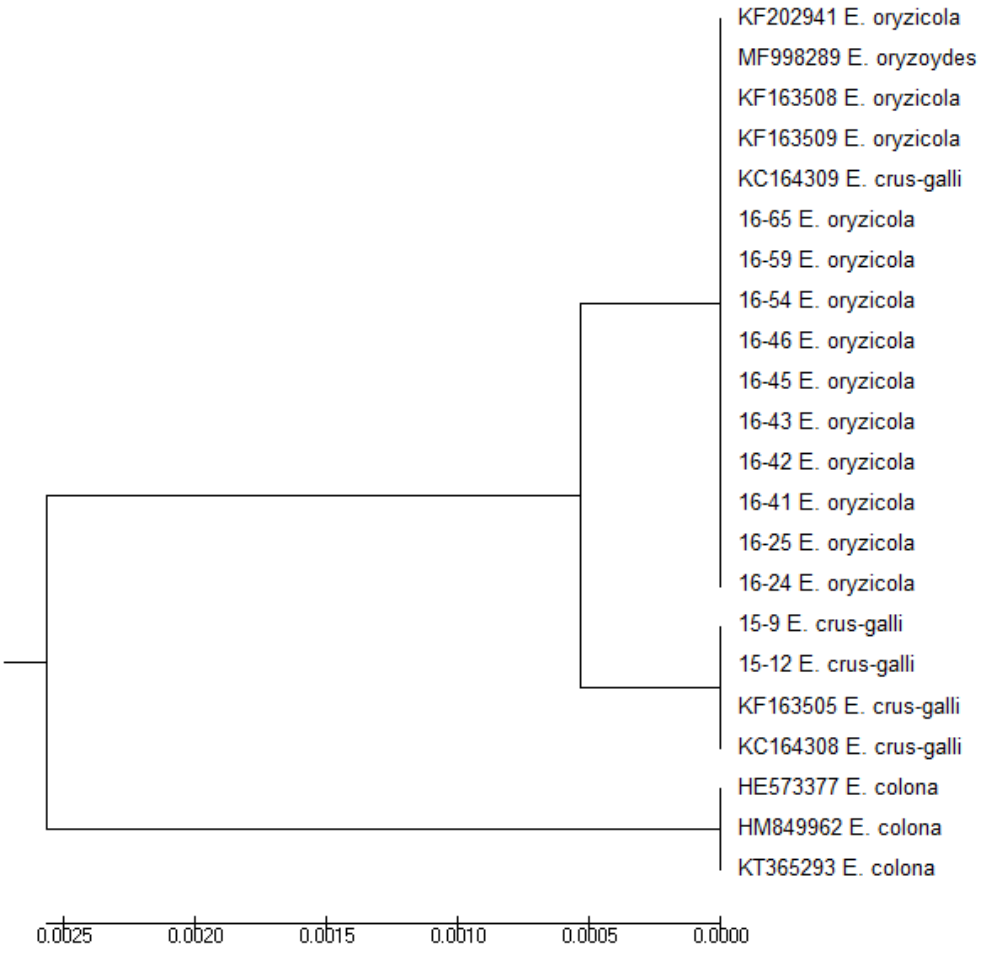


Fig. 31: UPGMA dendrogram of rbcL for reproduced accessions. Classification according to Costea&Tardif was added.

MatK discrimination reflects better than that of rbcL the complexity of morphological traits encountered among our *Echinochloa* spp. accessions. Using this molecular marker, it is possible to discriminate among three different species: *E. crus-galli*, *E. phyllopogon* and *E. oryzicola* (Tabacchi *et al.*, 2006) as described in Fig. 32.

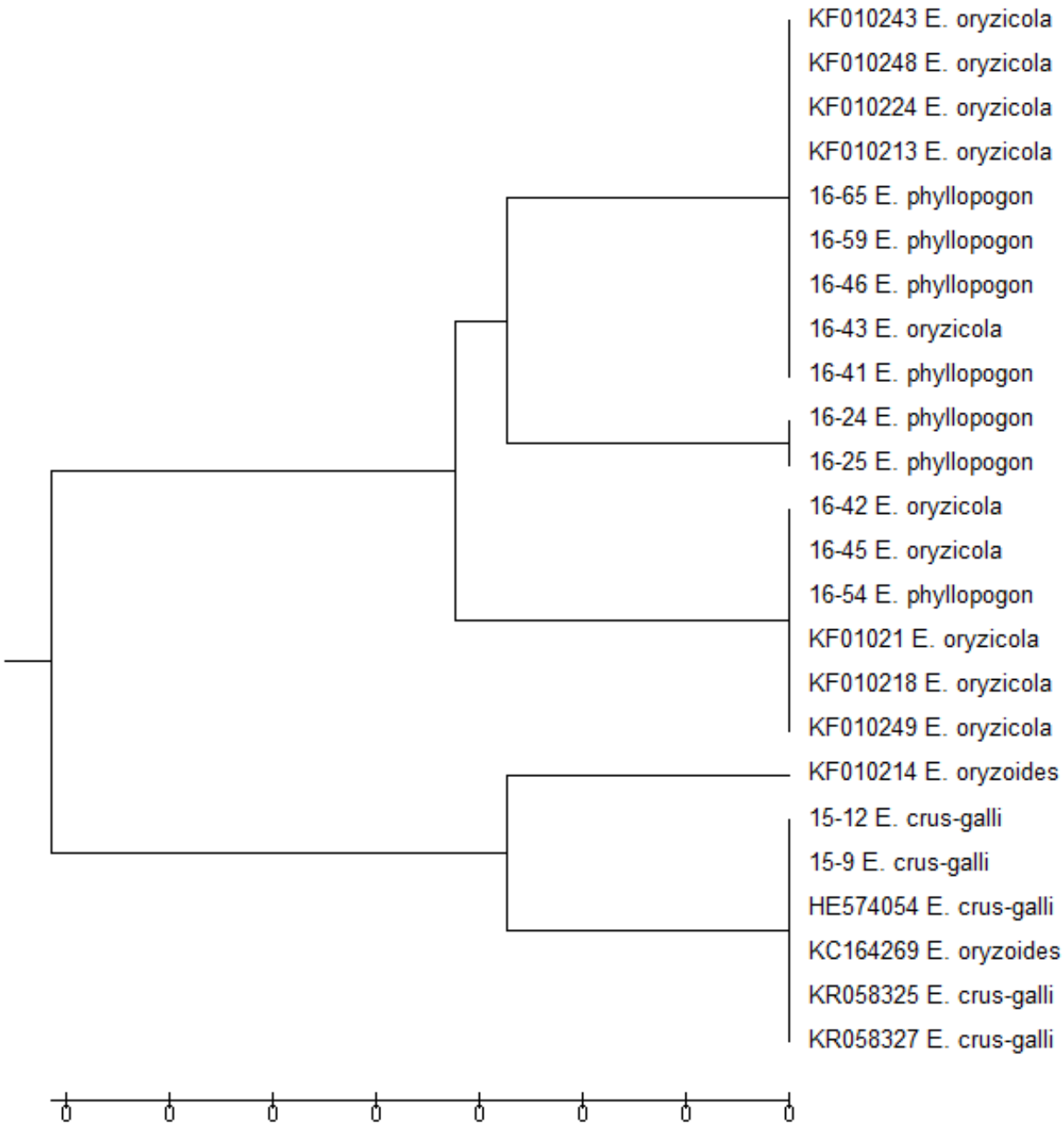


Fig. 32: UPGMA dendrogram of matK for reproduced accessions. Classification according to Tabacchi *et al.* (2006) was added.

The same discrepancies highlighted in matK discrimination on original plants were found in reproduced accessions, as expected: 16-43 was classified as *E. oryzicola*, but genetically clusterizes, in matK dendrogram, with *E. phyllopogon*. The opposite situation happened for accession 16-54: classified as *E. phyllopogon* it clusterizes with *E. oryzicola*.

A final sequence comparison was performed putting together sequences of matK, rbcL and psbA-trnH (CBOL Plant Working Group, 2009). The UPGMA tree built on reproduced accessions confirms the presence of multiple clusters: one containing the *E. phyllopogon* plus the accession 16-43, one containing the two accessions of *E. oryzicola* plus accession 16-54, one with accessions 16-24, 16-25 and 16-46 and one with *E. crus-galli* (Fig. 33).

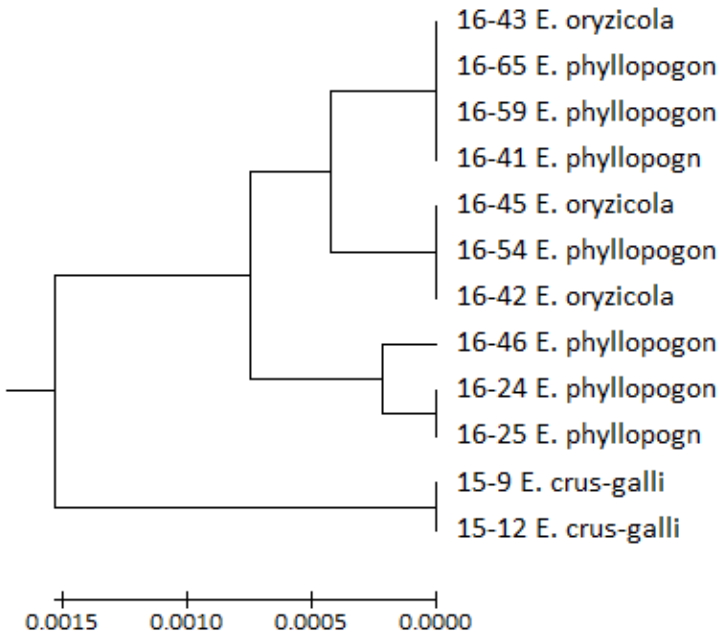


Fig. 33: UPGMA tree built on reproduced accessions for matK, rbcL and psbA-trnH together.

It was confirmed that *E. oryzoides* and *E. colona* are absent in our bulk of accessions.

Although more similar to *E. oryzicola* for many morphological traits such as the size of the spikelet and caryopsis (Costea & Tardif, 2002; Tabacchi & Viggiani, 2017), it is still debated whether *E. oryzoides* and *E. crus-galli* belong to the same species as multiple studies reached this conclusion using different molecular tools (Asíns *et al.*, 1999; Tabacchi *et al.*, 2006; Yamaguchi *et al.*, 2005; Ye *et al*., 2014). MatK gene and trnL analyses performed confirmed this result.

Accession 16-24 and 16-25 should be confirmed as "purple variations" of *E. phyllopogon* (Pignatti, 1981; Tabacchi *et al.*, 2006) or of *E. oryzicola* (Tabacchi & Viggiani, 2017; Costea & Tardif, 2002) meaning that the SNP highlighted in matK sequences are not significant for species discrimination. Results of psbA-trnH analyses confirmed this hypothesis, in fact the SNP found in accessions 16-24 and 16-25 were common to other accessions of *E. phyllopogon*: i.e. 16-46.

3.2.6 SS-PCR

Results of Specie-Specific (SS) – PCR made possible to discriminate *E. phyllopogon* from *E. oryzicola* and the "unclassified SE" *Echinochloa* in one single reaction of PCR.

Protocol was tuned on matK gene, using accessions 16-42 (*E. oryzicola*), 16-25 (*Echinochloa* SE), and 16-65 (*E. phyllopogon*).

