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Department of Agronomy, Food, Natural resources, Animals and Environment (DAFNAE)

PhD course in Crop Science

Cycle XXXII

Co-existence of ALS-resistant *Amaranthus* species in north-eastern Italy: how to manage them

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Declaration

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

September, 30th 2019 Andrea Milani

“It's the question, Neo.

It's the question that drives us.

It's the question that brought you here”

(The Matrix, 1999)

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Summary

This PhD thesis comprises 6 chapters: Chapter 1 and 6 provide, respectively, a general introduction on ALS resistance in Amaranths and some general conclusions, whereas research questions are addressed in Chapters 2 to 5. All chapters have been organized as standalone publications and they can be read independently, therefore, some repetitions above introduction and methods sections might occur. At the moment, only Chapter II is under review (Pest Management Science).

In the last ten years, a number of cases of *Amaranthus* spp. suspected ALS-resistant have been recorded in Italy, and in some cases more than one species appeared to be present in a single field. Three species were found: *Amaranthus retroflexus*, *Amaranthus hybridus* and *Amaranthus tuberculatus*, in one case living sympatrically. All populations were found to be ALS-resistant, and the main resistance mechanism was target-site mediated. A simplified identification key for weedy amaranths was devised. Herbicide resistant *A. tuberculatus* was found for the first time outside its native range (North America). The *ALS* substitution tryptophan 574 methionine was observed in dicots for the first time.

Population genetics tools (microsatellites) and haplotype analyses were used to infer the origin of some Italian *A. tuberculatus* populations and the evolution of resistance among them. Two ancestral lineages and mainly two resistant (carrying mutation 574) haplotypes were found. Very likely at least a resistant allele was introduced from outside Italy, which lately spread to some other populations. Another resistant allele could have been selected in Italy. The presence of the same haplotype among geographically separated population clearly indicates that resistance has a common origin. Birds and the use of non-certified (possibly infested) seeds might be the main causes of *A. tuberculatus* seeds dispersal.

The presence of more than one *Amaranthus* species in the same field can complicate weed management because different species can have different phenology and herbicide susceptibility. If *A. tuberculatus* is present in multiple *Amaranthus* species infestations, it should be the main target of herbicide treatments. Interventions should be rapid, because this species grows fast and therefore the herbicide application window is shortened.

In 2018, a population of *A. palmeri* was found infesting a soybean field in North Eastern Italy. Whole plant herbicide assays demonstrated that this population was also resistant to ALS, and molecular analysis revealed a point mutation at position 574. This is the first case of herbicide resistant *A. palmeri* in Europe.

Some *A. retroflexus* populations had a point mutation at position 376 of *ALS* gene, which conferred resistance to thifensulfuron-methyl, but not to imazamox. Further experiments clearly indicated that mutation 376 endows resistance to imazethapyr, but not to imazamox. Similar results were obtained with a *Sorghum halepense* population.

An *A. tuberculatus* population was resistant to thifensulfuron-methyl, but no endowing-resistance mutations were found in the whole *ALS* gene. Further experiments suggested that resistance was not due to either a known point mutation or enhanced metabolism, therefore the resistance mechanism remained unknown.

Four *Amaranthus* species were found to infest soybean fields in Italy: *A. retroflexus*, *A. hybridus*, *A. tuberculatus*, and *A. palmeri*. All these species have evolved resistance to ALS inhibitors. Herbicides with different SoA are still effective. Glyphosate (in the absence of a crop) and metribuzin can be used to control these resistant populations, whereas the use of bentazon should be further evaluated. Weed control should be focused on dioecious species (*A. palmeri* and *A. tuberculatus*), because they are associated with high risk of multiple-resistance evolution and

crop losses. If *A. palmeri* or *A. tuberculatus* are present in fields, integrated weed management (crop rotation, mechanical weeding, etc.) must be adopted to limit their impact. The use of non-certified seeds should be avoided.

Chapter I

General introduction

1. Introduction:

1.1. Humanity, agriculture and weeds

1.1.1. Weeds: why they matter?

Humans have struggled against the negative impact of weeds since the cultivation of crops commenced around 10,000 B.C.[1]. Weed is an anthropocentric concept that expresses the undesirability of some plant species that compete with any other cultivated human-desired species. Many plants species can grow within cultivated land, but luckily not all become weeds. Some intrinsic biological features distinguish weedy from non-weedy species: rapid growth, high progeny output, long seed persistence in the soil (seed bank), high genetic variability, high plasticity (rapid adaptation to environmental changes) and toxicity to crop[2]/animals[3]/humans[4]. In general, the presence of weeds increases cultivation costs and/or decrease crop value. Crop yield reduction is caused by competition for resources between the crop and the weed (e.g. *Amaranthus tuberculatus* can lower soybean yield by 73%[5]), whereas contamination of the final product can involve toxic compounds produced by the weed (e.g. *Datura* spp. in silage and vegetable crops[6]). Crop yield reduction and contamination of the final product are a threat to food security and food safety. Food security represents the availability of food and individuals' ability to access it: the aim of food security is to avoid hunger, making food available for everyone. Food safety represents the availability of healthy and hygienically clean food: the aim of food safety is to avoid food-borne illness. Crop losses can be caused by a number of possible interferences (pests, predators, weather conditions, etc.), but it has been estimated that weeds cause the highest potential crop loss (34%), with animal pests and pathogens being less important (losses of 18 and 16%)[7]. In a global perspective, this actually means a huge waste of food.

1.1.2. How to control weeds

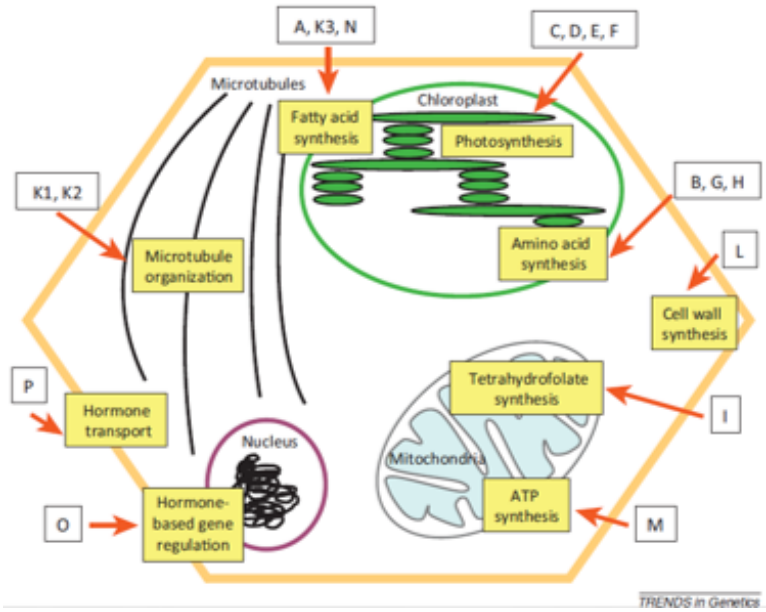
There are many possible approaches to manage weed infestations. They can be classified as physical, biological, ecological and chemical tools and they can have different impacts on crop, environment and society. Mechanical weeding is still very effective on weeds and has sustainable costs, that can be further lowered by the emergence of robots in agriculture[8]. On the other hand, mechanical weeding can have a high impact on the environment, causing decay of the soil structure (fostering soil erosion), fuel consumption and greenhouse gas emissions. Biological control, based on the introduction of natural enemies (pests or predators) of the target weed[9],

requires a huge amount of biological information to be adopted. This is not always easy to reach and therefore it is feasible only on small scale (home farming) and on specialized productions (e.g. the rice-duck farming[10], quite common in Asian countries). The integration of ecological and agronomic tools aims to make the cropping system unfavorable for weeds, by varying the agronomic environment and therefore counteract the adaptation of weeds. Crop rotation a well-known and common agronomic practice. It works because different crops have different life cycles (e.g. summer vs winter crops) and needs (water, fertilization, tillage), therefore they act as different habitats for weeds. Another agro-ecological tool is cover-cropping, i.e. keeping the field cultivated and covered most of the year. Despite the positive effect on weed control, agro-ecological tools have some significant limits: a) some soils/systems can be used only for one crop (e.g. flooded paddy rice fields), b) very often, only some crops are remunerative c) more knowledge and/or tools are necessary in comparison with monoculture. The chemical approach is based on "herbicides", products specifically designed to kill plants. Nowadays, this is the most cost-effective available tool to control and limit the expansion of weeds and therefore to increase food security and food safety. Nonetheless, herbicide use is associated with environmental issues, like soil and water pollution, and human health.

1.1.3. Herbicide classification

The first herbicides were developed by the Allied Power during the Second World War to destroy food productions and forests of the Axis Power (herbicidal warfare). Before the introduction and widespread use of chemical control in agriculture (around the 60's), mechanical tools and crop rotation were the main applied approaches to weed management. After their introduction, herbicides pushed the production of safe and cheap food to a level that would have never been achieved exclusively with traditional, non-chemical methods. As a consequence of this success, modern agriculture has been making extensive use of herbicides. There are several ways to classify herbicides, depending on which herbicide characteristics are considered: a) their site of action (their target within the plant) b) their selectivity c) the chemical structure d) the timing of application.

Figure 1. Cellular targets of herbicides and herbicide classification by sites of action according to the Herbicide Resistance Action Committee (HRAC). Picture taken from a published paper[11].

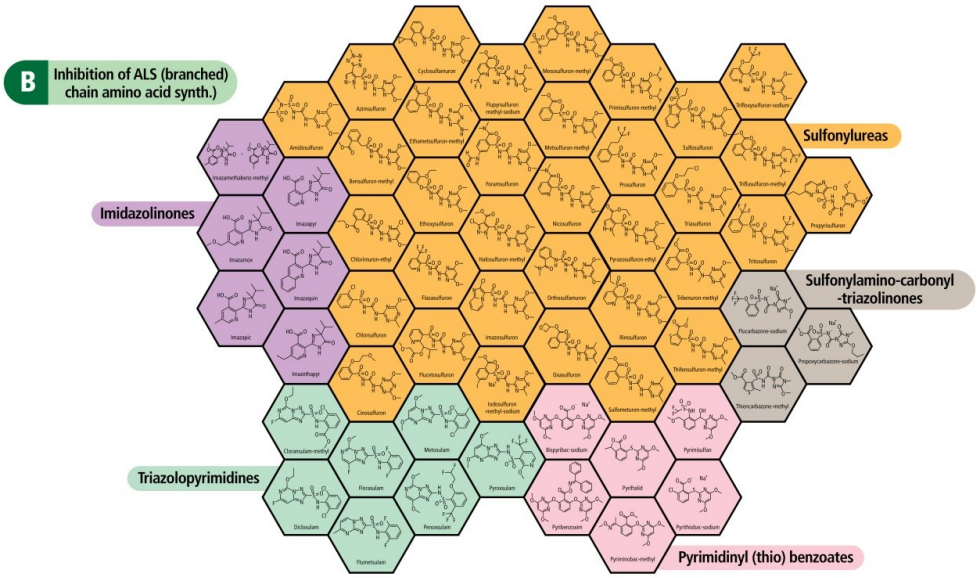


There are four classification systems based on the site of actions (SoA, molecular target of herbicides): Canada, Australia and United States used to have their own classification system, while Herbicide Resistance Action Committee (HRAC) proposed a classification that nowadays is widely accepted, based on codifying SoA with letters (or group of letters) (Fig. 1).

Another fundamental characteristic of herbicides is selectivity. A non-selective (total) herbicide can kill all weeds as well as the crop, if not properly managed. Instead, a selective herbicide is active only against specific weeds or weed categories (e.g. monocots vs dicots) and the crop can tolerate the herbicide treatment according to strict conditions of use. Selective herbicides are crop-specific. This characteristic has two related consequences: 1) crop rotation is necessary to change herbicides and 2) no-crop rotation would result in an extremely limited number of herbicides available for weed control.

The classification based on the chemical structure allows to cluster together those molecules that share a common chemical structure. Herbicides with a common chemical structure normally have the same site of action, but have different selectivity and therefore are used in different crops, against different weeds. As an example, five chemical families belong to ALS-inhibiting herbicides (classified as group B, according to HRAC classification system) (Fig. 2).

Figure 2. Section of HRAC panel representing ALS-inhibiting herbicides (group B) divided into five chemical families.



Pre-emergence herbicides kill weed when seeds are germinating and are therefore soil-applied prior to the emergence of weed seedlings. These herbicides have a certain persistence in the soil that allows them to perform their action for a relatively long period. Nevertheless, their proper activity strictly depends on the content of organic matter if the soil (that inactivate them) and weather conditions (that must favor weed germination and provide enough moisture in the soil). Instead, post-emergence herbicides are leaf-applied when weeds are actively growing and herbicide is leaf-absorbed within one hour after application. Post-emergence herbicides are the most used class of herbicides, because of their high efficacy, safer eco-toxicological and environmental profile and easier use.

1.1.4. Herbicide resistance

The over-reliance on a limited number of herbicides with the same SoA is the main cause of the evolution of herbicide resistance. According to the Herbicide Resistance Action Committee (HRAC)[12], herbicide resistance is defined as "the naturally occurring inheritable ability of some weed biotypes within a given weed population to survive a herbicide treatment that would, under normal use conditions, effectively control that weed population". The over-reliance on a limited number of herbicides is caused by the simplification of cropping systems (i.e. the lack of crop and herbicide rotation/monoculture, repeated use of a single herbicide, or use of several herbicides

with the same site of action) and the withdrawal of products considered obsolete/unsafe and the lack of registration of new chemicals.

Monoculture is a common practice and it is the result of market request, that favors the cultivations of a single -economically more profitable- crop rather than other -less profitable- ones, together with the undeniable benefits of an extremely simplified agriculture (easy laborer and land management, less machine investments, high efficiency etc.).

The withdrawal of products considered obsolete/unsafe is a result of the increased sensitivity of public opinion and authorities to the environmental problems of herbicides. While research and development (R&D) technologies regulatory approval and commercialization procedure costs is continuously increasing, the success rate to find a marketable new herbicide is dropping from the 90's, fallowing the fate of other chemicals: this -only apparent- paradox is explained by the 'Eroom's Law'[13,14]. European regulation (EC) No 1107/2009 first, and Directive 91/414/CEE then, fixed specific constrains on criteria used for the safety evaluation of new molecules and re-registration of new formulations containing "old" molecules. The reduction in number and diversity of active ingredients available, as well as the widespread use of highly-active herbicides (e.g. ALS-inhibitors) will increase the risk of resistance and make its management harder[15].

The abiotic stress exerted by herbicides acts on weeds as selective pressure does. Herbicides act positively selecting natural variation that allows plants to survive even in presence of the selective agent. The higher the pressure, the higher the risk of selecting sub-populations of weeds with not-complete herbicide susceptibility. Therefore, herbicide resistance is an evolutive process driven by herbicides that happens within cultivated fields. After a few years of selection with herbicides with the same site of action, herbicide resistance will rise and spread, if no countermeasures are taken. This is what exactly happens when a very effective molecule is continuously used to control weeds in a monoculture.

1.1.5. Resistance mechanisms

Natural selection acts on phenotypes, that are the composite of the observable characteristics of an organism. Phenotypes result from the interaction of the genotype -the inheritable characteristics- of the organism and the environment. Therefore, genotypes associated to positively selected phenotypes will increase in frequency in the population as a result of selective pressure. The genetic basis responsible for the positively selected phenotype may become more

common in a population. The genetic basis of resistance, herein called resistance mechanisms, can be classified as “target-site” or “non target-site”.

Target-site (TSR) mechanisms affect the interaction of the herbicide with its molecular target[16]. TSR can be due to a single point mutation or the altered expression of the gene encoding for the molecular target of the herbicide (gene duplication or a mutation in a regulatory sequence). Therefore, TSR is normally controlled by dominant or semi-dominant alleles at the target nuclear gene (monogenic control) following Mendelian inheritance[17,18].

Non target-site (NTSR) mechanisms contribute to avoid the herbicide to reach its molecular target[11]. Some examples are vacuolar sequestration of chemicals, altered translocation, reduced radical/foliar absorption and enhanced (faster) metabolism[19]. Non target-site mechanisms might arise after repeated selection with herbicide doses lower than the recommended[20], involve multiple genes[21] and are therefore more complex to study than TSR[22]. However, glutathione S-transferase (GST)-mediated NTSR to triazines in velvetleaf is inherited as a single nuclear gene[23]. Only a few examples of NTSR exist among broadleafs[24], while it is quite common among grass weeds[25,26].

At the same time, two broad cross-resistance categories can be recognized: target-site cross-resistance and non-target-site cross-resistance. Target site cross resistance occurs when a change at the biochemical site of action of one herbicide also confers resistance to herbicides from a different chemical family that inhibit the same site of action in the plant. Target-site cross-resistance is caused, e. g., by point mutation 574 at ALS locus, that confers resistance to a wide variety of ALS-inhibiting herbicides. Non target site cross resistance, instead, is defined as cross resistance to herbicides belonging to different classes conferred by a mechanism(s) other than target-site. An example of non-target site cross resistance is enhanced metabolism of both ALS and ACCase-inhibitors given by the same cytochrome P450 in *Lolium* spp[27].

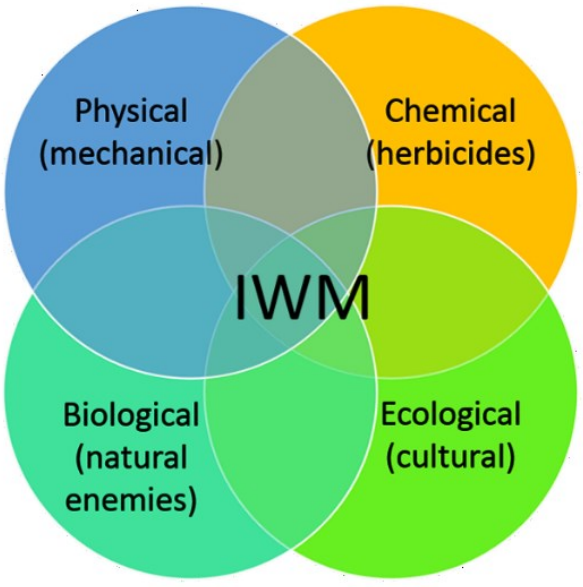
Multiple resistance is defined as the expression (within individuals or populations) of more than one resistance mechanism. The simplest cases are where an individual plant (or population) possesses two or more different resistance mechanisms which provide resistance to a single herbicide, or class of herbicides. For example, a biotype of *Lolium rigidum* was found having a mutation at ALS gene and also enhanced metabolism[28]. More complicated situations might occur when both target-site and non-target-site resistance mechanisms co-exist (at plant or population

level), endowing resistance to herbicide belonging to different classes. Grass weeds are particularly prone to this events and many examples exist[29,30,31,32]. The most complicated and difficult to control situations are where a number of resistance mechanisms, involving both target-site and non-target-site resistance mechanisms, are present within the same individual. Multiple resistance is the more problematic to be managed, because it lowers the possible alternative chemicals that can be used. The most recent bibliography clearly indicates that multiple resistance evolves more rapidly in cross-pollinated and highly genetically variable species[33], while it evolves slower in selfing species[34].

1.1.6. Integrated weed management (IWM)

Herbicide resistance problems demonstrate the weakness of over-reliance on a single weed management tool[1]. To manage weeds, it is important to integrate a range of weed control tools without excessive reliance on only one method[35]. This reduces the selective pressure imposed on weeds, mitigating their evolution process and diffusion. This holistic approach is commonly defined as "Integrated Weed Management" (IWM, Fig. 3), and is a specific interpretation of the broader concept of "Integrated Pest Management" (IPM). The long-term approach of integrated weed management is to increase/maintain crop yield/profitability and land/environment qualities. To achieve this point, integrate weed management should focus on the most economical and effective weed control while taking into account ecological considerations.

Figure 3. Integrated approach to weed management (IWM) takes into account all possible weed control tools.



Knowledge of weed biology is essential for proper application of integrated weed management principles. Weed biology relates to plant attributes such as morphology, seed dormancy, germination, physiology of growth, phenology, competitive ability and reproductive biology of the weed. Deep knowledge of strengths and weaknesses of the target weeds are crucial to define the best strategy to limit weed expansions and therefore it the basis of weed management programs[36]. From this point of view, integrated weed management resembles a war. In the Chinese military treatise "The art of war" written by the military strategist Sun Tzu (roughly 5th century BC) there is a sentence that well matches the concept of management:

"*If you know the enemy and know yourself,*

you need not fear the result of a hundred battles.

If you know yourself but not the enemy,

for every victory gained you will also suffer a defeat.

If you know neither the enemy nor yourself,

you will succumb in every battle"

An emerging issue in weed science is the spread of herbicide resistant weedy Amaranths worldwide. Twelve *Amaranthus* species have already evolved resistance to herbicides: of those, nine evolved resistance to ALS herbicides and five evolved multiple resistance (including ALS). These weeds are very common in summer crops and their presence should promptly be evaluated, but their identification is not easy.

The next paragraphs of the dissertation will focus on what we know, and what we don't, about some *Amaranthus* species and ALS-inhibiting herbicides.

1.2. Herbicide resistant Amaranths, an emerging issue

1.2.1. Botanical aspects of Amaranths

Amaranthus L. is a genus comprising about 70 -mostly annual- monoecious and dioecious species with worldwide distribution. Approximately 40 species are native to the Americas, the remaining ones to the other continents[37]. Some species are used as ornamentals (e.g. *A. hypochondriacus* L. cv 'Elephant Head', *A. caudatus* L. cv 'Mira'), as vegetables (*Amaranthus tricolor* L. cv 'Lal Sag' used in India or cv 'Tampala' used in southern United States) and as grain food (*Amaranthus cruentus* L.)[38]. All these species can easily adapt to most agricultural systems and therefore have the potential to cause economic impacts to agriculture.

This genus is critical from the taxonomical point of view due to its high phenotypic variability which led to nomenclatural disorder and misapplication of names[37,39]. *Amaranthus* includes 3 subgenera: subgenus *Acnida* (L.) Aellen ex K.R.Robertson with 3 sections, subgenus *Albersia* (Kunth) Gren. & Godr. with 4 sections, and subgenus *Amaranthus*, with 3 sections and 2 subsections. However, *Amaranthus* classification does not appears conclusive and new taxa (at section and subsection levels) could be described[39,40].

Being *Amaranthus* such a critical genus for classification, the species identification in the field is even more complicated, also because identification keys are indecipherable to the majority of non-specialists. This difficulty is a major obstacle for integrated weed management principles. The correct application of the scientific name to a certain species is crucial to exchange information concerning it and therefore to better know it. For this reason, easy to use, simplified -whenever possible-, identification keys would be of a great help in case of genus with many species that can live sympatrically.

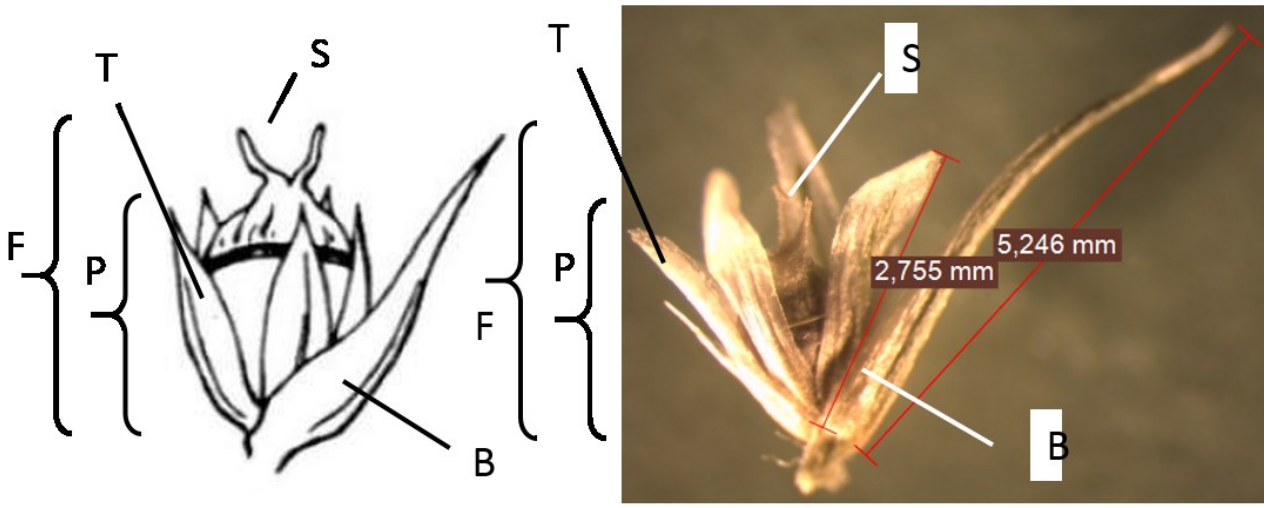
1.2.2. Inflorescence and flower

The structure of the inflorescence in the Amaranthaceae is very complex[41]. The flowers are unisexual and plants can be dioecious (subgenus *Acnida*) or monoecious (subgenus *Albersia* and subgenus *Amaranthus*). Classifications keys are based on the pistillate flower characteristics (Fig. 4), while staminate flower is taxonomically irrelevant. The pistillate flower is composed of 1−5 tepals (equal or unequal), subtended by 2−5 bracts (in *A. spinosus* L. bracts of the first flower in the first cyme is metamorphosed into a spine-like structure[42]. The tepals (in pistillate flowers) are very variable in shape (from linear to ovate, sometimes spathulate). The apex of bracts can be

truncate, obtuse, or acute, sometimes mucronate or awned (while awns do not occur in the tepals). Each bract has membranous -usually hyaline- borders: in the subgenus *Amaranthus* can be thinning to the apex or abruptly interrupted about at the half of the total length of the bract, depending on the species.

The result of this complex synflorescence is a spike- or panicle-like inflorescence and/or an axillary glomerular arrangement.

Figure 4. The structure of Amaranths flower (A. retroflexus, in the picture). S: stigma, F: fruit, T: tepal, B: bract, P: perianth.



1.2.3. Herbicide resistant Amaranths

As mentioned above, herbicide resistant Amaranths are spread worldwide, making this genus an emerging issue in weed science. Three *Amaranthus* species, namely *Amaranthus retroflexus* L., *Amaranthus tuberculatus* (Moq.) J.D.Sauer (synonym of *A. rudis*) and *Amaranthus palmeri* S.Watson are among the top 10 most troublesome weeds[43].

Twelve *Amaranthus* species have already evolved resistance to herbicides (in alphabetic order): *Amaranthus albus* L., *Amaranthus blitoides* S.Watson, *Amaranthus blitum* L. subsp. *oleraceus* (L.), *Amaranthus cruentus* L., *Amaranthus hybridus* L. (synonym: *A. quitensis*), *Amaranthus palmeri*, *Amaranthus powellii* S.Watson, *Amaranthus retroflexus*, *Amaranthus spinosus* L., *Amaranthus tuberculatus* and *Amaranthus viridis* L.

