EMPIRICAL INVESTIGATION OF *DE NOVO* MUTATIONS CONFERRING HERBICIDE RESISTANCE

BY

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THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Crop Sciences
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2018

Urbana, Illinois

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ABSTRACT

In a predictable natural selection process, herbicides select for adaptive alleles that allow weed populations to survive. These resistance alleles may be available immediately from the standing genetic variation within the population, as well as, may immigrate via pollen or seeds from other populations. Moreover, because all natural populations are constantly subject to new mutant genotypes by *de novo* mutations, resistant mutants may arise spontaneously in any herbicide-sensitive weed population. Recognizing that the relative contribution of each of these three sources deeply affect what strategies should be applied to counteract herbicide resistance evolution, we aimed to provide experimental information to the resistance evolutionary framework. In this sense, the objective of this experiment was to calculate the *de novo* mutation rate conferring herbicide resistance in a natural plant population, and, specifically, test the hypothesis that the mutation rate increases when plants are stressed by sub-lethal exposure to herbicides. For this purpose, we used a method to discover spontaneous herbicide-resistant mutants by screening millions of plants using grain amaranth and resistance to ALS herbicides as a model system. After screening 70,000,000 plants, no spontaneous resistant genotypes were detected, determining the probability to find a spontaneous ALS-resistant mutant in a given sensitive plant population as lower than 2×10^{-8}. This is lower than expected from theoretical calculations based on previous studies, setting a higher limit for the probability of herbicide-resistant mutants to arise spontaneously in natural plant populations. In addition, we found no evidence that herbicide stress

increased the mutation rate. The results found in this study imply that *de novo* mutations conferring herbicide resistance do not appear to occur at high frequency in plant populations.

To all who work for a more equitable and inclusive society.

ACKNOWLEDGEMENTS

This was a life-changing experience for me, so I would like to thank all the people for who I shared this way.

In the first place, thank you to my advisor Dr. Patrick Tranel. for giving me the tremendous opportunity of being part of this. You trusted in me when you didn't know me. I am in debt with you. And thank you for teaching me that weeds are cool. Indeed, they are.

Secondly, I would like to thank the other members of my committee. Thank you, Dr. Matthew Hudson and Dr. Kenneth Paige, your thoughts were great contributions to my project and inspiring to myself.

Third, I would like to thank all the Tranel's lab members. In special, Dr. Darci Giacomini, thank you for all your help and your kindness. You are super. Cristina Song, thank you for teaching me lot of things when I started at Tranel's lab, your patience was endless with me. Sebastian Sabaté, thank you for making the many Illinois grey days a little funnier. Sos crack amigo! Ahmed Sadeque thank you for opening your hearth and your beliefs to me. Brent Murphy, thank you for your great scientific questions and your delicious cakes. Lady, Kathryn Lillie, thank you for being my desk neighbor, I will miss your silent presence. Alvaro Larrán, Alvarillo, thank you for being my friend for 3 months, those 3 months were the best. Chenxi Wu, thank you for being super nice to me. Yaling Bi, it was fun trying to talk with you. Also, I would like to thank all the undergrad assistants that have helped with my project, Ivanna Yu, Emily Eul, Brittany Hermosillo, and Salman Khan. You worked hard guys!

In addition, I would like to recognize the University of Illinois for giving excellent education opportunities to international students. What you do here in Illinois is amazing. In this way, I would like to recognize the University of Buenos Aires, and the Argentinian society for investment in public education. I am proud to be a product of the public education of Argentina.

I would like to express my gratitude to my family, parents Liliana and Roberto, my sister Florencia, and my grandmother Pierina for supporting me in the distance. Thank you for investing money and years of your life in myself. I would not be here without your effort. Also, thank you to all my friends in Argentina and around the world for hacerme el aguante siempre!

In the end, thank you, Maru Bevilacqua, my future wife, for supporting me every day and making each moment memorable. I could not do anything without your love and your encouragement to surpass my limits. You are the best partner for this search journey that we decided to do with our lives. Gracias tana, te amo.

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CHAPTER 1: Introduction

1.1The problem of herbicide-resistant weeds

After the last ice age, more than 10,000 years ago, human beings began to settle in permanent places and rely on growing crops and raising cattle to support a more benign, sedentary way of life (Grimberg and Svanström 1967). From that beginning, the progress of civilization has increasingly demanded higher and higher food production, requiring the addition of more land under agricultural production and the increase of productivity per land unit. It has only been in the last 60 years, however, that the augmentation of the human population, along with increases per capita, consumption level, and life-expectancy, generated a dramatic increase in food demand that only could be compensated by the technological advance going on from the ‘’Green Revolution’’ until now. As these variables are expected to exponentially increase in the near future, agricultural production systems will be challenged as never before (Lanz et al. 2018; Prosekov and Ivanova 2018).

Since the dawn of agriculture, nature has challenged crop productivity in many different ways. One challenge that farmers have always had to face is the presence of weeds growing alongside and competing with the crop plants (Powles and Yu 2010). Aboveground, crops and weeds compete for light interception, while in the soil, they compete for the absorption of water and nutrients. This competition ultimately results in yield reduction, decreasing the economical returns of the crop. Approaches to mitigate this loss have been historically based on physical perturbations (hand weeding, hoeing, tillage, burning) as well as crop management techniques (row spacing, seeding rates,

planting date, and cover crops). However, with the development of the chemical industry after the Second World War, herbicides began to be mass produced, beginning a new era in weed control and imposing themselves as the most effective, easiest, and least expensive way to kill weeds (Heap 2014). During the last 65 years, more than 300 herbicides have been brought to the market, widely replacing any other method of control (Busi et al. 2013).

Although highly successful, herbicides raise concerns in relation to human and environmental safety, and now also are facing efficacy challenges. Within this latter group, the primary driving force for reduced efficacy on weeds is their evolved resistance to these chemicals (Burgos et al. 2013). Weed resistance is defined as ‘’the evolved capacity of a previously herbicide-susceptible weed population to survive an herbicide and complete its life cycle when the herbicide is used at its normal rate in an agricultural situation’’ (Heap 2014). In the same way that bacteria evolve resistance to antibiotics and insects evolve resistance to insecticides, weeds evolve resistance to herbicides. For natural populations, the continuity of life under changing circumstances is maintained by natural selection acting on genetic diversity (Kondrashov 1984). This explains how natural populations survive extreme events. In the context of weedy populations, herbicide applications are abrupt and extreme disturbances to targeted populations. The prior existence of a subset of a population that is genetically capable of surviving the perturbation, allows the population to persist in the presence of the chemical control (e.g. herbicides select for adaptive alleles).

Because herbicides select for adaptive alleles conferring resistance, whenever herbicides are applied, resistance evolution responds. That is why the prolonged reliance on herbicides for weed control has produced a constant stream of reports of resistant weed populations since the first documented case in 1968 (Ryan 1970). In an effort to understand and deal with this phenomenon, all published cases are summarized in the International Survey of Herbicide-resistant Weeds website, which is located at www.weedscience.com. As of March 2018, the survey includes 491 resistant cases corresponding to 254 weed species—148 dicots and 106 monocots—in 70 countries. Reports indicate evolved resistance to 23 of the 26 existing site-of-action (SoA) groups of herbicides.

The herbicide-resistance phenomenon has been exacerbated in the last 20 years. As with any other natural selection event, an increase in selection pressure increases the population frequency of the individuals that can survive selection. The overreliance on herbicides for weed control and the predominance of only a few herbicidal products meant farmers used the same products year after year, increasing the selection pressure for resistant genotypes in weed populations. The major reasons for this behavior were adoption of no- or low-tillage systems, lack of crop rotation (e.g. monocultures), and, perhaps most importantly, the predominance of herbicide-resistant crops in which most of the chemical control relies on just one product (e.g. glyphosate-resistant crops) (Powles 2008; Green 2014). Moreover, the presence of existing herbicide-resistant weeds in the fields led to farmers choosing between even fewer products for weed management (Beckie and Harker 2017). This reached the extreme in the cases of multiple resistance (i.e. weed populations resistant to herbicides targeting

different sites of action). In Illinois, one population of waterhemp (*Amaranthus rudis* Sauer) was shown to have evolved resistance to herbicides from four sites of action (Bell et al. 2013).

From an economical perspective, the reduced number of effective herbicidal options has increased the farmer's overall cost for weed management. This problem is magnified by the fact that the chemical industry has only brought a few new products with new SoAs to the market in the last quarter century. Previously, the continuous emergence of new active ingredients in the market kept the problem of herbicide-resistant weeds at bay. However, since the 80's when 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors were introduced—no new SoAs have arisen from the industry. Part of this can be explained by the devaluation of the herbicide market with the launch of glyphosate-resistant crops and the appearance of cheap generic herbicides, but the lack of new chemistries may also be attributed to the increased cost of bringing new products to the market, and the consolidation of the pesticide industry into a few large corporations (Duke 2012; Beckie and Harker 2017).

As most of these economic factors will not likely be resolved in the short-term, growers should not expect to have many new active ingredients available in the near future (Duke 2012). However, as the intensive use of herbicides is maintained, resistance issues will continue to occur. Therefore, good resistance management becomes crucial to preserve the usefulness of available herbicide products for as long as possible.

1.2 Herbicide-resistant weed management: an evolutionary subject

Because herbicides select for adaptive alleles, the design of resistance management strategies should be targeting the factors that selection depends on. These include characteristics of the weed species (fecundity, breeding system, generation time, seed longevity, gene flow by pollen and seed, and seed dormancy), genes conferring resistance (frequency, number, dominance, provided resistance-level, and fitness in the absence of herbicide), herbicide (site of action, chemical structure, residual activity), and operational factors (dose, frequency, operator accuracy, , equipment, and environmental conditions during operation) (Powles and Yu 2010, Heap 2014). A combination of these last factors determines the overall selection pressure placed on a population. For a particular herbicide, because the number of individuals treated over time can be managed by the frequency of application, and their fecundity can be affected by the rate level, the selection pressure is thought to be the factor that we can influence the most. In this sense, diminishing herbicide selection pressure is the cornerstone of management strategies to counteract resistance evolution. This approach should be applied, however, in agricultural production systems that highly base weed control on herbicides.