The best results were achieved using the combination of F3_ERE + F4_SE couple of forward primers.

Results of the SS-PCR show 2 amplicons: one of 122 bp for *Echinochloa SE* and one of 248 bp for *E. oryzicola*. No amplicons were produced – as expected – for *E. phyllopogon* (Fig. 34).

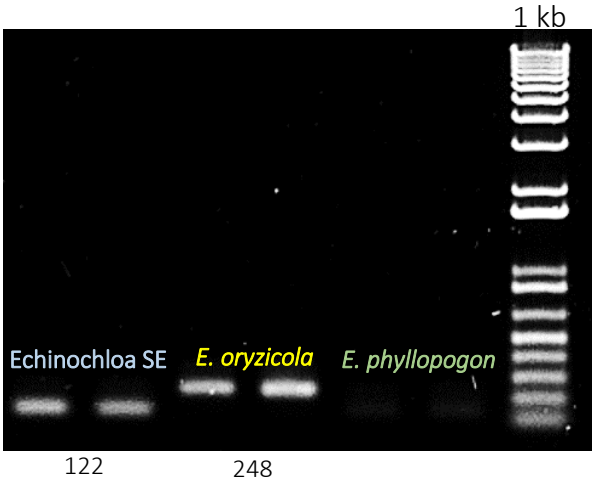


Fig. 34: results of SS-PCR. Different amplicons of different species are represented: 122 bp for *E. oryzicola*, 248 bp for *Echinochloa* SE and no amplicons for *E. phyllopogon.*

3.2.7 Dose-response experiments

3.2.7.1 Preliminary screening results

Graphics built on preliminary screening results are reported in Fig. 35 and 36.

For cyhalofop-butyl all accessions showed a high degree of susceptibility with percentage of plant survival inferior to 20% already at 1/4x dose (75 g a.s. ha^{-1}) of the recommended field dose and were completely controlled at 1x dose (300 g a.s. ha^{-1}). The only exception was accession 16-59 which showed 50% (±25.5%) of plant survival with 34.3% (±22.2%) of fresh weight at 1/4x dose, but it was completely controlled when the product was used at full dose.

Also using florpyrauxifen-benzyl all accessions were completely controlled when the product was applied at 1x dose (30 g a.s. ha^{-1}), with the exceptions of accessions 16-24, 16-54 and 15-65 which had 5.6% (±5.6%), 8.3% (± 8.3%) and 5.6% (± 5.6%) of plant survival, respectively. Florpyrauxifen-benzyl showed fresh weight percentage superior to that of cyhalofop-butyl, but this can be due to the different effect of a hormonic herbicide compared to ALS and ACCase inhibitors.

In case of penoxsulam, 15-9 and 15-12 were completely controlled with 10 g a.s. ha^{-1}, while accession 16-65 showed 94.4% (±5.6%) of plant survival. All other accessions showed intermediate plant survival values ranging from 20% to 65.5% at 1/2x dose (Fig. 35). Note that in this probe experiment the 1x dose for penoxsulam was not included, because it has been used in the previous tests (see section 3.2.2), in which all accessions proved to be well controlled at that dose of penoxsulam.

Fig. 35: results of plant survival obtained in the screening performed with cyhalofop-butyl, penoxsulam and florpyrauxifen-benzyl on the 12 selected accessions of *Echinochloa* spp. Colors indicate the different classification in which these accessions were divided according to matK division: red for *E. crus-galli*, yellow for the *E. phyllopogon*, green for *E. oryzicola*, blue for the "unclassified SE" *Echinochloa* and black for those which phenotype does not match with the matK separation.

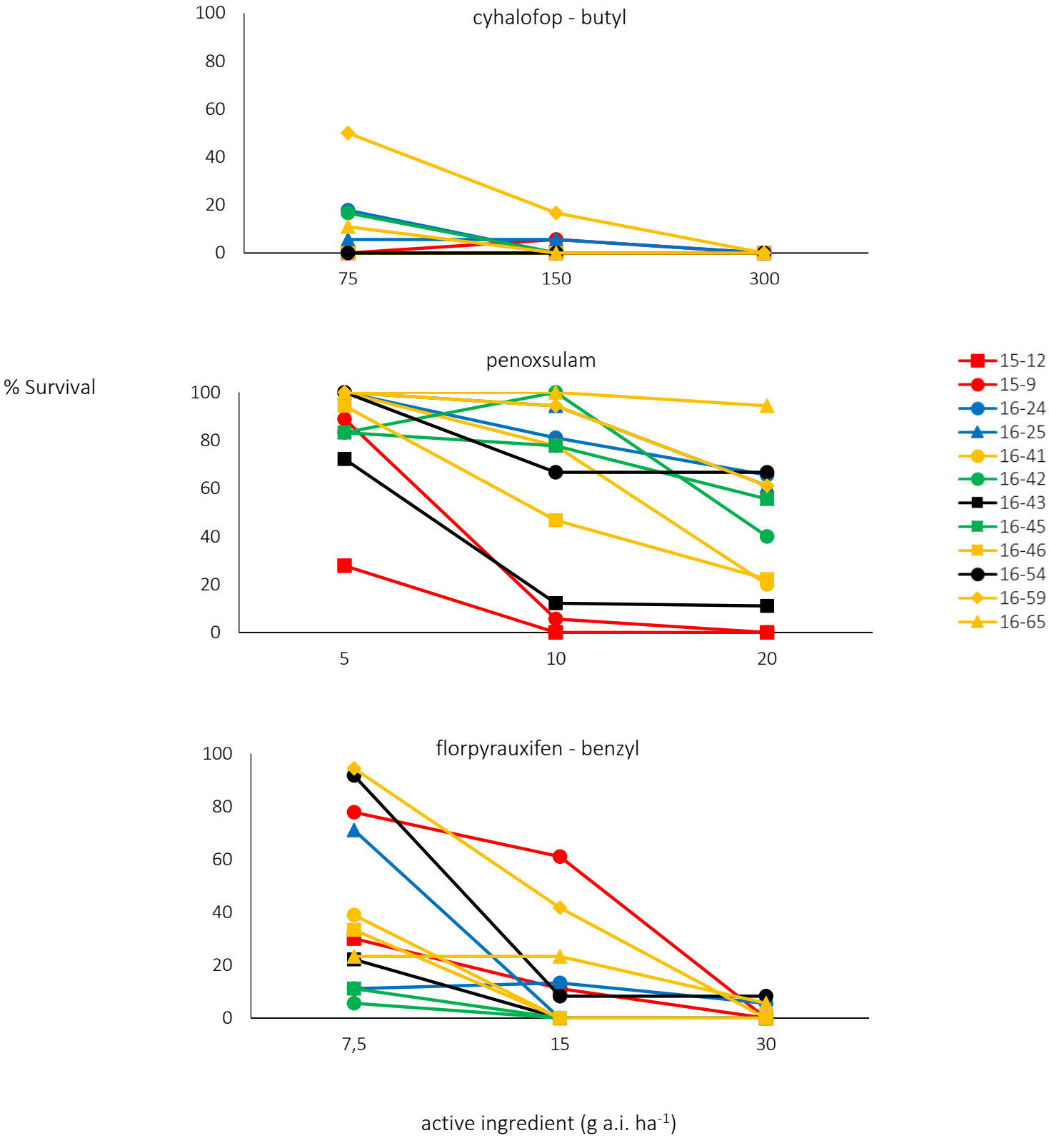
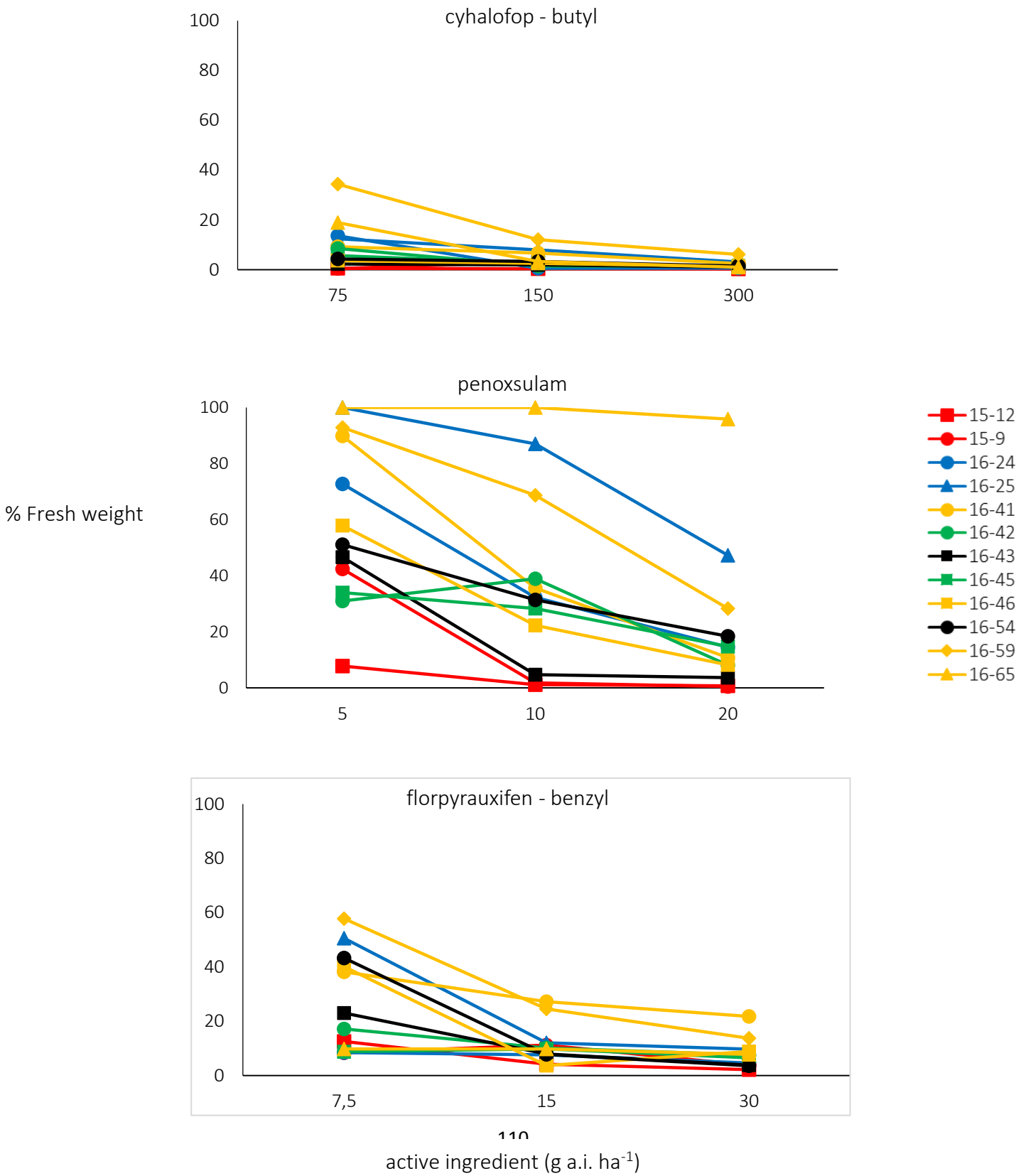


Fig. 36: results of fresh weightl obtained in the screening performed with cyhalofop-butyl, penoxsulam and florpyrauxifen-benzyl on the 12 selected accessions of *Echinochloa* spp. Colors indicate the different classification in which these accessions were divided according to matK division: red for *E. crus-galli*, yellow for the *E. phyllopogon*, green for *E. oryzicola*, blue for the "unclassified SE" *Echinochloa* and black for those which phenotype does not match with the matK separation.