Eleven sites of action are involved in *Amaranthus* resistance (HRAC classification, alphabetic order, common acronym): B (inhibition of acetolactate synthase, ALS), C1-C2-C3 (inhibition of photosynthesis at photosystem II, PSII, triazine-urea-nitrile), D (electron diversion of photosystem I, PSI), E (inhibition of protoporphyrinogen oxidase, PPO), F2 (inhibition of 4-hydroxyphenyl-pyruvate-dioxygenase, HPPD), G (inhibition of EPSP synthase, EPSP), K1 (inhibition of microtubule assembly), K3 (inhibition of very long chain fatty acid -VLCFA- biosynthesis) and O (synthetic auxins).

Monoecious species (*A. hybridus*, *A. powellii*, *A. retroflexus*) have evolved multiple resistance to -at most- two sites of action, whereas dioecious species evolved resistance up to five sites of action (*A. palmeri*: B+C1+F2+G+O and B+E+G+K1+K3) and six sites of action (*A. tuberculatus*: B+C1+E+F2+ G+O). Most cases of multiple resistance include resistance to ALS inhibitors.

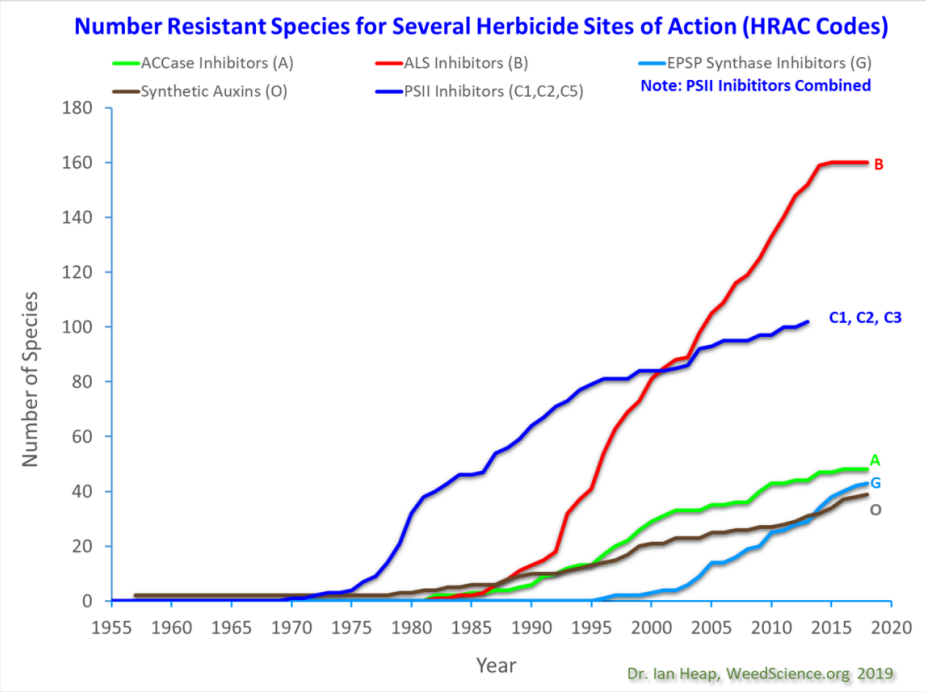
In Italy, the first case of resistant Amaranth was recorded in 1999, when *A. retroflexus* was found to be resistant to triazine. Some years later, in 2003, cross-resistance to imazamox and thifensulfuron-methyl was found in a population ascribed to *A. retroflexus* (later recognized as *A. hybridus* by the Authors, personal communication). Since then, the ALS-resistant *Amaranthus* cases are increasing[44] and this is of concern also because the area cultivated with soybean in Italy is increasing[45]. Indeed, in the last ten years, many *Amaranthus* spp. infestations were found in soybean fields treated with ALS inhibitors.

1.3. Acetolactate synthase (ALS) inhibitors

1.3.1. Evolution of resistance to ALS-inhibiting herbicides

The introduction of acetolactate synthase (ALS)-inhibiting herbicides, during the 80's, was a "game changer" in herbicide technology. The reasons of their immediate and durable success are their high efficacy on a broad spectrum of target species (both grasses and broadleaves), their very specific target site and the low toxicity to non-target organisms (including mammals, fish, insects and other invertebrates). ALS inhibitors (group B of HRAC classification) include five chemical families: the sulfonylureas (SU), the imidazolinones (IMI), the triazolopyrimidines (TP), the pyrimidinyl(thio)benzoates (PTB) and the sulfonylamino-carbonyl-triazolinones (SCT) (Fig. 2). ALS resistant weed have been reported all over the world (fig. 4A) and are the herbicides that caused resistance evolution in the greatest number of species (Fig. 5). In Italy, evolution of resistance to ALS-inhibiting herbicides involves 36 species, with *Alisma plantago-aquatica* being the first (1994). Since then, evolution of resistance to ALS-inhibiting herbicides evolved rather slowly in Italy, but included some cases of multiple-resistance[46] and non-target-site resistance[47]. Even if many *Amaranthus* species already evolved resistance to ALS-inhibiting herbicides worldwide, only a few cases were found in Italy[18], until recently.

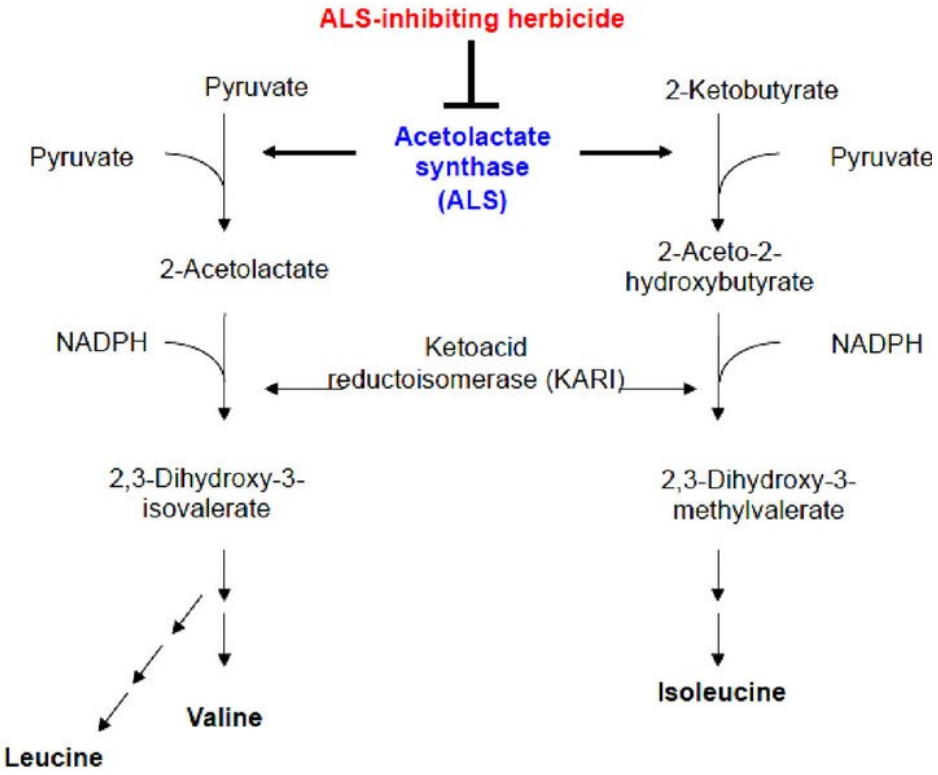
Figure 5. Chronological increase in the number of species that evolved herbicide resistance to 5 herbicide sites of action. Different line colors refer to different HRAC codes. This image belongs to [48]



1.3.2. Acetolactate synthase enzyme: the target of ALS inhibitors

Acetolactate synthase (ALS or AHAS, EC 2.2.1.6) is the target of ALS inhibitors and it is the first enzyme in the biosynthesis of branched-chain amino acids (Fig. 6). AHAS does not exist in animal cells, for this reason it is an attractive target for developing antimicrobials, antifungal agents, and herbicides[49,50,51]. Nevertheless, a human protein of yet unknown function, sharing some sequence similarity with bacterial ALS, is encoded by the ILVBL (ilvB-like) gene[52]. ALS enzyme catalyzes two main reactions: a) the condensation of two molecules of pyruvate to acetolactate and b) the condensation of pyruvate and 2-ketobutyrate to acetohydroxybutyrate. Acetolactate and acetohydroxybutyrate will be then used in parallel pathways to obtain valine/leucine or isoleucine, respectively. Thiamine pyrophosphate (TPP), flavin adenine dinucleotide (FAD) and Mg^{2+} are used as co-factors in the two condensation reactions[53].

Figure 6. Pathway of the branched-chain amino acid (BCAA) biosynthetic. ALS catalyzes first step of biosynthesis of BCAAs and is under feedback regulation by valine, leucine and isoleucine (not shown). ALS is the target for five -structurally distinct- classes of herbicide, that, inhibit the first enzymatic reaction. Picture taken from a published paper[54].



L-valine, L-leucine, or L- isoleucine has been observed to inhibit AHAS in different microorganisms with a feedback mechanism[55]. A synergistic inhibition of the enzyme is observed when a combination of valine and leucine is used, suggesting two separated binding sites for these amino acids. In plants, a pair of catalytic subunits form an intimate dimer containing two active sites, each of which lies across a dimer interface and involves both monomers[56]. ALS gene is nuclear encoded, but the protein is transferred to chloroplasts thanks to a transit peptide.

Whether the inhibition by herbicides is reversible and competitive or noncompetitive remains controversial and, probably, different herbicide families behave differently[56]. After the first crystal structure of yeast ALS was published, the localization of the herbicide binding site started to become clear[57]. Several structures of yeast ALS, complexed to sulfonylureas, were subsequently described[58]. Thanks to these herbicide-complexed crystal structures, it was possible to discover that herbicide-enzyme interaction sites were localized within a channel connected with the active site. Therefore, enzymes carrying point mutations in correspondence of these key sites could result in altered susceptibility to ALS inhibitors, by avoiding the herbicide to interfere with substrate-active site interaction.

1.3.3. Molecular basis of resistance to ALS inhibitors

The most common mechanism of evolution of resistance to ALS-inhibiting herbicides in plants is target-site based, due to point mutations conferring the resistance (amino acid substitution). So far, 15 point mutations were observed to alter the ALS herbicide sensitivity. Eight are known to confer resistance to weeds (Ala_{122}, Pro_{197}, Ala_{205}, Asp_{376}, Arg_{377}, Trp_{574}, Ser_{653}, Gly_{654})[59], while seven were discovered with artificial selection experiments (Gly_{121}, Met_{124}, Val_{196}, Arg_{199}, Asp_{375}, Val_{571} and Phe_{578})[60]. Some mutations apparently have specific resistance pattern: 1) Ala_{122} and Ser_{653} endow resistance to imidazolinones, but not sulfonilureas 2) Pro_{197} endow resistance to sulfonylureas and triazolopyrimidines, but not imidazolinones 3) Trp_{574} endow a broad-spectrum cross-resistance to all the five classes of ALS inhibitors. However, the association 'point mutation' and 'herbicide resistance pattern' is not always strict, as there are many exceptions, often specific to weed species. A possible explanation to these exceptions might be that most herbicide resistance assays aim to define the resistance level of populations, not the resistance pattern of specific mutations. The common practice in these assays is to test populations with the same herbicides that they might have experienced, therefore eventual differential effect of similar molecules is not detected. Indeed, resistance to one compound of a particular chemical family of

ALS-inhibiting herbicides does not guarantee cross-resistance to all members of that chemical family. A better approach to define herbicide resistance pattern associated with a point mutation would be a) purify sub-populations made of specimens carrying only a point mutation b) to test this purified sub-populations with many herbicides with the same site of action. For this kind of assays, two herbicide doses (field rate and three-times that) would be sufficiently informative. An example of a doubtful case is point mutation 376, that apparently confer cross-resistance to sulfonylureas and imidazolines, but *in vitro* assays demonstrated that -at least in some cases-, it does not confer resistance to imazamox[61]. Precisely define eventual resistance patterns associated to point mutations would be helpful to better understand the mechanism of herbicide resistance, but could also have practical impact. For example, in weed science differential resistance pattern could be used to purify population with more than one point mutations (or mechanisms).

1.4. Aims of the research

The research conducted during my Ph.D. was focused on the emerging issue of *Amaranthus* spp. resistant to ALS inhibitors in Italy. It started from the finding of a number of infested soybean fields, from 2010 and 2014. While it was clear that *Amaranthus* spp. was involved, the species was unclear, because plants were phenotypically highly variable, having different shape, height and habit. Once the species were identified, it appeared that an *Amaranthus* species relatively new for Italy was expanding rapidly, and this observation led to some new questions.

The research is developed in four chapters (II to V) in order to respond to the following main objectives:

1. identify the *Amaranthus* spp. species present in the Italian soybean fields, determine the resistance pattern and investigate the resistance mechanism involved in these mixed *Amaranthus* populations
2. Investigate the spread of ALS resistant alleles in *Amaranthus tuberculatus* populations through microsatellites markers and ALS amplicon sequencing via NGS
3. Elucidate the biology (phenology and height growth curve) of three *Amaranthus* species (*A. retroflexus*, *A. hybridus* and *A. tuberculatus*) and their sensitivity to ALS-inhibitors
4. Investigate the resistance pattern and molecular basis of resistance of an *Amaranthus palmeri* population appeared in 2018 in a soybean field. Test the efficacy of imazethapyr on several *A. retroflexus* populations. Elucidate the molecular bases of a suspected non-target-site resistant *A. tuberculatus* population.

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Chapter II

A family affair: resistance mechanism and alternative control of three *Amaranthus* species resistant to acetolactate synthase inhibitors in Italy

Abstract

BACKGROUND: several soybean fields in north-eastern Italy were found to be infested by several species of *Amaranthus* spp. not adequately controlled by acetolactate (ALS) inhibitor herbicides. The aims of this research were to create a simplified botanical key to identify weedy amaranth species, determine the resistance patterns and main mechanisms involved and evaluate alternative chemical control.

RESULTS: An easy-to-use botanical key was devised and successfully used to identify amaranth species present in the infested sites and the results were confirmed through a species-specific molecular marker. *Amaranthus retroflexus* L. (redrood pigweed) was found in three sites and the ALS-resistant plants contained an $Asp_{376}Glu$ substitution at the *ALS* gene endowing resistance to thifensulfuron-methyl. All the identified *Amaranthus tuberculatus* (Moq.) J.D.Sauer (waterhemp) and *Amaranthus hybridus* L. (smooth pigweed) accessions (identified in seven and two sites, respectively) were cross-resistant to thifensulfuron-methyl and imazamox and almost all the ALS-resistant plants had nucleotide mutations causing amino-acid substitution at codon 574 of *ALS* gene. One *A. hybridus* accession displayed two novel $Trp_{574}Met$ and $Trp_{574}Arg$ substitutions which have been detected for the first time in the *Amaranthus* genus. All the ALS-resistant *Amaranthus* accessions were adequately controlled by glyphosate and metribuzin while the efficacy of bentazon was not complete.

CONCLUSIONS: the simplified botanical key proposed herein could be a useful tool for farmers and weed scientists to reliably identify *Amaranthus* species in the field. The main resistance mechanism in the three *Amaranthus* species is target-site mediated. This is the first evidence of ALS resistant *A. tuberculatus* outside its native North American range.

Keywords: Acetolactate-synthase resistance; waterhemp; smooth pigweed; redroot pigweed; point mutations; sympatry

2.1. Introduction

Amaranthus is a genus comprising about 70 monoecious or dioecious species that are mostly summer annuals, competitive, have C_4 metabolism, infest summer crops such as soybean, maize, cotton, carrots, tomatoes and potatoes and are known to cause high yield losses.[1,2,3] They are spread worldwide, covering a number of different habitats.[4] Three *Amaranthus* species, namely *Amaranthus retroflexus* L., *Amaranthus tuberculatus* (Moq.) J.D.Sauer (formerly called *Amaranthus rudis* J.D.Sauer) and *Amaranthus palmeri* S. Watson are among the top 10 most troublesome weeds.[5]

Chemical control of these species mostly relies upon post-emergence herbicides such as acetolactate synthase (ALS) inhibitors, photosystem II (PSII) and 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitors (HRAC groups B, C and G, respectively). In addition, protoporphyrinogen oxidase (PPO) and 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors (HRAC group E and F, respectively) are used, although to a lesser extent. *Amaranthus* species are prone to evolve resistance after repeated exposure to herbicides having the same site of action (SoA). To date, monoecious *Amaranthus* species have evolved multiple resistance to two different SoAs,[6] *A. hybridus* to ALS and PSII inhibitors (HRAC group C1),[7] to ALS and EPSPS inhibitors,[8] and recently to EPSPS inhibitors and synthetic auxins.[9] *A. retroflexus* evolved multiple resistance to two classes of PSII (HRAC group C1 and C2),[10] to ALS and PSII inhibitors,[11] whereas another biotype proved to be resistant to ALS and PPO inhibitors.[12] Dioecious species evolved a number of complex two- and three-way multiple resistance[6] with *A. tuberculatus* that became multiple resistant to up to six SoA (ALS, PSII, EPSPS, PPO, HPPD inhibitors and synthetic auxins).[13] The ability of these latter species to rapidly evolve herbicide-resistant biotypes could be related to their reproductive system. Since they are obligate outcrossers, they have high genetic recombination rates leading to a higher genetic and phenotypic variability in comparison to monoecious (self-fertilization) *Amaranthus* species.[14] It appears that the risks of selecting herbicide resistance traits differ among *Amaranthus* species, so a rapid and reliable species identification is needed for a proper and timely implementation of integrated weed management.

The *Amaranthus* genus has been the subject of many taxonomic studies, but it is still not completely understood.[15] Species identification through conventional botanical keys can be complex, time consuming and frustrating for non-experts because of the use of specific jargon.[16] Regional botanical accounts (Floras) are of limited usefulness when species of recent introduction are present (i.e. alien species).[17] Monographs provide a complete and structured account of

specific genera and are thus crucial in understanding the classification of botanical groups.[18] A taxonomic revision of the *Amaranthus* genus in Italy has been recently conducted and twenty four species were characterized.[4] Hybridization events are quite common in this genus,[19,20] which makes *Amaranthus* identification even more complicated. A number of hybridization events among amaranths were documented in agricultural environments[21,22,23] and diverse molecular markers were developed to study horizontal gene transfer (HGT) of herbicide resistant traits through hybridization.[24,25] There is therefore a need to create a simplified botanical key usable by non-professional systematic botanists.

The first European ALS-resistant amaranth was collected in soybean fields in north-eastern Italy in 2006.[26] Despite the case being recorded as *A. retroflexus*, the authors have recently verified that the correct species is instead *Amaranthus hybridus* (Laura Scarabel, Maurizio Sattin and Andrea Milani, personal communication). Since then, the area cultivated with soybean in Italy has substantially increased,[27] resulting in a rising number of ALS-resistant *Amaranthus* cases. [28] Some *Amaranthus* infestations appeared as mixed populations, showing plants with huge variability in height, shape, color and habit. In 2010, 2014 and 2017 seeds from *Amaranthus* spp. populations that survived ALS treatment were collected in soybean fields and investigated with the following objectives: (1) identify the *Amaranthus* species present in the collected populations (2) confirm the suspected ALS resistance (3) elucidate the molecular mechanism of ALS resistance and (4) determine the chemical options to control ALS-resistant amaranths.

2.2. Materials and Methods

2.2.1. Simplified identification key for weedy amaranths

To design the simplified dichotomous key, six *Amaranthus* species were considered because of their propensity to evolve herbicide resistance in maize-soybean cropping systems worldwide:[10] *A. palmeri*, *A. tuberculatus*, *A. retroflexus*, *A. hybridus*, *Amaranthus. spinosus* L. and *Amaranthus powellii* S. Watson. The most recent, simple and comprehensive literature was used to select four discriminatory morphological characteristics:[4] plant reproductive morphology (monoecy vs dioecy), presence/absence of spiny bracts, pistillate flower tepals (number and length) and the membranous border of flower bracts. Some authors formerly considered *A. tuberculatus* and *A. rudis* as separate species,[19] others considered synonymous the two taxa,[29] whereas others considered them as varieties of a single species.[30] Despite that, all authors agreed that these two entities differ basically in tepal number and fruit dehiscence. Similarly, some authors considered *A. bouchonii* and *A. powellii* as different species,[4] whereas others proposed a single species with two subspecies: *A. powellii* subsp. *powellii* and *A. powellii* subsp. *bouchonii*.[31] For all these entities there have been reports of herbicide resistant development.[32,33,34,35] For the purpose of this work, the simplest classification was used: *A. tuberculatus* and *A. rudis* were considered as a single species, as well as *A. powellii* and *A. bouchonii*. The authors did not mean to question the current classifications of the *Amaranthus* genus, but rather to propose a simplified key as an easy-to-use tool for non-professional systematic botanists to identify amaranths of high agronomic impact in annual cropping systems.

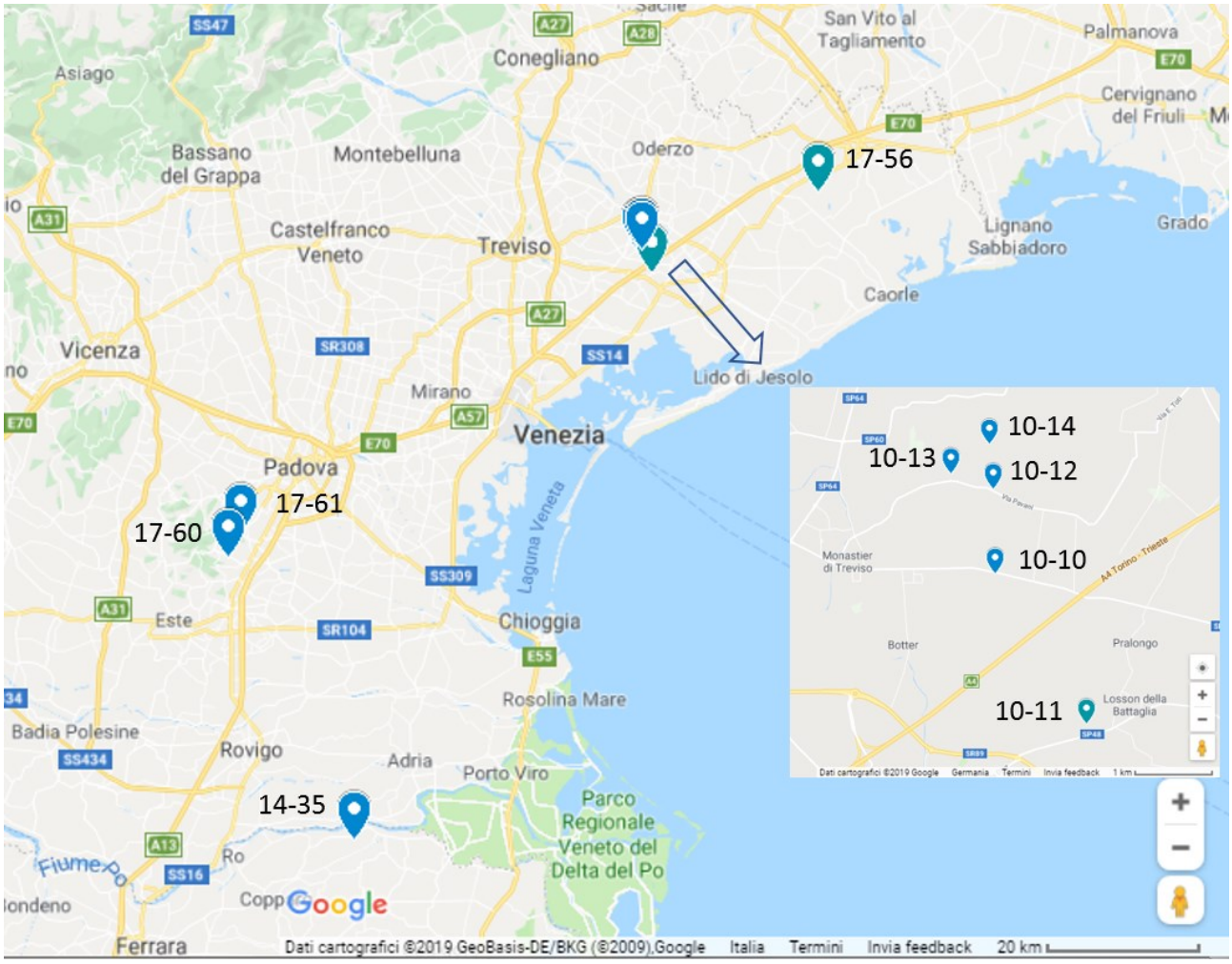
2.2.2. Origin of plant material and species identification

Seeds from *Amaranthus* spp. plants that had survived an ALS treatment were collected from soybean fields where resistance to ALS inhibitors was suspected. Five populations were collected in 2010, one in 2014 and three in 2017. Since these populations appeared to be a mix of *Amaranthus* species, an identification of the species present was performed. Fifty seeds per sampling site (2010 and 2014) were germinated following a previously described protocol[26] and seedlings were grown in the greenhouse until flowering. When the first flowers started to open, the simplified botanical key was used to identify the species. At least 20 plants of the same species from the same site were then enclosed in non-woven fabric cages and left to produce seeds. A male/female plant ratio of 1:1 was kept for the dioecious species *A. tuberculatus*. The seed progenies were used for all subsequent experiments. For the three populations collected in 2017, the identification of *Amaranthus* species was performed directly in the field using the simplified

botanical key. In addition, three susceptible populations that had never been treated with ALS inhibitors were included in herbicide screening experiments, one per *Amaranthus* species: 17-53 for *A. hybridus*, 17-52 for *A. retroflexus* and 17-65 for *A. tuberculatus.*

Accession codes refer to year of sampling and sampling sites (Fig. 1), letter "L" means "identified and reproduced in greenhouse" and a third letter refers to species found within the same site (R: *A. retroflexus*, H: *A. hybridus*, T: *A. tuberculatus*).

Figure 1. Sampling map with sampling site codes (adapted from Google Maps[36]).



2.2.3. Whole-plant herbicide sensitivity assessment

Seed germination procedure was the same as in section 3.2.[26] Seedlings growth and herbicide treatment were done following an established and protocol.[37] Experimental layout was a complete randomized design with two replicates (trays). Twenty seedlings per population, at very similar growth stage, were transplanted into plastic trays (325x265x95 mm) with a standard potting mix (60% silty loam soil, 15% sand, 15% perlite and 10% peat) and watered daily as required. The experiment was conducted in a greenhouse and repeated twice. Just prior to treatment, plants of

each pot were counted. Plants were treated at 12-14 BBCH [38] at the recommended field rate (1x) and three times that (3x) with ALS-inhibitors and only at field rate with alternative herbicides, along with recommended surfactants. ALS-inhibitor herbicides field rates: thifensulfuron-methyl was applied at 6 g a.i. ha^{-1} (Harmony 50 SX, DuPont™, 50 g a.i. 100 g^{-1}), imazamox was applied at 40 g a.i. ha^{-1} (Tuareg®, DuPont™, 40 g a.i. L^{-1}). Alternative control herbicides field rates: glyphosate was applied at 480 g a.i. ha^{-1} (Roundup Platinum®, Monsanto, 480 g a.i. L^{-1}), metribuzin was applied at 210 g a.i. ha^{-1} (Feinzin® 70 DF, Adama, 70 g a.i. 100 g^{-1}), bentazon was applied at 870 g a.i. ha^{-1} (Basagran® SG, Basf, 87 g a.i. 100 g^{-1}). Herbicides were applied using a precision bench sprayer delivering 300 L ha^{-1} at a pressure of 215 kPa and speed of about 0.75 m s^{-1}, with a boom equipped with three flat-fan (extended range) hydraulic nozzles (Teejet, 11002). Four weeks after herbicide application, the number of surviving plants and the visual estimation of their biomass (VEB) were assessed. The VEB scores, ranging from 10 (for plants not affected by the herbicide compared to the untreated control) to 0 (when the plants were clearly dead, sensitive), were given to each treated tray. On the basis of herbicide efficacy, the accessions were ascribed to four categories as follows: susceptible (S) if survivors were fewer than 5% at 1x rate; moderately resistant (MR) if survivors were between 5% and 20% at 1x rate; resistant (R) if survivors were more than 20% at 1x rate; highly resistant (HR) if survivors were more than 20% at 1x rate and more than 10% at 3x rate. Greenhouse temperature varied between 15 and 20 °C and from 25 to 34 °C, during the night and day, respectively. Standard error (SE) was calculated for each data mean.