Along these lines, in addition to cultural and mechanical methods, recommended resistance-management strategies usually propose methods that include the effective use of herbicides. Rotating herbicides with different sites of action is a common strategy to delay the evolution of herbicide resistance provided there is no cross-resistance (i.e. alleles that provide resistance to more than one site of action) (Norsworthy et al. 2012). However, the mixture of herbicides with different sites of action is considered a better

step than rotation to delay resistance (Owen et al 2015). In addition, the introduction of crop cultivars with alternative single or stacked herbicide resistance traits appear as one of the most promising strategies available to farmers in the near future to delay resistance (Meyer et al 2015). It should be considered, however, that these herbicide-resistant crops will be based on SoA groups which have been extensively used, already existing many documented resistant-weed populations to these chemistries, several with multiple resistance (Beckie and Harker 2017). Although probably successful in the short-term, any of these approaches do not appear to be lasting solutions for mitigation of herbicide resistance (Green and Owen 2011).

In order to devise sustainable resistance management strategies, it is crucial to consider the whole ecological dynamic that results in the appearance of resistance issues. As its core, herbicide resistance is an evolutionary process, thus management strategies should aim to destabilize resistance evolution (Heap 2014). Therefore, it is necessary to understand the evolutionary forces underpinning the phenomenon, and accordingly, it is increasingly being argued that more evolutionary biology research should be incorporated in the design of any strategy for mitigating herbicide resistance (Neve 2007). Moreover, herbicide resistance is recognized as a valuable phenomenon to address general theories of evolutionary ecology such as rapid adaptive change (Busi et al. 2013). In consequence, this project aims to provide experimental information to the resistance evolutionary framework.

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CHAPTER 2: Empirical investigation of the mutation rate conferring herbicide resistance

2.1 Introduction

2.1.1 The mutation rate

Novel mutation is the ultimate source of genetic variation because it is the only process that creates completely new genetic variants (i.e. new genes and alleles) (Lynch 2010; Long et al. 2013). Once these primary changes are produced, other evolutionary forces such as selection, drift, and migration can act to spread and select these variants, generating adaptive traits and increased differentiation (Hollister et al. 2010; Long et al. 2015). But only mutations are properly considered to be the raw material of evolution (Freeman and Herron 2007).

Basically, mutations are understood to be any type of permanent change in the base sequence of the DNA molecule. These can occur *de novo* through a number of mechanisms including single nucleotide substitutions, short insertions or deletions (indels), and even larger or more complex changes (chromosomal rearrangements) like large deletions, duplications, translocations or inversions (Drake et al. 1998; Hollister et al. 2010). Within these, because of its high frequency, single nucleotide substitutions (e.g. point mutations) are thought to underlie many of the phenotypic differences within and among species.

A point mutation alters a single point (i.e. one nucleotide) in the base sequence of a gene (Freeman and Herron 2007). Several mechanisms have been proposed that are capable of producing point mutations (DeRose-Wilson and Gaut 2007; Yang et al.

2015). Out of all these mechanisms, the occurrence of errors during DNA replication and DNA repair are believed to be the main generators of single nucleotide changes in the genome (Freeman and Herron 2007). Both types of changes result from reactions catalyzed by DNA polymerase. When the mistaken substitution was from a purine (adenine, A or guanine, G) to another purine or from a pyrimidine (T or C) to another pyrimidine, it is called a transition. Conversely, a transversion is when a purine is substituted for a pyrimidine or vice-versa (Freeman and Herron 2007). Analyses of the mutation rates from a wide range of species indicate that transitions are much more common than transversions in nature (Long et al. 2015). The main explanation for this phenomenon supposes that transitions are less perturbing to DNA synthesis, decreasing the chance to be recognized for correction (Freeman and Herron 2007). When a point mutation occurs in the coding region of a gene resulting in amino acid change, it may alter protein function, so it is referred to as a nonsynonymous substitution. Differently, when a base substitution produces no change in the amino acid sequence, it is called a synonymous substitution. Both types create new alleles.

Furthermore, in relation to fitness, mutations can be categorized as deleterious, neutral or beneficial (Baer et al. 2007). In theory, only non-neutral mutations are under direct selection. Considering that proteins have been subjected to selection for millions of years, random changes in their amino acid sequences would hardly improve their function, therefore, most of the mutations occurring in the genome are believed to be deleterious or neutral (Sung et al. 2016). Because deleterious mutations lead to a decrease in an individual's overall fitness, natural selection purges out the vast majority of new alleles created by mutation in the population. Under this reasoning, natural

selection must also screen against mutation rate modifiers (i.e. mutator alleles) that increase the frequency of deleterious alleles each generation (Baer et al. 2007). In this sense, the mutation rate should evolve toward zero (Freeman and Herron 2007).

However, the mutation rate is always greater than zero in nature (Kraemer et al. 2016). From a logical survival standpoint, it is commonly argued that if all loci in a population are fixed for the fittest allele in a current environment, in the absence of mutation, a population would not be able adapt to any new environment, ultimately leading to extinction. Following this idea, natural selection should have optimized mutation rates to guarantee species survival in the evolutionary long-term. Despite this, direct empirical or theoretical evidence adding support to this theory has not yet been found (Lynch et al. 2016). Alternatively, there are two main explanations that have been proposed to understand non-zero mutation rates (Drake et al. 1998; Sung et al. 2016).

The ‘cost of fidelity’ theory asserts that increasing DNA replication or repair accuracy has an energetic or kinetic cost which has a negative impact on fitness, so fitness is optimized at a non-zero mutation rate (Drake et al. 1998; Lynch et al. 2016). For example, in the well documented trade-off between polymerase accuracy and polymerase speed in bacteria, speeding up the replication rate is a means to increase fitness, but in doing so, selection for this increased rate would lead indirectly to an increase in the mutation rate (Freeman and Herron 2007). Therefore, an optimum for fitness is established by the rate between mutator and antimutator alleles. Supporting the ‘cost of fidelity’ is the fact that the DNA replication and repair fidelity in nature is lower than the biochemical potential limit as evidenced in many species. In addition, it is alleged that there may be a point in the progressive lowering of the mutation rate at

which it became so small in terms of fitness that it is randomly eliminated by genetic drift (Lynch 2010; Sung et al. 2012; Kraemer et al. 2016; Sung et al. 2016). When this ‘’selection drift’’ barrier is met also depends on the balance between mutation rate modifiers. In an attempt to link both ideas, it has been argued that the mutation rate divergence across the tree of life is a result of how well selection successfully optimizes replication accuracy within the limit defined by random genetic drift (Lynch et al. 2016).

To sum up, the variation of the mutation rate across living beings is determined by the interplay of mutation with selection and drift (Sung et al. 2016). Mutation rates can be defined as the number of mutations at any genomic scale (from a nucleotide to genome level) per cell division, per generation or per unit time. Intuitively, as fitness refers to the contribution of one individual to the next generation, natural selection acts directly on the mutation rate per genome per generation (Baer et al. 2007). Inversely, the mutation rate influences both the speed at which populations respond to natural selection and the rate at which fitness may decline due to inbreeding, thus comprehending the mutation rate is key to understand the genetic structure of populations over time (Ness et al. 2012; Long et al. 2013). In spite of its importance, information about the rate of spontaneous mutations is scarce because its empirical determination is extremely hard (Lynch et al. 2008; Saxer et al. 2012).

2.1.2 Review of mutation rate determination methods

Direct calculation of the de novo mutation rates is highly challenging due to the rarity of the event (e.g. the very low rate of spontaneous mutagenesis) and because deleterious mutations are often purged by selection in natural populations (Lynch 2010; Ness et al. 2012).

Traditionally, most estimates have been indirect phenotypic screens. Phenotypic screens aim to estimate mutation rates by studying large populations of organisms and counting the number of offspring that had observable mutant phenotypes in each generation. In most cases, these observable mutant phenotypes were due to loss-of-function mutations, i.e. changes in DNA that inactivate a specific gene leading to a complete lack of the encoded protein (Drake et al. 1998). Lambert and Alexander (1968) estimated the spontaneous mutation rate of the dominant allele at the opaque-2 locus in four homozygous inbred corn lines. A frequency of about one mutation in every 300,000 female gametes was found. More recently, gene-specific estimates were performed by using plant transformations at 'reporter genes'. Kovalchuk et al. (2000) modified *Arabidopsis thaliana* plants by incorporating stop codons at different positions of the b-glucuronid-ase (uidA) gene to prevent its translation into the active protein. The reversion of the modified stop codons back to the original codons, via spontaneous mutations, was detected by quantifying the restoration of uidA activity in following generations. Similarly, Filkowski et al. (2003), using a reporter transgenic gene, estimated the spontaneous mutation rate per nucleotide in *Nicotiana tabacum* and *Arabidopsis thaliana*. Moreover, the rate of spontaneous mutation may be estimated by measuring mutagen-induced mutation rates, by using mutagens to which the relation between the spontaneous and mutagen-induced mutation rates is known (Kondrashov and Kondrashov 2010). Also accomplished by measuring observable traits, the rate of deleterious mutations is commonly calculated by evaluating the decline in fitness-related traits in mutation accumulation (MA) lines (explained below). Several experiments of this type have been performed *in Arabidopsis thaliana* (Schultz et al.

1999; Shaw et al. 2000; Shaw et al. 2002; Rutter et al. 2010). Schoen (2005) compared the deleterious mutation accumulation between two species with distinctive mating systems of the *Amsinckia* genus. Bobiwash et al. (2013) discovered early-acting inbreeding depression manifested as a high rate of fruit abortion, by measuring the rate of deleterious somatic mutation in lowbush blueberry (*Vaccinium angustifolium*). Although, gene-specific approaches offer experimentally clever and straightforward methods for estimating mutation rates, they also have some important deficiencies. Collecting data on observable phenotypes results in an underestimate of the actual mutation rate because mutations leading to less detectable changes than a complete gain or loss-of-function, including silent site mutations, are likely to be missed using these approaches. Additionally, because the mutation rate varies across the genome, estimations based on specific-loci might lead to erroneous conclusions if extrapolated to genome-wide estimates or to different loci.