3.2.7.2 Dose-Response pot experiments

Based on the results of the probe screening, nine accessions were chosen for the dose-response experiments. The choice was driven by three factors:

- response to herbicides in the screening: e.g. accession 16-65, which showed the highest rate of plant survival for penoxsulam, was excluded from the dose-response experiments
- morphological and molecular classifications: it was decided to include in the dose-response two populations for each species identified, therefore two accessions of *E. phyllopogon* were excluded
- Field of origin of the accession

Two accessions classified as *E. phyllopogon* (16-41 and 16-59), two classified as *E.oryzicola* (16-42 and 16-45) and two with a "cross classification" (16-43 and 16-54), i.e. 16-43 showed the phenotype of *E.oryzicola*, but matK and psA-trnH clusterisation classified it as *E. phyllopogon*, the opposite happened for 16-54.

Accession 16-25 was also included in the experiment on the base of molecular markers discrimination results and peculiar morphologic characteristics and treat it as a separate species even if it was demonstrated that it is not. Accessions 15-12 and 15-9 *E. crus-galli* were also chosen for the tests.

The two experiments were performed in different environments, so results cannot be mediated.

Results of 2017 experiment were very variable, for this reason only 2018 results will be discussed in details. Graphs of plant survival and fresh weight data of 2017 and 2018 dose-response experiments are included in Appendix I.

Standard errors of 2018 experiment, calculated with the non-linear logistic regression were <10% for all ED_{50}, indicating that the log-logistic equation fitted the data without any data transformation (λ=1) and the herbicides dose range used in the test was appropriate (Tab. XXIV).

Tab. XXIV: results of 2018 dose-response analyses. ED_{50} (GR_{50}) and slopes are displayed for % of plant survival and for % of fresh weight, respectively. Standard error (SE) is reported in brackets. For each accession is shown the species into which it was classified, the herbicides applied and the type of assessment - % survival or fresh weight – conducted. **(*)**: for these accessions the phenotype and clusterization for matK and psbA-trnH did not match.

Accession code	Classification	cyhalofop - butyl		penoxsulam		fluorpyrauxifen - benzyl	
		ED_{50} (g a.s. ha^{-1})	Slope	ED_{50} (g a.s. ha^{-1})	Slope	ED_{50} (g a.s. ha^{-1})	Slope
15-12	*E. crus-galli*	32.3 (2.7)	4.9 (2.0)	5.9 (0.4)	2.9 (0.4)	21.9 (1.1)	4.6 (0.6)
15-9	*E. crus-galli*	51.9 (1.4)	6.2 (0.9)	6.0 (0.7)	2.0 (0.4)	18.3 (0.7)	4.6 (0.6)
16-25	*Echinochloa* spp.	104.4 (4.5)	4.2 (0.5)	22.0 (0.8)	3.6 (0.5)	14.7 (0.6)	4.6 (1.3)
16-41	*E. phyllopgon*	63.4 (2.4)	5.2 (0.9)	2.8 (0.4)	2.8 (0.4)	11.9 (1.2)	2.4 (0.5)
16-59	*E.phyllopogon*	300.1 (11.3)	4.8 (1.8)	23.2 (3.1)	2.5 (0.3)	54.3 (6.5)	3.1 (0.7)
16-42	*E. oryzicola*	43.4 (3.4)	2.4 (0.5)	7.4 (0.4)	2.7 (0.6)	13.3 (1.0)	2.3 (0.3)
16-45	*E. oryzicola*	33.2 (2.0)	3.1 (0.6)	10.9 (0.8)	3.1 (0.7)	8.7 (1.4)	1.9 (0.5)
16-43	*E. oryzicola* (*)	36.8 (3.9)	2.8 (0.6)	5.4 (0.5)	3.1 (0.5)	15.6 (0.9)	2.6 (0.4)
16-54	*E. phyllopgon* (*)	108.5 (10.1)	4.5 (1.1)	19.1 (1.1)	1.9 (0.4)	24.4 (2.0)	2.8 (0.4)

Accession code	Classification	cyhalofop - butyl		penoxsulam		fluorpyrauxifen – benzyl	
		GR_{50} (g a.s. ha^{-1})	Slope	GR_{50} (g a.s. ha^{-1})	Slope	GR_{50} (g a.s. ha^{-1})	Slope
15-12	*E. crus-galli*	29.1 (3.8)	4.7 (1.1)	1.3 (0.2)	1.3 (0.2)	6.9 (0.3)	2.0 (0.1)
15-9	*E. crus-galli*	40.9 (3.8)	7.5 (7.6)	2.4 (0.2)	1.4 (0.2)	7.5 (0.3)	3.0 (0.4)
16-25	*Echinochloa* spp.	76.5 (3.5)	2.3 (0.2)	17.6 (1.3)	2.4 (0.4)	13.6 (0.7)	3.1 (0.5)
16-41	*E. phyllopgon*	47.9 (2.7)	2.3 (0.3)	5.4 (0.3)	2.0 (0.2)	7.6 (0.6)	2.7 (0.5)
16-59	*E.phyllopogon*	212.8 (9.8)	1.5 (0.2)	10.4 (1.5)	1.4 (0.2)	16.7 (1.4)	2.9 (0.4)
16-42	*E. oryzicola*	20.4 (1.6)	1.6 (0.2)	1.7 (0.2)	1.5 (0.2)	8.5 (0.5)	2.2 (0.2)
16-45	*E. oryzicola*	24.7 (0.7)	4.7 (0.4)	3.7 (0.4)	1.3 (0.2)	5.2 (0.3)	2.3 (0.3)
16-43	*E. oryzicola* (*)	17.6 (1.3)	2.1 (0.4)	2.1 (0.2)	1.4 (0.2)	5.0 (0.3)	2.0 (0.3)
16-54	*E. phyllopgon* (*)	88.0 (7.8)	2.5 (0.2)	4.8 (0.5)	1.5 (0.3)	8.0 (0.6)	1.5 (0.2)

For cyhalofop–butyl (Fig. 37), it was assessed the highest degree of variability in the response of the different species. No difference in susceptibility were assessed among species, contrary to previous publication (Vidotto *et al*., 2007) where accessions of *E. crus-galli* resulted significantly more sensitive to this herbicide when applied at field dose. The variability was similar in the two experiments. The two accessions of *E. oryzicola*, 16-42 and 16-45, showed susceptibility levels closer to that of *E. crus-galli* and both species are in the lowest part of the graphic, seemingly more sensitive than the other accessions. The highest variability was detected in *E. phyllopogon* (16-41 and 16-59) accessions. Accession 16-59 showed ED_{50} and GR_{50}, respectively, five times and four times higher than 16-41: the first had ED_{50} and GR_{50} equal to 300.1 (±11.3) and 212.8 (± 9.8) g a.s. ha^{-1}, while these values in accession 16-41 were equal to 63.4 (±2.4) and 47.9 (±2.7) g a.s. ha^{-1}. Similar differences were registered between the two "crossing" accessions, i.e. 16-43 and 16-54. In this case ED_{50} and GR_{50} of 16-54 were, respectively, three and five times higher than those of 16-43.

Florpyrauxifen–benzyl response was less differentiated among species (Fig. 38), only accession 16-59 showed ED_{50} value of 54.3 (±4.5) sensibly superior to the others and 16-45 inferior to the others and equal to 8.7 (±1.4). Accessions 15-9 and 15-12 responded similarly to each other, but their susceptibility was not different from the "white" accessions.

Dose response of accessions in 2018 experiment was less variable than in 2017: all accessions here, but 16-59, were controlled at 2x rate of this herbicide (60 g a.s. ha^{-1}).

The high variability assessed in 2017 experiment could be due to the high temperatures recorded during the course of the trial in June-July of 2017 (See Appendix II).

For penoxsulam (Fig. 39), *E. crus-galli* seemed slightly more sensitive than the other species: i.e. accessions 15-9 and 15-12 had ED_{50} of 5.6 and 6.0 g a.s. ha^{-1}, respectively. A similar response was given by accessions 16-41, 16-42 and 16-43, which ED_{50} was respectively of 2.8, 7.5 and 5.4 g a.s. ha^{-1}, whereas the value of ED_{50} for other accessions was on average equal to 18.8 g a.s. ha^{-1}.

We cannot affirm that there is a specie-specific response of *Echinochloa* to penoxsulam. However, our results seem to reflect the penoxsulam herbicide strategy used in rice fields: treatment is anticipated at 2-3 leaves stage in case of mixed infestation of "white" and "red"

Echinochloa, while *E. crus-galli* alone is sensitive to this herbicides up to two tillers stage (i.e. *E. crus-galli*, in the field, is more sensitive to penoxulam than other species).

In general we can say that, relatively to the performance of the different species, the two experiments provided similar results. In both cases, variability of response among species was high and it was not possible to find a common pattern for each combination species x herbicide.

It is worth to highlight that in 2017 experiment, for all of the three herbicides, fresh weight of plants decreased more rapidly than in 2018 (See Appendix I).

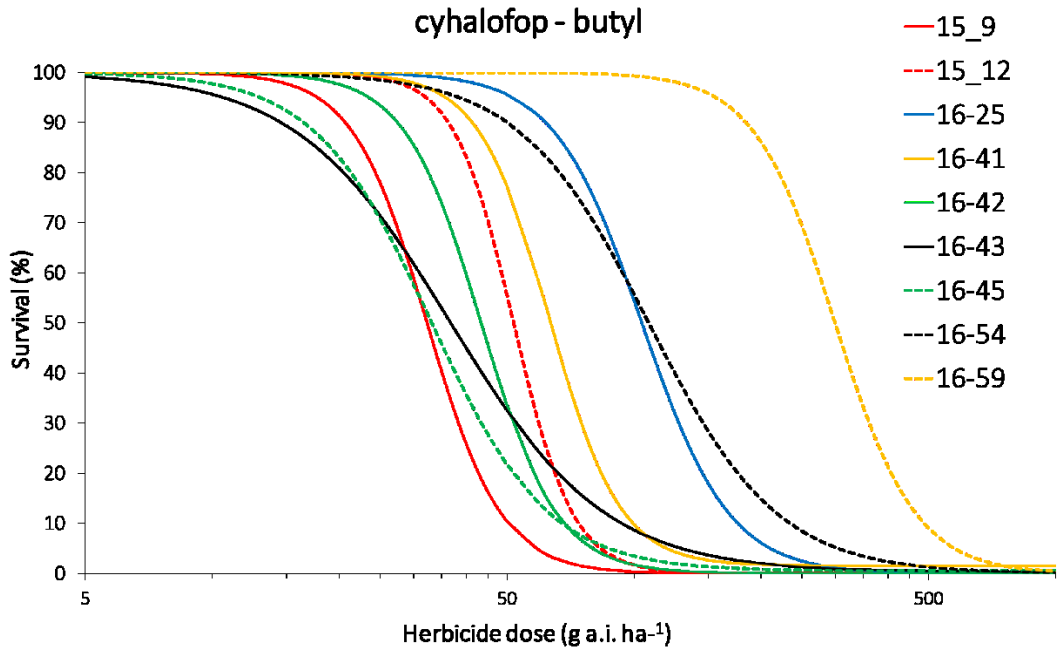
In general, accession 16-59 showed a higher ability in herbicides detoxification in comparison with the other accessions. As a trend, we can say that *E. crus-galli* and *E. oryzicola* seem more sensitive to the three herbicides tested, as proved by their, on average, lower values of ED_{50} and GR_{50}.