2.2.4. Molecular analyses

2.2.4.1.Genomic DNA extraction and ALS amplification

Genomic DNA was extracted from young leaf tissue of five plants that survived the treatment with thifensulfuron-methyl at 1x rate, plus two non-treated plants per susceptible check. Extraction was done using the CTAB (cetyltrimethylammonium bromide) method.[39] Amplification of *ALS* gene was obtained using the primers as described[26]. PCR mixes were performed using GoTaq® G2 Hot Start Polymerase (Promega, USA) in a 25 µL mixture including 5 µL of 5x Green GoTaq Flexi Buffer, dNTPs mix (0.2 mM each), $MgCl_2$ (1.5 mM), forward and reverse primers (0.2 µM), 0.125 µL GoTaq DNA Polymerase, and 50 ng DNA. Amplification conditions: 2 min at 95 °C; 35 cycles of 30 s at 95 °C, 30 s at 58 °C, 40 s at 72 °C; 5 min at 72 °C. PCR products were purified with NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co., Germany) following the manufacturer's

instructions. Once purified, both strands of the PCR products obtained from each plant were Sanger-sequenced by BMR Genomics (Italy) and edited with FinchTV 1.4.0.

3.4.1.1.Species-specific molecular marker

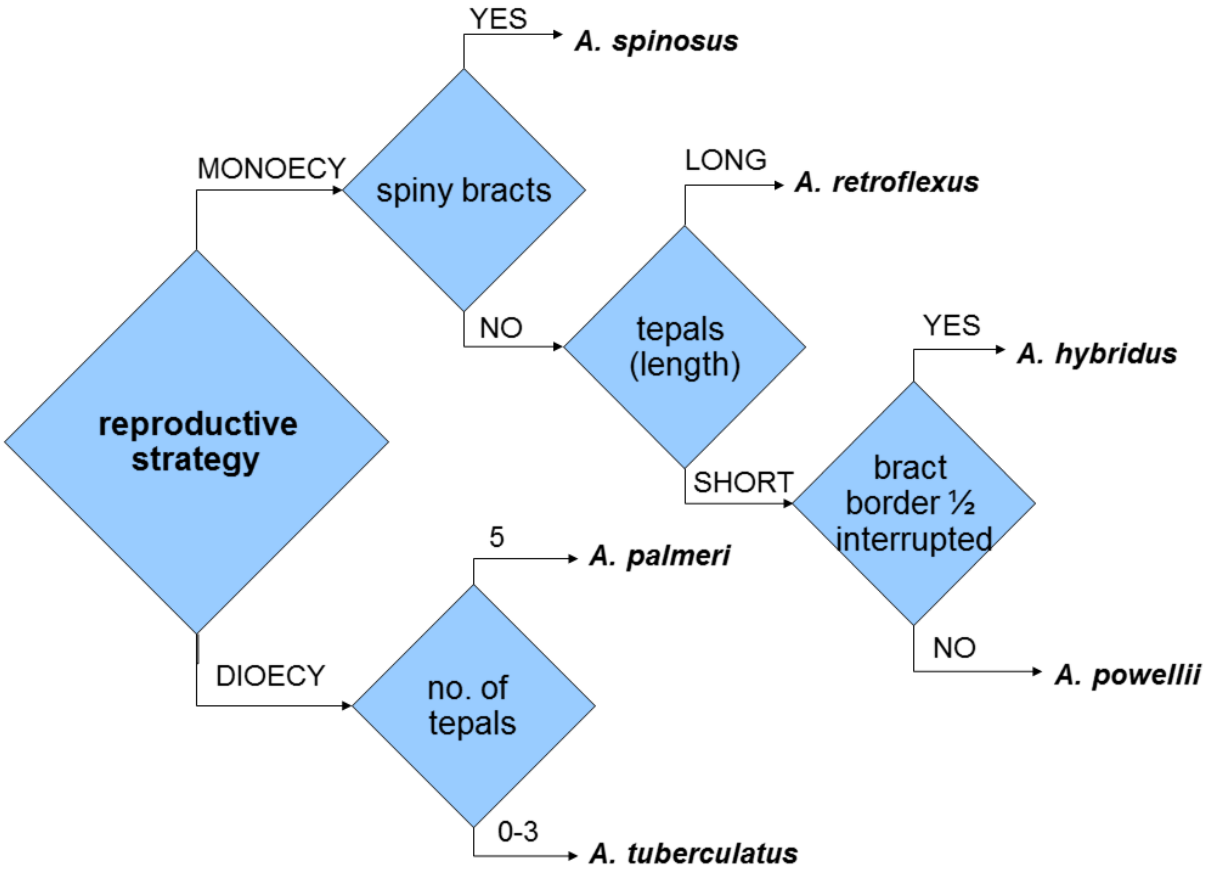
A recently published *Amaranthus* species-specific molecular marker was used to further confirm the species identity and to find possible hybrids.[24] Primers to specifically recognize and amplify species-specific polymorphisms within an intronic region of EPSPS gene were designed. This permitted the identification of *Amaranthus* species after PCR amplification with different primers in separate PCR reactions. Four plants of two accessions per species (24 plants in total) were PCR tested. The accessions chosen were those that had been found together in the field, plus the most phenotypically variable accession of *A. tuberculatus* (10-13 L). gDNA samples were randomly selected among those extracted for *ALS* amplification. PCR mixes were performed using GoTaq® G2 Hot Start Polymerase (Promega, USA) in a 25 µL mixture including 5 µL of 5x Green GoTaq Flexi Buffer, dNTPs mix (0.2 mM each), $MgCl_2$ (1.5 mM), forward and reverse primers (0.2 µM), 0.125 µL GoTaq DNA Polymerase, and 50 ng DNA. Amplification conditions: 2 min at 95 °C; 35 cycles of 30 s at 95 °C, 30 s at 55/56/58 °C (depending on primer couple), 2 min at 72 °C; 5 min at 72 °C. Primer couples AW471 × AW482 were used to amplify gDNA from *A. retroflexus*, (1616 bp); AW468 × AW469 for *A. tuberculatus* (992 bp); AW473 × AW483 for *A. hybridus* (1623 bp). [24]

2.3. Results

2.3.1. Species identification with the simplified botanical key

Identification of *Amaranthus* species was performed following the simplified botanical key reported in Fig. 2. Plants from site 10-12 were monoecious, spine-like structures were absent and tepals were as long as stigmas, so the accession was classified as *A. retroflexus* (10-12 L). Plants from sites 10-10, 10-13, 10-14 and 14-35 were dioecious and had up to three tepals, so the accessions were classified as *A. tuberculatus* (10-10 L, 10-13 L, 10-14 L and 14-35 L). Plants from site 10-11 were monoecious, spine-like structures were absent, but tepal length was not the same for all plants. Tepals of 27 out of 50 plants were as long as stigmas, so these plants were classified as *A. retroflexus* and reproduced separately (10-11 R-L). Tepals of 23 out of 50 plants were shorter than stigmas and bracts had membranous borders interrupted mid-way, so these plants were classified as *A. hybridus* and reproduced separately (10-11 H-L).

Figure 2. The simplified botanical key for weedy amaranths



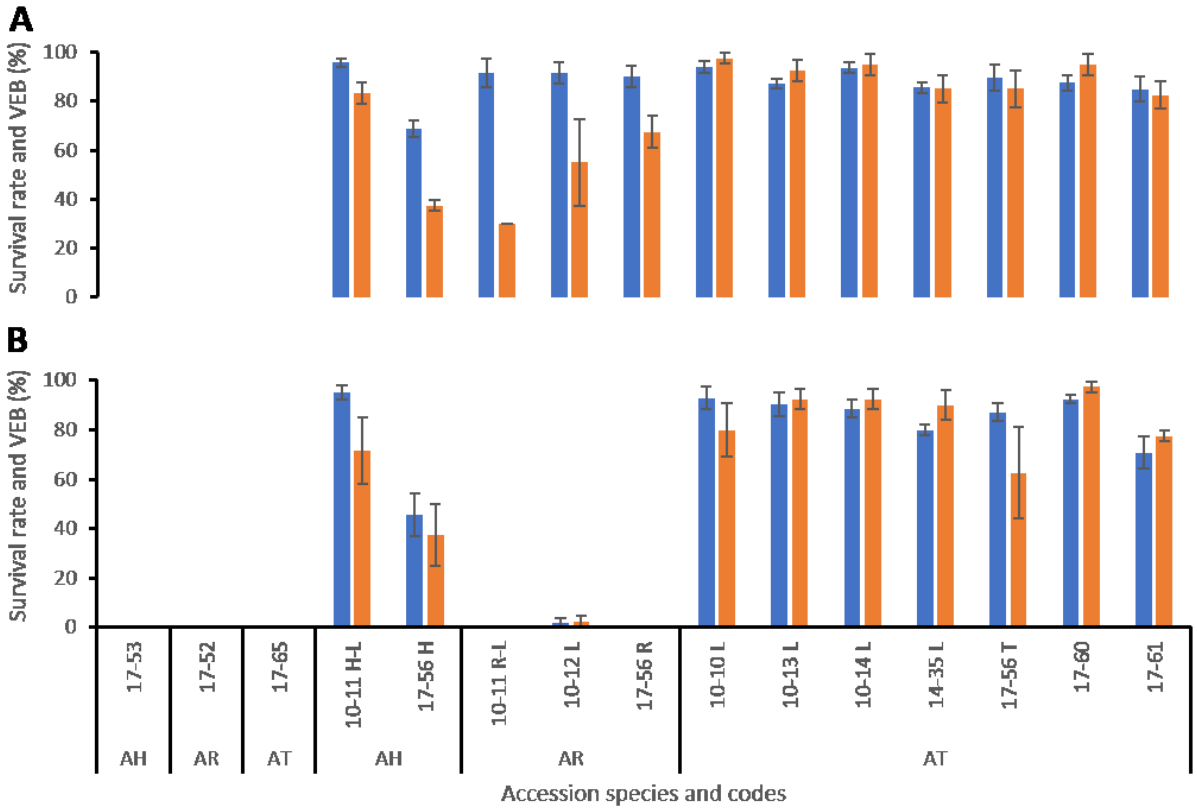
Species identification of populations collected in 2017 was done directly during the sampling and seeds were collected separately depending on the species. Plants of sites 17-60 and 17-61 were dioecious and had up to three tepals, so the accessions were classified as *A. tuberculatus*. In site 17-56, three species of *Amaranthus* were found, *A. retroflexus*, *A. hybridus* and *A. tuberculatus*.

2.3.2. Herbicide efficacy

2.3.2.1.ALS-inhibitors

Survival rate and VEB mean values did not vary significantly between the first and second experiment (t-test with $\alpha=0.05$) and therefore the data were pooled and averaged. Survival rates and VEB did not vary significantly between dose 1x and 3x for both herbicides. Thus, for clarity, only results relative to 1x rates are reported for both herbicides (Fig. 3). Herbicide susceptible accessions (17-53, 17-52, 17-65) were completely controlled by both ALS-inhibitor herbicides, imazamox and thifensulfuron-methyl, indicating that herbicide rates and application protocol were effective

Figure 3. Effect of thifensulfuron-methyl (A) and imazamox (B) treatments at respective field rates. Blue bars refer to the percentage of surviving plants and orange bars to VEB. Vertical bars represent the standard errors. Acronyms AH, AR, AT refer to A. hybridus, A. retroflexus and A. tuberculatus, respectively. The first three accessions on the left are the susceptible checks. 80 plants were treated per each accession.



All accessions were resistant to thifensulfuron-methyl, with survival rates ranging from 69% to 98%. The *A. tuberculatus* and *A. hybridus* accessions were also resistant to imazamox, with survival rates of 71 to 93% and 45 to 95%, respectively.

Visual estimation of biomass did not highlight significant differences among most accessions, generally varying from 30% to 98%. Population 17-56 H had the highest VEB loss (62%) with both herbicides.

Since *Amaranthus* species are diploid and have one functional copy of ALS gene, this behavior was compatible with target site-based resistance mechanism. Furthermore, because different resistance patterns were observed among *Amaranthus* species, different resistant ALS alleles should be involved.

2.3.2.2.Alternative control herbicides

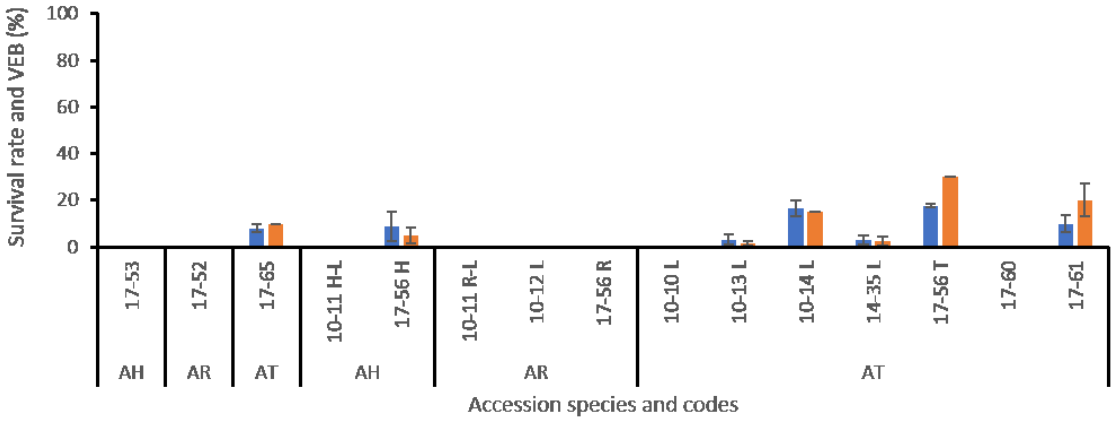
No plants of susceptible and ALS-resistant accessions survived glyphosate or metribuzin at the recommended field rate (data not shown). Bentazon treatment (Fig. 4) completely controlled *A. retroflexus* and *A. hybridus* susceptible checks and ALS resistant accessions, with the exception of accession 17-56 H that had 9% of survivors and 5% VEB. Plants of the *A. tuberculatus* susceptible check survived (8%) the treatment with bentazon but their biomass was very low (10% with respect to non-treated control). The ALS resistant accessions of *A. tuberculatus* 10-10 L, 10-13 L, 14-35 L and 17-60 were completely controlled by bentazon while accessions 10-14 L, 17-56 T and 17-61 had a survival rate of 17, 18 and 10%, respectively and a VEB of 15, 30 and 20%, respectively.

2.3.3. *ALS* point mutations

Mutated *ALS* alleles were detected in almost all plants (Table 1), except one plant for each accession 10-14 L, 17-56 T and 17-61 (*A. tuberculatus*), 17-56 R (*A. retroflexus*) and 17-56 H. No mutation was detected in susceptible checks.

A. retroflexus resistant plants had a point mutation at position 376 of the *ALS* gene and were homozygous for aspartic to glutamic acid (GA**T** to GA**A**) substitution. Instead, all *A. tuberculatus* and *A. hybridus* resistant plants had a point mutation at position 574 of the *ALS* gene. In 8 out of 9 accessions all resistant plants had a tryptophan to leucine change (single substitution of G to T, T**G**G to T**T**G) (Table 1).

Figure 4. Survival rate and VEB of plants treated with bentazon at field rate. Blue bars refer to survival rate, orange bars to VEB. Vertical bars represent standard errors. The first three accessions are the susceptible checks. Acronyms refer to different species: AH-A. hybridus, AR-A. retroflexus, AT-A. tuberculatus.



Different amino acid substitutions were observed in accession 17-56 H (*A. hybridus*): five out of seven plants were homozygous for a tryptophan to methionine change (double substitution of TG with AT, **TG**G to **AT**G), one out of seven was heterozygous: an allele encoded for tryptophan to methionine change, the other for tryptophan to arginine change (single substitution of T with A, **T**GG to **A**GG). One out of seven plants was homozygous for a tryptophan to leucine change.

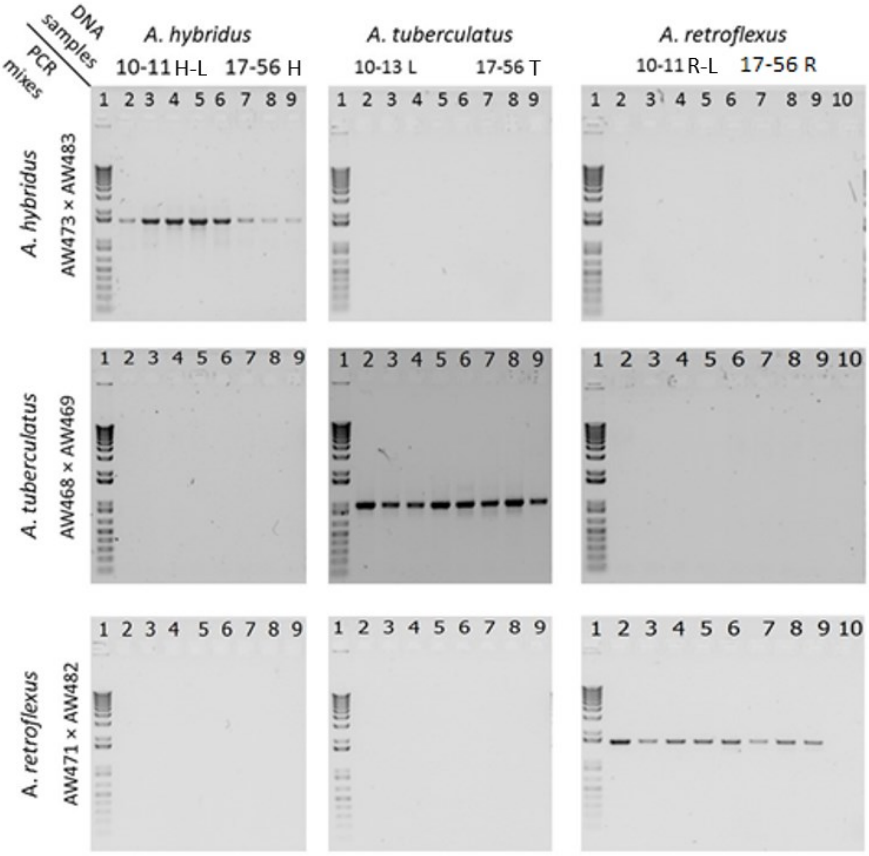
Table 1. *ALS* point mutations associated with resistance in the 3 *Amaranthus* species. S means susceptible and HR highly resistant. ‡ means not all plants were mutated.

Amaranthus species	Accession code	No. mutated plants/ no. analyzed plants	*ALS* point mutation	Resistance pattern	
				imazamox	thifensulfuron-
A. retroflexus	10-11 R-L	7/7	$Asp_{376}Glu$	S	HR
	10-12 L	7/7	$Asp_{376}Glu$	S	HR
	17-56 R	5/6‡	$Asp_{376}Glu$	S	HR
A. tuberculatus	10-10 L	7/7	$Trp_{574}Leu$	HR	HR
	10-13 L	7/7	$Trp_{574}Leu$	HR	HR
	10-14 L	6/7‡	$Trp_{574}Leu$	HR	HR
	14-35 L	7/7	$Trp_{574}Leu$	HR	HR
	17-56 T	6/7‡	$Trp_{574}Leu$	HR	HR
	17-60	5/5	$Trp_{574}Leu$	HR	HR
	17-61	4/5‡	$Trp_{574}Leu$	HR	HR
A. hybridus	10-11 H-L	7/7	$Trp_{574}Leu$	HR	HR
	17-56 H	5/7	$Trp_{574}Met$	HR	HR
		1/7	$Trp_{574}Leu$	HR	HR
		1/7	$Arg_{574}Met$	HR	HR

2.3.4. Species-specific molecular marker

Each population was tested with all three possible PCR mixtures, each one designed to give a PCR product only if species-specific sequences were recognized. The eight *A. hybridus* samples had an amplicon of the expected size of ~1600 bp after amplification with primers AW473 and AW483 (Fig. 5, on the left). The eight *A. tuberculatus* samples had an amplified fragment of ~1000 bp when primers AW468 and AW469 were used and the eight *A. retroflexus* samples had an amplicon of 1600 bp when using the couple of primers AW471 and AW482 (Fig. 5, on the right). Visible bands were well defined and none of the DNA samples was amplified by more than one primer couple (PCR mix).

Figure 5. Patterns of Amaranthus species-specific molecular marker from eight plants for each Amaranthus species. In each gel picture: lane 1 is 1 Kb Plus DNA Ladder (Invitrogen™, ninth band from the bottom is 1650 bp, 16 ng), lanes 2 to 9 are DNA samples. Line 10 is a negative control (no DNA). The eight DNA samples for each species (from left to right, 24 samples in total), were PCR amplified with three different PCR mixes (from top to bottom), each containing species-specific primers.



2.4. Discussion

Comprehensive botanical keys can be tricky to use by non-experts and errors in species classification can generate confusion. Correct species identification is crucial for the implementation of proper integrated weed management, because different species are differently prone to evolve herbicide resistance and react differently to various control tools. The simplified botanical key presented herein focuses on six *Amaranthus* species mostly found in soybean crops. It was used successfully in complex situations, where up to three *Amaranthus* species were found co-living. Easier to use identification tools designed to recognize pests of specific agronomic habitats can be useful for agronomists and pest scientists.

All tested accessions were resistant to ALS inhibitors and most surviving plants had a point mutation, thus the main resistance mechanism appeared to be target-site mediated. However, the presence of a few resistant plants without mutation suggested that other mechanisms contributed to the overall resistance status. In *A. hybridus* and *A. tuberculatus* the most common point mutation was $Trp_{574}Leu$ that caused cross-resistance to thifensulfuron-methyl and imazamox, regardless of the species. This mutation is widely known to endow cross-resistance to sulfonylureas and imidazolinones,[10,40,41] thus results were consistent with what was previously reported. $Trp_{574}Met$ substitution, found in accession 17-56 H of *A. hybridus*, was previously described in *Apera spica-venti* L., however the authors tested chlorsulfuron resistance, but not other ALS-inhibitors.[42] It is worth mentioning that this amino acid substitution is caused by a double mutation, an overall rare and intriguing event.[43,44] $Trp_{574}Arg$, which was detected in only one plant of 17-56 H, had previously been found in *Digitaria sanguinalis* (L.) Scop. causing a broad-spectrum resistance to nicosulfuron, imazethapyr and flumetsulam.[45] Notably, survival rates to thifensulfuron-methyl and imazamox of each cross-resistant accession, i.e. *A. hybridus* and *A. tuberculatus*, had comparable values. The only exception was *A. hybridus* 17-56 H where the efficacy of the sulfonylurea (about 70%) was significantly higher than the imidazolinone (about 50%). It could be speculated that this behavior is associated to the presence of variant $Trp_{574}Met$ in about 50% of the treated plants, i.e. only $Trp_{574}Met$ homozygous individuals survived both herbicides.

All *A. retroflexus* accessions carrying the $Asp_{376}Glu$ point mutation were highly resistant to thifensulfuron-methyl, but susceptible to the imidazolinone imazamox. This result apparently disagrees with the initial characterization of this mutation,[46] as well as with further investigations in different species,[47,48,49] which demonstrate that $Asp_{376}Glu$ causes resistance to imidazolinones.

In these studies, the imidazolinone herbicide was imazethapyr. Nevertheless, some authors have recently observed that both *Lolium perenne* L. and *Raphanus raphanistrum* L. carrying $Asp_{376}Glu$ had different responses to different imidazolinones herbicides.[50,51] ALS *in vitro* assays conducted on *Sorghum halepense* (L.) Pers., also demonstrated that imazamox inhibits the enzyme even if mutated at position Asp_{376}.[52] Other experiments conducted on the same *A. retroflexus* accessions analyzed in this study proved that this mutation endows cross-resistance to thifensulfuron-methyl and imazethapyr (unpublished data, Andrea Milani et al.).

Generalization on the cross-resistance patterns to ALS herbicides endowed by specific *ALS* mutations cannot be based on response to one or two herbicides from a particular ALS herbicide chemistry.[51] This is of particular importance for resistance management recommendations as to which ALS inhibitors remain effective for weed control.[52,53] Multiple molecules of the same chemical family should be tested at the same time when describing a new mutation/substitution, possibly choosing among the ones that the weed population might have experienced.

Our results proved that herbicides with different modes of action are effective against ALS-resistant *A. retroflexus*, *A. hybridus* and *A. tuberculatus*, so they could be used to manage these resistant *Amaranthus* biotypes in soybean fields. Given that *A. retroflexus* populations carrying $Asp_{376}Glu$ mutation were perfectly controlled by imazamox, it could also be used. Glyphosate and metribuzin were effective at the field rate on all species, whereas bentazon was only effective on *A. hybridus* and *A. retroflexus*. Post-emergence application of bentazon resulted in poor control of some *A. tuberculatus* accessions, likely because some were slightly bigger than others when treated. It had been observed that bentazon poorly controlled *A. tuberculatus* plants at 6-10 leaf growth stage:[54] higher efficacy could be obtained at earlier growth stage (BBCH 10-12, early post-emergence application). However, it might also be possible that different *Amaranthus* species respond differently to bentazon treatment.

A. tuberculatus naturalization in Italy dates back to the 1980s,[55] but its habitat was limited to river beds and banks and it was unknown in agricultural habitats until this report. No wild type *A. tuberculatus* infestation in soybean was reported in Europe until now and, so far, all populations found in soybean crops in Italy proved to be ALS-resistant. Further research is ongoing to understand whether these populations evolved resistance traits independently or not. *A. hybridus* is quite common in agricultural habitats, but because of its overall resemblance to *A. retroflexus* its presence might be underestimated. Since the most widely used post-emergence treatment in

soybean is (tank-mixed) thifensulfuron-methyl and imazamox, species carrying $Trp_{574}Leu$ or $Trp_{574}Met$ mutations might be rapidly selected, having a selective advantage over plants carrying $Asp_{376}Glu$. Hybridization among these three co-living species, is not likely in this case, because they had different mutations. In fact, no hybrids were detected using a species-specific molecular marker.

Results confirmed the species identification obtained with the simplified botanical key and excluded that tested samples contained hybrids. This is of particular interest, knowing that inter-fertile species had been found living together (i.e. *A. tuberculatus* and *A. hybridus*). It was observed that *A. tuberculatus* can gain *A. hybridus* resistance traits under field conditions through hybridization,[22,56] whereas in our case each species likely evolved resistance traits independently.