Alternatively, more reliable, though also indirect, estimation methods of the mutation rate rely on measuring the level of divergence of DNA neutral sites between species. DNA sites that are assumed to be neutral (or very nearly) in terms of fitness are non-coding sites (intergenic regions) and synonymous positions in a protein-coding gene at which a nucleotide substitution has no influence on the protein sequence ('silent sites') (Bromham et al. 2015). Because natural selection is not likely to affect them, these are thought to evolve at the mutation rate, or at least, be largely determined by mutation (Lynch 2010; Bromham et al. 2015). Therefore, the mutation rate can be estimated from the measurement of molecular divergence at neutral sites between species (Keightley et al. 2009; Kucukyildirim et al. 2016). In plants, DeRose-Wilson and

Gaut (2007) calculated genetic divergence within and between *Arabidiopsis thaliana* and *Arabidopsis lyrata* based on intergenic and synonymous coding-region sites. Neutral-sites approaches, however, require several assumptions that are difficult to validate, including neutrality (Keightley et al. 2009; Kondrashov et al. 2010). For example, DNA polymerase is able to more efficiently transcribe certain codons than others (codon bias), so even silent sites can be subject to selection (Freeman and Herron 2007).

The drawbacks to the methods described above have led to efforts to directly estimate the mutation rate by sequencing the complete genomes of mutation accumulation (MA) lines of parents and their offspring (Keightley et al. 2014). Mutation accumulation (MA) experiments are a direct way to study mutational variation (Keightley et al. 2009) and have been successfully performed in many different organisms including complex eukaryotes. Mutation accumulation lines are lines derived from a common founder that are maintained for multiple generations. After an adequate number of generations, the complete genomes of each evolved lineage are sequenced and compared with the founder to identify de novo mutations, thus revealing the per-generation mutation rate occurring over the course of the experiment (Dillon et al. 2015; Lynch et al. 2016). By bottlenecking experimental populations, this method minimizes the effectiveness of natural selection (i.e. makes transmission to the next generation random with respect to fitness) allowing nearly all mutations to accumulate with the exception of the most strongly deleterious that cause complete sterility or lethality (Lynch et al. 2008; Kraemer et al. 2016). In the past, this approach was used to calculate the deleterious mutation rate by direct measurement of fitness-related traits

(as explained above), or to calculate the mutation rate by directly sequencing specific loci or genomic regions. Recently, however, with the emergence of new high-throughput sequencing technologies, it is possible to perform genome-wide direct estimates of the mutation rate (Keightley et al. 2009; Ness et al. 2012; Schrider et al. 2013). This approach provides a molecular spectrum of spontaneous mutations which do not suffer from the biases affecting indirect estimations of the mutation rate. The first experiment using this approach was published by Lynch et al. (2008), reporting the spontaneous mutation rate of the yeast *Saccharomyces cerevisiae*, and since then similar studies in many other species have been published. In plants, Ossowski et al. (2010) report the mutation rate of *Arabidopsis thaliana* to be on the order of 6.5×10^{-9} per site per genome per generation. Later, Jian et al. (2014) confirmed these numbers. Several published studies on the unicellular green algae *Chlamydomonas reinhardtii* revealed a mutation rate at least an order of magnitude lower than in *Arabidopsis thaliana* (Sung et al. 2012; Ness et al. 2012; Kraemer et al. 2016). Although MA experiments provide a more accurate estimate of the mutation rate versus indirect methods, they also suffer from potential difficulties. For example, a recessive mutator allele might become fixed by inbreeding in the MA line generating an over-estimation of the actual mutation rate in natural populations.

Furthermore, the applicability of MA lines experiments is limited because inbred lines cannot be produced for most species (Keightley et al. 2015). In this sense, an alternative way to directly estimate the mutation rate is by whole-genome sequencing of parents and their offspring. Because mutational events can be detected in a few generations and in a well-defined framework (pedigree), this is believed to be the most

straight-forward approach for mutation rate calculation (Baer et al. 2007; Kraemer et al. 2016). For this reason, this is the most popular approach to measure the mutation rate in hominids including humans (Roach et al. 2010; Kong et al. 2012; Venn et al. 2014). This has also been also used in *Drosophila melanogaster* and in the tropical butterfly *Heliconius melpomene* (Keightley et al. 2014; Keightley et al. 2015). In the plant kingdom, the parent-offspring approach has been performed in *Arabidopsis thaliana, Oryza sativa,* and *Prunus persica* (Yang et al. 2015; Xie et al. 2016).

2.1.3 Mutation rate variation in nature

Direct measurements of the mutation rate revealed that mutations per site per generation (u) ranges from 10^{-11} to 10^{-8} across the tree of life (see Appendix, Table A.1). This finding confirmed mutations introduce a great deal of genetic variation into populations in every generation. In addition, mutation rates vary between species, within species, within an organism, and across the genome (Baer et al. 2007; Lynch et al. 2016).

Between-species mutational variation is significant and correlates with species-specific characteristics. First, there is a strong relationship between the mutation rate per nucleotide site per generation (u) and total genome size (Lynch 2010). As proposed by Drake (1991), u varies inversely with genome size (G) in microbes (Drake's rule), implying that the mutation rate per genome per generation (uG) is mostly constant across all microbial life. Inversely, when analyzing only eukaryotes, mutation rates scale positively with genome size (Lynch 2010; Sung et al. 2012; Sung et al. 2016). Differences in mutation rates between dicots were attributed to differences in genome sizes as a function of their DNA repair machinery efficiency. Studies performed in

Arabidopsis thaliana showed that, for a specific gene, it has a lower mutation rate than *Nicotiana tabacum*, for which the genome is 20 times larger than *Arabidopsis* (Kirik et al. 2000). Contrastingly, Bromham et al. (2015), by analyzing sequences from 130 families of angiosperms corresponding to a specific nuclear gene, found that greater family-average genome size is associated with lower mutation rate. Second, as spontaneous mutation is mostly caused by DNA polymerase errors, the accumulation of mutations per generation directly correlates with the generation time, with a higher accumulation of mutations occurring in species that have shorter lengths of time between generations as a consequence of more cell-divisions per time unit (Baer et al. 2007; Jiang et al. 2014).

With few exceptions, relatively little work has been done to directly estimate the spontaneous mutation rate in plants. As stated earlier, Ossowski et al. (2010) estimate the mutation rate across the genome of *Arabidopsis thaliana* to be in the order of 6.5 x 10^{-9}. In another MA study, Jiang et al. (2014) estimated a similar rate (5.2 x 10^{-9}) for *Arabidopsis*. Performing parent-offspring analysis, Yang et al. (2015) calculated a mutation rate of 7.4 x 10^{-9} and 3.2 x 10^{-9} for *Arabidopsis thaliana* and *Oryza sativa*, respectively. The unique experiment conducted in perennials was published by Xie et al. (2016) revealing a mutation rate of 7.7 x 10^{-9} in peach (*Prunus persica*). These numbers place the mutation rate per site per generation of vascular plants (10^{-9}) in the middle of the eukaryotes' range. Additionally, there are some MA studies conducted in *Chlamydomonas reinhardtii*, a single-celled chlorophyte that has been extensively used as a model organism in plant physiology, which has a genome as large as *Arabidopsis thaliana* (~120 Mb). Different direct estimations place its mutation rate between 10^{-11}

and 10^{-10}, similar to other unicellular organisms such as many bacteria and yeast species (Ness et al. 2012; Sung et al. 2012; Kraemer et al. 2016). However, to make a fair comparison between unicellular and multicellular organisms, we must divide the per-generation rate by the number of reproductive cell divisions per generation; in that case *Arabidopsis thaliana* would also have a rate in the order of 10^{-10} assuming 30-40 cell divisions per generation. The strikingly similarity of these reported rates suggests that the mutation rate is robust across the plant kingdom and is potentially tightly constrained by natural selection (Ness et al. 2012).

In addition, there is strong evidence indicating that mutation rates may vary substantially among individuals within species (Schrider et al. 2013). Within-species mutational variations has been attributed to: (1) genetic differences among individuals, (2) environmental differences experienced by the individuals, and (3) evolution of the mutation rate itself (Kondrashov et al. 2010). Interestingly, Yang et al. (2015) found a greater than 3-fold higher mutation rate in hybrids (F1) than in homozygous individuals of *Arabidopsis thaliana* and *Oryza sativa*. Another group (Jiang et al. 2014) demonstrated that stress accelerates the accumulation of mutations and epimutations in *A. thaliana* lineages. Many other stress-induced changes in the mutation rate are detailed in Chapter 3.

Within an individual, mutation rate also varies from one cell to another. In animals, somatic mutation rates are notably higher than in germline cells (Kovalchuk et al. 2000; Lynch 2010). This has been demonstrated across a wide range of species. In humans, the mutation rate in different somatic tissues is on average 17-fold higher than germline rates (Lynch 2010). Furthermore, there is ample evidence of mutation variation

between nuclear and organellar genomes. In many well-studied organisms, including *D. melanogaster*, *C. elegans*, and *S. cerevisiae*, mitochondrial substitution rates are much higher than those in nuclear DNA (Denver et al. 2000; Lynch et al. 2008; Haag-Liautard et al. 2008). Mitochondria lack some of the DNA-repair enzymes found in the nucleus, thus more mutational events would occur in mitochondrial DNA as a result of fewer errors being repaired (Freeman and Herron 2007). In plants, however, the few studies performed are erratic and non-conclusive around this matter. For example, Bromham et al. (2015) identified significant pairwise correlations between substitution rates across nuclear, mitochondrial, and chloroplast genomes.