Results were confirmed by the lack-of-fit F test performed on both plant survival and fresh weight. For all of the three herbicides testes, in many cases it was possible to simplify the regression to a model with common slopes, but ED_{50} always resulted statistically different, except in the case of response to penoxsulam of *E. crus-galli* (15-9 and 15-12) accessions for both plant survival and fresh weight.

This indicated that accessions belonging to the same species equally respond to the increment of the dose of herbicides, but the dose required to achieve the control of plants is different. Therefore, it was not possible to demonstrate that different species of *Echinochloa* have different response to herbicides, independently from the dichotomous key used for plant classification. If we use Costea & Tardif (2017) classification instead of Tabacchi *et al.* (2006), we can verify that all accession classified as *E. oryzicola* respond differently to the various three herbicides tested: i.e. ED_{50} and GR_{50} are always statistically different.

This is the first case that this type of experiment was conducted on purified accessions of *Echinochloa* spp. previously tested for their susceptibility.

Fig. 37: Dose response curves of cyhalofop–butyl in 2018 experiment using the log-logistic model. Both survival (Top) and Fresh Weight (Bottom) are displayed. Lines of different color represent the different species of *Echinochloa*: red for *E. crus-galli*, green for *E. oryzicola*, blue for the "unclassified SE", yellow for *E. phyllopogon*, black for the two accession for which molecular marker discrimination did not match with the phenotypic classification.



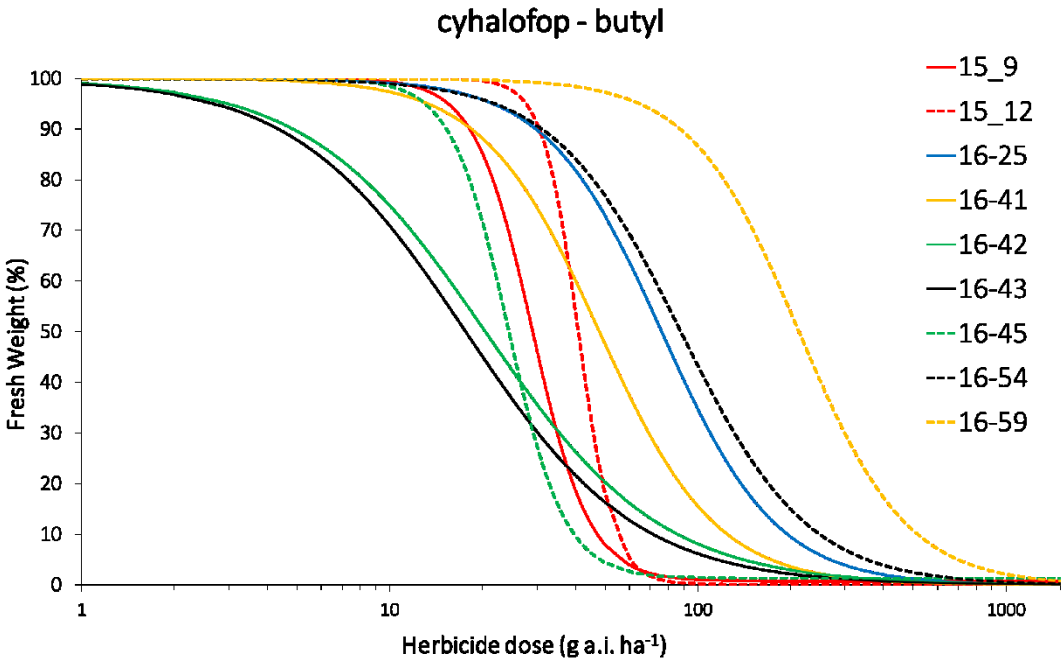
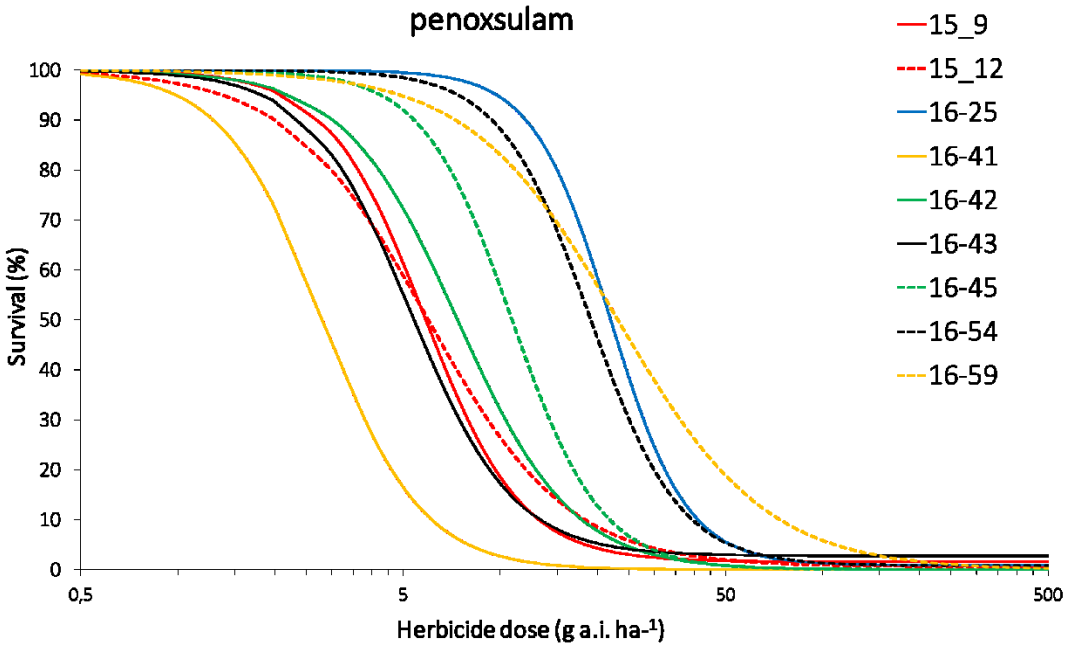


Fig. 38: Dose response curve of penoxsulam in 2018 experiment using the log logistic model. Both survival (Top) and Fresh Weight (Bottom) are displayed. Lines of different color represent the different species of *Echinochloa*: red for *E. crus-galli*, green for *E. oryzicola*, blue for the "unclassified SE", yellow for *E. phyllopogon*, black for the two accession for which molecular marker discrimination did not match with the phenotypic classification.



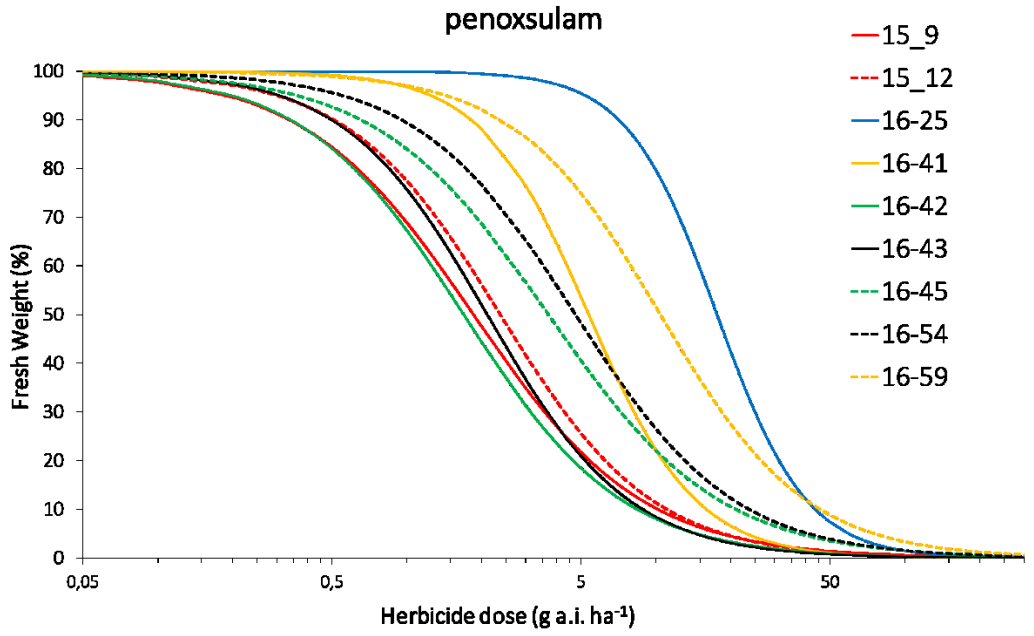
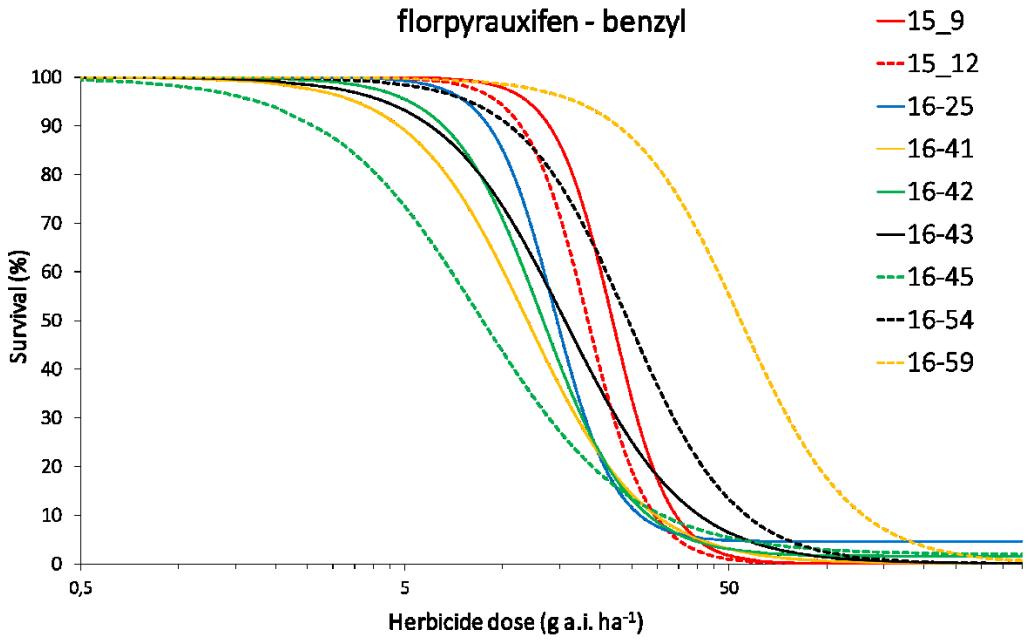
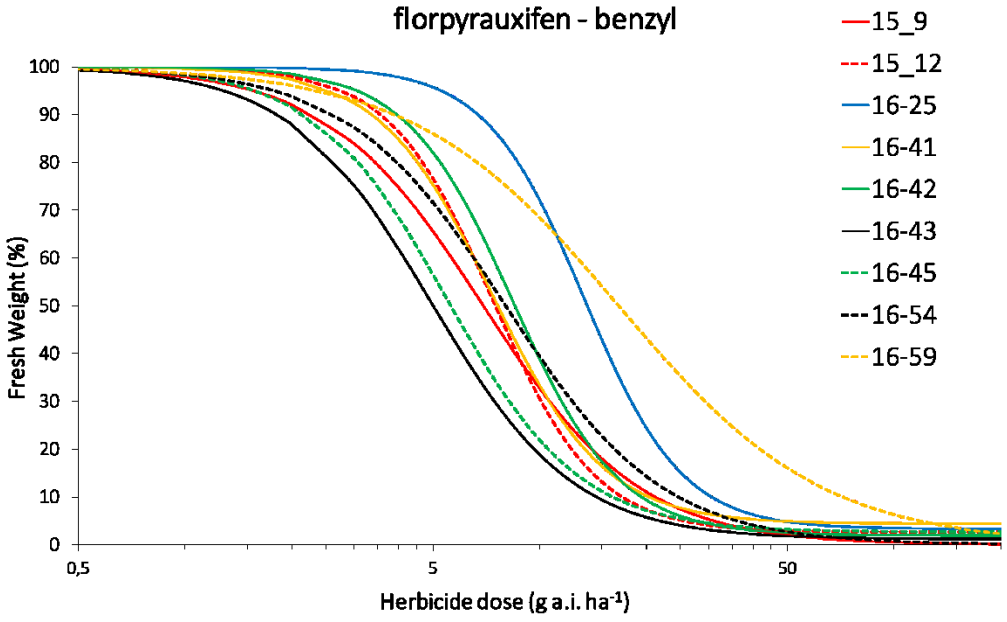