In addition to using herbicides with different SoAs, all existing alternatives to chemical control should be adopted to delay/slow down resistance evolution. Crop rotation, mechanical control, stale seed bed, denser soybean sowing (narrow-rows) and crop cultivar choice should be part of proper integrated weed management strategies.

2.5. Conclusion

The simplified botanical key proposed herein could be a useful tool for agronomists, weed scientists and non-professional systematic botanists. It allowed three different *Amaranthus* species living either in different fields or sympatrically to be identified reliably. All tested accessions were resistant to ALS inhibitors and most surviving plants had a point mutation, so the main resistance mechanism appeared to be target-site mediated. $Trp_{574}Leu$ was the most common point mutation and caused *A. hybridus* and *A. tuberculatus* accessions to be cross-resistant to thifensulfuron-methyl and imazamox. The *ALS* substitution $Trp_{574}Met$, new for *Amaranthus* genus, was found, and likely allowed *A. hybridus* to resist both imazamox and thifensulfuron-methyl. Glyphosate and metribuzin can be used to effectively control these populations, whereas the use of bentazon should be more carefully evaluated.

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Chapter III

Population structure and ALS resistance evolution of *Amaranthus tuberculatus* in Italy

3.1. Introduction

A. tuberculatus is an invasive weed species that is threatening agriculture worldwide. It is of concern because of its high propensity to expand its habitat and evolve herbicide resistance. Its habitat is typically riparian, and it infests riverbanks and floodplains. *A. tuberculatus* is native of United States and Canada, has now been recorded as casual alien in many countries all over the world and have become naturalized somewhere. The first cases of *A. tuberculatus* resistant to ALS-inhibiting herbicides were recorded in its native range (1993 in USA and 2002 in Canada). Since then, resistant cases had increased, as well as the number of herbicide site of action involved. Herbicide resistance patterns, resistance mechanisms and their inheritance have been deeply investigated in the last twenty years. Minor attention has received the study of population genetics approaches aimed to understand the dynamics of *A. tuberculatus* invasion in agricultural habitats. In the earlier study, microsatellites (short sequence repeat, SSR) were used to trace habitat expansion of *A. tuberculatus* var. *rudis*, demonstrating its expansion from west to east USA[1]. In the second study, a population genomics approach showed that glyphosate resistance in Canada occurred because of both introduction of resistant populations from USA and independent selection[2]. Both studies referred to North American populations and there is no information about *A. tuberculatus* population structure outside its native range.

In Italy, *A. tuberculatus* became invasive from the 80's, starting from the West side of Po Valley and then spreading along the Po river, until its delta[3]. *A. tuberculatus* forms very dense populations that tend to exclude native flora. It is considered invasive in Lombardia and Emilia-Romagna regions and naturalized in Veneto, Toscana and Marche. Despite its invasiveness, floristic records in Italy were limited to its habitat[4] until recently. In 2010, the first European populations of agricultural weedy waterhemp were found in Veneto region. This earlier finding, was then followed by several others, including one population in Emilia-Romagna in 2014 and some populations in 2017 in Veneto. Remarkably, all those populations were found in soybean fields, far from the habitat of this species. During the sampling, no wild type populations of this weed were observed in the surroundings and they were supposed to be isolated populations. Furthermore, all stakeholders agreed that susceptible population of this species were never observed infesting fields before that period. Post-emergence application of acetolactate synthase (ALS)-inhibitors is the most adopted weed control strategy in soybean in Italy and whole plant herbicide essays confirmed that seven *A. tuberculatus* populations collected in Italy were cross-resistant to thifensulfuron-methyl and imazamox (10-10, 10-13, 14-35, 17-56, 17-60 and 17-61; *cf.*

Chapter II, where populations 10-10, 10-13 and 14-35 were named 10-10L, 10-13L and 14-35L, respectively). Partial sequencing of *ALS* gene revealed that those populations had a tryptophan to leucine substitution at position 574 of *ALS* gene, known to cause broad cross-resistance to inhibitors of acetolactate synthase in *A. tuberculatus*[5]. Even if all populations had the same point mutation, it was unclear whether they originated from single or multiple selection events and how they spread across the Country. The disruptive potential of *A. tuberculatus* is well-known and understanding how herbicide resistance evolves is crucial for its management.

Most studies on pesticide resistance aim to identify the mechanism conferring reduced sensitivity to the compound of interest and how to control eventual resistant populations. This approach is fundamental for agronomy and integrated pest management, but these studies do not help to unravel how resistance originates and spreads. However, origin and spread of resistance are human-driven evolutionary processes[6,7]: a deeper understanding of the evolutionary mechanisms responsible for resistance can greatly benefit resistance risk assessment and management strategies[8,9]. Herbicide resistance is a fascinating example of evolution in action[10,11], with rapid adaptation fallowing abrupt environmental changes -the selective pressure imposed by herbicides- and does have the potential to contribute to a broader understanding of evolutionary processes. If a point mutation gives a selective advantage, its frequency within a population will rise as a result of selective pressure. After some generations, the variation (i. e. the polymorphism level) among the nucleotides near the point mutation would be heavily reduced, or even completely eliminated (i. e. selective sweep)[12]. Neutral (not under selection) loci that are genetically (physically) linked with the point mutation would be inherited together with it, because of genetic hitch-hiking[13]. The sequence of genetically linked alleles that are inherited together -the haplotype- is, therefore, expected to be very different among resistant populations, in particular if non-coding sequences are also taken into consideration. For this reason, the analysis of resistant haplotypes among different pest populations gives information on whether an endowing-resistance point mutation arose -independently- multiple times or from a single mutation event. While this approach has been quite exploited in resistance to drugs and insecticides, there are only a few examples of haplotype analysis in studying herbicide resistance (*cf.* 'The evolutionary origins of pesticide resistance'[14] for a wider discussion).

The aim of this study was to investigate the origin and spread of resistance to ALS-inhibiting herbicides among eight *Amaranthus tuberculatus* populations collected in Italian soybean fields.

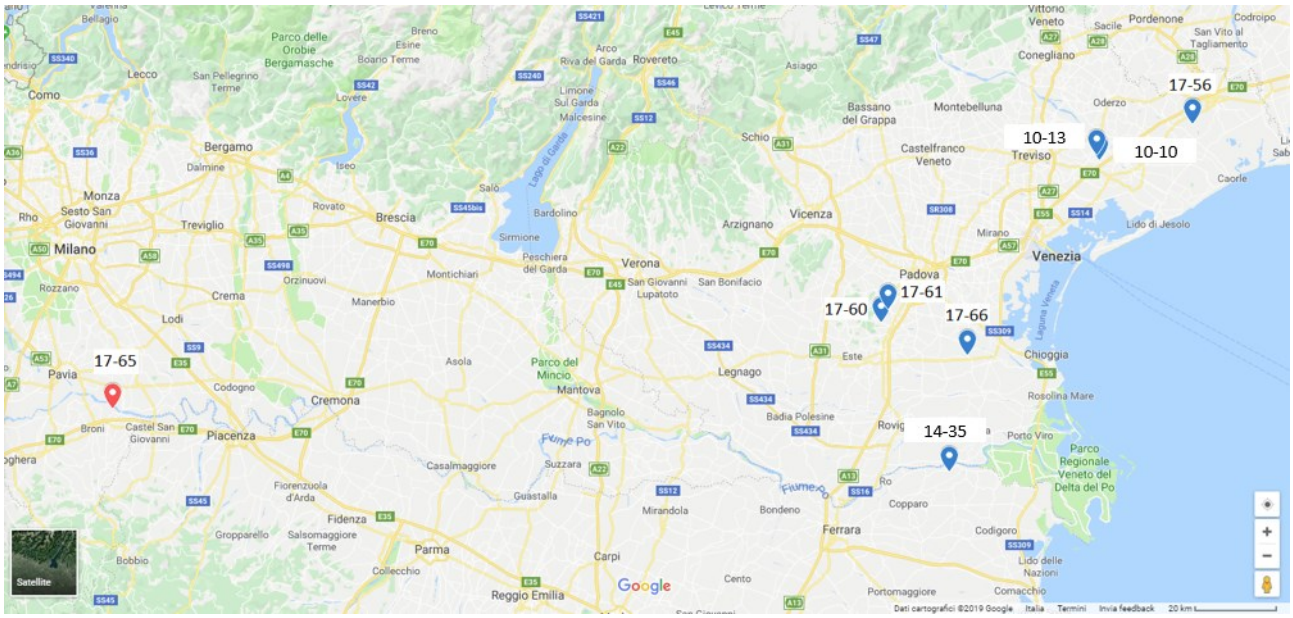
Population structure has been performed with a microsatellite (previously described[1]) protocol, while haplotypes has been inferred from *ALS* amplicon sequencing data. These analyses would give an insight to the comprehension of herbicide resistance evolution and contribute to better weed management strategies.

3.2. Materials and methods

3.2.1. Plant material

Seven *A. tuberculatus* populations were found in North-Eastern Italy soybean fields treated with ALS-inhibiting herbicides and seeds of each population were collected at plant maturity: population 10-10 and 10-13 were collected in 2010, population 14-35 in 2014, population 17-56, 17-60, 17-61 and 17-66 in 2017. Population 17-65 was collected in a Po River floodplain in 2017, where it presumably had never been treated with herbicides. Populations 10-10, 10-13 and 14-35 were reproduced in non-woven fabric cages (from at least 20 plants). All, but one, field-collected populations were highly cross-resistant to imazamox and thifensulfuron-methyl and had a tryptophan to leucine mutation at position 574 of *ALS* gene (*cf*. Chapter II). In addition, another population (17-66) included in this study was highly resistant to thifensulfuron-methyl only (A. Milani, L. Scarabel and M. Sattin, unpublished data).

Figure 1. Sampling sites and population codes. Blue pointers refer to ALS-resistant populations A. tuberculatus, red to susceptible ones. Modified from Google Maps [15].



3.2.2. Microsatellite genotyping and analysis

Twenty seeds per each population were germinated following a protocol previously described[6] and seedlings were grown in the greenhouse until flowering. 12 plants per population (1:1 male:female ratio) were randomly chosen and leaves samples were collected and conserved at -80 °C for subsequent analysis.

3.2.2.1.DNA extraction

DNA was extracted from 100 mg of freeze leaves samples using the CTAB (cetyltrimethylammonium bromide) method [7]. DNA integrity was estimated by electrophoresis on

a 0.8% agarose/1× TAE gel containing 1× SYBR Safe DNA stain (Thermo Scientific, Pittsburgh, PA, USA). Both purity and quantity of DNA extracts were assessed with a NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific, Pittsburgh, PA, USA). Each DNA sample was used for microsatellite analyses and *ALS* amplicon sequencing.

3.2.2.2.Amplification of microsatellite loci

To genotype 96 *A. tuberculatus* samples, a modified version of a previously described protocol [2] was used. The same loci were analyzed, but primers were designed to be multiplexed in two multiplex PCR (mPCR) reactions. Four universal (tagged) primers were used: Hill (TGACCGGCAGCA-AAATTG)[8], Tail_D (CGGAGAGCCGAGAGGTG)[9], D8S1132 (GGCTAGGAAAGGTTAGTGGC)[10] and PAN3 (TGTAGAAAGACGAAGGGAAGG, designed by G. Galla). Universal primers were 5' labeled with different dyes (6FAM™, VIC™, NED™ and PET™ respectively) and all forward locus-specific primers were added with the tag sequences at 5' end. mPCR mixes were performed using GoTaq® G2 Hot Start Polymerase (Promega, USA) in a 10 µL mixture including 2 µL of 5x GoTaq Flexi Buffer, dNTPs mix (0.2 mM each), MgCl2 (1.5 mM), forward (tailed) primers 0.2 µM, reverse (PIG-tailed) primers 0.3 µM, fluorescent primers 0.1 µM, 0.05 µ µL GoTaq DNA Polymerase, and 50 ng DNA. Amplification conditions: 95°C*2', 5 cycles of [95°C*30", 61°C*30", 72°C*20"], 10 cycles of [95°C*30", 64°C*30", 72°C*20"], 20 cycles of [95°C*30", 58°C*30", 72°C*20"], 72°C*5'. mPCR mix 1 amplified loci C1140, C3695, AAC1, C4097 e C0745 and mPCR mix 2 amplified loci C4999, ATC9, C3561, TAG5 e C9333. mPCR products were then run into capillary electrophoresis with an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). LIZ500 was adopted as molecular mass standard. Primer combination of multiple PCR mixes, primer sequences, repeat motif and size of each locus, dyes used for visualization are ported in supplementary material (Table 4).

3.2.2.3.Microsatellite data analysis

Peak size was determined using Peak Scanner™ Software Version 1.0 (Applied Biosystems). Descriptive statistics were obtained using GenAlEx 6.5 [11]. The software POPGENE 1.32 (Yeh *et al*., 1997) was used to compute the dendrogram based on Nei genetic diversity. The model-based Bayesian analysis implemented in STRUCTURE v2.3.4 [12] was used to explore the putative population genetic structure. The analysis was carried out using a burn-in of 500.000 iterations and a run length of 500,000 Markov Chain Monte Carlo (MCMC) replications in ten independent runs. Prior knowledge about the number of populations was not included. The number of populations (K) in the dataset was determined by the averaged likelihood at each K and the variance between replicates was determined by running a continuous series of K = 1–9 to

determine the optimal number of populations present within the 96 individuals. The optimum number of clusters was predicted following the *ad hoc* statistic ΔK [13] implemented in Structure Harvester v0.6.94 [14]. Simulations were performed with no a priori assumptions concerning the admixture model and correlation in the allele frequencies (all combinations were tested).

3.2.3. Amplicon sequencing of acetolactate synthase gene

3.2.3.1. Primer design and PCR amplification

DNA samples are the same used for SSR genotyping (Par. 2.2.1). Concentrations were quantified with Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific, Massachusetts, USA) and adjusted to 3 ng ul^{-1}. Primers were designed using a reference *A. tuberculatus* genome (kindly provided by Prof. P. Tranel, data not published). *A. tuberculatus* acetolactate synthase gene, complete cds (GenBank: EF157818.1), was used to identify the contig containing *ALS* locus. Primers were designed with Benchling (https://benchling.com) to amplify a region of 4-4.5 kbp including *ALS* coding sequence (2 kbp). Primer sequences are ported in supplementary material (Table 5). A pool of 36 random samples were used for initial primer tests and PCR optimization. After that, primers Fw_3 and Rev_4 were chosen because of higher specificity. 10 samples out of 96 were amplified with primers Fw_4 and Rev_3 because amplification with Fw_3 and Rev_4 failed. PCR were performed using Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific, Massachusetts, USA) in 30 µL mixture including 6 µL of 5X Phire Green Reaction Buffer, dNTPs mix (0.2 mM each), forward and reverse primers 0.625 µM each, 0.4 µL Phire Hot Start II DNA Polymerase, and 9 ng DNA. Amplification conditions: 1 min at 95 °C; 35 cycles of 5 s at 95 °C, 5 s at 60 °C, 60 s at 72 °C; 5 min at 72 °C.

3.2.3.2.Dual-indexed library preparation

Libraries were prepared using in-house Tn5 transposase and following a tagmentation procedure previously described [16]. In brief a) in-house Tn5 transposase conjugation to streptavidin magnetic beads (NEB, USA) b) addition of conjugated beads to each DNA sample c) brief incubation to allow tagmentation reaction d) Tn5 transposase stripping. Dual-indexed libraries were prepared using i7 and i5 index adapters (Illumina, USA), Q5® High-Fidelity DNA Polymerase (NEB) and tagmented DNA as template, following manufacturers' instructions. After PCR amplification/indexing, single samples were pooled together and size-selected (~450 bp) with BluePippin (Sage Science, Inc., USA). Actual size and quality of libraries were evaluated using a Bioanalyzer High Sensitivity Chip and run on 2100 Bioanalyzer (Agilent Technologies, Inc, USA). The libraries were then sequenced

to a coverage depth of 500X on an MiSeq™ (Illumina) instrument using a paired-end 150 base read chemistry.

3.2.3.3.SNP-calling pipeline and haplotypes handling

After sequencing, each MiSeq™ reads 1 and reads 2 of each sample were a) de-multiplexed (removal of indexing primers) b) aligned to a reference *ALS* sequence (obtained from a previous *A. tuberculatus* genome assembly, kindly provided by P. Tranel) with BWA-MEM [17] (a paired-end alignment tool) c) sorted, indexed and then merged with SAMtools [18] to get a sorted multiple alignment file. Variants were called with the Bayesian genetic variant detector freebayes [19]. Complex variants (or multi-nucleotide variants, MNPs) were decomposed to more basic/primitive alleles (single-bp) SNPs with vcflib [20]. Genetic variants were annotated with SnpEff [21], a tool that annotates and predicts the effects of genetic variants (such as amino acid changes) using a reference sequence (*A. thaliana ALS*). Haplotype estimation (phasing) was computed with SHAPEIT [22,23]. *In silico* phased haplotypes were aligned using MEGAX [24], and a Neighbor-joining tree was obtained with the same software (bootstrap: 1000). Tree was drawn with Interactive Tree Of Life (iTOL) v4 [25]. Phased haplotypes were also used to infer TCS Networks [26] with the software PopART [27] and the same software was used to draw the georeferenced haplotype map.

3.3. Results

3.3.1. Genetic diversity and population structure

Descriptive statistics of genetic diversity were calculated across the investigated markers and populations. Of the 10 microsatellite loci considered in this study, two loci (C9333 and C3561) did not provide consistent amplification profiles and were excluded in the following investigations. The mean number of observed alleles (Na) across the investigated loci was equal to 4.06, ranging from 1.4 to 6.1 (Table 1). The effective number of alleles ranged from a minimum value of 1.14 to a maximum value of 4.12. For all investigated loci, the effective number of alleles, which was estimated based on allele frequencies across all populations, was lower than the observed number of alleles. Shannon information index across these loci was relatively low, as its estimated ranged from 0.15 to 1.54. Noticeably, comparable values for observed and expected heterozygosity were recorded for most loci, as also indicated by the F statistics performed across the investigated loci. Accordingly, the inbreeding coefficient F, which was estimated as 1 - (Ho / He), was on average as low as 0.07 across the investigated loci. The only exception to this was represented by the locus AAC1, which had a pronounced defect of heterozygosity, as indicated by a fixation index (F) equal to 0.56 (Table 1).

At population level, the mean number of observed alleles ranged from 3.4 to 5.6, while the effective number of alleles ranged from 1.8 to 3.9. The Shannon information index was on average equal to 0.99, ranging from 0.6 to 1.3. The observed heterozygosity (Ho), whose average value was equal to 0.48, was found to be remarkably variable across the investigated populations. More in detail, while heterozygosity exceeded 0.50 in most populations (consistently with the outcrossing reproductive strategy of A. tuberculatus), its estimate was found remarkably low in populations 10-10 (Ho: 0.26) and 14-35 (Ho: 0.37). High variability was also found in our estimates of gene diversity (He), which ranged between 0.37 in population 10-10 and 0.63 in population 17-65. As shown in Table 1, populations 10-10 and 14-35 displayed signs of moderated inbreeding, as indicated by a fixation index (F) higher than 0.2.

Pairwise Population Nei unbiased genetic distance and Fst values among populations are reported on Table 2. The investigated populations were characterized by low genetic distances (mean uHe: 0.203, ranging from 0.019 to 0.393). The estimated value of FST averaged across all comparisons was as low as 0.095, indicating that most genetic variation is found within populations and genetic differentiation among populations is low overall. Genetic flow, which was estimated from FST in all pairwise comparisons, was on average equal to 3.023, with a range of 1.134 – 9.622 for individual

comparisons (Table 3). A high genetic flow was estimated for the pairwise comparisons 17-66 and 17-65 (value 9.62) as well as 14-35 and 17-65 (6.63). Interestingly, both indices highlighted a certain degree of differentiation between the populations: 17-56, 10-10 and, to a lesser extent, 10-10 and the remaining populations (which were overall characterized by lower genetic differentiation as expressed by uHe and FST). This scenario is graphically represented by the neighbor-joining tree and principal coordinate analysis (Figure 2, A and B, respectively), which were generated from the pairwise estimates of uHe. Accordingly, both clustering approaches grouped 14-35, 17-60, 17-61 and 17-66 together with 17-65, and apart from 10-10, 10-13 and 17-56. Consistently with this population clustering, a Mantel test performed by using genetic and geographic distances confirmed the lack of correlation between the two matrices and suggested no isolation by distance across the considered sampling range.

By considering the geographic distribution and main reproductive strategy of A. tuberculatus, we decided to investigate the genetic structure of the populations by adopting multiple computational strategies concerning the admixture model and correlation of allele frequencies. All simulations estimated the most likely number of populations (K) as 2. The clustering of individual samples in two main populations (K:2, Figure 3) allowed the consistent grouping of individuals belonging to the wild population (17-65) within the same ancestral population. Although very low admixture levels (membership: > 80%) were detected for most individuals, several populations (namely, 10-10, 10-13, 17-56 and 17-61) included individuals with contrasting population assignments, indicating, to some extent, admixture at population level.

Interestingly, multiple simulations involving both ancestral and allele frequency models identified an additional level of genetic structure for K:4. In this case, we noticed that populations sampled in fields located in close geographic proximity such as 10-10 and 10-13 and, to a lower extent, 17-60 and 17-61, were clustered apart.

Table 2. Descriptive statistics of genetic diversity calculated across markers and waterhemp accessions, including sample size, No. Alleles (Na), No. Effective Alleles (Ne), Shannon Information Index (I), Observed Heterozygosity (Ho), Unbiased Expected Heterozygosity (uHe), and Fixation Index (F). The fixation index was calculated as 1 - (Ho / He).

Locus	N		Na		Ne		I		Ho		uHe		F	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
AAC1	10.625	0.625	3.625	0.420	2.526	0.299	1.004	0.124	0.284	0.087	0.588	0.058	0.557	0.126
C3695	11.875	0.125	5.375	0.944	3.704	0.723	1.308	0.230	0.674	0.107	0.652	0.101	-0.087	0.061
C0745	11.875	0.125	5.375	0.420	3.691	0.491	1.397	0.126	0.687	0.071	0.713	0.055	0.008	0.046
C1140	11.750	0.164	6.125	0.611	4.122	0.492	1.536	0.094	0.712	0.053	0.772	0.023	0.032	0.078
C4097	11.750	0.164	3.250	0.559	1.698	0.177	0.660	0.116	0.401	0.108	0.384	0.064	-0.040	0.156
C4999	11.250	0.412	3.875	0.350	2.707	0.244	1.085	0.082	0.558	0.057	0.633	0.044	0.088	0.050
TAG5	11.375	0.324	3.500	0.327	1.797	0.182	0.748	0.101	0.399	0.055	0.419	0.064	-0.031	0.064
ATC9	10.750	0.250	1.375	0.183	1.138	0.081	0.153	0.083	0.093	0.058	0.099	0.056	0.011	0.120
Population														
10-10	11.375	0.183	2.500	0.423	1.808	0.282	0.603	0.164	0.263	0.095	0.367	0.099	0.228	0.140
10-13	11.125	0.125	3.750	0.559	2.577	0.422	0.979	0.182	0.508	0.102	0.542	0.095	0.036	0.077
14-35	11.250	0.366	3.500	0.567	2.189	0.331	0.849	0.169	0.373	0.089	0.479	0.091	0.204	0.111
17-65	12.000	0.000	5.625	1.051	3.864	0.895	1.285	0.254	0.604	0.109	0.628	0.107	-0.006	0.069
17-60	11.875	0.125	4.375	0.565	2.685	0.305	1.096	0.139	0.598	0.083	0.602	0.068	-0.051	0.084
17-61	11.000	0.423	4.375	0.800	2.855	0.562	1.009	0.250	0.411	0.109	0.514	0.127	0.112	0.083
17-66	11.500	0.378	5.000	0.655	3.184	0.598	1.178	0.206	0.504	0.106	0.597	0.098	0.097	0.111
17-56	11.125	0.639	3.375	0.460	2.223	0.248	0.890	0.122	0.546	0.117	0.531	0.058	0.021	0.188
Loci and Population	11.406	0.121	4.063	0.250	2.673	0.183	0.986	0.068	0.476	0.037	0.533	0.033	0.075	0.040

4. *Figure 2. Neighbor joining tree and principal coordinate analysis (A), which was generated from the pairwise estimates of uHe between populations (B)*

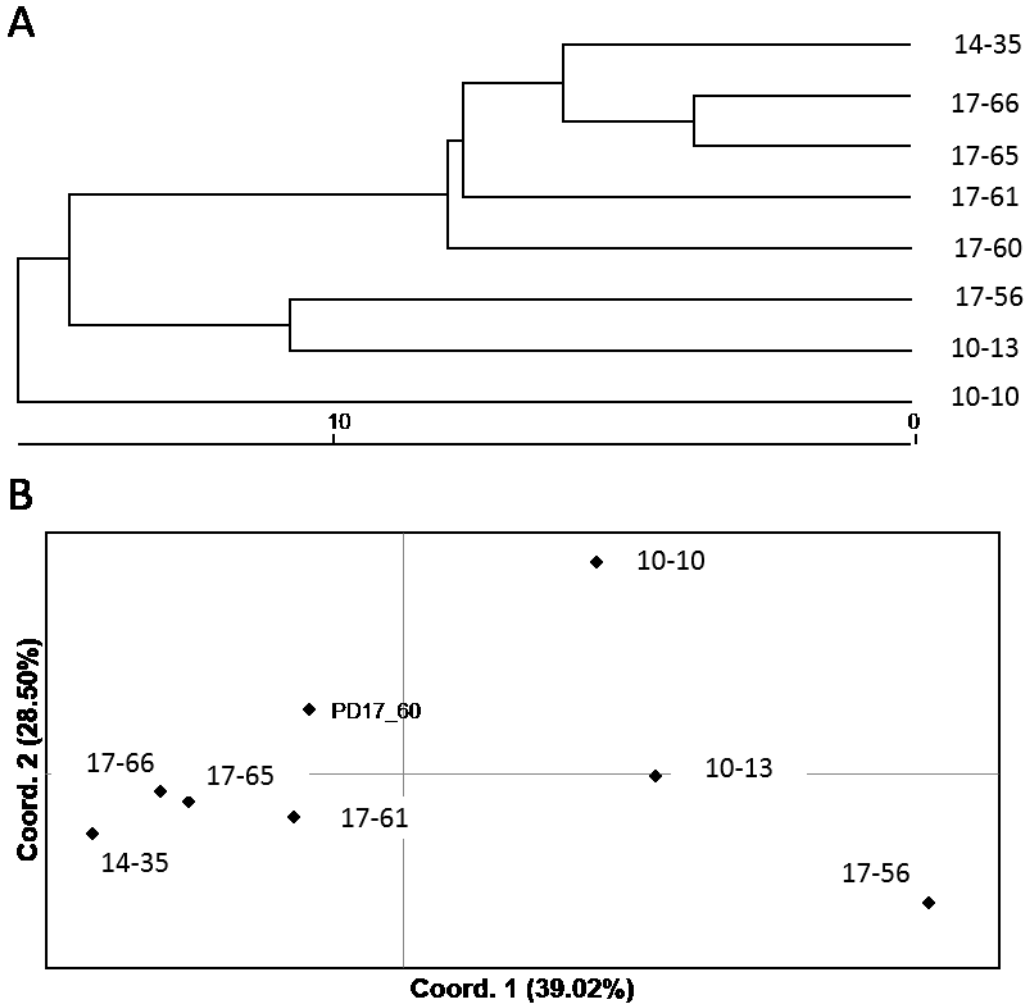


Figure 3. Population assignment by STRUCTURE of individuals for K=2 (upper graph) and K=4 (bottom graph), for the 8 genotyped populations. Populations 14-35, 17-60, 17-61 and 17-66 clustered together with the wild Italian population 17-65, separately from populations 10-10, 10-13 and 17-65. Population names and single plant codes are shown below the bar graph.