Moreover, the mutation rate varies across the genome (Kraemer et al. 2016), most notably with a difference in the spontaneous mutation rate in coding regions compared to non-coding regions. In the previously cited MA experiment in *Arabidopsis thaliana* conducted by Ossowski et al (2010), intergenic mutations were found more frequently than mutations in the genic region. This was attributed to a higher mutation rate in pericentromeric regions of chromosomes where gene density is lower. In addition, a study based on the divergence of orthologous regions between *Arabidiopsis thaliana* and *A. lyrata* estimated a higher rate in non-coding regions than in synonymous coding sites (DeRose-Wilson and Gaut 2007). Previously, Kovalchuk et al. (2000) suggested that the mutation frequency calculated by transgene reversions may be a function of the chromosomal position of the transgene because that defines accessibility for damage and accessibility for repair. If the mutation rate depends on the genome position, it can be inferred than the mutation rate also varies between genes. Also, Lynch et al. (2016) suggests that a gene-specific mutation rate may be related with the

transcriptional activity, so highly expressed genes might have a higher rate than less expressed ones.

At the scale of individual sites, transitions are more common than transversions and G:C positions tend to mutate at higher rates than A:T positions, thus transitions from G:C to A:T are the most common point mutation in nature (Ness et al. 2012; Kong et al. 2012; Schrider et al. 2013). Particularly, in *Arabidopsis thaliana*, Ossowski et al. (2010) found that transitions were 2.4 times more frequent than transversions, and G:C → A:T transitions were by far the most frequent type of mutations. As many G:C sites are known to be methylated, the high rate of transitions at G:C sites is partially explained by the spontaneous deamination of methylated cytosines, which leads to thymine substitution (DeRose-Wilson and Gaut 2007; Ossowski et al. 2010). However, transitions at G:C sites are also found to be overabundant at un-methylated sites in *Arabidopsis thaliana*, thus factors in addition to methylation may contribute to this bias. One of these factors include the bases flanking a particular site; regions that are referred to as the ‘’sequence context’’ and are known to be a good predictor of mutation rate (Kraemer et al. 2016). A common explanation for this phenomenon is that the bases flanking a particular nucleotide are thought to play a role in its susceptibility to DNA-damaging agents. In nature, mutations are shown frequently to cluster around pyrimidine dimer sites creating hotspots for mutation (Kovalchuk et al. 2000). Indeed, the vast majority of mutations caused by ultraviolet (UV) light, in both prokaryotes and eukaryotes, are G:C→A:T transitions at sites where the C is adjacent to another C or T (dipyrimidine sites) (Ossowski et al. 2010). The increased rate of transitions at G:C sites in plants, therefore, can be also explained in part by the effect of UV-induced

mutagenesis. More recently, Kiselev et al. (2018) reported an increase of G:C→A:T transitions after induced mutagenesis by UV-C treatment in *A. thaliana*, reinforcing this theory. Accordingly, data available on the unicellular algae *Chlamydomonas reinhardtii* mimic the trends found in vascular plants: mutations occurring at C:G sites were more frequent than mutations at A:T sites, C:G→T:A transitions are over-represented, and the surrounding GC content strongly influenced the mutability at a site (Ness et al. 2012; Kraemer et al. 2016). The strong correlation between G:C content and SNP polymorphisms suggests that GC content is a major determinant of evolutionary rate variation across different genome regions (DeRose-Wilson and Gaut 2007).

2.1.4 Mutations conferring resistance to ALS-herbicides

Acetolactate synthase (ALS), also referred as acetohydroxyacid synthase (AHAS), is the first enzyme in the branched-chain amino acids biosynthesis pathway and catalyzes the formation of both aceto-hydroxybutyrate and acetolactate, ultimately leading to the production of leucine, isoleucine, and valine (Tranel and Wright 2002; Powles and Yu 2010). The ALS enzyme has a catalytic subunit and a regulatory subunit that governs the activity of the former. The substrate-binding catalytic site is situated deep within a channel on the catalytic subunit. ALS-inhibiting herbicides do not bind to the catalytic site, but bind next to the entry of the channel, blocking substrate access to the catalytic subunit (Duggleby et al. 2008). This stops synthesis of branched-chain amino acids, starving the plant and leading to plant death. Secondarily, ALS inhibition may lead to the accumulation of 2-ketobutyrate and the disruption of the photo-synthetized products transported in the plant (Tranel and Wright 2002).

When ALS-inhibiting herbicides were first developed, they signified a great advance for agriculture. While other herbicides were typically applied at kilograms per hectare, ALS-inhibiting herbicides were applied at grams per hectare. In addition, ALS inhibitors control a broad-spectrum of weeds, present selectivity for major world crops, have application-time plasticity (PRE and POST-emergence), soil residual activity, and low mammalian toxicity (Tranel and Wright 2002). These superb qualities made ALS inhibitors one of the most popular classes of herbicides around the world, being used in many crops over huge areas for more than 30 years. From 1982, when the first ALS-inhibiting herbicide (chlorsulfuron) was brought to the market, 54 ALS-inhibitors were registered globally, more than in any other SoA group of herbicides (Heap 2014). This long list of compounds are composed of five chemical families: sulfonylureas (SUs), imidazolinones (IMIs), triazolopyrimidines (TPs), pyrimidinyl-thio(or oxy)benzoates (PTBs), and sulfonyl-amino-carbonyl-triazolinones (SCTs).

In spite of their initial success, the use of ALS-inhibiting herbicides rapidly led to the evolution of resistance. Impressively, the first discovered case of resistance dates just five years after chlorsulfuron was released, with many reports following this initial case (Mallory-Smith et al. 1990). To date, there are 171 reported cases of ALS-inhibitor resistance, making it by far the group of herbicides with the most cases reported, followed by photosystem II-inhibitors, and ACCase-inhibitors. More importantly, in many troublesome weed species, ALS-inhibitor resistance is not confined to a few populations; rather, it is widespread across huge regions, reducing the usefulness of this group of herbicides. That is the case for waterhemp (*Amaranthus rudis* Sauer) in the Midwest of the United States (Patzoldt and Tranel 2007).

Resistance to ALS inhibitors may occur as a result of reduced sensitivity to the herbicide at the site of action (target site resistance), or by an increased detoxifying metabolism in the plant (non-target site resistance). Among these two, lack of sensitivity in the target site is the main cause of ALS-resistance events, with multiple mutations conferring resistance particularly likely to evolve in the ALS codifying gene (Powles and Yu 2010). Until now, 29 different amino acid substitutions have been reported in plants, distributed across eight amino acid sites in the ALS protein (Table 2.1).

Among the eight amino acid sites, Ala122, Pro197, and Ala205 are situated near the amino-terminal end of the ALS protein, Asp376 and Arg377 are in the center, and Trp574, Ser653, and Gly654 are located near the carboxy-terminal end (Figure 2.1). The residue Pro197 presents the highest number of known amino acid substitutions providing resistance (11), with Pro197Ser substitution reported the most (Powles and Yu 2010). Substitutions of Pro197, however, usually presents strong resistance to sulfonylureas only (Guttieri et al. 1995; Yu et al. 2008, Yu and Powles 2014). Similarly, many substitutions at Ala122 and Ser653 sites provide resistance to imidazolinones (Trucco et al. 2006; Patzoldt and Tranel 2007; Riar et al. 2013). In this sense, some amino acid substitutions may cause resistance to one chemical family of ALS-inhibiting herbicides, but not to others. Moreover, a mutation providing resistance to one herbicide may not confer resistance to other compounds of the same chemical family (Tranel and Wright 2002). That is because ALS-inhibiting herbicides bind to different positions across the binding domain, depending on the herbicide's chemical structure. Nevertheless, mutations often provide cross-resistance to more than one ALS-inhibitor-chemical family as a consequence of the overlapping orientation of different herbicides

around the binding site (Yu and Powles 2014). Particularly, Trp574Leu mutation is known to provide a broad-spectrum of resistance across different ALS-inhibitor chemical families (Kaloumenos et al. 2013; Panozzo et al. 2013; Pandolfo et. al 2016). Beyond these considerations, it should be pointed out that ALS-inhibitor resistant weed populations often present more than one resistance mutation (Yu et al. 2008).

The very frequent occurrence of resistance mutations in the ALS gene has been a leading factor in ALS-resistance evolution. The rapid resistance evolution to ALS-inhibiting herbicides may be partially explained by multiple factors—high lethality of sensitive biotypes, repeated use/no rotation, infrequent inclusion in mixes, and soil residual activity—that imposed a high selection pressure for resistance alleles. However, these factors do not completely explain why ALS-inhibitor resistance developed faster than resistance to other herbicide groups. In essence, the reasons for the more rapid evolution of ALS-inhibitor resistance versus that of other groups of herbicides lie within its genetics. Nucleotide substitutions providing ALS-inhibitor resistance are dominant over sensitive alleles, thus herbicides select for heterozygous as well as homozygous resistant plants. Also, even though the ALS protein functions in plastids, the ALS-encoding gene is in the nuclear genome. Therefore, it is not disseminated only by seeds, but via pollen as well. Comparatively, in photosystem II-inhibitors, in which resistance evolved slower, resistant alleles are solely inherited by seeds because the encoding gene is located in the chloroplast which is maternally inherited in plants (Tranel and Wright 2002).

Nonetheless, the most remarkable factor responsible for the high incidence of ALS-resistant mutations is believed to be the high tolerance for amino acid substitutions

in the herbicide-binding site of the ALS protein. Mutations at this site do not produce deleterious consequences for its enzymatic activity. This is best explained by the fact that the ALS binding site is separated from the catalytic site of the ALS enzyme, thus mutations at the binding site can prevent herbicide binding without affecting enzyme functionality (Duggleby et al. 2008; Powles and Yu 2010). For further evidence of this, several studies tried to determine if resistance mutations in the ALS gene are associated with a fitness cost in the absence of herbicide selection. As expected, some mutations presented none to insignificant cost on fitness, including substitution Pro197Ser, which helps to explain why this mutation is so frequent in nature (Vila-Aiub et al. 2009). In respect to very frequent mutation Trp574Leu, different studies showed contrasting fitness penalties associated with it, depending on the weed species analyzed (Tardif et al. 2006; Yu et al. 2010; Wu et al. 2018). Low-fitness cost means that after they have been herbicide-selected, resistance mutants will remain in the population regardless of the selective-agent presence.