Fig. 39: Dose response curve of florpyrauxifen - benzyl in 2018 experiment using the log logistic model. Both survival (Top) and Fresh Weight (Bottom) are displayed. Lines of different color represent the different species of *Echinochloa*: red for E. crus-galli, green for *E. oryzicola*, blue for the "unclassified SE", yellow for *E. phyllopogon*, black for the two accession for which molecular marker discrimination did not match with the phenotypic classification.





3.2.8 SPECIFIC CONCLUSIONS

Results of the combination of classic taxonomic classification, molecular markers discrimination and dose-response experiments on multiple purified and sensitive *Echinochloa* spp. accessions led us to multiple conclusions.

Molecular markers utilized in this study provided a good, but not complete discrimination among the different species of *Echinochloa* spp.: rbcL was the one that provided the best match with two of the classifications included in this study: i.e. Costea&Tardif (2002) and Tabacchi & Viggiani (2017) which only take into account very small traits of the plants, such as spikelets and caryopses, thus not always suitable for field classification.

Discrimination provided by matK, is the one which betters describes the complexity of composition of *Echinochloa* spp. populations in Italian rice fields, although discrepancies are present. When approaching species discrimination a higher number of accessions must be considered in the study, especially when studying a genus like *Echinochloa*, which shows a high degree of plasticity in each morphological trait. DNA barcoding approach has proved to be a reliable tool for weed genome analyses and we wish that it will be implemented in the near future.

Dose-response experiments results suggest that differences in the response to herbicides of the different *Echinochloa* species might be more related with tolerance/resistance issues or other phenotypical and physiological traits (e.g. germination delay), than to plant morphology (Vidotto *et al.*, 2007). On the base of our results, planning a field herbicide strategy on the base of *Echinochloa* species composition might be misleading, both for technicians and farmers. Furthermore, herbicide susceptibility cannot be considered a discrimination trait for *Echinochloa* spp. with the herbicides now available.

CHAPTER IV

GENERAL CONCLUSIONS

4 RESEARCH CONCLUSIONS

The research tackled two important aspects of herbicide resistance in Italian rice fields: epidemiology and classification of *Echinochloa* spp. as well as the interaction between *Echinochloa* species and the most commonly used herbicides.

The epidemiological study, based on a large area of about 200,000 ha, revealed that the main predictors associated with the presence of herbicide resistance are water seeding and crop rotation and that soil texture has also an impact, even if to a lesser extent.

Resistance is more frequent in areas were diversity in space and time is low and where traditional rice cropping system is more frequent, i.e. where water seeding and lack of crop rotation are common practices. Stochastic maps based on neural network analyses confirm that the risk of resistance evolution is higher in those areas and also exists, even if lower, in areas where resistance had never been recorded.

Random sampling followed by resistance screening confirmed the outcome of the neural network analyses. Resistant *Echinochloa* are present in those areas where previous monitoring based on farmers' complaints had not recorded any case. However, *Echinochloa* spp. density assessed on field was medium-low, likely not alarming farmers that can manage resistance with practices that keep it at an acceptable level: e.g. crop rotation.

Echinochloa spp. study highlighted that it is not possible to find a univocal classification for this genus: published dichotomous keys consider different morphological traits, so the use of one or another leads to different conclusions.

Barcoding proved to be a helpful tool for the discrimination of *Echinochloa* species, although further studies are needed. Molecular marker species discrimination based on different genes matched with different dichotomous keys. Given the complexity of the situation, where multiple *Echinochloa* species often coexist in the same field, the most suitable match between molecular and phenological classifications appeared to be matK gene and the classification proposed by Tabacchi *et al.* (2006) because it allowed to distinguish three species, *E. crus-galli*., *E. phyllopogon* and *E. oryzicola*.

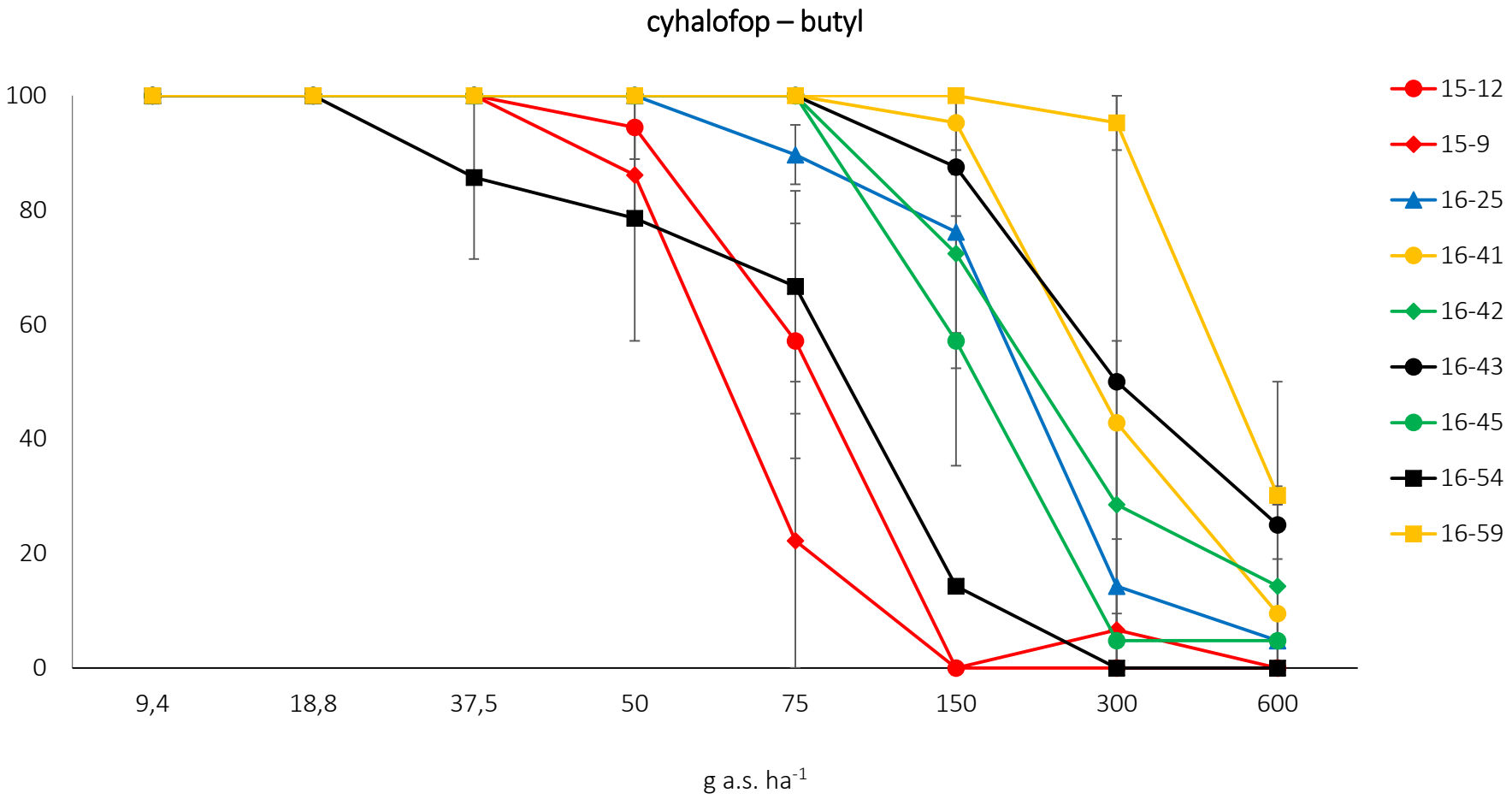
Dose-response studies on susceptible purified accessions highlighted that there is no clear interaction between herbicide efficacy and *Echinochloa* species. *E. crus-galli*, i.e. "red"

Echinochloa, seems to be slightly more susceptible than the “white” species. Nevertheless, variability is high and the average herbicide efficacy is similar among species.

The results suggest that tuning herbicide strategies according to subtle morphological differences of *Echinochloa* species present in the field is wrong, especially in case of resistance management.

APPENDIX I

Fig.I: Data of plant survival assessment in 2017 Dose – Response experiment expressed as percentage (%) of the untreated check for cyhalofop butyl,penoxsulam and florpyrauxifen - benzyl. Vertical bars represent the standard errors. Different colors indicate the different species: red for *E. crus. galli*, green for *E. oryzicola*, blue for the "unclassified SE", yellow for *E. phyllopogon*, black for those accesson for which morphological traits did not match with matK discrimination.