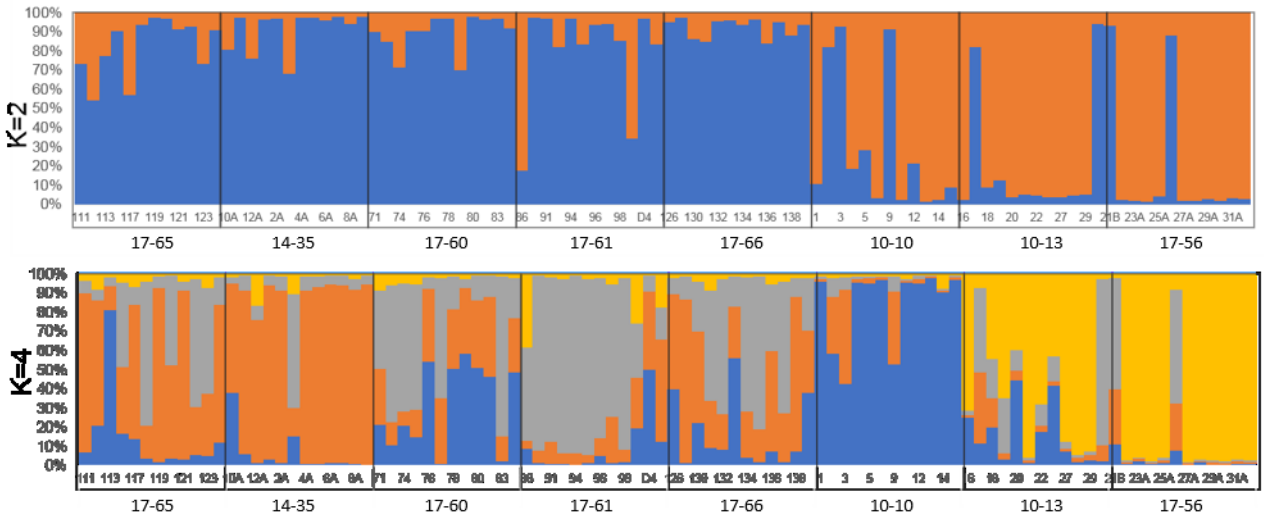


Table 3. Pairwise Population Nei unbiased genetic distance (above diagonal) and Fst values (below diagonal)

	17-56	10-10	10-13	17-60	17-61	17-66	14-35	17-65
17-56		0.393	0.205	0.329	0.292	0.328	0.373	0.315
10-10	0.181		0.223	0.182	0.296	0.265	0.347	0.251
10-13	0.093	0.127		0.188	0.155	0.206	0.256	0.150
17-60	0.112	0.108	0.086		0.121	0.073	0.163	0.122
17-61	0.128	0.153	0.085	0.072		0.066	0.143	0.099
17-66	0.114	0.122	0.075	0.048	0.045		0.085	0.019
14-35	0.142	0.176	0.106	0.084	0.075	0.048		0.032
17-65	0.111	0.130	0.065	0.064	0.062	0.025	0.036	

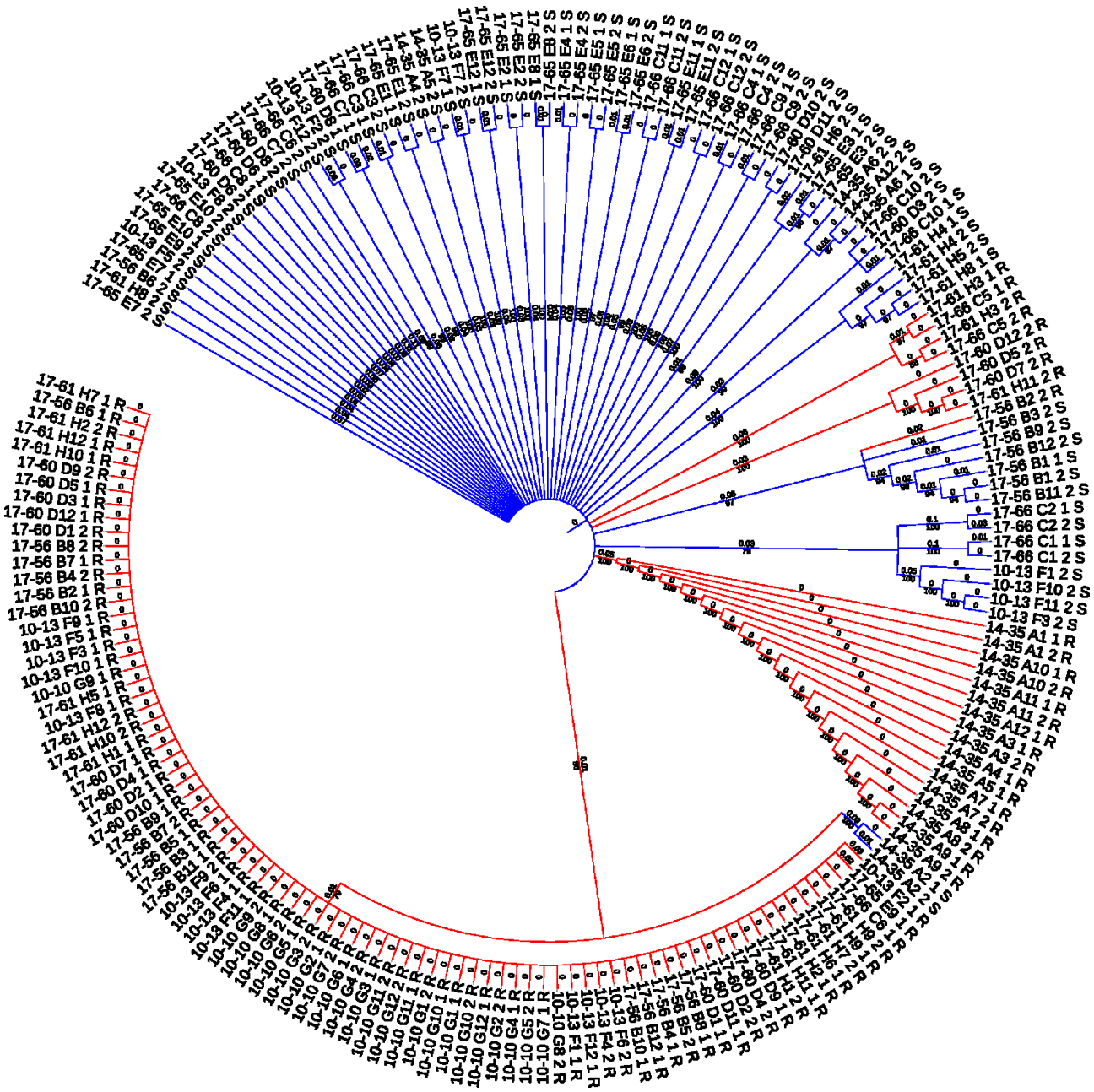
Table 4. Summary of gene flow (Nm) estimates for the investigated populations. Gene flow was estimated as Nm = [(1 / Fst) - 1] / 4

	17-56	10-10	10-13	17-60	17-61	17-66	14-35	17-65
17-56								
10-10	1.134							
10-13	2.430	1.719						
17-60	1.976	2.073	2.648					
17-61	1.700	1.379	2.702	3.218				
17-66	1.941	1.797	3.081	4.943	5.332			
14-35	1.513	1.174	2.107	2.716	3.104	5.001		
17-65	1.995	1.672	3.571	3.667	3.795	9.622	6.633	

3.3.2. Amplicon sequencing of acetolactate synthase gene

389 SNP were found along an amplified sequence of 3956 bp, after filtering for high quality SNP. 112 SNPs were found in the upstream sequence (700 bp), 128 within the coding region (2016 bp) and 149 in the downstream region of the coding sequence (1238 bp). It should be noted that no introns are predicted within the coding region of *ALS*. Within the coding region, we found 54 non-synonymous SNPs. All sequences were checked for the presence of the resistance-endowing mutations (Ala 122, Pro 197, Ala 205, Asp 376, Arg 377, Trp 574, Ser 653, Gly 654)[28]. Additionally, sequences were screened for the presence of a set of additional mutations which were shown to be involved in changes in herbicide sensitivity in artificial selection experiments (Gly 121, Met 124, Val 196, Arg 199, Asp 375, Val 571 and Phe 578)[29]. Remarkably, from this set of non-synonymous mutations potentially associated with herbicide resistance, W574L was the only resistance-endowing mutation found across the sequences. Surprisingly, this mutation was found also in plant E9 belonging to the herbicide susceptible (wild) population 17-65.

Figure 4. Neighbor joining haplotype tree of ALS SNPs. Red clades refer to resistant haplotypes (carrying the $Trp_{574}Leu$ point mutation), while the blues to the susceptible ones. Note that branch lengths are not respected (because of graphical needs), but they are reported along with bootstrap value.



After *in silico* phasing, haplotypes were used to build a Neighbor-joining haplotype tree of *ALS* SNPs (Figure 4). The tree showed three main branches: one grouping mainly susceptible haplotypes (blue clades in Fig. 4) and two separate branches grouping mainly resistant haplotypes (red clades in Fig. 4). Sequences without the W574L mutation scattered from the tree, indicating the absence of specific haplotypes, therefore they were excluded from subsequent analysis. 111 resistant haplotypes (containing the W574L point mutation) were analyzed to define identical sequences and to create a TCS haplotype network and a georeferenced map of haplotype diversity. 12 unique-sequence haplotypes were found, as also the tree highlighted. Among these,

one was found 16 times (14-35_A1_1_R), one 81 times (10-10_G1_1_R), and 10 with frequencies varying from 1 (singletons) to 4. The two most frequent resistant haplotypes (10-10_G1_1_R and 14-35_A1_1_R) diverged for 45 SNPs, therefore they are highly genetically different. Other less frequent haplotypes diverged each other for a very variable number of SNPs (Fig. 5) and were likely result of recombination events. The georeferenced map highlighted distribution and frequencies of each haplotype among populations (Fig. 6). The most frequent haplotype (10-10_G1_1_R) is shared among populations geographically distant, while the other -less frequent- (14-35_A1_1_R) haplotype is found only in population 14-35.

Figure 5. Haplotype network at ALS locus (coding and non-coding sequence). All selected haplotypes had the point mutation $Trp_{574}Leu$. Names of haplotypes refers to the original name of the sequence, reported also in the haplotype tree and in the text. Different colors refer to populations where sequences were found. Sizes of the circles correspond to the number of identical sequences representing each haplotype. Tick marks along a branch indicate the number of mutations between two neighboring haplotypes.

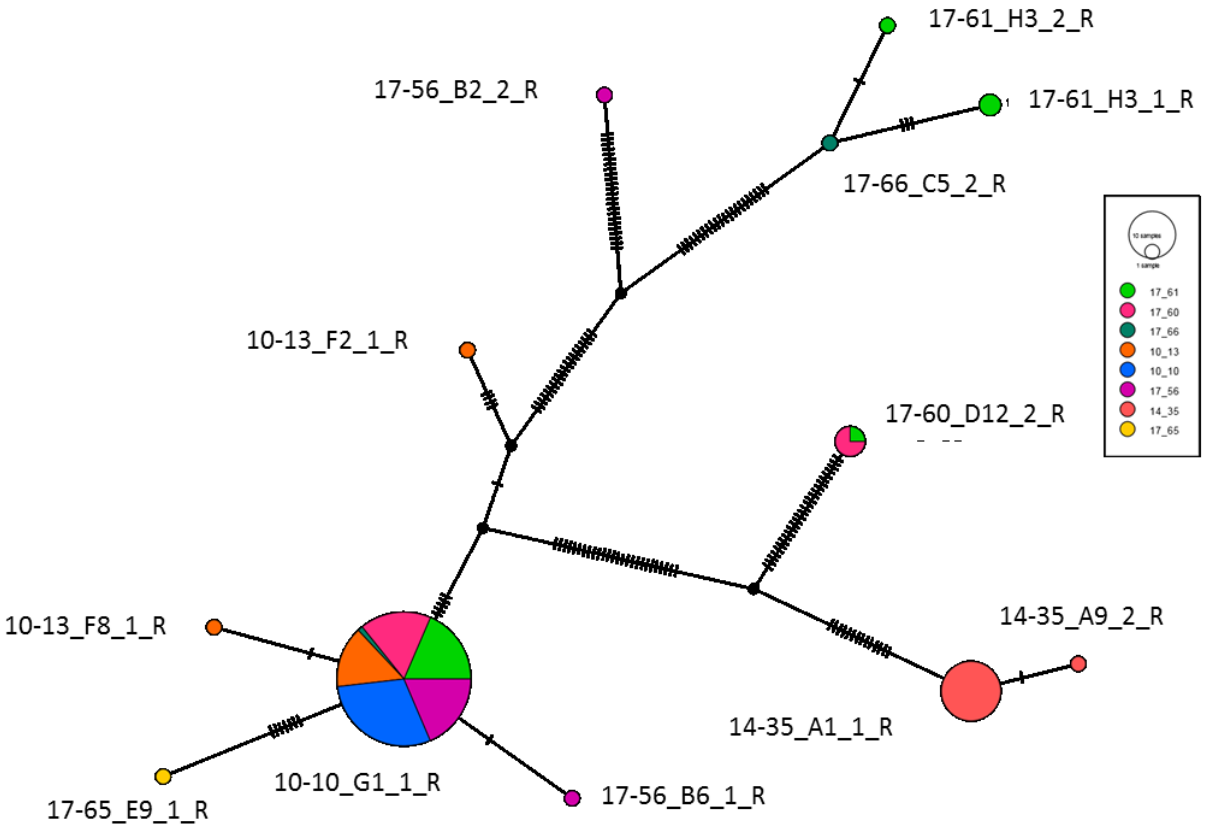
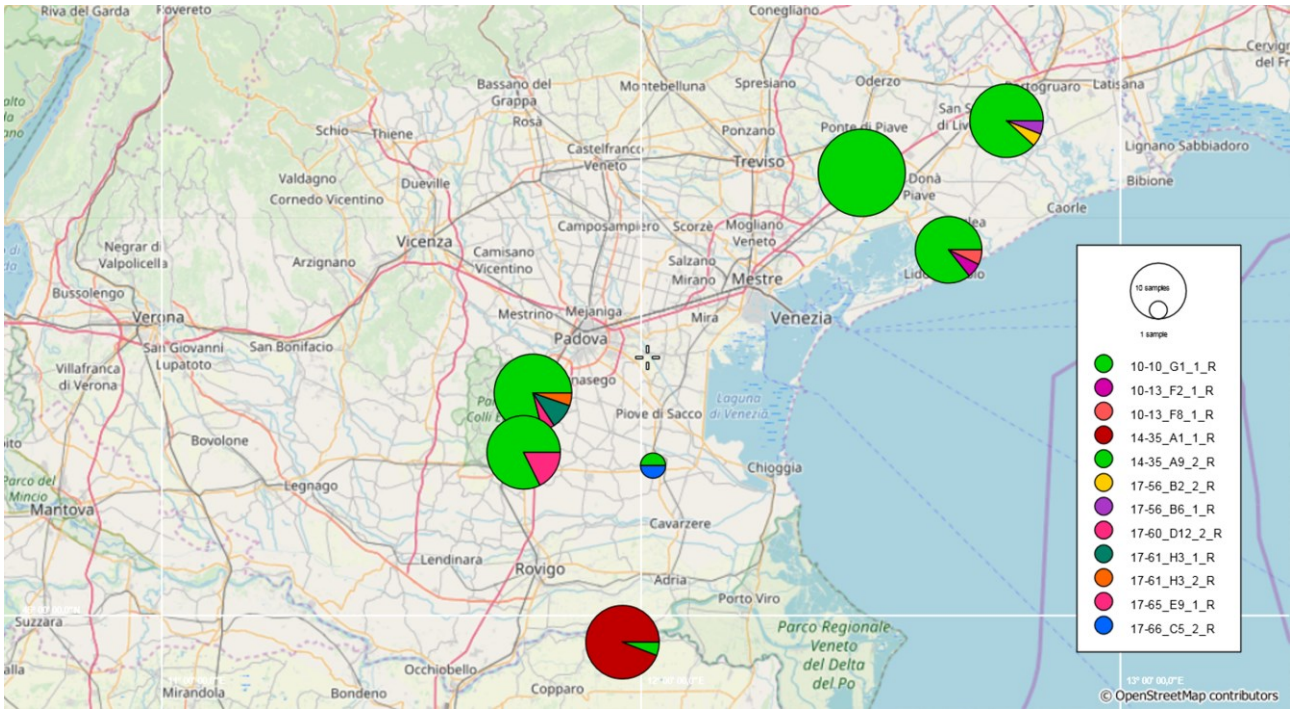


Figure 6. Georeferenced map of haplotype diversity. Each color corresponds to different haplotypes. Note that only haplotypes 14-35_A1_R and 10-10_G1_1_R are present at high frequency. For better map visualization, the singleton haplotype 17_65_E9_1_R found in population 17-65 is not displayed and the real positions of populations 10-10/10-13 and 17-60/17-61 are slightly modified (cf. Fig. 1).



3.4. Discussion

3.4.1. Genetic diversity and population structure

Overall, genetic diversity data were consistent with what previously observed for this species within its native range. Gene diversity (He) was on average equal 0.48 and ranged between 0.37 and 0.63 for individual populations, indicating high within-population genetic diversity. High genetic diversity within-populations and Fst values suggested low genetic differentiation between populations, as it was already been reported[1]. Differences between observed and expected heterozygosity were on average low. In fact, F values suggesting a possible deficiency of heterozygotes was only observed in populations 10-10 (F: 0.228) and 14-35 (F: 0.204).

A dioecious mating system and wind pollination are expected to promote extensive gene flow, potentially leading to genetic admixture and homogenization across large geographical areas[1]. Reasonably, the size of this geographical area should be limited by the pollen dispersal of each species. Pollen dispersal of *A. tuberculatus* is limited to 800 m[30], but a field study demonstrated that pollen-mediated gene flow (PMGF) in glyphosate-resistant *A. tuberculatus* declined by 90% at 88 m (depending on the direction of the pollen-receptor blocks in a concentric donor-receptor design)[31]. As distances between the eight sampling locations were greater than those reported expected for effective pollen mediated gene flow, it seems reasonable to consider the eight populations as isolated and presumably undergoing independent evolution. 17-60/17-61 and 10-10/10-13 were the nearest accessions (3.5 and 1.4 km far as the crow flies, respectively). While uNei genetic distance between populations 10-10 and 10-13 was relatively high, low genetic differentiation was found for populations 17-60 and 17-61. Furthermore, population 17-65 and 17-66 had the lowest value of uNei distance (0.025) although they were found in geographical areas 200 km apart. As a result, the clustering of populations pictured by the two statistical methods employed in this study appeared to be largely independent from the sampling location. Consistent with these observations, the Mantel test confirmed the lack of correlation between the two matrices and suggested no isolation by distance across the considered sampling range.

All simulations estimated the most likely number of populations (K) as 2. Populations 10-10, 10-13 and 17-56 clustered together and apart from a second cluster represented by remaining populations (i.e. 14-35, 17-60, 17-61, 17-66 and 17-65). Although very low admixture levels (membership: > 80%) were detected for most individuals, several populations (namely, 10-10, 10-13, 17-56 and 17-61) included individuals with contrasting population assignments, indicating, to some extent, admixture at population level. This genetic population structure, which indicates a

relatively low level of differentiation between the sampled populations and involving the clustering of populations from distinct geographical regions is consistent with the estimated high intra-population genetic diversity and low inter-population genetic differentiation.

Amaranthus tuberculatus is an alien invasive species, native of North America, where the presence of two ancestral populations has been widely discussed[32,33,1,2]. First observations led to conclude that two separate species existed, namely *A. tuberculatus* and *A. rudis*, with different morphological characters and habitat[32]. Lately, a study revealed that morphological characters initially observed were inconsistent, and only one, highly morphologically variable species was proposed[33]. More recently, thanks to both molecular markers and morphological characters, the species was further investigated and the presence of two varieties of *A. tuberculatus*, namely *A. tuberculatus* var *rudis* and *A. tuberculatus* var *tuberculatus*, has been accepted[1,2]. In this work, microsatellites were the same previously developed to study population structure of American *A. tuberculatus* populations, that led to recognize the presence of both varieties across USA. Our microsatellite data might had identified the same ancestrals and therefore the introduction of this species in Italy might have involved both American native populations. Future investigations performed by using populations derived from both sites and a larger number of SSR loci will possibly help in clarifying the relationship existing between North American and Italian populations.

3.4.2. Amplicon sequencing of acetolactate synthase gene

Amplicon sequencing of *ALS* gene and its flanking non-coding regions was used to compare single nucleotide polymorphisms (SNPs) variation at this locus among populations. 389 SNPs were found within a region of 4 kbp. *In silico* phased haplotypes were used to build a maximum likelihood haplotype tree. Three main branches were found: one including all resistant haplotypes of population 14-35, one grouping all resistant haplotypes belonging to all other populations and a third branch including mostly susceptible haplotypes of all accessions (and some resistant haplotypes). Haplotypes having the W754L mutation were used to draw the TCS haplotype network and to create a georeferenced map of haplotype diversity. The haplotype network identified two distinct and highly represented resistant haplotypes, together with 10 less represented haplotypes. Genetic differentiation (expressed as the number of polymorphic sites) between haplotype 14-35_A1_1_R and haplotype 10-10_G1_1_R is consistent with their independent evolution. Since less frequent haplotypes highly differed from all other sequences,

they evolved independently, indicating a high number of recombination event. The presence of a single resistant haplotype (10-10_G1_1_R) shared by most populations, clearly indicate that genetic exchange happened among them. As already stated, genetic exchange among these populations cannot be happened through pollen migration, because of the high distance among populations. Instead, it must have occurred through multiple seed migration events.

Two resistant populations displayed moderate inbreeding, but it is not clear whether it was caused by the strong selection imposed by herbicides or by other factors (e.g. the artificial population reproduction). Intriguingly, seed migration caused little (if no) effect on population admixture, likely because only the *ALS* alleles undergone herbicide selective pressure, but not microsatellite markers.

3.4.3. Origin of resistant populations

Assuming that resistance roughly appeared following the timeline of findings, 10-10 and 10-13 were the first populations evolving resistance among both clusters. No wild population belonging to their cluster was observed in the surroundings, thus it might be possible that one or both these populations were already resistant when introduced into Italy. Among the possible scenarios regarding the early introduction and spreading of resistant genotypes in north Italy, we might consider the followings alternatives: a) the two resistant populations 10-10 and 10-13 were introduced together (e.g. with contaminated sowing seed) or b) the introduction one population was followed by the establishment a second population (e.g. contaminated agricultural machineries). Noteworthy, while population 10-13 did not differ significantly from the other populations for most genetic indices, the population 10-10 had the lowest genetic diversity (uHe: 0.367) and the highest fixation index (F: 0.228) observed in this study, possibly indicating a bottleneck effect. If these populations were not introduced together, our genetic data might suggest that population 10-13 derived from population 10-10. The resistant population 17-56, clearly belongs to the same cluster of 10-10 and 10-13 it possibly originated from population 10-10 or 10-13.

Among populations belonging to the cluster that includes the Italian wild type population 17-65, herbicide resistance was firstly observed in population 14-35. Population 14-35 was collected not far away from the Po River, where *A. tuberculatus* is widespread. Populations 14-35 and 17-65 (collected in the Po River) are genetically similar, but it is not possible to infer if population 14-35 directly evolved from population 17-65, because no wild type populations were observed to infest

fields. At the same time, it possible that population 14-35 was introduced already resistant. Indeed, it had unbiased expected heterozygosity (uHe) and fixation index (F) values similar to that of population 10-10. Indeed, it evolved resistance independently from all other populations, because its resistant haplotype is clearly different from the others.

Populations 17-60 and 17-61 had the genetic background different to that of populations 10-10, 10-13 and 17-56, but their most common resistant haplotype was 10-10_G1_1_R, evidently because of seed migration from one of the Northern populations. Since admixture in population 17-60 was lower than that in population 17-61, the latter likely was the initial site of introduction of the resistant 10-10_G1_1_R haplotype. Population 17-60 and 17-61 might also be the result of selection on a susceptible, undetected, population that evolved its own resistant haplotypes, but also undergone seed introduction from other populations.

Population 17-66 evolved resistance to ALS independently from all other resistant populations for two reasons: 1) the low number of mutated plants (16%) did not justify the high resistance level to thifensulfuron-methyl (70%) and 2) specific haplotypes were found. Resistance mechanism had to be different (i.e. non- target site- mediated) and thus the evolutionary history. Population 17-66 and 17-65 had the lowest Nei unbiased genetic distance (0.025), suggesting a strong relationship between the two. The field where population 17-66 was sampled was really far from *A. tuberculatus* habitat, and no wild population was found in the surroundings, thus it is unlikely that this population has been selected in the nearby. Even if only two plants had the W574L point mutation, three haplotypes were observed: two were similar to the ones found in populations 17-60 and 17-61, and one was 10-10_G1_1_R, possibly indicating a seed introduction from these populations.

Our results support the hypothesis that resistance to ALS-inhibiting herbicides in A. tuberculatus populations occurred because of both independent/recurrent selection and spread of resistant haplotypes from resistant populations. Indeed, seed migration played a major role in herbicide resistance evolution, but it is not clear how it occurred. Invasive species seeds could be spread in a number of ways, both human- and/or natural-mediated. There are two main scale of spreading: a long-distance dispersal, likely responsible for the introduction of populations 10-10 and 10-13 from outside of Italy, and short-/medium-distance dispersal, responsible for the spreading of the resistant haplotype across the North East Italy. Long-distance seed dispersal (LDD) could have occurred through contaminated sowing seeds or animal feed used for sowing. Short-distance

dispersal (0-30 km) could have occurred through contaminated machineries or manures[34], whereas medium-distance dispersal could have occurred through irrigation and rainfall events[35], as well as transportation through migrating wildlife such as ducks and geese[36]. The latter hypothesis is particularly intriguing because germinable *Amaranthus tuberculatus* and *Amaranthus palmeri* were found in fecal deposition (endozoochory) of the duck *Anas platyrhynchos* (Mallard) in Missouri (USA)[36]. Available seeds of *A. retroflexus* were also found in *Perdix perdix* (Grey Partridge)[37] and *Emberiza schoeniclus* (Reed Bunting)[38] droppings (excrements) in Poland[39]. All these species live in the Po River plain and might account for the extensive seed migration of *A. tuberculatus* in Italy[40], but no specific data on seed consumption among these species in Italy are currently available. On the other hand, soybean is a genetically stable cleistogamous species, thus some farmers still self-produce their next-season seeds: this practice is not prohibited in Italy -although not encouraged-, but can only explain the migration of seeds within the boundaries of the farmer's properties. Another possible practice -this time forbidden-, is to sow soybean intended for uses other than sowing: in this case introducing exotic weeds would be possible. Sowing non-certified soybean seeds could also explain the spread of (eventually resistant) weed populations. A recent -alarming- report stated that this phenomenon account for 30% of the total soybean sowing seeds in Italy[41].