Importantly, if ALS-resistance mutations have a fitness cost, it is crucial to understand what the frequency of resistant alleles was prior to selection in the population. The probability of a given weed population to evolve ALS resistance depends on the frequency of ALS-resistance alleles in the standing genetic variation of the population. That initial genetic diversity constrains the outcome of herbicide selection leading to resistance evolution (Jasieniuk et al. 1996). It is already known that although highly conserved, the ALS gene presents different levels of variability within specific weed populations (Tranel et al. 2004). Moreover, the selection for different resistance-alleles in close populations suggests that they existed at a high frequency in

unselected populations (Trucco et al. 2006). In this sense, as genetic variation is ultimately generated by spontaneous mutations, an elevated mutation rate in the ALS gene might play an important role in the high incidence of ALS-resistant mutants. However, the mutation rate leading to mutations conferring ALS-resistance is not known.

2.1.5 The origin of alleles conferring herbicide resistance

Herbicides act as selective agents that, over time, increase the frequency of initially rare resistance alleles (Heap 2014). As in other evolutionary processes, the capacity of a population to evolve in response to a novel environment requires previous genetic variation (Trucco et al. 2006; Mimura et al. 2013). Consequently, it is not surprising that weed populations overcoming changes in agricultural ecosystems often have a correlative abundance of genetic polymorphisms (Jasieniuk and Maxwell 2001).

The origin of these adaptive alleles is a cause of debate in evolutionary theory. As it is available immediately, the standing genetic variation within the population is thought to be the primary source of resistance alleles, and so the main factor upon which adaptation depends (Mimura et al. 2013; Délye et al. 2013a). In addition, a sensitive weed population could be contaminated by gene flow, via pollen or seeds from a resistant population (i.e. immigration). Furthermore, as any natural plant population is constantly loaded with mutant genotypes via *de novo* mutations, herbicide-resistant mutants may arise spontaneously in a given sensitive population (Jasieniuk et al. 1996; Délye et al. 2013a).

The relative importance of each of these three sources deeply affect what strategies should be applied to counteract herbicide resistance evolution. If the standing

genetic variation was the main source of alleles conferring resistance in a weed population, these alleles have been conserved in the population before herbicide selection, so their fitness cost must be insignificant. Thus, they are expected to remain in the population in the absence of selection. Alternatively, if resistance evolves by new mutations, as most mutations are expected to be deleterious, natural selection should purge out most of the new resistance alleles in the absence of herbicide selection. Moreover, when adaptive alleles are derived from the standing genetic variation, adaptive evolution is determined by the amount of variation before selection. Therefore, the evolutionary outcome of the current selection process would also depend on how previous selection events constrained genetic variants in the population. In this case, the application of only one herbicide, by bottlenecking genetic diversity, will reduce the probability of the target weed population evolving resistance to another herbicide if applied. Contrastingly, if alleles providing resistance are mainly a product of new mutations, selection by one herbicide will not reduce the likelihood for herbicide-resistance to other subsequent herbicides because new genetic variants will arise after the onset of selection, reducing its bottlenecking power.

As an aside, it should be mentioned that there are many cases where a single gene provides cross-resistance to more than one herbicide of different site of action (Délye et al. 2013a). That is often the case when the resistance is not target-site-based but instead resulted from a decrease in herbicide translocation inside the plant, or the enhancement of one of the plant herbicide detoxification mechanisms, including glutathione S-transferases (GSTs), cytochrome P-450s (P450s), and UDP-dependent glucosyl-transferases (UGTs) (Powles and Yu 2010; Yuan et al. 2006; Huffman et al.

2015). Additionally, herbicide-target genes may be linked and segregate together (Tranel et al. 2017). In that case, selection for one resistance trait will also select for the linked trait. Moreover, it has been proposed that herbicide applications may favor herbicide-resistance evolution by selecting for mutator genes that increase the mutation frequency leading to increased resistance in the population (Gressel 2011).

The interaction between factors leading to resistance evolution is commonly simulated by models with the purpose of providing insights to herbicide-resistance management (Renton et al. 2014). However, because estimations of parameters such as the standing genetic variation or the mutation rate conferring resistance are extremely difficult to calculate empirically, they are often estimated based mostly on assumptions, which limits the reliability of the models. For example, Jasieniuk et al. (1996) presentated the relationship of the initial frequency of resistant individuals in an unselected population to the mutation rate as following:

$$qe = u/hs$$

(qe: the equilibrium frequency of a resistance allele, u: is the mutation rate, h: is the degree of dominance, and s: is the fitness penalty of the resistance allele in the absence of selection).

However, empirical determinations of these parameters are scarce. Preston and Powles (2002) measured the frequency of ALS-inhibitor resistance in unselected populations of rigid ryegrass (*Lolium rigidum*) in Australia to be in the order of 10^{-4}-10^{-5}. Délye et al. (2013b) calculated the frequency of one mutation providing resistance to ACCase-inhibitors in unselected French populations of the grass weed *Alopecurus*

myosuroides to be in the order of 10^{-4} by DNA analysis of herbarium specimens collected previous to the use of herbicides.

To our knowledge, there is no peer-reviewed published data on the empirical determination of the spontaneous mutation rate providing herbicide-resistance in plants. Stannard and Fay (1987), by screening 20 million individuals of alfalfa (Latin binomial), selected 15 individuals resistant to chlorsulfuron. This brings a hypothetical resistance mutation rate of 7.5×10^{-7}, assuming a sensitive genetic background of the parents and no contamination during the experiment; however, neither the cause of resistance was characterized nor the origin of plant material well-detailed. It has been speculated that an elevated mutation rate in weed populations, possibly mediated by mutators, may be one of the causes of their rapid evolution to herbicide resistance as well as to their high adaptiveness in general (Gressel 2011).

2.2 Objective

Taking into account the necessity of adding more evolutionary biology research in the design of herbicide-resistance-mitigation strategies, this project aimed to generate empirical data on the relative contribution that each source of resistant alleles adds to the genetic background of weed populations. In this sense, the purpose of this experiment was to calculate the *de novo* mutation rate conferring herbicide resistance to ALS-inhibitors in a plant population.

To achieve this goal, we performed a method to discover spontaneous herbicide-resistant mutants by screening millions of plants. We used grain amaranth and resistance to ALS-inhibiting herbicides as a model system. Unraveling how resistance arises spontaneously in an amaranth population holds special interest because two of

the most troublesome weeds in Illinois are amaranths: waterhemp (*Amaranthus rudis* Sauer) and Palmer amaranth (*Amaranthus palmeri* S Wats.). More importantly, determining the mutation rate leading to ALS-inhibitor resistance is crucial, not only to unravel the causes of the high occurrence of ALS-inhibitor-resistant mutants in particular, but to increase the general understanding of the origin of genetic variants leading to herbicide-resistance evolution.

2.3 Materials and methods

2.3.1 Experiment system

We used grain amaranth cv. 'Plainsman' (*Amaranthus hypochondriacus* L. *x A. hybridus* L.) and resistance to ALS-inhibiting herbicides as a model system (Baltensperger et al. 1992). As in other *Amaranthus* species, grain amaranth produces millions of seeds per mother plant, but has limited seed dormancy and higher genetic homogeneity than its weedy counterparts. More importantly, grain amaranth is not known to have herbicide resistance in its genetic background, which decreases the probability of contamination from the field. In contrast, ALS-inhibitor resistance is widespread within the weedy amaranth species. Additionally, grain amaranth also produces pale seeds, which can be used as a contamination checkpoint to make sure no black-seeded amaranth weed seeds are present. ALS-inhibitor-resistant mutations are functionally dominant, enabling the selection of newly-arisen spontaneous mutations, which are expected to be present only in one chromosome during the first generation (heterozygous mode). Furthermore, ALS mutations provide a high-level resistance, thus high rates can be used to strongly select resistant mutants from a sensitive population, diminishing the occurrence of false positives. In addition, the

extended soil activity of ALS inhibitors permits the selection of plants at the seedling stage, allowing for the screening of millions of individuals in a reduced space.

The experiment consisted of producing millions of seeds, planting them at a very high density, screening them at the seedling stage with a pre-emergence herbicide, and looking for surviving resistant individuals (Figure 2.2). Because producing enough seed required several independent batches, the cycle was repeated five times.

2.3.2 Plant material

The plant population used in this study corresponds to grain amaranth cv. 'Plainsman' (*Amaranthus hypochondriacus* L. *x A. hybridus* L.) obtained from the North Central Regional Plant Introduction Station (Ames, IA). All plant batches were initiated from sibling seeds of the same generation, all of which originated from a unique self-pollinated plant. That plant was grown during the summer of 2014 in an isolated greenhouse room to avoid contamination with ALS-resistant *Amaranthus* weeds.

2.3.3 Seed production

Seeds were produced in independent batches of around 200 grain amaranth plants. Five batches were produced in total from the spring of 2015 to the spring of 2017 (Table 2.2). To initiate a batch, seeds were planted in 12.3 x 12.3 cm inserts filled with a medium (WeedMix:LC1) that is 3:1:1:1 (LC1: soil: peat: torpedo sand). Flats were sub-irrigated overnight prior to sowing. Sowing density was 100 seeds per insert. Inserts were placed into 27.4 x 53.9 cm flat with holes that were then placed into a display flat for bottom watering. Germination rate of the seeds was between 90-95%. Plants were allowed to grow in the 12.3 x 12.3 cm inserts until each plant had its first true leaf longer

than a quarter of an inch. Then, seedlings were transplanted into 10.1 cm diameter pots filled with WeedMix:LC1 and pre-watered prior to transplanting. Between 400 and 500 seedlings were germinated each time to select a uniform group of around 200 plants for each batch. When plants reached 12-15 cm in height, they were transplanted into 9.4 L pots filled with a medium that is 1:1:1 (soil: peat: perlite) plus 30 grams of 13-13-13 Osmocote® (ICL Specialty Fertilizers, Dublin, OH) and pre-watered prior to transplanting. The pots were placed on benches laying directly on the floor. Plant density varied between 4 and 10 plants/m^2 depending on the batch. The greenhouse room was maintained at 28/22 C day/night. Natural sunlight was supplemented with General Electric 208 volt fixtures with metal halide lamps to provide a 16:8 h photoperiod during the entire plant cycle. Plants were watered twice daily by an automatic irrigation system that deliver water directly via an independent emitter located in each pot. Liquid fertilization was provided once a week with a starting dose of 300 ppm (20-20-20) increasing to 400 ppm after flowering had started. Flowering started approximately 45 days after planting in all batches. Plants were harvested at least 10 days after it was noted that seeds were able to be detached from the plants without pushing them. Inflorescences were cut manually and stored in paper bags for a month to allow drying. Dry plants were cleaned manually to obtain seed. The seed from each parental plant was weighed and stored in separate bottles. Seed bottles were stored in a cold room at 4°C.