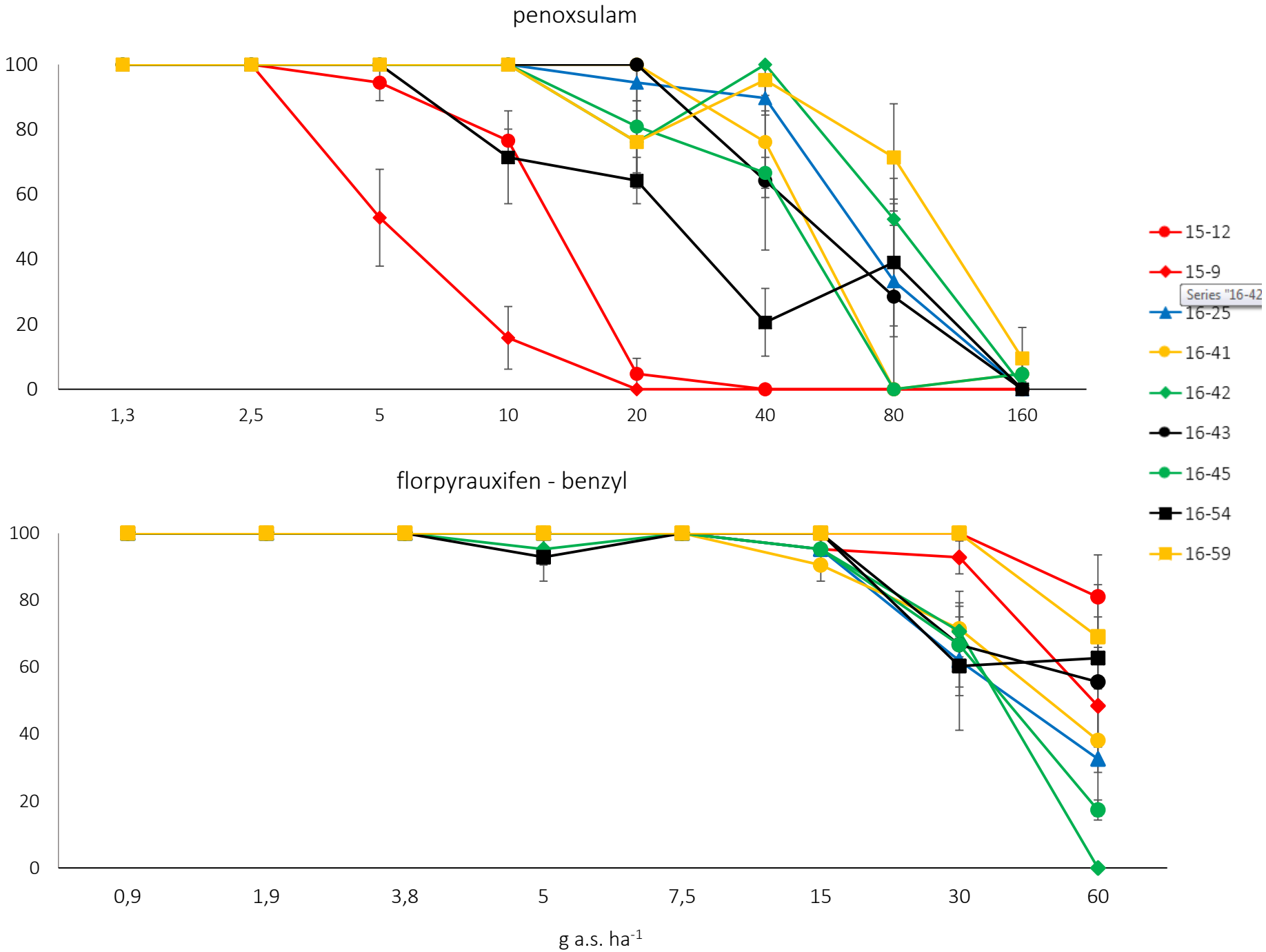

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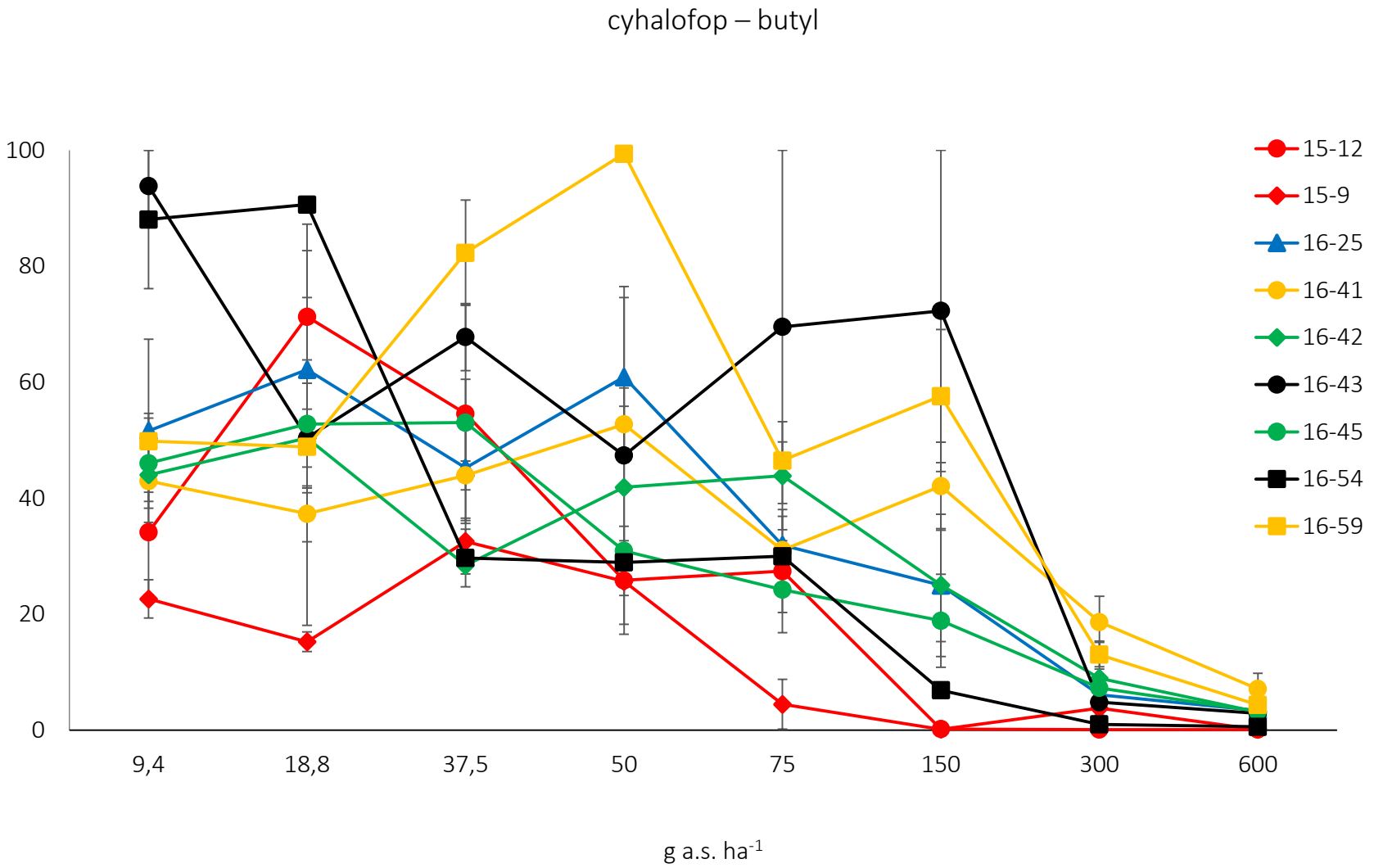
Fig.II: Data of fresh weight assessment in 2017 Dose – Response experiment expressed as percentage (%) of the untreated check for cyhalofop butyl, penoxsulam and florpyrauxifen - benzyl. Vertical bars represent the standard errors. Different colors indicate the different species: red for *E. crus. galli*, green for *E. oryzicola*, blue for the "unclassified SE", yellow for *E. phyllopogon*, black for those accesson for which morphological traits did not match with matK discrimination.