3.5. Conclusions

Combining population structure information and *ALS* gene sequencing, it was possible to reconstruct a hypothetic evolutionary history of resistance to ALS-inhibiting herbicides in *Amaranthus tuberculatus*, an invasive and disruptive weed that infest summer crops, in Italy.

Our data support the hypothesis that resistance evolution in Italy started, at least, from three geographically separated populations. Six out of seven ALS-resistant populations had the point mutation W574L, and five out of six had the same resistant haplotype, indicating a common origin of resistance. Among these five populations with a common resistant haplotype, the first collected one likely was the origin of the resistant haplotype, while other populations evolved resistance as a consequence of seed migration from that one. Furthermore, there is the suspect that this original resistant population was already resistant when it was introduced in Italy. One out of six resistant populations had a different resistant haplotype, indicating a separate evolution. Only one, out of seven resistant population, did not have any known endowing-resistance mutation along the whole *ALS* gene. Further experiments are ongoing to elucidate the resistance mechanism of this population, but it was clear that it evolved separately from the others. For both of these last two populations, there was no element to exclude that they derived from a susceptible Italian population.

Seed migration among *Amaranthus tuberculatus* population appeared to be quite common. Zoochory, human activities and/or bad farming practices remain the most likely causes, but there were no clear explanations for that. Indeed, further efforts should be made to understand the mechanisms that cause the seed migration, as it causes the rapid expansion of this weed.

Supplementary material

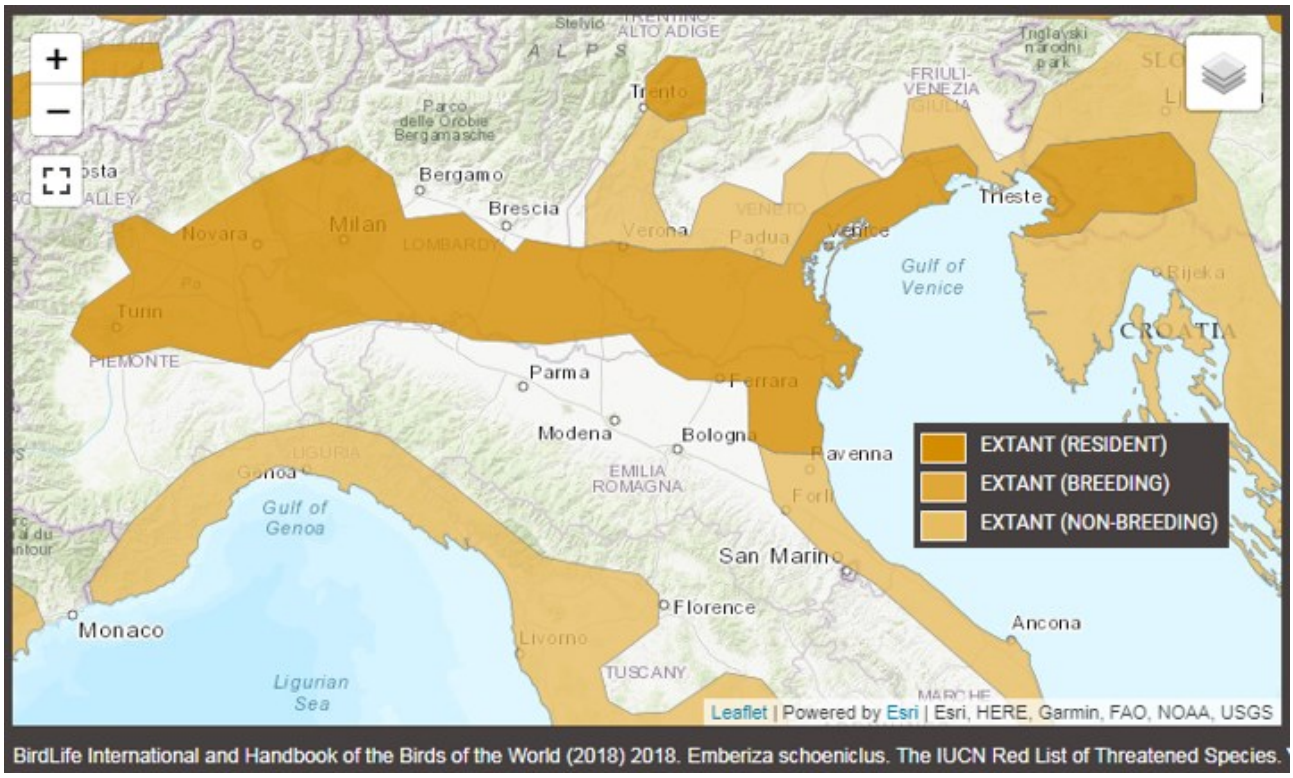
Table 4. Primer combination of multiple PCR mixes, primer sequences, repeat motif and size of each locus, dyes used for visualization. † indicates information reported in the original paper from which loci were taken [1].

PCR mix	Dye	Repeat†	Size range† (bp)	primer code	tagged locus-specific primer sequence (5'-3')
1	PET	$(GAT)_{10}$	113-181	PAN3_C1140F	TGTAGAAAGACGAAGGGAAGGTTGAAGACGACGATCTTTCTGGAT
				C1140R	GTTTCTTCCCCTCTGTACACCATAATCGAAC
	VIC	$(TGA)_8$	127-174	Tail_D_C3695F	CGGAGAGCCGAGAGGTGTCAACTTCTTATTCTTGGGTTGCTTC
				C3695R	GTTTCTTCCTTACCTTCTCTCAAAAGCACCA
	FAM	AAC	112-130	Hill_AAC1F	TGACCGGCAGCAAAATTGCCCACCAAGGATGATCATTTAGAC
				AAC1R	GTTTCTTTCATCATTATTTGTTGGCGTTGAC
	FAM	$(ACC)_8$	164-179	Hill_C4097F	TGACCGGCAGCAAAATTGATCATCTTCTGCTAAGGCTGTTGG
				C4097R	GTTTCTTATATCTTCCCCAATTGGACTCCTC
	NED	$(TGA)_{10}$	130-164	D8S1132_C0745F	GGCTAGGAAAGGTTAGTGGCTAGGAAGTTCATCCATAAGCTCGG
				C0745R	GTTTCTTCAATTCCAAGGAATCATCCTCATC
2	FAM	$(ACC)_8$	120-141	Hill_C4999F	TGACCGGCAGCAAAATTGCCACCCAATGACCCATACCTACTA
				C4999R	GTTTCTTGATGAGGTTGATAATTGGGGTTCA
	PET	ATC	142-160	PAN3_ATC9F	TGTAGAAAGACGAAGGGAAGGTAGCCATTTCAACCTTACGAGGAA
				ATC9R	GTTTCTTACCGTTGATTGATTTTATGGCATC
	VIC	$(CCA)_8$	123-141	Tail_D_C3561F	CGGAGAGCCGAGAGGTGCCATAAACCATTTTCCCAGACC
				C3561R	GTTTCTTACTTCTGGCCCAATTAGGAAGTC
	NED	TAG	132-163	D8S1132_TAG5F	GGCTAGGAAAGGTTAGTGGCGTCGCTGAATTGTTTTAGCTTGGT
				TAG5R	GTTTCTTTGGGAATTCTCTCTTGTGACACAGT
	FAM	$(GAT)_8$	165-199	Hill_C9333F	TGACCGGCAGCAAAATTGAACTAAACGCATTTGCCATTGAA
				C9333R	GTTTCTTTGTTCATCTAACCACATCATAATGGAA

Table 5. Codes and sequences of primers used for ALS amplicon sequencing

Code	Sequence (5'-3')
Fw_6	TGCTGAAGGATATTTGTTGTGCT
Fw_5	TTTAATGGGCTGGGCTTGAGATA
Fw_4	CGTGTTCAATCTCAGCTGCTTAG
Fw_3	TTTGTCAAAGACCCTTGCGTTTT
Fw_2	AAAACGACAAGTCAACCCATCAC
Fw_1	TTAAGCGCCTCCACTCATTTCT
Rev_1	GTCAAGCAATGTGAGACAGACTT
Rev2	AACATAAGGCCTCAAAGACCACA
Rev_3	TGCAATGTTGACTCGTTTCTGTC
Rev_4	ACCGTGACGAAGCCAAATTTAAG
Rev_5	GCCGAAAGTGATGATGAAGATGG

Figure 7. Geographic range of Emberiza schoeniclus (Reed Bunting). Note the overlapping with the findings of ALS-resistant A. tuberculatus population (cf. Fig.1). Modified from The IUCN Red List of Threatened Species 2018[38].



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Chapter IV

Phenology, height curve, ALS dose-response and seed predation of three *Amaranthus* species

4.1. Introduction

Amaranthus is a complex genus, comprising 74 species[1], having high phenotypic variability. Many amaranths are weeds in summer crops that can cause high yield losses in soybean[2], maize[3], cotton[4], tomatoes[5] and potatoes[6] when not adequately controlled. They are spread worldwide, covering several different habitats. Four *Amaranthus* species rank among the 15 weed species that have already evolved resistance to more than five herbicide sites of action[7]. These four species, namely *Amaranthus palmeri* S. Watson, *Amaranthus tuberculatus* (Moq.) J.D.Sauer (formerly called *Amaranthus rudis* J.D.Sauer), *Amaranthus hybridus* L. and *Amaranthus retroflexus* L., are widely considered troublesome weeds[8,9].

Three of them *A. retroflexus*, *A. hybridus* and *A. tuberculatus* are considered as invasive alien species in Europe and are widespread in North Eastern Italy. The first two were the most common amaranth species infesting crop fields, whereas *A. tuberculatus* habitat was limited to floodplains and riverbanks[1]. However, recently, *A. tuberculatus* has jumped "out of the swamp"[10] and has started to infest Italian soybean crops (*cf.* Chapter II). This *Amaranthus* species is becoming a significant weed problem because of its rapid growth, huge phenotypic variability and dense infestations.

Phenology and height growth rate are very useful weed biology aspects for proper application of integrated weed management. They are essential to determine the most effective herbicide application timing and the extent of the application window (e. g. the presence of a fast-growing species might shorten the application window). Flowering phenology might clarify if hybridization among inter-fertile species is biologically feasible in field conditions. Pollen viability in the dioecious and wind-pollinated species, *Amaranthus tuberculatus*, has been estimated to be 5 days[11], and it is expected to be even less for monoecious species[12]. Therefore, flowering overlapping is an essential condition for effective inter-specific hybridization and eventual horizontal gene transfer (HGT). Horizontal gene transfer of *ALS* resistant alleles among *A. tuberculatus* and *A. hybridus* had already been proven under field conditions[13], but it was not verified in the case described in Chapter II, where *A. retroflexus*, *A. hybridus* and *A. tuberculatus* were found living sympatrically in the same soybean field, and have independently evolved different point mutation at the acetolactate synthase (*ALS*) locus conferring resistance to ALS inhibitors (*cf.* Chapter II). A possible explanation could be that phenology of the three species was sufficiently different to avoid cross-fertilization. Indeed, specific information about phenology and

height growth rate of these three species, related to cumulative growing degree days in a Mediterranean climate was lacking.

Another intriguing aspect of considering the presence of different species of the same genus within a common agricultural environment, is whether they have similar herbicide susceptibility or not. Stakeholder observed a lack of control in many *A. tuberculatus* infestations and lower herbicide susceptibility was suspected. From a practical point of view, herbicide must be applied according to the dose that control the less susceptible species. Indeed, precise estimation of acetolactate synthase (ALS)-inhibiting herbicides efficacy on these three species was lacking.

The ability of a species to colonize a new habitat can be partially explained by the lack of an efficient predator[14]. Carabids are frequently indicated as playing a major role in post-dispersal weed seed predation, thanks to their generalist diet and common presence in agricultural ecosystems[15]. Post-dispersal weed seed predation could play a significant role in reducing the weed seed bank, and, in combination with other factors, may contribute to effective weed suppression, resulting in a reduced reliance on synthetic chemical control practices[16,17,18]. The potential of carabids in weed suppression in agricultural systems has been widely investigate[19,15,20,21]. Carabids are known to predate many *Amaranthus* species in agricultural environment, including *Amaranthus tuberculatus*, within its native range (North America). Indeed, no information is available on predation of *A. tuberculatus* outside its native range, where it is considered a treat for agriculture and biodiversity, being an emerging invasive exotic species.

The aims of this research were: 1) to define flowering phenology and the height growth curve of *Amaranthus retroflexus*, *Amaranthus hybridus* and *Amaranthus tuberculatus* in a common garden experiment 2) to estimate the ALS-inhibiting herbicides susceptibility among the three species and 3) to verify if the exotic invasive species *Amaranthus tuberculatus* would be predated less than the other species in conventional and conservative systems.

We found that the dioecious species *Amaranthus tuberculatus* flowered and matured later than did the two populations of *Amaranthus retroflexus* in all sowing dates. In all sowing dates, the absolute height growth rate of *Amaranthus tuberculatus* was 0.6 cm GDD^{-1}, barely the double than that of the other two species. No significant differences were found among species regarding susceptibility to ALS-inhibiting herbicides, but a few *Amaranthus tuberculatus* plants were observed to survive two- and four-fold the recommended field dose of thifensulfuron-methyl.

During the three days field trials, invertebrates (mainly carabids) predated a mean of 67% of all the three *Amaranthus* species.

4.2. Materials and methods

4.2.1. Plant material

Table 1. Amaranthus Species, code, origin, site of sampling and GPS coordinates of all the six popultions used in this study.

Species	Code	Origin	Locality	Municipality	Province	GPS
Amaranthus retroflexus	17-52	urban	Parco degli	Roma	Roma	41°51'01.3"N
	17-37	country	private farm	Berra	Ferrara	44°57'40.6"N
Amaranthus hybridus	17-53	urban	Parco della	Roma	Roma	41°52'01.0"N
	11-01	country	University farm	Legnaro	Padova	45°20'48.7"N
Amaranthus tuberculatus	17-64	river	Cascina Vinzasca	Gombito	Cremona	45°14'45.3"N
	17-65	flood		Portalbera	Pavia	45°06'26.5"N

Two accessions per each of the *Amaranthus* species were collected in 2017 except for accession 11-01 that has been reproduced in 2011 from original population collected in 1999, (Table 1). This latter accession was previously ascribed to *A. retroflexus*[22], but it has now been identified as *A. hybridus*. To cover a higher amplitude of variation, accessions of the same species were chosen from different latitudes and origin ("agricultural" vs "non-agricultural"). Both *A. tuberculatus* accessions were limited to non-agricultural areas because all *A. tuberculatus* accessions of agricultural origin found in Italy were ALS-resistant (*cf.* Chapter II). For each accession, at least 30 heathy and mature plants were collected and air dried in the greenhouse; after one month, seeds were hand threshed, cleaned and conserved at 4°C.

4.2.2. Phenology and height growth curve

Plastic pots (32 cm diameter, 20 cm tall) were filled with 20 kg of non-sterile substrate composed of 60% silty loam soil, 15% sand, 15% perlite and 10% peat. Non-sterile soil was covered with about 4 cm of sterile silty loam soil to avoid contamination with foreign seeds. About a hundred seed of a single accession per pot was hand sowed and gently covered with a few millimeters of sterile soil. Seeds were conserved at 4°C until sowing, thus avoiding dormancy and allowing fast germination. Automatic drip irrigation assured the soil to be always at maximum capacity. Once germinated, plants were hand thinned, in order to have one healthy plant per pot.

Experiment took place outside, in a semi-controlled environment, in summer 2018. To simulate late May, early and late June seedbed preparation periods, three *Amaranthus* sowing dates were done: May 25, June 8 and 22 (the day after the summer solstice). The experiment was arranged as

a split-plot design, with planting date as the main plot, species as secondary plots and population as subplots.

Rainfall precipitations, relative air humidity and temperature data were daily collected by a weather station close to the experimental site (45°20'49.0"N 11°57'07.6"E). Data were analyzed and released by Regional agency for environmental protection of Veneto (ARPAV). All data are freely available online[23]. To calculate the growing degree-days, the equation

$$GDD = [(T_{max} + T_{min})/2] - T_b$$

was used[24], where T_{max}/T_{min} are daily maximum/minimum air temperatures, respectively, and T_b is the base temperature. Since no species specific base temperature data were available for all three *Amaranthus* species, 10°C was used, as suggested by bibliography[25].

4.2.3. Dose-response experiments

A preliminary two-doses whole-plant herbicide screenings were conducted to confirm the susceptibility of the accessions, following a robust protocol previously described with slightly modifications[26]. Seeds were germinated as previously described[22], but the germination temperature for *A. tuberculatus* was increased to 18°/28°C night/day, while it was 15/25°C for the other two species. Seedlings were treated at same condition of the dose-response described above, but only with the recommend field dose and three times that of thifensulfuron-methyl and imazamox. For both two-doses and dose-response assays, after germination seedlings at similar growth stage were then transplanted into 11 cm square pots (3 liters volume) filled with a standard potting mix (60% silty loam soil, 15% sand, 15% perlite, and 10% peat) and watered daily to maintain the substrate at or near field capacity. Two ALS-inhibiting herbicides were tested: thifensulfuron-methyl was applied at 1x field rate of 6 g a.i. ha^{-1} (Harmony 50 SX, DuPont™, 50 g a.i. 100 g^{-1}) plus surfactant 0.3% (DASH® HC, BASF, 37.5 g a.i. 100 g^{-1}), imazamox was applied at 1x field rate of 40 g a.i. ha^{-1} (Tuareg®, DuPont™, 40 g a.i. L^{-1}). The experimental layout was a completely randomized design with three replicates (two pots per replicate) and six plants per pot. Each population was treated with six doses of each herbicide (plus an untreated control). All doses were calculated using a geometric progression, ranging from 1/32 to 4-times the recommended field dose. Plants were sprayed at BBCH stage 12−14 (2 to 4 leaf stage). Herbicides were sprayed using a precision bench delivering 300 L ha^{-1} at a pressure of 215 kPa and speed of 0.75 m s^{-1}, with a boom equipped with three flat-fan (extended range) hydraulic nozzles (TeeJet, 11002). Irrigation

was stopped a few hours before herbicide treatment until 24 h after treatment. Plant survival and shoot fresh weight per pot were recorded 4 weeks after treatment (WAT). Plants were assessed as being dead if they showed no active growth, regardless of color. Survival rates per replicate were expressed as a percentage of the untreated control. The first experiment was conducted outside, in summer 2018. Since one population flowered two weeks after the herbicide treatment, the experiment was repeated in the greenhouse the next year (2019), adding artificial light 2 hours a day.

4.2.4. In field seed predation

Seed predation was monitored using seed cards[27], which were prepared using 6 × 4 cm pieces of sandpaper (3M®, grit 80) lightly sprayed with an aerosol glue (Ferrario, Ripo Spray), to which 30 seeds of each *Amaranthus* species were applied (one card per species). Since half of each seed card surface was occupied by seeds, an average of 25000 seeds m^{-2} were placed, that was within the range of *Amaranthus* seedbank in a summer crop[28]. To avoid vertebrate's interference, seed cards were enclosed in cages made of metal wire (1 cm^{-1} mesh); a plastic plate on top, stuck on the ground with long nails, protected seed cards from eventual rain. Four cages with three seed cards each (one per *Amaranthus* species), four meters spaced, were placed 20 meters far from borders of each field. Cages were placed within soybean rows, avoiding intentional modification of the crop canopy.

Trials were conducted in 8 sites randomly distributed within two localities of Udine province (North-Eastern Italy), Orsaria (46°02'21.8"N 13°22'53.3"E) and Rivignano (45°52'40.4"N 13°02'24.6"E), during summer 2018. In each site, a conventional tillage field was close to a conservation tillage field. Environmental conditions were the same at the two neighboring sites. All fields were cultivated with soybean following the usual techniques of the adopted soil management: conventional tillage fields were ploughed 40 cm depth, (immediately followed by one or two tills for seedbed preparation), whereas conservation tillage included all techniques characterized by non-inversion of soil for at least 5 years.

Seed cards were placed *in loco* on August 27 and were collected 4 days later, as this period had been previously recognized to be enough to obtain a good predation rate (F. Lami, personal communication). The main arthropod species involved in predation and considered in the present work were carabids belonging to Harpalini tribe: *Harpalus (Pseudoophonus) rufipes* (DeGeer,

1774), *Harpalus (Pseudoophonus) griseus* (Panzer, 1796) and *Harpalus distinguendus* (Duftschmid, 1812), unpublished data).

4.2.5. Statistical analyses

The dose−response data were analyzed using a nonlinear regression analysis based on the log−logistic equation[29] $Y = C+[(D - C)/[1 + (x/LD_{50})b]$ where Y is the fresh weight or survival, C and D are the lower and upper asymptotes at high and zero doses, respectively, LD_{50} is the dose giving the 50% response, b is the slope, and x the herbicide rate. Doses giving the 50% response, that is, LD_{50} (based on survival data) and relative standard errors, were calculated using the macro BIOASSAY97. For biological reasons and to improve the estimates of the parameters, the upper and lower asymptotes were forced to 100 and zero, respectively, only for survival data. The data were first analyzed separately as single curves to define slopes and inflection points. Data of the same species, but different accessions, were then regressed together, to verify if they fitted the same model. The complex model was compared with progressively simplified models having common parameters among curves. The lack-of-fit F-test was performed at each step, and the simplification stopped when a significant lack-of-fit occurred (α=0.01).

Plant height growth curve were also analyzed using a log−logistic equation with BIOASSAY97. The equation $H = C+[(D - C)/[1 + (x/H_{50})^b]$ was used, where H is the plant height, function of the growing degree-days (GGD) x, C and D are the lower and upper asymptotes (minimum and maximum height, respectively), H_{50} is the cumulative thermal unit giving the 50% response and b is the slope. The lower asymptote was fixed to zero, whereas the others were determined. Absolute height growth rate (AGR) was calculated using values from the growth fitted curve, according to equation $AGR=(H_2-H_1)/(X_2-X_1)$, where H_2-H_1 is the difference in height between two sampling time point and X_2-X_1 is the time elapsed between them (expressed in GDD).

4.3. Results and discussion

4.3.1. Phenology and height growth curve

4.3.1.1.Phenology

The main BBCH stages investigated were the initiation of flowering (BBCH 51), the full flowering (BBCH 65) and seed maturation (BBCH 89). Table 2 contains a detailed description of these stages. The description is in agreement with a recently published work that specifically described BBCH scale for monoecious *Amaranthus* species[30].

A. hybridus plants of population 17-53 started flowering before all the others, in all sowing dates, with GDD accumulated ranged between 312 and 318. Also *A. retroflexus* plants of population 17-52 initiated to flower sooner with GDD accumulated ranged between 312 and 369, depending on the sowing data. Conversely, *A. tuberculatus* plants from both populations 17-64 and 17-65 initiated flowering later than all and this delay was evident also in the other phenological stages (full flowering and ripe seed), Overall, the cumulative growing degree days necessary to reach each phenological state decreased with later sowing apart from the *A. hybridus* plants of population 11-01. This effect is particularly pronounced for both *A. tuberculatus* populations where the GDD accumulated to reach the full flowering or ripening seed decreased widely at the later sowing data (Table 3). This observation is consistent with short-day species, that flowers in relation to photoperiod[31,32].

In all sowing dates, populations collected in agricultural environment (namely, 17-37 and 11-01) had later flowering and maturation compared to that collected in non-agricultural environment, of the same species. Different phenology between agricultural and non-agricultural populations of the same weed species could be related to weed mimicry[33]. Weeds tend to adapt to crop phenology, to better exploit the time they have available in the growing season and therefore increase their fitness (e.g. producing more seeds). Having a growing season perfectly compatible to most summer annuals crops, *Amaranthus tuberculatus* wild populations might have a competitive advantage in comparison with non-agricultural biotypes of *A. retroflexus* and *A. hybridus*.

Table 2 Description of the phenological growth stages of Amaranthus sp. according to the BBCH scale.

BBCH	Description
51	Beginning of main panicle emergence, inflorescence is visible from above
65	main inflorescence full flowering: anthers and/or stigmas are visible and spread along the whole
89	main inflorescence ripe grain: the utricle of main inflorescence's flowers is darkened, perfectly

Table 3. Cumulative growing degree days (GDD) necessary to reach three main phenological states: visible inflorescence (BBCH 51), full flowering (BBCH 65) and ripe seed (BBCH 89) for each sowing date, species and population. Values are referred to the mean of four plants-replicates and standard errors are reported in brackets.

Sowing	Species	Population	Inflorescence is	Full flowering	Ripe seed
25th May	*A. retroflexus*	17-52	350 (0)	495 (0)	688 (0)
		17-37	520 (25)	714 (26)	791 (0)
	A. hybridus	11-01	521 (60)	707 (18)	1118 (89)
		17-53	318 (0)	495 (0)	791 (0)
	A. tuberculatus	17-64	627 (19)	795 (62)	1030 (0)
		17-65	660 (46)	853 (73)	1030 (0)
8th June	*A. retroflexus*	17-52	369 (0)	505 (0)	608 (0)
		17-37	505 (0)	719 (0)	847 (0)
	A. hybridus	11-01	411 (0)	719 (0)	993 (0)
		17-53	312 (0)	411 (0)	608 (0)
	A. tuberculatus	17-64	664 (32)	783 (37)	993 (0)
		17-65	664 (32)	783 (37)	993 (0)
22nd June	*A. retroflexus*	17-52	312 (0)	415 (0)	653 (0)
		17-37	415 (0)	653 (0)	800 (0)
	A. hybridus	11-01	568 (43)	653 (0)	912 (0)
		17-53	312 (0)	415 (0)	653 (0)
	A. tuberculatus	17-64	526 (0)	653 (0)	912 (0)
		17-65	526 (0)	653 (0)	1205 (0)

4.3.1.2.Height analysis

The height of the plants was significantly different among the different *Amaranthus* species and the maximum height for each species remained unchanged at the different sowing dates (table 4). *A. tuberculatus* plants were far the tallest among all, reaching a maximum height of about 3 meters (Table 4). The height of the *A. retroflexus* plants ranged between 95 to 152 cm while that of the *A. hybridus* plants resulted rather different, 53-86 cm and 180-211 cm, for population 17-53 and 11-01 respectively.