2.3.4 Contamination control

Each parental plant was tested for the presence of both waterhemp DNA and the most common mutation providing ALS resistance in weedy amaranths, Trp574Leu.

Waterhemp DNA can be detected by the presence of a restriction site recognizable by EcoRV endonuclease in the ALS gene region A that is absent in grain amaranth. Similarly, the Trp574Leu mutation is detected by MfeI endonuclease in the ALS gene region B (Foes et al. 1998). DNA was extracted from young leaf samples according to a modified version of the CTAB procedure indicated by Doyle and Doyle (1990). DNA content of each sample was measured using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and diluted to 10 ng/ul. Polymerase chain reaction (e.g. PCR) consisted of 12.3 ul H_2O, 5 ul 5X GoTaq® green buffer (Promega corporation, Madison, WI), 2 ul dNTP (2.5 mM), 2.5 $MgCl_2$ (25 mM), 1 ul (0.4 uM) of each primer, 0.2 ul GoTaq® DNA polymerase (5 U/uL; Promega corporation, Madison, WI), and 1 ul (10 ng/ul) sample DNA in a total reaction of 25 ul. Primers used for the amplification of ALS region A were as follows: alsf1, 5'-AGCTCTTGAACGTGAAGGTG; alsr1, 5'-TCAATCAAAAGAGGTCCAGG, and for the ALS region B amplification: alsf2, 5' TCCCGGTTAAAATCATGCTC; alsr2, 5'-CTAAACGAGAGAACGGCCAG, as described by Foes et al. 1998. The thermo-cycling program began with 3 min at 95°C; then 35 cycles of 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C; finalizing by 5 min at 72°C. DNA amplification was checked with a 1% agarose gel. A volume of 10 ul product of each PCR reaction was subjected to its respective digestion: 16.5 ul H_2O, 3 ul CutSmart™ buffer (10x; New England Biolabs, Ipswich, MA), and 0.5 ul ECoRV (20 U/ul; New England Biolabs); 2.2 ul H_2O, 2.5 ul CutSmart™ buffer (10x), and 0.3 ul MfeI (20 U/ul; New England Biolabs), and incubated overnight at 37°C. Digested products were run on a 2% agarose gel. For both digestions, negative samples produced a band at 500 bp, while positive samples produced a band at 400 bp.

Because hybridization between grain amaranth and weedy amaranths is common, batches were grown in a greenhouse room to minimize plants contact with the outside environment's air. Neighboring rooms did not have weedy amaranth populations growing inside. By managing the planting date, the growing season was set to avoid flowering during times of the year when waterhemp pollen is most prevalent in the air in central Illinois (from June to September).

2.3.5 Seed screening

The seed from each parental plant was planted separately in 12.3 x 12.3 cm inserts filled with the same medium described above and covered with a layer of 2 mm of the same growth medium. The sowing density had a maximum of 23,000 seeds per insert, in order to respect the upper limit of 160 seeds/cm^2 previously determined as innocuous to germination rate. Heterozygous waterhemp plants with the Trp574Leu mutation were included in each screening session to check resistant survivorship. Inserts were placed into 27.4 x 53.9 cm flat with holes that were then placed on to a display flat for bottom watering. To moisten the growth medium prior to sowing, flats were sub-irrigated overnight. Right after sowing, flats received an application of imazethapyr (Pursuit®, 240 g a.i. /L; BASF, Florham Park, NJ) at a 10x rate (2.9 L/ha) which was previously determined to effectively discriminate between heterozygous resistant mutants (carrying the Trp574Leu substitution) and sensitive grain amaranth plants. Herbicide was applied using a research spray chamber (De Vries Manufacturing, Hollandale, MN) calibrated to deliver 185 L/ha at 276 kPa. The flats were located 60 cm below the nozzle (80015 EVS; Teejet Technologies, Wheaton, IL). After spraying, 1 mm of water was sprayed over the flats to incorporate the herbicide. Then flats were

watered from the top daily. Seeds started to germinate on the same day. Most seedlings emerged from the second to the seventh day after treatment (DAT). Sensitive seedlings stopped their growth about 10 DAT. At 15 DAT, surviving plants were evident and bigger than the rest and were transplanted to 10.1 cm diameter pots filed with WeedMix:LC1.

2.3.6 Germination rate

Germination rate was calculated in separated 7.7 x 5.5 cm inserts filled with WeedMix:LC1 to calculate the germination rate of the seed bulk. From each parental plant, 160 seeds were sowed in a cm^2 of soil to simulate the real density. This density was maintained by sowing the seeds with a tube that had a constant measured diameter. These flats were not treated with herbicide, but allowed to grow normally. After germination, seedlings were counted manually.

2.3.7 Resistance confirmation

Once a surviving seedling was detected, it was firstly examined visually to detect obvious waterhemp traits. Any waterhemp-DNA contamination was then checked by PCR as described above. Similarly, Palmer amaranth contamination was checked by PCR that consisted of the mix described above with the following primers: fwd, 5′-GCGAACATGTTTATCATACCTGG-3′; rev, 5′ -CTCAATACTGGGTGCATCCAC-3′ (Murphy et al. 2017). Thermo-cycling started with 5 min at 95°C; then 27 cycles of 1.5 min at 95°C, 1 min at 59°C, and 2 min at 72°C; finalizing by 5 min at 72°C. DNA amplification was checked on a 1% agarose gel for direct confirmation of any Palmer amaranth DNA presence. When a plant was verified to be none of these weed species,

a post application of imazethapyr at 1x rate (0.29 L/ha) was sprayed when the plant was 10 cm tall. If the plant survived, it was allowed to produce seed. DNA was extracted in order to sequence the ALS gene to look for the presence of mutations. Sanger sequencing was performed by the UIUC Core Sequencing facility (334 ERML, 1201 W. Gregory, Urbana, IL). When the plants finished their cycle, the seed was cleaned manually and stored in the cold room at 4°C. At least 10 offspring from each resistant plant was planted independently and grown up. When offspring plants were 10 cm tall, imazethapyr at 1x was applied in order to check for resistance inheritance.

2.4 Results and Discussion

The five batches of grain amaranth produced more than 87,000,000 seeds in total (Table 2.3). Seed production between batches was mostly homogeneous with the exception of one batch that had more than triplicate the yield of the others. The seed produced per parental plant was highly variable, ranging from less than 1,000 to more than 300,000 seeds with an average of 77,000 seeds per plant. The average seed weight remained mostly constant across batches and plants with a mean of 0.06 grams per 100 seeds.

Based on each batch's germination rate, we determined that at least 70,000,000 plants were screened (Table 2.4). Our screening procedure was demonstrated to be sufficiently robust to identify resistant individuals within a sensitive population, because we recovered 25 resistant individuals during this experiment. Following the procedures described above (2.3.7 Resistance confirmation), all the recovered individuals were determined to be contaminations from ALS-resistant *Amaranthus* weeds: waterhemp and Palmer amaranth. The contamination was produced via either the pollination of

grain amaranth flowers with *Amaranthus* weed pollen (i.e. hybridization) or the direct presence of weed seeds in the planted grain amaranth. In this sense, no spontaneous resistant genotypes were detected. This gives an estimate of the probability to find a spontaneous ALS-resistant mutant in a given sensitive plant population to lower than 2 x 10^{-8}.

In a theoretical approach to this matter, we calculated the possibility of the nucleotide substitutions known to provide strong resistance (>10 times the label rate) to imidazolinones (Table 2.1) to occur in grain amaranth based on its ALS gene sequence (GenBank accession EU024568) and the spontaneous mutation frequencies in *Arabidopsis thaliana* reported by Ossowski et al. (2010). Assigning the average mutation rate of 6.5 x 10^{-9} per site per haploid genome per generation observed in *Arabidopsis thaliana* and considering 8 possible sites to be substituted in the ALS gene of grain amaranth (Table 2.5), the probability of any given plant spontaneously mutating to be resistant is 5.2 x 10^{-8} (Table 2.7). This signifies that we should have detected at least 3 resistant mutants in 70 million screened plants, with the chance of finding at least one at 97.37% (SE=1.9). Accounting for the difference in mutation type rate found in *Arabidopsis thaliana* and adjusting based on the C:G bias in the genome, the probability of an ALS-resistant mutant discovery ascended to 7.9 x 10^{-8} per plant (Table 2.6), so at least 5 resistant individuals should be found in 70 million seedlings, and the chance of detecting at least one resistant plant increased to 99.6 % (SE=2.35) (Table 2.7). It should be considered, though, that part of C:G→T:A mutations found in *Arabidopsis thaliana* were produced in C:T sites that are known to be methylated. Spontaneous deamination of methylated cytosine leads to thymine substitution being an

important source of mutation (Schmitz et al. 2011). However, we do not know anything about the methylation status of C:T sites in the ALS gene of grain amaranth. Consequently, by not computing the C:G→T:A mutations at methylated C:G sites in *Arabidopsis thaliana*, the probability diminishes to 5.8×10^{-8} (Table 2.6), i.e. 4 mutants screened from 70 million plants (Table 2.7). More importantly, the mutation rate between genic and intergenic regions varies substantially in *Arabidopsis thaliana*. This was attributed to a higher mutation rate in pericentromeric regions where gene density is lower than further away from the centromere. Although we do not know the exact location of the ALS gene in relation to the centromere, we may speculate that it is situated far away from the centromere as that is generally the case for most genes. Considering the mutation rate within genic regions only and subtracting the mutations originating in mobile elements, the probability for selecting an ALS-resistant mutant in a sensitive population decrease to 1.38×10^{-8} which is less than 1 individual (0.96) in 70 million plants. In that case, the chance of getting at least one resistant plant lowers to 61.93% (SE=0.98). If this estimation is close to reality, we should have screened at least 72.5 million plants to find at least 1 resistant individual to ALS-inhibitors (Table 2.7).