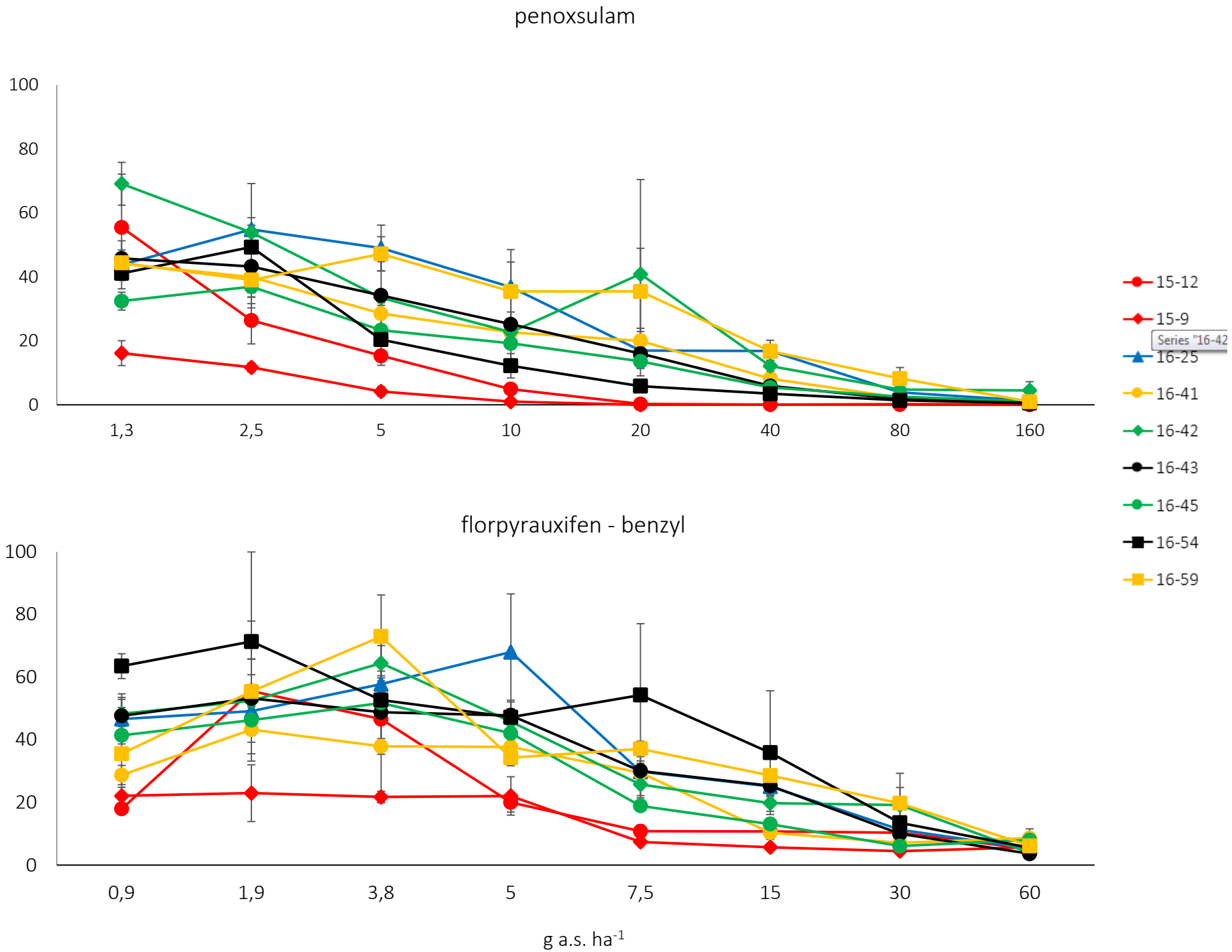

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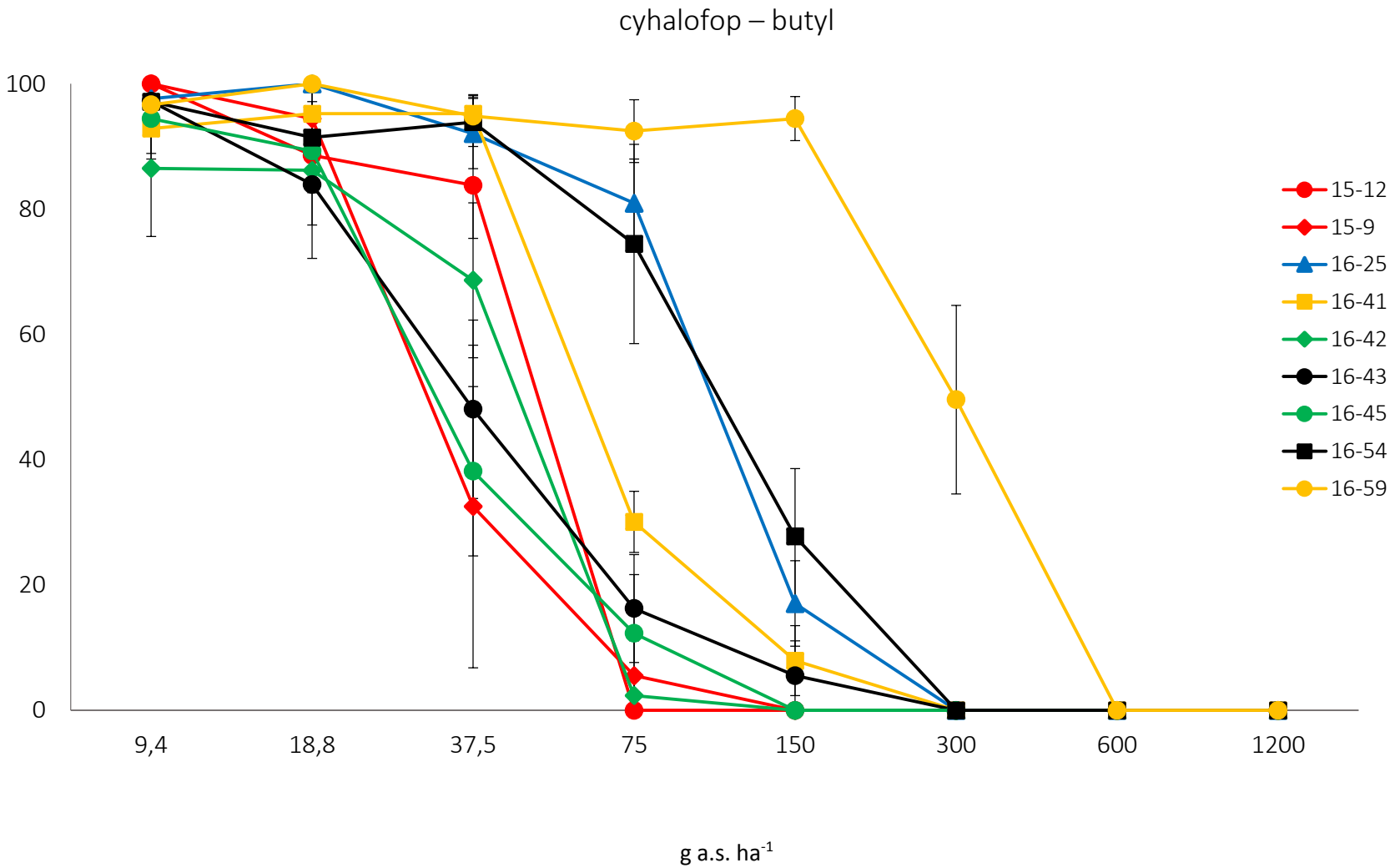
Fig. III: Data of plant survival assessment in 2018 Dose – Response experiment expressed as percentage (%) of the untreated check for cyhalofop butyl, penoxsulam and florpyrauxifen - benzyl. Vertical bars represent the standard errors. Different colors indicate the different species: red for *E. crus. galli*, green for *E. oryzicola*, blue for the "unclassified SE", yellow for *E. phyllopogon*, black for those accesson for which morphological traits did not match with matK discrimination.



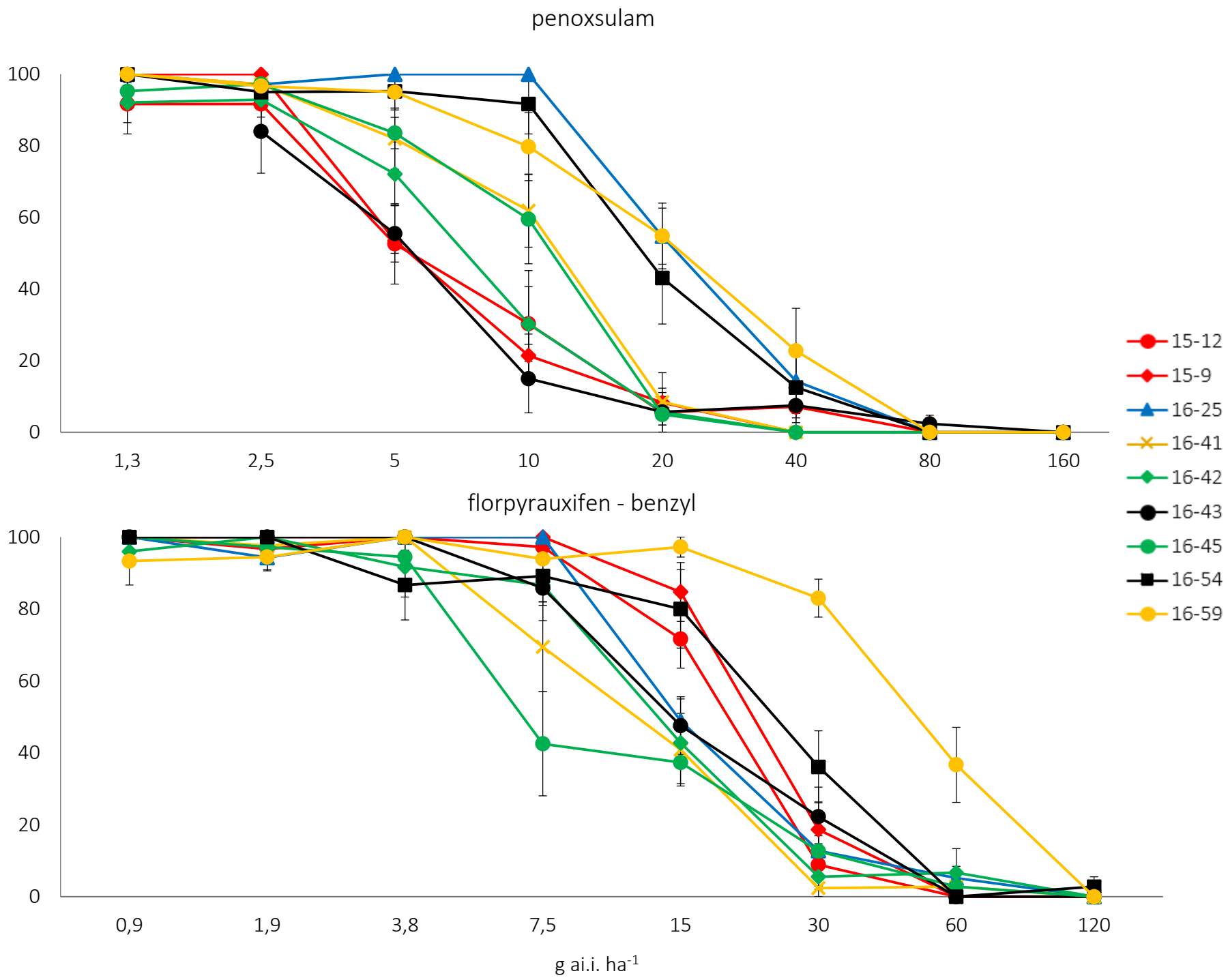

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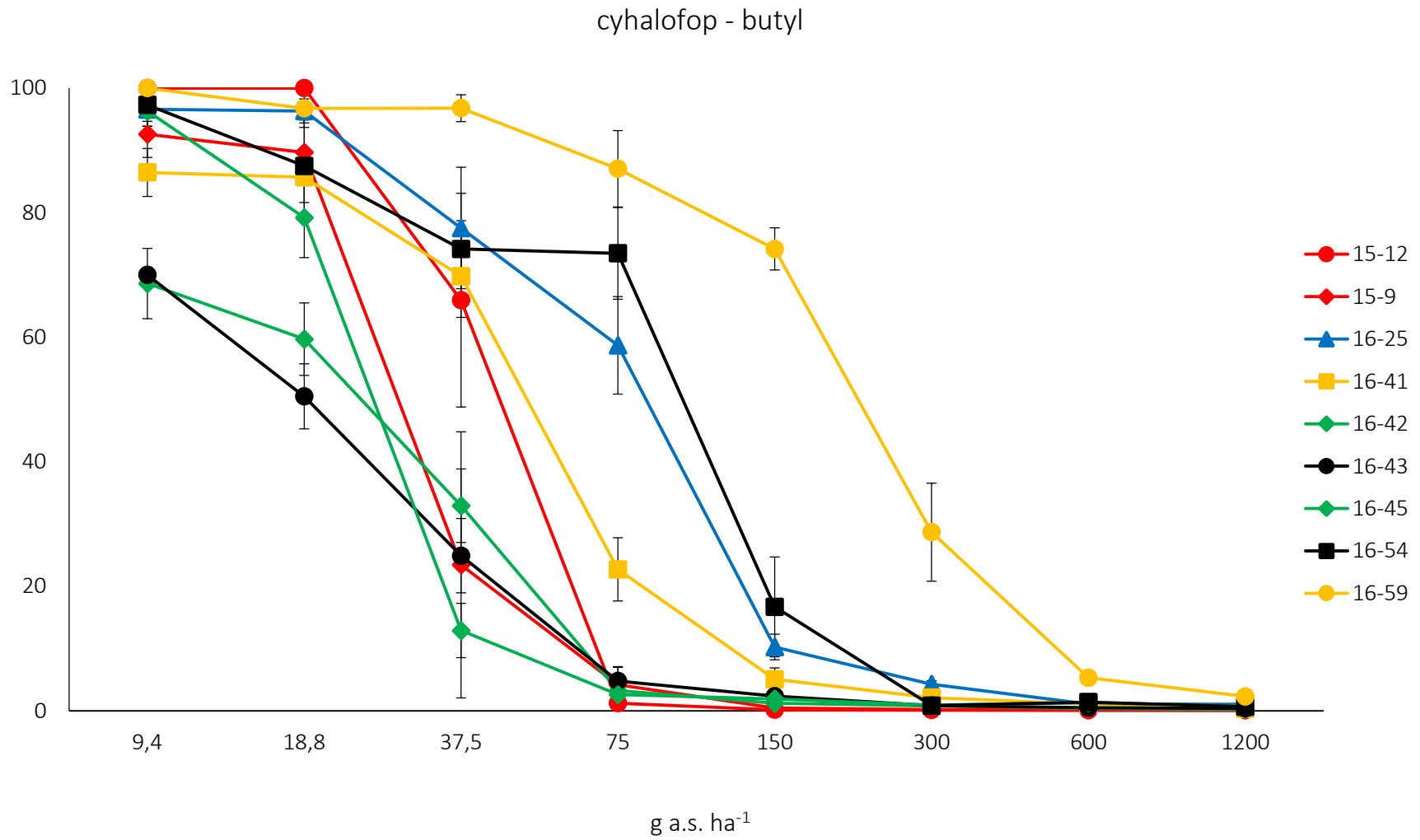
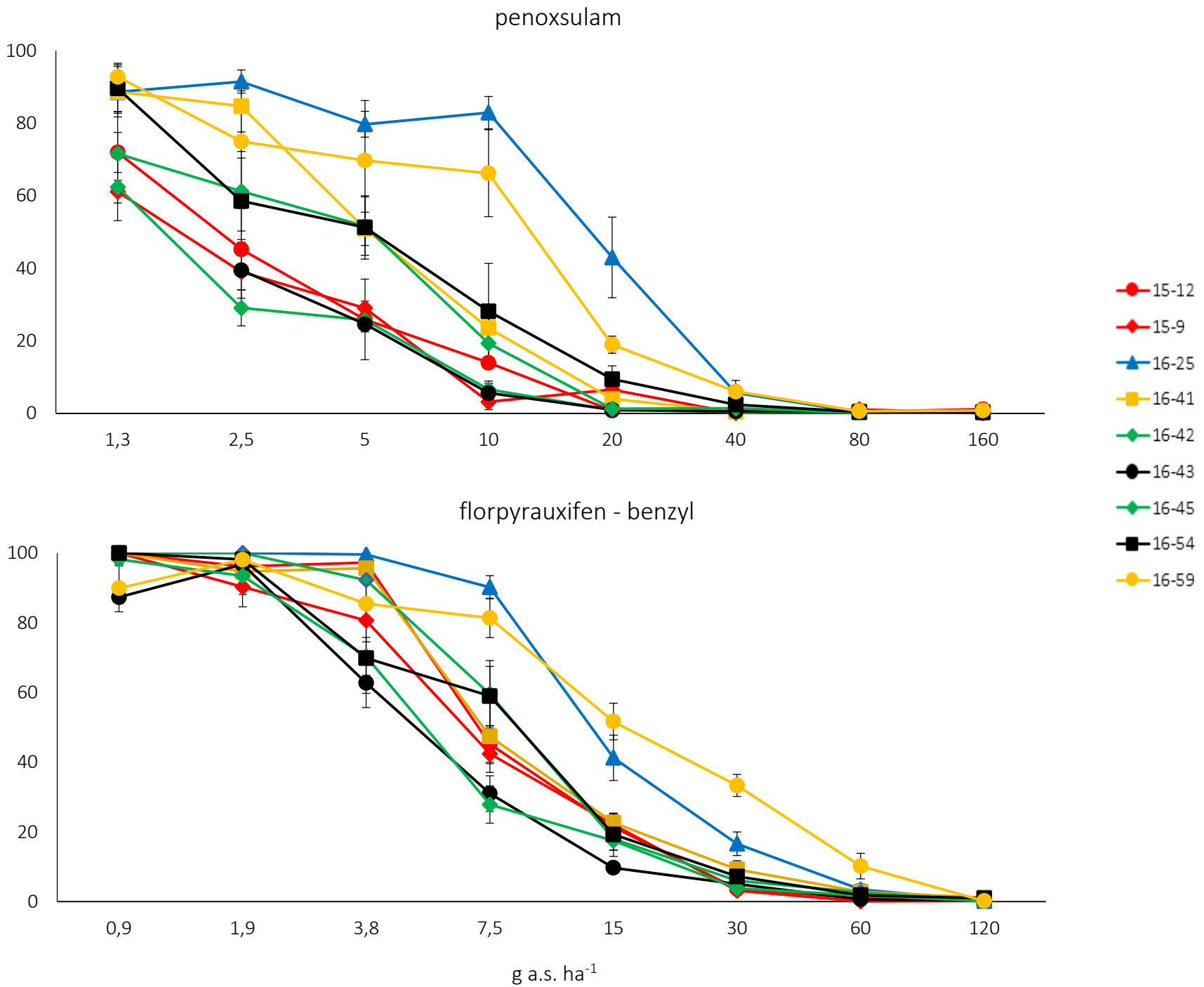
Fig. IV: Data of fresh weight assessment in 2018 Dose – Response experiment expressed as percentage (%) of the untreated check for cyhalofop butyl, penoxsulam and florpyrauxifen - benzyl. Vertical bars represent the standard errors. Different colors indicate the different species: red for *E. crus. galli*, green for *E. oryzicola*, blue for the "unclassified SE", yellow for *E. phyllopogon*, black for those accesson for which morphological traits did not match with matK discrimination.