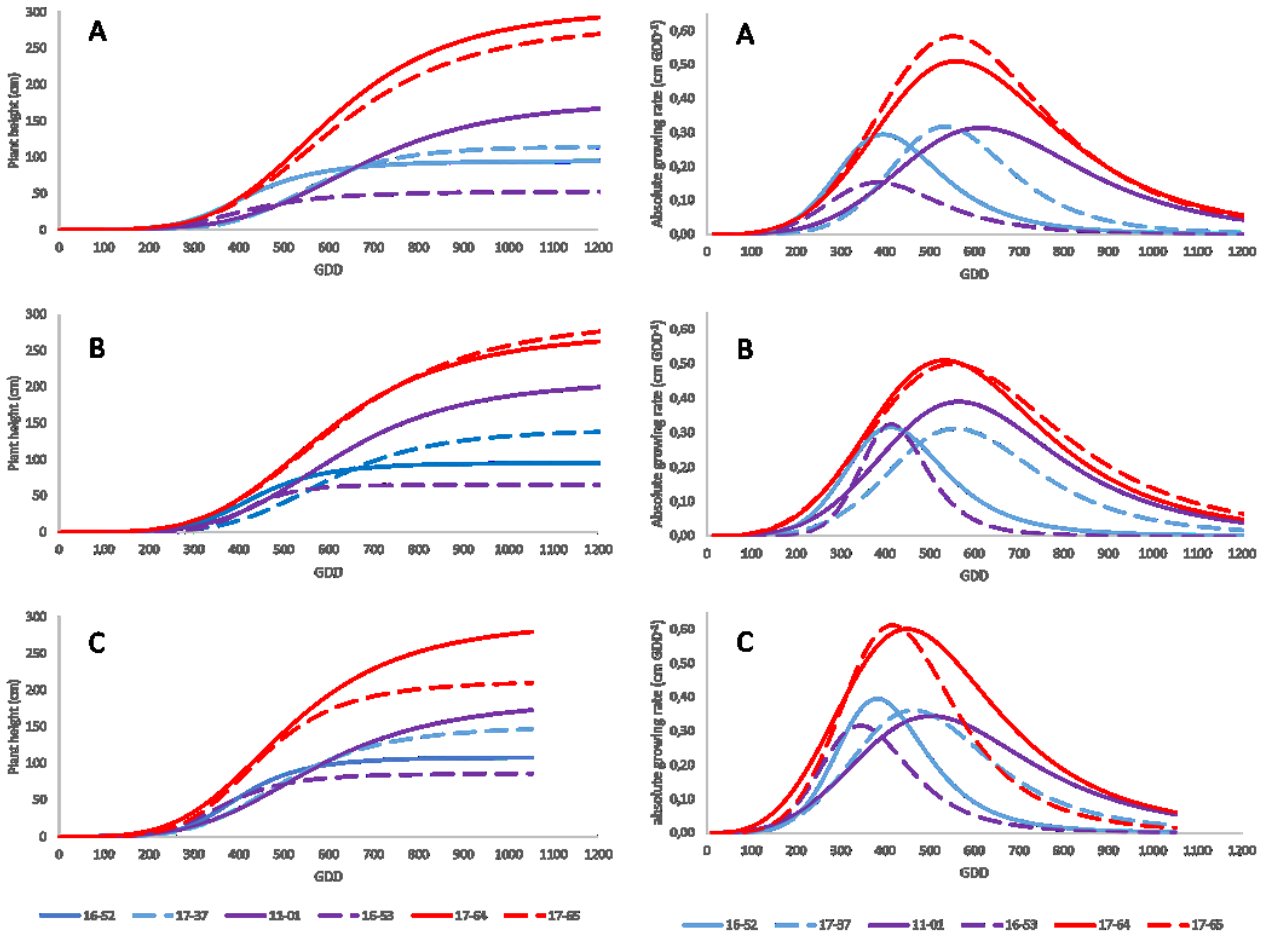
The cumulative growing degree day necessary to reach half the maximum height decreased markedly in population 10-11 (*A. hybridus*), 17-64 and 17-65 (*A. tuberculatus*). It is notable that the two populations of different species, collected in non-agricultural environment, had very similar values of H_{50}, even if having very different maximum heights. H_{50} has already been estimated for *A. tuberculatus*, but time was indicated in weeks after transplant[34], making the comparison among studies difficult.

Table 4. Estimated maximum height (C), rate of change (b), inflection point H_{50}) and goodness of fit (residual sum of squares, RSS) from a four-parameter logistic function for A. retroflexus, A. hybridus and A. tuberculatus populations in a common garden experiment. Standard errors are reported in brackets. Maximum values for absolute growth rate are derived from the log-logistic function.

Sowing	Species	Population	C [cm]	b [cm GDD^{-1}]	H_{50}	RSS	Maximum AGR
25th May	*A. retroflexus*	17-52	95 (2)	-5 (0,5)	421 (9)	1022	0,3
		17-37	116 (2)	-6 (0,5)	558 (8)	1861	0,3
	A. hybridus	11-01	180 (13)	-4,5 (0,7)	672 (33)	19380	0,3
		17-53	53 (2)	-4,6 (0,6)	415 (13)	1192	0,2
	A. tuberculatus	17-64	307 (10)	-4,4 (0,4)	606 (15)	15233	0,6
		17-65	286 (11)	-4,2 (0,4)	621 (18)	16499	0,5
8th June	*A. retroflexus*	17-52	95 (2)	-5,6 (0,4)	433 (6)	857	0,3
		17-37	142 (4)	-5,1 (0,4)	598 (11)	1743	0,3
	A. hybridus	11-01	211 (11)	-4,4 (0,5)	622 (22)	9495	0,4
		17-53	65 (1)	-8,2 (0,8)	420 (6)	593	0,3
	A. tuberculatus	17-64	277 (13)	-4,1 (0,4)	594 (21)	14927	0,5
		17-65	297 (13)	-3,9 (0,3)	622 (19)	10086	0,5
22nd June	*A. retroflexus*	17-52	108 (3)	-5,7 (0,5)	402 (8)	1240	0,4
		17-37	152 (4)	-4,6 (0,3)	504 (9)	1181	0,4
	A. hybridus	11-01	189 (13)	-3,9 (0,6)	570 (30)	5970	0,3
		17-53	86 (2)	-5,2 (0,5)	366 (8)	899	0,3
	A. tuberculatus	17-64	296 (10)	-3,9 (0,3)	510 (13)	4808	0,6
		17-65	213 (7)	-4,9 (0,5)	447 (11)	5444	0,6

Maximum values of absolute growth rates (calculated at H_{50}) of *A. retroflexus* and *A. hybridus* were similar, and barely half of that of *A. tuberculatus* populations. Absolute height growth rates for *A. tuberculatus* has been estimated[25], but not the maximum value (that is related to H_{50}), therefore a direct comparison across studies is not feasible. Indeed, also previous studies reported *A. tuberculatus* being faster growing than *A. retroflexus*.

Figure 5. A, B, C, left: height growth curve of two A. retroflexus (blue lines), two A. hybridus (violet lines) and two A. tuberculatus (red lines) populations in a common garden experiment. Lines are the response curves predicted from non-linear regression with a four-parameter logistic function. A, B, C, right: absolute growth rate (AGR) derived from the height growth curve fitted equation. Letter refers to three sowing dates: A 25th May, B 8th June, C 22nd June



Post-emergence ALS-inhibiting herbicides should be applied within the 4-true leaves stage of broadleaf weeds, or 3 inches (7.5 cm) if expressed as height, and the application period for optimum control should be adapted accordingly to the fastest-growing species (or biotypes). The height reached by each population when population 17-52 (*A. retroflexus*) is 7 cm tall is reported in Table 5. While for the first sowing (25th May) mean values are equal or lower than 7 cm, for the next sowings the values increased. In particular, *Amaranthus tuberculatus* populations were much

taller that the other populations. Absolute growing rates, calculated at the same timepoints are comparable with those previously observed by other authors[25].

In this study, results might be influenced by experimental conditions. The maximum plant height, at least that of the bigger plants, could be limited by the size of pots, that was equal for all. Real temperature aboveground might be different than that of the soil inside the pots, leading to different cumulative growing degree days. Basal temperature might vary among species and/or population, while a constant value was used for GDD estimation. Similarly, the formula did not consider eventual ceiling temperatures. The very high maximum values of absolute height growth rates observed in this study could be a result of the constant availability of water during the experiment. After emergence, seedlings were chosen randomly, among the bigger and healthier available in each pot: this could have selected fast-growing sub-populations.

Table 5. Comparison of height and absolute growing rates among populations, estimated from the log-logistic curve and its derivative, respectively. Height of population 17-52 was fixed to 7 cm and the cumulative growing degree days necessary to reach this height was obtained from the log-logistic equation, for all sowing dates. This value was then used to report the corresponding height and absolute growing rate for each population. Populations reached the reported values after 257 GDD for the first sowing, 274 GDD for the second, and 252 for the last sowing. H refers to height (cm) and AGR to absolute growth rate (cm GDD^{-1}).

sowing date	17-52		17-37		11-01		17-53		17-64		17-65	
	H	AGR	H	AGR	H	AGR	H	AGR	H	AGR	H	AGR
25th May	7	0,12	1	0,02	2	0,04	5	0,08	7	0,11	7	0,10
8th June	7	0,12	3	0,04	6	0,08	2	0,05	11	0,15	11	0,15
22nd June	7	0,14	6	0,10	8	0,11	11	0,18	18	0,25	12	0,21

4.3.2. Dose-response experiments

In the outside experiments, LD_{50} values for imazamox were at least four times lower than the recommended field rate (corresponding to 40 g a.i. ha^{-1}), with the exception for population 17-53 for which the LD_{50} was higher 22.2 g a.i. ha^{-1} (Table 6). Similarly, the LD_{50} values for thifensulfuron-methyl were at least half the recommended rate of 6 g a.i. ha^{-1}, with the exception for population 17-53.

It was not possible to fit the data of the fresh weight into the logistic equation because yet at one fourth of imazamox or thifensulfuron-methyl field rate 90% fresh weight reduction was recorded for most accessions, confirming the high susceptibility of these accessions to both herbicides. The

unique exception was population 17-53 (*A. hybridus*), that reached 90% fresh weight reduction at the half of imazamox field rate and at two-times the thifensulfuron-methyl field rate (data not shown).

Population 17-53 was collected within a city park in Rome, where, very likely, it has never been subjected to ALS herbicides selective pressure. The high LD_{50} values and the low fresh weight reduction observed might be explained by the fact that this population was the most early-flowering among all, as shown also by the phenology experiment. In fact, it started flowering two weeks after herbicide application, whereas none of the other populations was flowering at the end of the experiment.

Greenhouse experiments confirmed the trend of outside experiments, even if absolute LD_{50} values were generally lower and weight reduction higher. Population 17-53 partially conformed to the other populations, but still flowered before that the survival and fresh weight were recorded.

An interesting observation, from a weed management perspective, is that plants survived to recommended field rates had different size, depending on the species. Even if standardized fresh weight of *A. tuberculatus* survivors was similarly low as the other species, the absolute size of these plant was bigger.

Table 6. Effect of imazamox on survival rate of two A. retroflexus, two A. hybridus and two A. tuberculatus populations, data elaborated by the log-logistic model on the dose-response experiments. Slope of curve and LD_{50} are reported, separated by experimental set-up (outside vs greenhouse). LD_{50} is the herbicide rate (g a.i. ha^{-1}) causing 50% reduction in survival rate; standard errors are given in brackets. Curves belonging to the same species were regressed together, if the lack-of-fit test allowed to do so, and resulting slope and/or LD_{50} are reported.

year	pop	slope	LD_{50}	species	slope	LD_{50}
2018 (outside)	17-52	4,9 (0,7)	6,9 (0,3)	*A. retroflexus*	4,8 (0,4)	7,1 (0,2)
	17-37	4,6 (0,5)	7,2 (0,3)			
	11-01	4,4 (0,7)	7,6 (0,4)	*A. hybridus*	4,7 (0,6)	7,7 (0,3)
	17-53	6,2 (2,3)	21,9 (0,9)			22,2 (0,8)
	17-64	3,4 (1,0)	9,8 (0,8)	*A. tuberculatus*	3,2 (0,5)	10,7 (0,5)
	17-65	3,2 (0,3)	11,5 (0,3)			
2019 (greenhouse)	17-52	1,8 (0,3)	5,8 (0,7)	*A. retroflexus*	1,8 (0,2)	5,8 (0,4)
	17-37	1,8 (0,2)	5,8 (0,6)			
	11-01	1,9 (0,3)	4,7 (0,5)	*A. hybridus*	2,2 (0,3)	4,5 (0,4)
	17-53	2,7 (0,5)	11,4 (1,0)			11,5 (1,1)
	17-64	2,4 (0,4)	5,1 (0,4)	*A. tuberculatus*	2,4 (0,3)	5,1 (0,3)
	17-65	2,4 (0,4)	5,2 (0,4)			

Table 7. Effect of thifensulfuron-methyl on survival rate of two A. retroflexus, two A. hybridus and two A. tuberculatus populations, data elaborated by the log-logistic model on the dose-response experiments. Slope of curve and LD_{50} are reported, separated by experimental set-up (outside vs greenhouse). LD_{50} is the herbicide rate (g a.i. ha^{-1}) causing 50% reduction in survival rate; standard errors are given in brackets. Curves belonging to the same species were regressed together, if the lack-of-fit test allowed to do so, and resulting slope and/or LD_{50} are reported.

year	pop	slope	LD_{50}	species	slope	LD_{50}
2018 (outside)	17-52	2,7 (0,6)	1,7 (0,1)	*A. retroflexus*	2,2 (0,3)	1,8 (0,1)
	17-37	1,9 (0,3)	1,9 (0,2)			
	11-01	2,2 (0,7)	1,8 (0,3)	*A. hybridus*	2,3 (0,4)	1,8 (0,2)
	17-53	2,5 (0,5)	6,3 (0,6)			6,3 (0,8)
	17-64	1,5 (0,2)	2,9 (0,3)	*A. tuberculatus*	1,4 (0,2)	3,0 (0,3)
	17-65	1,3 (0,2)	3,2 (0,4)			
2019 (greenhouse)	17-52	2,5 (0,6)	0,8 (0,1)	*A. retroflexus*	1,7 (0,2)	0,9 (0,1)
	17-37	1,5 (0,3)	0,9 (0,1)			
	11-01	1,4 (0,2)	0,6 (0,1)	*A. hybridus*		
	17-53	5,1 (0,6)	3,9 (0,1)			
	17-64	1,6 (0,3)	0,9 (0,1)	*A. tuberculatus*	1,2 (0,2)	0,9 (0,1)
	17-65	0,9 (0,2)	0,8 (0,2)			

Figure 6 Dose-response curves of two A. retroflexus (blue lines), two A. hybridus (violet lines) and two A. tuberculatus (red lines) populations to imazamox (A, C) and thifensulfuron-methyl (B, D). Survival data were analyzed using the log-logistic model, lines are the response curves predicted from non-linear regression. A and B refer to the experiment conducted outside (2018), whereas C and D to the experiment conducted in the greenhouse (2019).

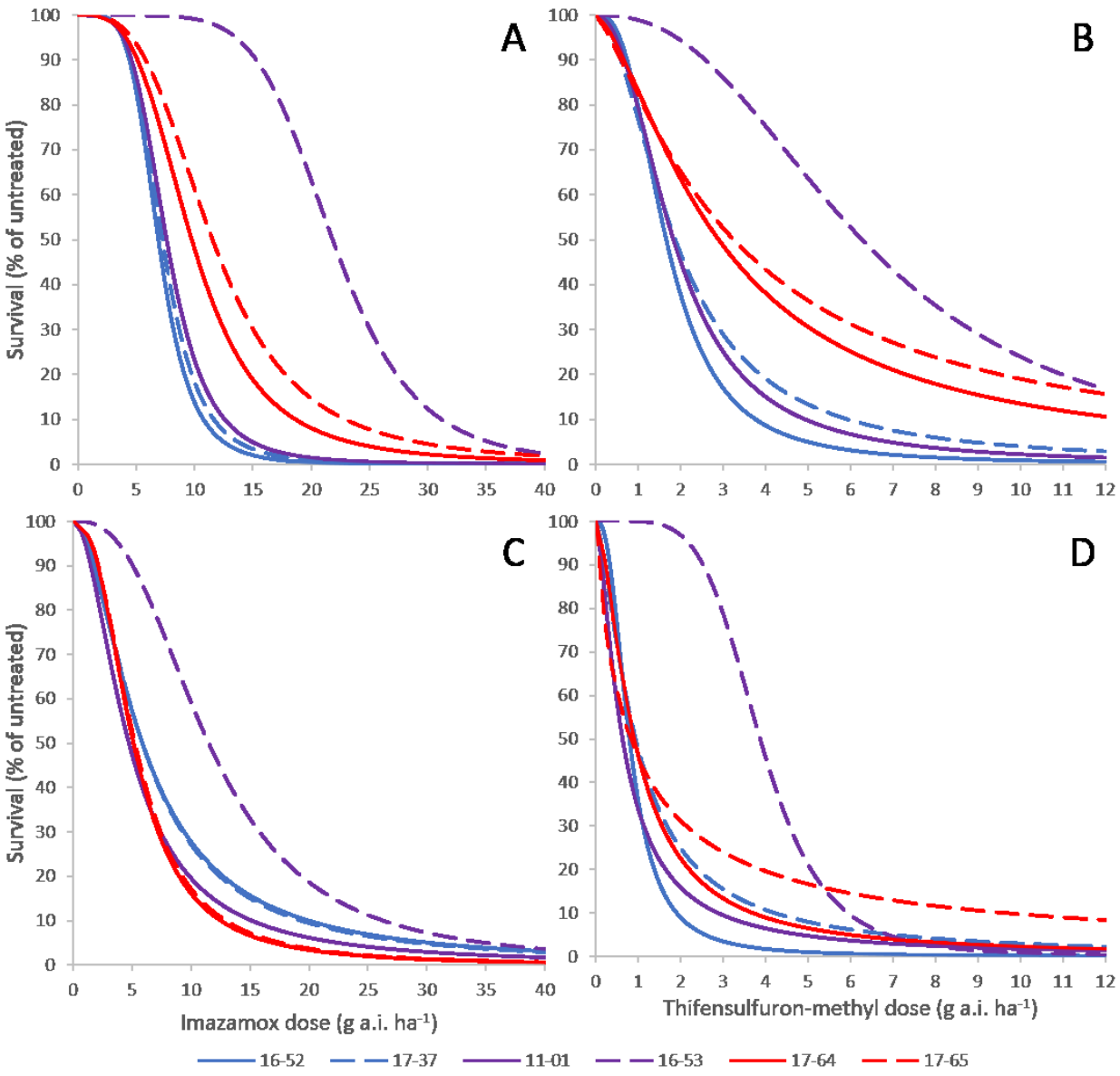
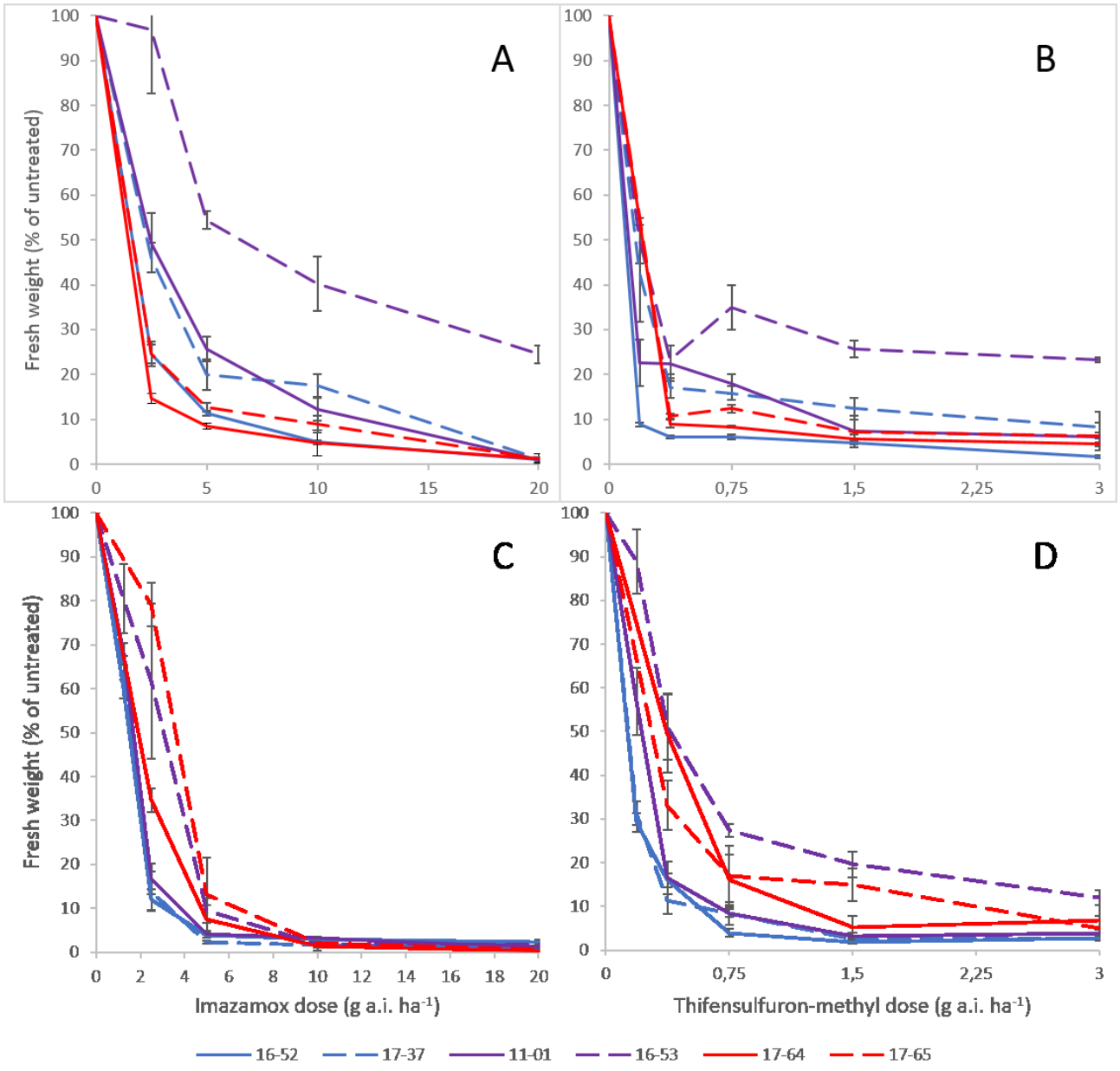


Figure 7. Dose-response curves of two A. retroflexus (blue lines), two A. hybridus (violet lines) and two A. tuberculatus (red lines) populations to imazamox (A, C) and thifensulfuron-methyl (B, D). Fresh weight data expressed as a percentage of the fresh weight of the un-treated control. A and B refer to the experiment conducted outside (2018), whereas C and D to the experiment conducted in the greenhouse (2019). Note that 20 and 3 g a.i. ha^{-1} are half the recommended field doses of imazamox and thifensulfuron-methyl, respectively.



4.3.3. In field seed predation

After three days of seed card exposure, the mean field predation was 67%. No differences were found (t-test, α=0.05) either between treatments (conventional or conservative fields) or among species (*Amaranthus retroflexus*, *Amaranthus hybridus* and *Amaranthus tuberculatus*). This result is intriguing because it clearly indicates that carabids do have the potential to mitigate the expansion of an invasive exotic weed species like *Amaranthus tuberculatus*, even if they were not used to that feed. Having found no difference in predation between conventional and no-till fields was quite surprising, but not completely. First studies on this issue showed that predation in conservation tillage was higher than on conventional tillage, but a more recent study showed no differences among tillage management[35]. Research is ongoing on this issue, and some experiments suggested that carabids activity and seed predation could be affected also by the size/perimeter rate of the fields (F. Lami, personal communication).

Even if predation of *Amaranthus tuberculatus* seeds were predated the same of the other tested *Amaranthus* species in experimental conditions, there is no evidence that carabids can effectively have access to *A. tuberculatus* seeds all year round. This is because seeds of *Amaranthus retroflexus* and *Amaranthus hybridus* mature in summer, while the activity of the carabids is high. Furthermore, mature seeds immediately drop from the plants. Instead, *Amaranthus tuberculatus* seeds mature in October, when carabids are much less active. Furthermore, mature seeds of *A. tuberculatus* do no easily drop from the plant, but they will be available only after crop harvesting. Plowing the land immediately after crop harvesting would limit even more the time available for predation of new seeds. Instead, conservation tillage practices would allow a longer predation period.

Some of the possible biases of using seed cards to measure predation are[36]: 1) some predators may avoid the exclusion cages, even if their size would allow them to enter 2) glued seeds could result difficult to remove to some predators 3) seeds are easily available on a flat -easily accessible- surface. However, all these issues should be considered as systematic and would not challenge the comparisons between the treatments.

4.4. Conclusion

Phenology varied a lot among populations. *Amaranthus retroflexus* was the earliest flowering and maturing species, whereas *Amaranthus tuberculatus* the latest. The two *Amaranthus hybridus* populations flowered and matured very differently, one similarly to *A. retroflexus*, while the other later. Therefore, flowering overlapping between *A. retroflexus* and *A. tuberculatus* is not likely. Instead, partial flowering overlapping is likely to occur between *A. retroflexus* and *A. hybridus*, and among *A. hybridus* and *A. tuberculatus*.

A. tuberculatus grew in height two times faster than the other two species, therefore, if herbicide recommendations are based on *Amaranthus* height, *A. tuberculatus* would be the target species to define herbicide rates and timing in case of sympatry with other Amaranths. Furthermore, the herbicide application windows might be shortened by the presence of *A. tuberculatus*.

Similar susceptibility to ALS-inhibiting herbicides was found among populations. 90% weight loss was caused by 10 and 1.5 g a.i. ha^{-1} of imazamox and thifensulfuron-methyl, respectively, in all populations. Notably, a few plants of *A. tuberculatus* survived to two- and four-fold the recommended field dose of thifensulfuron-methyl. Survivors of *A. tuberculatus* might have a competitive advantage in comparison to eventual survivors of *A. retroflexus* and *A. hybridus*, because of faster growing rates and longer growing season.

Post dispersal seed predation among species was not different, either between treatments or among species. Due to the delayed maturation of *A. tuberculatus* compared to the other two species, it is likely that conventional tillage reduces post-dispersal seed predation of this species, because of seed burying.

Amaranthus is unique example of genus with more than ten species invading the same agricultural habitats. This is interesting for weed management, but also for ecology and evolution. All *Amaranthus* species have C4 metabolism and therefore could be favored in the long term, climate-changing perspective. Consequently, their frequency within summer crops could increase in the near future, and the deep knowledge of their biology will be of extreme help for effective control of their expansion. Among all *Amaranthus* species, the dioecious ones will be -very likely- favored. In united states, *Amaranthus tuberculatus* has gone from virtual anonymity to become one of the most significant troublesome weed[10] and it apparently undergo the same fate in Europe. Its

presence must be readily ascertained, and all control tools should be implemented to avoid its expansion.

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Chapter V

Latest findings and future perspectives

5.1. Introduction

In this Chapter, three experiments -not directly linked each other- will be considered. These experiments were designed to answer specific questions that emerged during the PhD project, although they have not been completely developed yet. The first experiment concerned the findings of the first suspected ALS resistant *Amaranthus palmeri* outside its native range, the second experiment focused on resistance pattern associated with point mutation at codon 376 of the ALS gene and the last experiment tried to elucidate the resistance mechanism suspected to be NTSR in an *Amaranthus tuberculatus* population.