In addition, it must be pointed out that in most of the published ALS-resistance cases, mutations checked to survive a 10x rate (strong resistance) were duplicated in their respective genomes (homozygous). Newly-arisen spontaneous mutations are expected to be mostly in one chromosome only (heterozygous) during the first generation. Therefore, while screening at 10x rate, we might have eliminated individuals containing some of the mutations known to confer resistance. We previously confirmed

that this herbicide rate effectively selected heterozygous waterhemp plants containing the Trp574Leu mutation from sensitive plants. We estimate the probability of one of these mutants to arise spontaneously in grain amaranth to be 1.71×10^{-9}. Thus, if it were true that we had been selecting for that mutation only, we had a chance of 11.28% (SE=0.34) to find at least one resistant plant in this study (Table 2.7). However, we also confirmed that heterozygous smooth pigweed *(Amaranthus hybridus*) plants containing the Ala122Thr mutation survived a 10x rate of imazethapyr. Moreover, we checked that waterhemp plants homozygous for the Ser653Asn mutation survived a 20x rate, so we may speculate that a heterozygous plant could survive half of that rate. Supposing that we were selecting for these three mutations only, the probability of finding an ALS-resistant plant with one of these three mutations was 2.51×10^{-8} which is less than 2 individuals (1.76) in the 70 million plants screened in this study. In that case, the chance to find at least one resistant mutant in this experiment was of 82.74% (SE=1.32) (Table 2.7). The probability to find any of these mutants decrease if adjusted by methylation and genic regions as described above (Table 2.7).

Regardless of these theoretical considerations, an empirically-determined probability of 2×10^{-8} per individual is established as a higher limit for the occurrence of spontaneous ALS-resistant mutants in an *Amaranthus* sensitive population. This implies that spontaneous mutations conferring herbicide resistance do not appear to arise at high frequency in plant populations. In that case, the design of resistance mitigation strategies should focus more on the standing genetic variation as the main source of resistant alleles in weed populations. Nonetheless, it should be recognized that plant populations in the field may have a higher mutation load than in a greenhouse. Different

studies demonstrated that natural stresses, like drought, flood, cold, salinity, and UV light may cause severe DNA damage in plants (Yao and Kovalchuk 2011; Jiang et al. 2014). Particularly, UV light may be an important source of spontaneous mutations (Filkowski et al. 2003). However, UV rays in this project were partially blocked by greenhouse panels potentially decreasing its damaging effect on DNA (Figure 2.3). Moreover, recently it has been proposed that non-lethal herbicide applications may increase the mutation rate in survival plants, and even lead to the selection of mutator genotypes in weed populations (Gressel 2011). All these factors may account for an underestimation of the mutation rate impact measured by this study. In this sense, future work looking into herbicide-resistance mutation rate determination should consider scaling up the total number of screened plants—increasing the chance of spontaneous-mutant discovery—and using a more realistic environmental setting.

2.5 Tables and figures

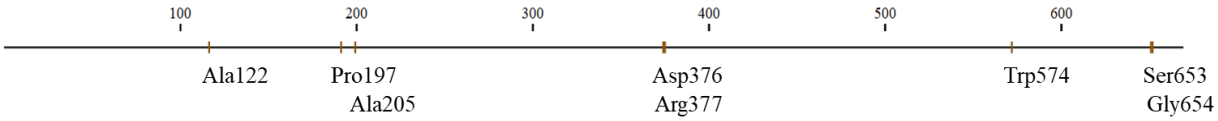

Figure 2.1. Sites in the ALS protein at which substitutions providing resistance have been identified in plants. Numbering of amino acids are based on the precursor ALS from *Arabidopsis thaliana*.

Table 2.1. Amino acid substitutions conferring resistance to ALS inhibitors (Heap 2018). Resistance level keys: S = sensitive, r = weak resistance, R = strong resistance (>10 times the label rate), nd = not determined.

Original site	Substitution	Resistance level		Weed species
		SUs	IMIs	
Ala 122	Thr	S	R	6
	Val	R	R	2
	Tyr	R	R	1
	Ser	nd	nd	1
	Asn	R	R	1
Pro 197	Thr	R	S/r	13
	His	R	S/r/R	8
	Arg	R	S/r	5
	Leu	R	S/r/R	12
	Gln	R	S/r	7
	Ser	R	S/r	26
	Ala	R	S/r	11
	Ile	R	r	1
	Asn	R	nd	1
	Glu	R	R	1
	Tyr	R	nd	1
Ala 205	Val	S/r/R	r/R	5
	Phe	R	R	1
Asp 376	Glu	r/R	r/R	12
Arg 377	His	R	nd	1
Trp 574	Leu	R	R	36
	Gly	R	ND	1
	Met	R	ND	1
	Arg	R	R	1
Ser 653	Thr	S/r	R	6
	Asn	S/r	R	7
	Ile	r	R	1
Gly 654	Glu	nd	R	1
	Asp	r	R	1

1-Seed production

2-Seed harvest and cleaning

Figure 2.2. Experiment cycle. 1- A batch of grain amaranth plants is grown in the greenhouse for seed production. 2- Inflorescences are harvested manually and seed is cleaned. 3- Seed is planted at a high density in flats. 4- Herbicide is applied on flats before seedlings emergence. 5- Herbicide-resistant plant screening.

Figure 2.2. (cont.)

3-Seed planting

4-Pre-emergence herbicide application

5-Herbicide-resistant plants screening

Table 2.2. Labor dates per seed production batch. To initiate a batch, seeds were planted in 12.3 x 12.3 cm inserts. Plants were allowed to grow in the inserts until each plant had its first true leaf longer than a quarter of an inch. Then, seedlings were transplanted into 10.1 cm diameter standard pots. Between 400 and 500 seedlings were germinated each time to finally select a uniform group of around 200 plants for each batch. When plants reached 5-6 inches in height, they were transplanted into 9.4 L pots. The pots were placed on benches laying directly on the floor. Flowering started approximately 45 days after planting in all batches. Plants were harvested at least 10 days after it was noted that seeds were able to be detached from the plants without pushing them.

Batch	Planting in inserts	Days in inserts	Transplant to 10.1 cm pots	Days in 10.1 cm pots	Transplant to 9.4 L pots	Days in 9.4 L pots	Flowering date	Harvest	Total days
A	1/24/2015	3	1/27/2015	16	2/12/2015	160	3/9/2015	7/22/2015	179
B	7/31/2015	10	8/10/2015	14	8/24/2015	136	9/14/2015	1/7/2016	160
C	1/7/2016	11	1/18/2016	28	2/15/2016	121	2/22/2016	6/15/2016	160
D	7/19/2016	15	8/3/2016	30	9/2/2016	144	9/3/2016	1/24/2017	189
E	1/27/2017	12	2/8/2017	40	3/20/2017	117	3/13/2017	7/15/2017	169

Table 2.3. Yield information per seed production batch.

Batch	Number of plants	Seed yield (g)	Seed yield/plant (g)	Ave. 100 seeds (g)	Yield (seeds)	Seeds/Plant
A	234	6,903	30	0.0612	11,287,902	48,239
B	214	6,717	31	0.0602	11,154,941	52,126
C	210	23,927	114	0.0625	38,282,720	182,299
D	294	6,282	21	0.0545	11,536,084	39,238
E	237	9,668	41	0.0651	14,844,573	62,635
Total	**1,189**	**53,497**			**87,106,221**	
Ave./batch	238	10,699	47	0.0607	17,421,244	**76,907**

Table 2.4. Calculated number of screened plants per seed batch based on their respective germination rates.

Batch #	Yield (seeds)	Germination rate (%)	Screened plants
Batch A	11,287,902	0.67	7,562,895
Batch B	11,154,941	0.63	7,027,613
Batch C	38,282,720	0.90	34,263,034
Batch D	11,536,084	0.83	9,574,950
Batch E	14,844,573	0.84	12,454,597
Total	**87,106,221**		**70,883,089**
Ave/batch	17,421,244	**0.77**	14,176,618