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APPENDIX II

Fig I: Values of maximum, minumim and average temperature (T) recorded during the course of the dose response trial in June and July 2017.. Plants were transplanted on 15th June 2017, treated on 22nd June 2017 and assessments were performed on 20th (cyhalofop – butyl and penoxsulam) and 26th (florpyrauxifen – benzyl) July 2017.

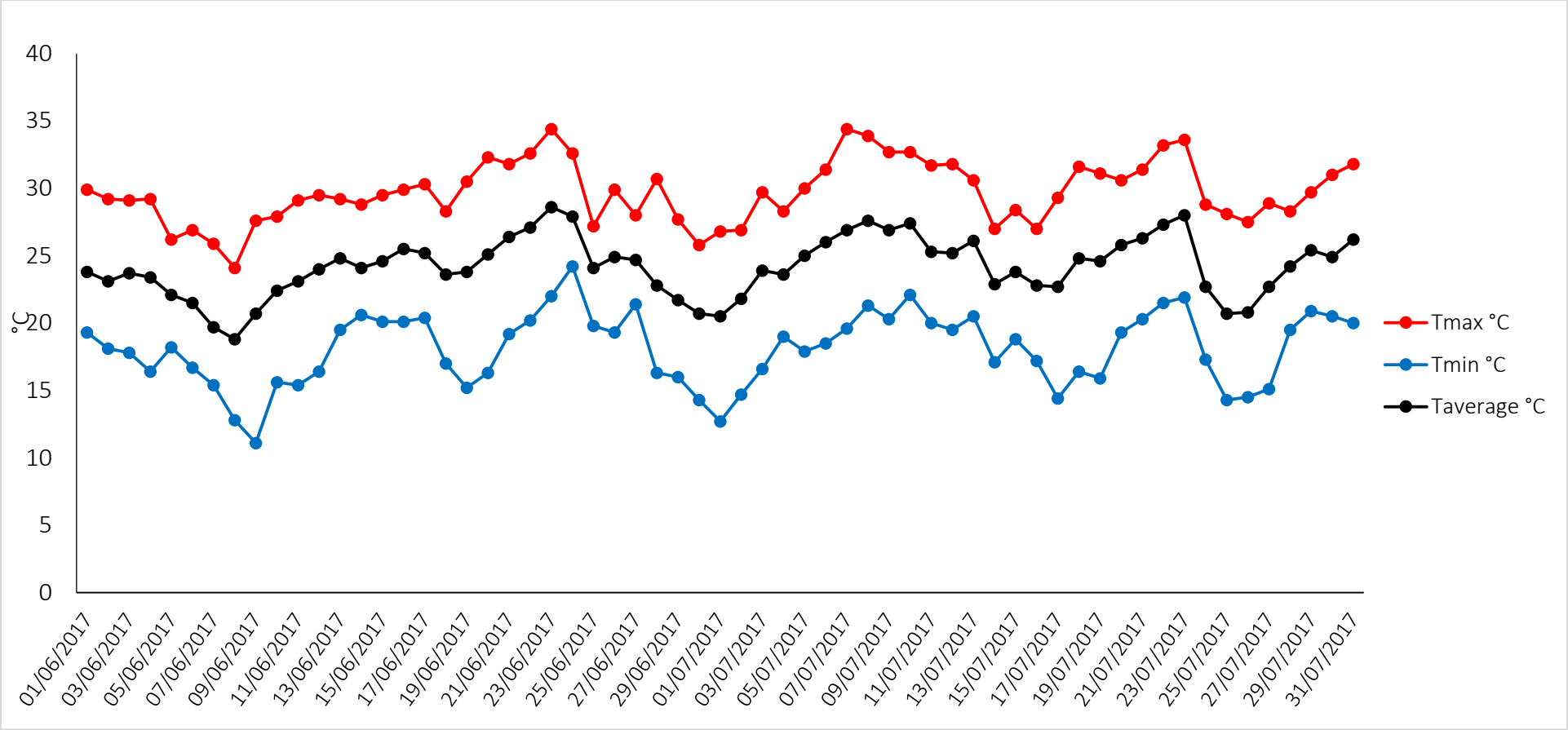
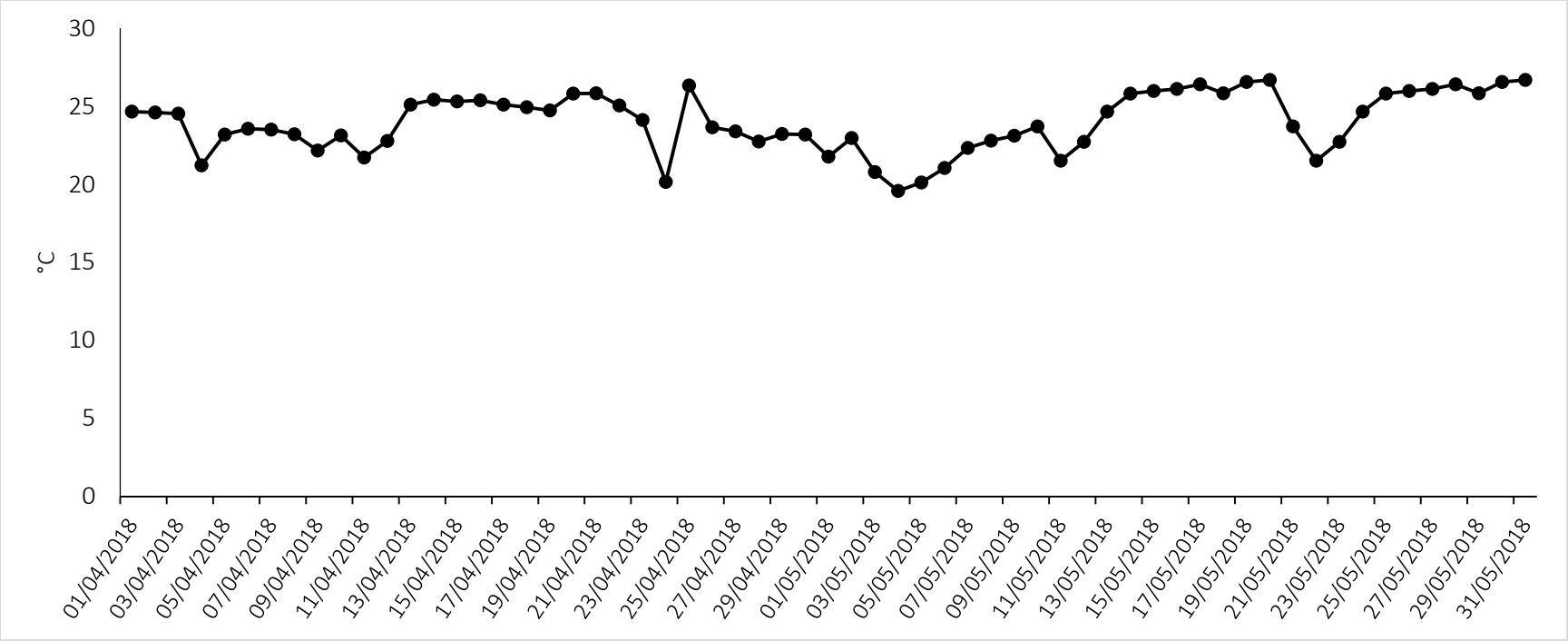


Fig II: Values average temperature (T) recorded in the greenhouse during the course of the dose response trial in April – May 2018. Plants were transplanted on 9th April 2018, treated on 20th April 2018 and assessments were performed on 16th (cyhalofop – butyl and penoxsulam) and 21st (florpyrauxifen – benzyl) May 2017.



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