Each experiment will be briefly introduced in the next paragraphs. Materials and methods will be described together, while results/discussion and conclusion will be discussed separately.

5.1.1. Presence of *Amaranthus palmeri* in Italy

Amaranthus palmeri is widely recognized as the most troublesome weed in broadleaf crop and one of the most prone to evolve herbicide resistance. *A. palmeri* is a dioecious species that has evolved resistance to eight sites of action[1] (HRAC classification, alphabetic order, common acronym): B (inhibition of acetolactate synthase, ALS), C1 (inhibition of photosynthesis at photosystem II, PSII, triazine), E (inhibition of protoporphyrinogen oxidase, PPO), F2 (inhibition of 4-hydroxyphenyl-pyruvate-dioxygenase, HPPD), G (inhibition of EPSP synthase, EPSP), K1 (inhibition of microtubule assembly), K3 (inhibition of very long chain fatty acid -VLCFA- biosynthesis) and O(synthetic auxins). Dioecious species are prone to evolve different resistance mechanisms, therefore multiple resistance is more common among them. *A. palmeri* evolved multiple resistance up to five sites of action[1] (B+C1+F2+G+O and B+E+G+K1+K3) and the management of these populations is certainly challenging, because of the low number of alternative chemicals. In North America (the native range of *A. palmeri*), where it is widespread, it represents a treat to many agricultural systems. Recently, herbicide resistant *A. palmeri* has been found in Israel[1], Argentina[2] and Brazil[3]. Early detection of herbicide resistance and proper integrated weed management are essential to avoid the spread of resistance as well as the control of the use of certified seeds.

Amaranthus palmeri was found for the first time in Italy in October 2014, along a roadside in Ravenna (North Eastern Italy) and it was recorded as casual alien. In 2018, plants of *A. palmeri* not

adequately controlled by ALS inhibitors were found in a soybean field near Padova and seeds were collected and used in the experiment.

The aim of this experiment was to confirm the suspected ALS resistance, define the resistance mechanism involved and evaluate alternative herbicides to control this *A. palmeri* population.

5.1.2. Resistance pattern associated to the mutant ALS allele at codon 376

As discussed in Chapter II, three *A. retroflexus* populations were found resistant to thifensulfuron-methyl and susceptible to imazamox. Plants resistant to thifensulfuron-methyl had the point mutation at codon 376 of the ALS gene. This resistance pattern was in apparent disagreement with the current bibliography[1], in particular if referred to Amaranths[4,5,6], because mutation 376 is normally described as endowing cross resistance to sulfonilureas and imidazolinones. According to bibliography, most resistance confirmation experiments were done with imazethapyr, and therefore the resulting cross-resistance pattern might be biased by the choice of this specific imidazolinone herbicide.

To test the hypothesis that this specific mutation confers resistance to imazethapyr, but not to imazamox, four weed populations, containing 376-mutated plants, were treated with imazamox and imazethapyr. To verify if this hypothesis can also be extended to another weed species, the assay was conducted on *A. retroflexus* (three populations) as well as on the monocot species *Sorghum halepense* (one population). These two species are well controlled by both imazethapyr and imazamox, according to the main herbicide formulation labels.

5.1.3. Different mechanism of resistance to ALS inhibitors in *Amaranthus tuberculatus*

As mentioned in Chapters I and III, *Amaranthus tuberculatus* is the most common and troublesome weed in soybean and has already evolved resistance to seven sites of action (*cf*. Chapter I and II). The vast majority of resistant cases to ALS inhibitors reported to date are due to mutant ALS alleles, especially in dicot weed species. Only some cases of NTSR to ALS inhibitors have been reported in dicots weed species[7,8,9], while it is more frequent in monocot weeds[10]. TSR is easier to detect through DNA-based techniques respect to NTSR, that instead requires in vivo herbicide metabolism experiments. However, to date, participation in non-target herbicide resistance has been well established for only four gene families: P450s, GSTs, glycosyltransferases and ABC transporters[11]. Among them, the most investigated is the enhanced metabolism of herbicides mediated by cytochrome P450, probably because its activity can be easily reversed and

observed using CYP450 inhibitors[12],[13]. A well-known and the most used P450-inhibiting molecule is the insecticide malathion, an insecticide.

A. tuberculatus population 17-66, resulted highly resistant to thifensulfuron-methyl and the resistance mechanism involved cannot be attributed only to TSR given that 85% of the resistant plants had no mutated ALS allele (*cf.* Chapter III). In order to assess the involvement of cytochrome P450 monooxygenase in thifensulfuron-methyl resistance, a bioassay at whole plant level with the use of malathion was performed on this *A. tuberculatus* population. Furthermore, a bioassay to evaluate the likely cross resistance of this population to imazethapyr was conducted.

5.2. Materials and methods

5.2.1. Plant material

All the *Amaranthus* populations used in this study (reported in Table 1) were already tested for herbicide resistance during this PhD thesis, except for *A. palmeri* population 18-100. *A. retroflexus* populations were resistant to thifensulfuron methyl, but not to imazamox, and had mutation $Glu_{376}Asp$ of ALS gene (*cf.* Chapter II). Population 17-66 (*A. tuberculatus*) was found to be resistant to thifensulfuron-methyl, but not to imazamox, and no known endowing-resistance mutation was found along the whole ALS gene, except for 15% of resistant plants, having point mutation 574 (*cf.* Chapter III). *A. palmeri* population 18-100 was found in a soybean field in North Easter Italy (GPS:45°34'32.3"N 11°54'43.9"E) where poor control with ALS herbicides was reported. Seeds from a number of mature plants were collected, air dried, cleaned and used for herbicide screening. The *Sorghum halepense* population 08-16H was reported to be resistant to nicosulfuron, but susceptible to imazamox, and was heterozygous for mutation $Glu_{376}Asp$ of ALS gene[14].

Table 4. Weed species, population codes, known resistance pattern and point mutation of each population involved in this study. SU (sulfonilureas: thifensulfuron-methyl for Amaranthus spp. and nicosulfuron for Sorghum halepense).

Weed species		Population	Imazamox	SU	Mutation
Amaranthus	*retroflexus*	10-11 R-L	S	R	$Glu_{376}Asp$
	retroflexus	10-12 L	S	R	$Glu_{376}Asp$
	retroflexus	17-56 R	S	R	$Glu_{376}Asp$
	retroflexus	17-52	S	S	No
	tuberculatus	17-66	S	R	No
	tuberculatus	17-65	S	S	No
	palmeri	18-100	-	-	-
Sorghum	*halepense*	08−16H	S	R	$Glu_{376}Asp$
	halepense	08−19SH	S	S	No

5.2.2. Whole-plant herbicide sensitivity assessment

Seed germination of the *S. halepense* seeds were done following a previously described protocols[14], while for *Amaranthus* spp. the seed germination protocol was described in Chapter II [15]. Seedlings growth and herbicide treatment were done following an established and robust protocol[16]. Experimental layout was a complete randomized design with two replicates (trays). Twenty seedlings per population, at very similar growth stage, were transplanted into plastic trays (325x265x95 mm) with a standard potting mix (60% silty loam soil, 15% sand, 15% perlite and 10% peat) and watered daily as required. Just prior to treatment, plants of each pot were counted. Plants were treated at 12-14 BBCH Scale[17]. ALS-inhibitor herbicides field rates: thifensulfuron-methyl was applied at 6 g a.i. ha^{-1} (Harmony 50 SX, DuPont™, 50 g a.i. 100 g^{-1}), imazamox was applied at 40 g a.i. ha^{-1} (Tuareg®, DuPont™, 40 g a.i. L^{-1}), imazethapyr was applied at 35 g a.i. ha^{-1} (Pursuit® 240, BASF, 240 g a.i. L^{-1}). Alternative control herbicides field rates: glyphosate was applied at 480 g a.i. ha^{-1} (Roundup Platinum®, Monsanto, 480 g a.i. L^{-1}), metribuzin was applied at 175 g a.i. ha^{-1} (Feinzin® 70 DF, Adama, 70 g a.i. 100 g^{-1}), bentazon was applied at 870 g a.i. ha^{-1} (Basagran® SG, Basf, 87 g a.i. 100 g^{-1}). The three experimental protocols were summarized in Table 2. All experiments were conducted in greenhouse and the first was repeated twice.

Table 5. Species, population code, herbicides and herbicides doses used in the three experiments. Thif (thifensulfuron-methyl); Mala + thif (thifensulfuron-methyl plus a pretreatment with malathion); Gly (glyphosate); Met (metribuzin); Ben (bentazon). 1x and 3x are the recommended field dose and three-times that, respectively.

Exp	Weed species	Population	Imazethapyr	Imazamox	Thif	Mala	Gly	Met	Ben
1	*Amaranthus palmeri*	18-100	-	1x-3x	1x-	-	1x	1x	1x
	Amaranthus	17-65	-	1x-3x	1x-	-	1x	1x	1x
2	*Sorghum halepense*	08−16H	1x-3x	1x-3x	-	-	-	-	-
		08−19SH	1x-3x	1x-3x	-	-	-	-	-
	Amaranthus retroflexus	10-11 R-L	1x-3x	1x-3x	-	-	-	-	-
		10-12 L	1x-3x	1x-3x	-	-	-	-	-
		17-56 R	1x-3x	1x-3x	-	-	-	-	-
		17-52	1x-3x	1x-3x	-	-	-	-	-
3	*Amaranthus*	17-66	1x	1x-3x	1x-	1x-3x	-	-	-
	tuberculatus	17-65	1x	1x-3x	1x-	1x-3x	-	-	-

A. palmeri plants were treated at the recommended field rate (1x) and three times that (3x) with ALS-inhibitors and only at field rate with alternative herbicides, along with recommended surfactants (experiment 1).

Plants having the mutant ALS allele at codon 376 were treated with imazethapyr and imazamox at the recommended field rate (1x) and three times that (3x) (experiment 2).

For cytochrome P450 inhibition experiment, *A. tuberculatus* plants were sprayed with malathion at 1140 g a.i. ha^{-1} (laboratory sample, 570 g L^{-1}); two hours later, the same plants were sprayed with thifensulfuron-methyl at the recommended field rate (1x) and three times that (3x) (experiment 3).

For all experiments, agrochemicals were applied using a precision bench sprayer delivering 300 L ha^{-1} at a pressure of 215 kPa and speed of about 0.75 m s^{-1}, with a boom equipped with three flat-fan (extended range) hydraulic nozzles (Teejet, 11002). Four weeks after herbicide application, the number of surviving plants and the visual estimation of their biomass (VEB) were assessed. The VEB scores, ranging from 10 (for plants not affected by the herbicide compared to the untreated control) to 0 (when the plants were clearly dead, sensitive), were given to each treated tray. For experiment 3, also fresh weight was recorded. On the basis of herbicide efficacy, the accessions were ascribed to four categories as follows: susceptible (S) if survivors were less than 5% at 1x rate; moderately resistant (MR) if survivors were between 5% and 20% at 1x rate; resistant (R) if survivors were more than 20% at 1x rate; highly resistant (HR) if survivors were more than 20% at 1x rate and more than 10% at 3x rate. Greenhouse temperature varied between 15 and 20 °C and from 25 to 34 °C, during the night and day, respectively. Standard deviation (SD) was calculated for each data mean. DNA was extracted from five plants of *A. palmeri* survived to thifensulfuron-methyl 1x and ALS gene was PCR amplified following the protocol and primers used for *A. tuberculatus* (described in Chapter II).

5.3. Results and discussion

5.3.1. Confirmation of ALS-resistant *Amaranthus palmeri* in Italy

A. palmeri population 18-100 resulted highly cross resistant to thifensulfuron-methyl and imazamox at both the recommended field rate and the higher rate tested. The survival rates ranged from 81 to 82% and from 100 to 95% for imazamox and thifensulfuron-methyl, respectively. The visual estimation biomass of the ALS-treated plants of this population were comparable to the non-treated control plants (data not shown), indicating a negligible effect of herbicides on them. In four out of five plants one mutant ALS allele (tryptophan to leucine substitution) at codon 574 of the ALS gene was identified. This mutation is known to confer broad spectrum resistance to ALS inhibitors. Therefore, the main resistance mechanism involved is target-site mediated. In contrast, this accession was perfectly controlled with glyphosate and metribuzin, whereas a poor control was observed with bentazon at the Italian recommended field rate (1x = 870 g a.i. ha^{-1}). Since *A. palmeri* was an almost unknown weed in Italy, no susceptible accession was found to be used in the assay. Therefore the *A. tuberculatus* population 17-65 was used as susceptible control and was fully controlled by all herbicides. This is the first report of herbicide resistant *Amaranthus palmeri* outside its native range (North America) and it should be carefully managed. Glyphosate and metribuzin are still effective to control this ALS-resistant population in the field, while bentazon failed to control it.

5.3.2. Resistance pattern of 376-mutated plants

Imazamox showed an efficient control of the mutant ALS plants of all tested populations of both species, *A. retroflexus* and *S. halepense*. Only some survivors were observed for population 08-16H at 1x dose while no plants were survived at the higher dose. Instead, imazethapyr failed to control them even at the higher dose tested. The initial hypothesis that point mutation 376 confers resistance to imazethapyr, but not to imazamox, is therefore confirmed in two species.

The reason of this unexpected different efficacy of imazamox and imazethapyr on 376-mutated plants might be related to the slightly different chemical structure of these two herbicides. Imazamox (5-ethyl-2-(4-methyl-5-oxo-4-propan-2-yl-1H-imidazol-2-yl)pyridine-3-carboxylic acid) has an additional methoxy group respect to imazethapyr (5-(methoxymethyl)-2-(4-methyl-5-oxo-4-propan-2-yl-1H-imidazol-2-yl)pyridine-3-carboxylic acid). This methoxy might interact with the

glutamic acid ($C_5H_9NO_4$) of $Asp_{376}Glu$ mutated enzyme, but not with the wild type residue (aspartic acid, $C_4H_7NO_4$).

Indeed, results should be confirmed using a wider range of species and including *in vitro* ALS activity assays. However, these results have highlighted that the generalization on the cross-resistance patterns to ALS herbicides endowed by specific *ALS* mutations cannot be based on response to one or two herbicides from a particular ALS herbicide chemistry. When a new mutation or substitution is described, multiple molecules of the same chemical family should be tested at the same time, choosing among the ones that the plant might have experienced. The detailed knowledge of resistance pattern associated with specific point mutations would be useful, for example, to easily purify populations with different resistance patterns/mutations.

Table 6. Survival rates of each population to different herbicide treatments, in the three experiments. Thif(thifensulfuron-methyl); Mala + thif (thifensulfuron-methyl plus a pretreatment with malathion) Gly (glyphosate); Met (metribuzin); Ben (bentazon). For each herbicide treatment, 1x and 3x are reported, according to the scheme shown in Table 1. Standard deviation of means is reported in brackets (if no standard deviation is indicated, it is equal to zero).

exp	Species	population	imazethapyr	imazamox	thif	mala + thif	gly	met	ben
1	AP	18-100	-	81(12)-82(6)	100-95(6)	-	0	4(6)	89(9)
	AT	17-65	-	0-0	0-0	-	0	0	0
2	SH	08−16H	83(4)-10(14)	11(7)-0	-	-	-	-	-
		08−19SH	8(4)-0	0-0	-	-	-	-	-
	AR	10-11 R-L	100-71(15)	0-0	-	-	-	-	-
		10-12 L	93(1)-57(10)	0-0	-	-	-	-	-
		17-56 R	84(2)-61(25)	0-0	-	-	-	-	-
		17-52	0-0	0-0	-	-	-	-	-
3	AT	17-66	78(6)	24(15)-12(8)	88(18)-83(11)	95(7)-77(11)	0	11(7)	29(6)
		17-65	0	0-0	15-13(4)	3(4)-3(4)	0	0	0

5.3.3. Mechanism of resistance to ALS inhibitors in *Amaranthus tuberculatus*

Only 24% of plants of *Amaranthus tuberculatus* population 17-66 survived at the recommended field dose of imazamox, confirming the results obtained in Chapter III. Resistance to

thifensulfuron-methyl, instead, was a lot higher (more than 80%), with comparable values at the recommended field dose and three-times that (Table 3). Furthermore, no differences were found in survival rates between plants treated with thifensulfuron-methyl alone and plants pre-treated with malathion, at both thifensulfuron-methyl doses (Table 3). Treatment with malathion alone had no effect on fresh weight (data not shown), but plants treated with thifensulfuron-methyl alone or both malathion and thifensulfuron-methyl had a fresh weight loss of about 50%, with no difference between 1x and 3x doses (data not shown). Resistance to imazethapyr was also similar to that of thifensulfuron-methyl (78%), but fresh weight loss was higher (about 60%, data not shown).

The results obtained are not compatible with cytochrome P450 metabolic resistance given that no significant effect on survival and fresh weight has been registered following malathion pre-treatment and thifensulfuron-methyl. The malathion dose used in others works ranged from 400[18] to 1000[19], up to 2000[20] g a.i. ha^{-1}, so having used 1140 g a.i. ha^{-1} it cannot be excluded that a higher dose is needed to inhibit the cytochrome P450 pathway in the *Amaranthus* plants. Indeed, further tests are needed by using different doses of herbicides and/or insecticide, or introducing a positive control population to definitely exclude the presence of a P450-mediated enhanced metabolism in this population.

Non target site resistance can also be due to altered translocation, glutathione S transferase (GST) detoxification or vacuolar sequestration among all, but none of these mechanisms have been described yet in *A. tuberculatus*. Other possibilities can be: a) the presence of acetohydroxyacid synthase homologs, as already observed to confer resistance in sunflower (*Helianthus annuus* L.)[21] b) the presence of a mutated enzyme with activity similar to that of ALS, like catabolic ALS (CALS)[22], that could partially remedy ALS inhibition c) the presence of a mutated ALS-interacting proteins, like AIP1 and AIP3[23].

To further investigate resistance mechanisms in this population, *in vitro* ALS activity assays and plant crosses will be performed.

5.4. Conclusion

Experiment 1 confirmed that the *Amaranthus palmeri* population infesting Italian soybean fields was ALS-resistant. As far as we know, this is the first report of herbicide resistance *A. palmeri* outside the Americas. A similar situation was observed with the congeneric weed species, *Amaranthus tuberculatus*, that once appeared in agronomical habitats, its spread was rather fast. Indeed, numerous ALS resistant biotypes were appeared in multiple locations in Italy (*cf.* Chapters II and III). Differently from *A. palmeri*, *A. tuberculatus* was historically present in Italy since the 80's, therefore evolution of resistance from resident -unnoticed- populations is still possible, although unlikely. Instead, this is -exactly- the second record of *A. palmeri* in Italy, and having found it already resistant is a clear proof that this population evolved resistance outside Italy and that its introduction is linked with agriculture habitat.

Experiment 2 confirmed that 376-mutated plants can survive to imazethapyr, but not to imazamox treatments. This experiment shed light on the resistance pattern of 376-mutated plants explaining the reason of the apparent disagreement with current bibliography (*cf.* Chapter II). Where imazethapyr resistance is detected, and mutation 376 is recognized as the cause of resistance, imazamox might be used instead of imazethapyr to control these populations (according to local laws and agricultural practices).

Experiment 3 found no evidences that thifensulfuron-methyl resistance in population 17-66 (*A. tuberculatus*) was due to enhanced cytochrome P450 metabolism. Resistance mechanism in this population remained unclear.

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Chapter VI

Overall conclusions

6.1. Overall conclusions

The focus of this PhD thesis was to acquire more insight into the biology and evolution of ALS-resistant *Amaranthus* spp. infesting soybean crops, an emerging problem for Italian agriculture, in order to devise proper resistant management strategies. The project began after the finding of a number of infested soybean fields from 2010 to 2017. *Amaranthus* spp. were clearly involved, but the species were unclear due to the extreme phenotypic variability. Since then, many other cases of resistance were recorded and studied. Results of the first part of the project highlighted the presence of many ALS-resistant populations of *Amaranthus tuberculatus*, a previously unknown species for the Italian agriculture. The second part of the project was therefore focused on better understanding the evolution of ALS resistance among populations of that new entity. The third part, instead, focused more on some biological traits of the three *Amaranthus* species that were identified at the beginning. The last part included some experiments not directly linked with each-other, which contributed to the overall understanding of herbicide resistant *Amaranthus* in Italy and also suggested possible future perspectives of research. The following paragraphs summarizes the results gathered during the three-year doctoral project and attempts to make final observations by integrating all this information.

6.1.1. Weedy Amaranths in Italy: old and new enemies

A simplified, easy-to-use, identification key was designed to classify the six *Amaranthus* species that were very prone to evolve herbicide resistance. Four *Amaranthus* species were found to infest soybean fields in Italy: *A. retroflexus*, *A. hybridus*, *A. tuberculatus* and *A. palmeri*. *A. retroflexus* and *A. hybridus* are two monoecious species, historically known to infest summer crops in Italy. *A. tuberculatus* and *A. palmeri*, instead, are two dioecious species, both coming from North American plains, but with a different history of invasion in Italy. *A. tuberculatus* was known to be present in Italy from the 80's, but its presence was limited to its typical habitat, riverbanks and floodplains. Before this study, there was no record of *A. tuberculatus* infesting fields in Italy. Even more surprisingly, before this study the presence of *A. palmeri* in Italy had only been recorded once and the observation was limited to a few specimens along the roadside. In this study the presence of resistant *A. palmeri* is therefore reported for the second time in Italy, but as a weed infesting soybean field, not a casual alien.

6.1.2. ALS resistance in Italian weedy Amaranths is target-site mediated

In total, 14 *Amaranthus* populations were found to be ALS-resistant: three belonged to *A. retroflexus*, two populations to *A. hybridus*, eight to *A. tuberculatus* and one to *A. palmeri*. All, except one population, had a point mutation at the ALS gene, conferring specific resistance patterns. Both *A. hybridus* and seven out of eight *A. tuberculatus* populations were cross-resistant to thifensulfuron-methyl and imazamox, and had a point mutation at position 574 of the ALS gene. In most plants of a single population of *A. hybridus,* the mutant *ALS* allele Met_{574}, were identified while in all other plants, in all populations, the mutant *ALS* allele Leu_{574} were detected. The substitution $Trp_{574}Met$ was reported in dicots for the first time. All *A. retroflexus* populations were only resistant to thifensulfuron-methyl, and had a point mutation at position 376 of the *ALS* gene (aspartic to glutamic acid, $Asp_{376}Glu$). Furthermore, an experiment involving both *A. retroflexus* and *S. halepense* plants having the mutant *ALS* $allele_{376}$ highlighted that this point mutation caused resistance to imazethapyr, but not to imazamox. Intriguingly, *A. retroflexus* ($Asp_{376}Glu$), *A. hybridus* ($Trp_{574}Met$) and *A. tuberculatus* ($Trp_{574}Leu$) were found living sympatrically within the same field. Only one population of *A. tuberculatus* was resistant to thifensulfuron-methyl and imazethapyr (not to imazamox), had no mutations, and that metabolic resistance was not likely to occur, and it remained unclear. Herbicide resistance in this intriguing population will be further explored.

6.1.3. *Amaranthus tuberculatus* is a "bad guy"

The presence of two 574-mutated *ALS* haplotype indicated that resistance evolved independently in at least two spatially separated populations. The spread of one of these 574-mutated *ALS* haplotypes among five other *A. tuberculatus* populations spatially far apart, clearly demonstrated that a number of seed migration events occurred in north-eastern Italy during the last decade.

Phenology experiments highlighted that *A. retroflexus* was the earliest flowering and maturing species, whereas *A. tuberculatus* the latest. Therefore, flowering overlapping between *A. retroflexus* and *A. tuberculatus* is not likely to occur. Instead, partial flowering overlapping is likely to occur between *A. retroflexus* and *A. hybridus*, and among *A. hybridus* and *A. tuberculatus*. Another characteristic of *A. tuberculatus* that emerges from this study is that these plants grow in height twice

as fast as *A. retroflexus* and *A. hybridus*. This can contribute to the successful invasion of this species, because even if it flowers later it is able to recover and overgrow the crop. Moreover, ALS herbicides were found to be very effective even at low doses on *A. retroflexus*, *A. hybridus* and *A. tuberculatus*. Nevertheless, some *A. tuberculatus* plants survived even with high doses of ALS herbicides. Seeds of all *Amaranthus* species were equally predated.

6.1.4. Keep an eye on the dioecious

Given that both the dioecious amaranths *Amaranthus tuberculatus* and *Amaranthus palmeri* were found to be ALS-resistant in Italy, their eventual presence in a field must be promptly recognized. *A. tuberculatus* is a very fast-growing species, prone to evolve herbicide resistance, and *A. palmeri* is expected to behave similarly. Therefore, if one of these two species were to be found, proper weed management would have to be considered. First of all, in mixed populations the herbicide treatment has to focus on these species, taking into account that the treatment window would be short, because they grow faster. In case of suspected ALS resistance, a key measure is to use herbicides with different SoA at different times. Glyphosate can be used when the crop is not present (in Europe the cultivation of Roundup ready crop varieties is not allowed), metribuzin can be used to effectively control ALS-resistant populations in soybean, whereas the use of bentazon might lead to unsatisfactory results. The introduction of pre-emergence herbicides, followed by a single post-emergence herbicide application, should be evaluated wherever possible. Given the prolonged germination period of Amaranths, another possibility is to split the post-emergence herbicide treatment into two applications, to control also late-germinating *Amaranthus* biotypes. To prevent soil seed bank enrichment, eventual escapes of late-germinated *A. tuberculatus* biotypes should be manually eradicated, wherever possible. The best time to do this, would be from mid-July to mid-August, when most *A. tuberculatus* plants would be at full bloom. This is mainly for three reasons: a) during flowering, female plants are easily to recognize and can therefore be the target of eradication efforts (saving half the work) b) seeds won't be ripe yet and specimens can therefore be left on the ground c) after mid-July, soybean canopy would avoid all eventual late-germinating biotypes.

The presence of carabids might contribute to post-dispersal seed predation of *Amaranthus* spp. seeds, especially in case of conservation agriculture.

6.1.5. Final remarks

Resistance can spread also because of seed dispersal. Even if no clear explanations were found for that, zoochory, human activities and/or bad farming practices (e.g. not cleaning harvesting machinery when moved from one field to another) remain the most likely causes. As a preventive measure and in compliance with good agronomic practices, it is necessary to use only certified seed.

Amaranthus is unique example of genus with more than ten species invading the same agricultural habitats. This is interesting not only from an agronomic point of view, but also from ecological and evolutionary perspectives. All *Amaranthus* species have C4 metabolism and therefore could be favored in the long term, climate-changing perspective. As a consequence, their frequency within summer crops could increase in the near future and the deep knowledge of their biology will be of extreme help for effective control of their expansion. Among all *Amaranthus* species, the dioecious ones will be very likely favored. In the USA, *Amaranthus tuberculatus* has gone from virtual anonymity to become one of the most significant troublesome weeds, together with *Amaranthus palmeri*. It is very likely that both weeds were introduced already ALS resistant in Italy. This eventuality should be taken in serious consideration, because caused the introduction of noxious exotic invasive weed species and herbicide resistance alleles at the same time. Specific efforts should be taken to avoid the diffusion of already resistant populations and further possible introduction of new “resistant” seeds through the use of not certified crop seeds.