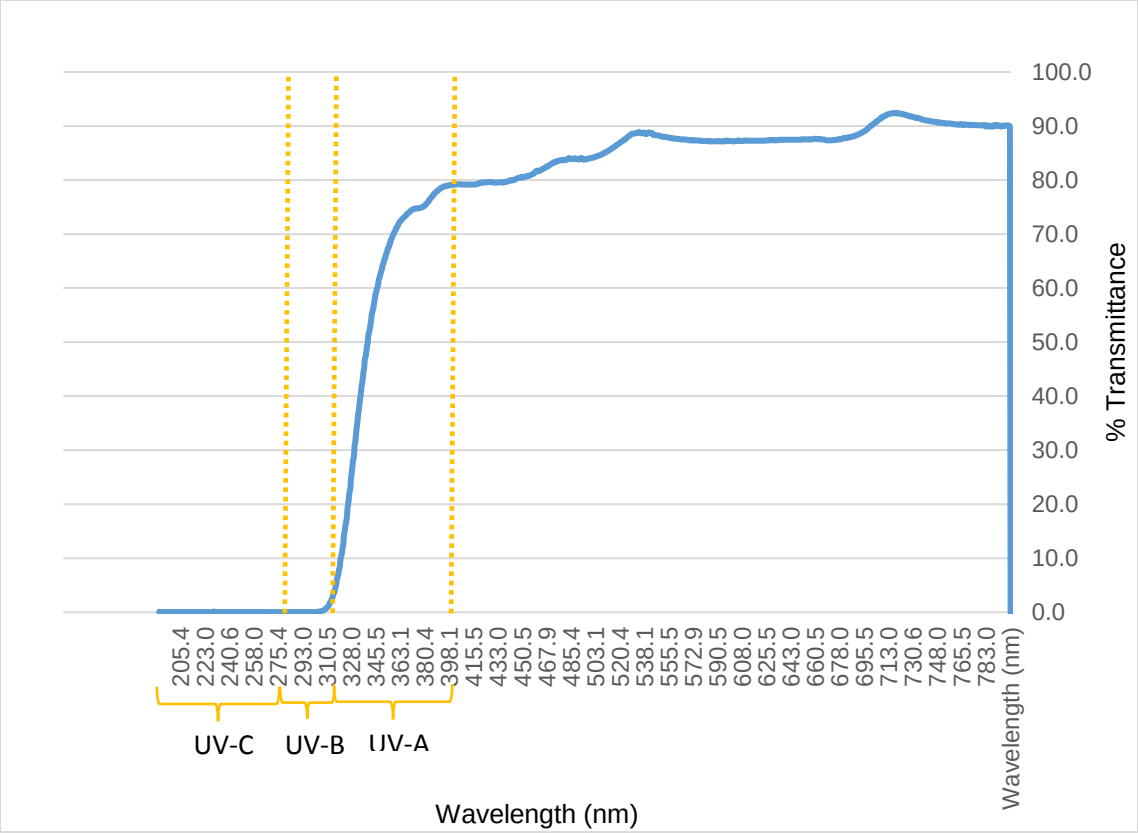

Figure 2.3. Light transmittance of Turner Hall greenhouse's glazing (University of Illinois). Blue line indicates the percentage of light transmitted per wavelength. Yellow dotted lines indicate ultraviolet light wavelength ranges. Ultraviolet types B (280-315 nm) and C (100-280 nm) are mostly blocked by the ozone layer. UV-A (315 to 400 nm) reaches the ground, but is partially reflected (not totally absorbed) by the greenhouse glazing.

Table 2.5. Possible resistance-conferring nucleotide substitutions in the ALS gene of grain amaranth. Because only one of three possible mutations confers resistance, sites are counted as a third of a site. Therefore, to calculate the mutation rate conferring ALS-inhibitor resistance in grain amaranth using the average mutation rate per site in *Arabidopsis thaliana*, 8 sites should be considered.

Original codon	Amino acid substitution	Nucleotide substitution		Probability
		Original	Substitute	
GCA	Ala122Thr	1-G	A	1/3
GCA	Ala122Val	2-C	T	1/3
CCC	Pro197Leu	2-C	T	1/3
GCT	Ala205Val	2-C	T	1/3
GAT	Asp376Glu	3-T	A/G	2/3
TGG	Trp574Leu	2-G	T	1/3
TGG	Trp574Arg	1-T	C	1/3
AGC	Ser653Asn	2-G	A	1/3
AGC	Ser653Thr	2-G	C	1/3
AGC	Ser653Ile	2-G	T	1/3
GGT	Gly654Asp	2-G	A	1/3
Total sites in haploid genome				4
Total sites in diploid genome				**8**

Table 2.6. Theoretical calculation of the mutation rate conferring ALS-inhibitor resistance per plant per generation based on mutation type frequencies found in *Arabidopsis thaliana*. Each base substitution probability per plant per generation was determined by assigning the mutation type frequencies of *Arabidopsis thaliana* to the ALS gene base substitutions known to provide resistance. * Same probabilities adjusted by the proportion of mutations originated in C:G methylated sites in the *Arabidopsis thaliana* genome. *[2] Numbers indicate base position in DNA codon.

Amino acid substitution	Nucleotide substitution		Mutation probability	Mutation probability*
	Original*[2]	Substitute		
Ala122Thr	1-G	A	5.86E-09	4.14E-09
Ala122Val	2-C	T	5.86E-09	4.14E-09
Pro197Leu	2-C	T	5.86E-09	4.14E-09
Ala205Val	2-C	T	5.86E-09	4.14E-09
Asp376Glu	3-T	A/G	6.15E-10	6.15E-10
Trp574Leu	2-G	T	8.57E-10	8.57E-10
Trp574Arg	1-T	C	1.28E-09	1.28E-09
Ser653Asn	2-G	A	5.86E-09	4.14E-09
Ser653Thr	2-G	C	7.14E-10	7.14E-10
Ser653Ile	2-G	T	8.57E-10	8.57E-10
Gly654Asp	2-G	A	5.86E-09	4.14E-09
Mutation rate in haploid genome			3.94E-08	2.91E-08
Mutation rate in diploid genome			**7.89E-08**	**5.83E-08**

Table 2.7. Theoretical calculation approaches for determining the ALS resistance mutation rate in grain amaranth. Average genome: average mutation rate per site per genome per generation in *Arabidopsis thaliana* assigned to the 8 possible resistance sites in the ALS gene of grain amaranth (Table 2.5). Adjusted per mutation type: mutation rate conferring ALS-inhibitor resistance per plant per generation based on mutation type frequencies found in *Arabidopsis thaliana.* Adjusted per methylated sites: the adjusted per mutation type calculation further adjusted by the proportion of C:G methylated sites. Adjusted per genic regions: the average genome approach adjusted by the proportion of mutations in genic regions of *Arabidopsis*. Trp574Leu: the probability of one C:G→A:T mutation in *Arabidopsis thaliana* in a diploid genome in one generation. Trp574Leu, Ala122Thr, and Ser653Asn: the probability of one C:G→A:T, and two C:G→T:A mutations in *Arabidopsis thaliana* in a diploid genome in one plant generation. * Adjusted per methylated sites and genic regions.

Calculation approach	Probability of finding 1 mutant	Reciprocal (1/ probability of finding 1 mutant)	Number of mutants to be expected in 70,000,000 plants	Probability of finding 1 mutant in 70,000,000 plants
Average genome	5.20E-08	19,230,769	3.640	0.9737
Adjusted per mutation type	7.89E-08	12,667,038	5.526	0.9960
Adjusted per methylated sites	5.84E-08	17,131,024	4.086	0.9832
Adjusted per genic regions	1.38E-08	72,485,207	0.966	0.6193
Trp574Leu	1.71E-09	583,333,333	0.120	0.1128
Trp574Leu, Ala122Thr, and Ser653Asn	2.51E-08	39,772,727	1.760	0.8274
* Trp574Leu	4.55E-10	2,198,717,952	0.032	0.0313
* Trp574Leu, Ala122Thr, and Ser653Asn	4.88E-09	204,935,797	0.342	0.2893

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CHAPTER 3: Experimental investigation of low-dose herbicide effect on the mutation rate conferring herbicide resistance

3.1 Introduction

3.1.1 Stress-induced mutagenesis in plants

Mutations to be passed on to the next generation must be present in gametes. However, most of the mutations produced in multicellular organisms occur in somatic cells (i.e. somatic mutations), and fewer occur in the germinal cells (i.e. germinal mutations) from which gametes rise. In this sense, not all of the mutations that arise during the life-span of an organism will potentially generate new genetic variants in the population. In animals particularly, genetic information cannot pass from soma to germplasm (Weismann's barrier), so the offspring inherit germinal mutations only. Conversely, in plants, reproductive structures start from somatic meristems, allowing any mutation in the soma to be potentially passed on to the next generation (Bobiwash et al. 2013). For this reason, somatic mutations are believed to have a particular significance in the plant kingdom (Kovalchuk et al. 2000). Several studies showed the accumulation of somatic mutations in the *Arabidopsis thaliana* genome during its life cycle (Boyko et al. 2006; Golubov et al. 2010; Kiselev et al. 2018). Going a step further, it can be inferred that any factor altering the mutation rate in plants may affect the occurrence of new genetic variants in the population.

In that regard, because most of the studies determining the mutation rate in plants have been performed in relatively sheltered and artificial laboratory environments, they might not reflect the real frequency of the phenomenon in nature

(Ossowski et al. 2010). Natural environments are rarely as benign as greenhouse-based experimental settings, and often expose plants to varying combinations of environmental stresses that may alter the mutation rate (Jiang et al. 2014). Furthermore, because more pathways are expected to be active in nature, and more genes expressed, the mutation rate should have a higher effect on fitness than in less-changing experimental settings (Rutter et al. 2010).

What is referred to as the general term “stress” can be translated into a variety of physical, chemical, and biological agents capable of affecting the DNA molecule and producing mutations, generally referred to as mutagens. Although mutagens can affect the DNA molecule in several ways, the direct damage of nucleotides and the break of the sugar-phosphate backbone are the most common ones. Damaged nucleotides are repaired via one of three mechanisms: base excision repair (BER), nucleotide excision repair (NER), or mismatch repair (MMR) (Schröpfer et al. 2014). These may result in single base substitutions or indels if mistakes occur during the repairing process. More critically, if the sugar-phosphate backbone is damaged, double-strand breaks in the DNA molecule are generated, making repair critical for cell viability (Migicovsky and Kovalchuk 2012). Double-strand breaks are repaired via non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), or homologous recombination (HR) (Yao and Kovalchuk 2011; Darracq et al. 2018). NHEJ is not a very precise repairing process, commonly generating point mutations, insertions, and deletions. HR, on the other hand, is an accurate process that uses the homologous sequence from the sister chromatid or the homologous chromosome as a template to fix the break (Schröpfer et al. 2014). However, homologous recombination may lead to unequal

crossing-over that generates gross chromosomal rearrangements, potentially producing gene duplications, gene deletions, or even the formation of new genes (Migicovsky and Kovalchuk 2012).

In 1928, Lewis Stadler was the first to demonstrate that x-rays induced mutations in barley. After that, many agents, including gamma rays, alkylating agents, and endonucleases, were identified by their mutagenic effects on plants, even using induced mutagenesis in a common technique to generate new genetic variants. From the 1930s when the first mutant wheat cultivars came to light, more than 3000 mutant crop varieties have been brought to the market (Pacher and Puchta 2017).

Additionally, many studies performed on plant populations grown in areas affected by the Chernobyl accident showed high numbers of DNA alterations. Firstly, Abramov et al. (1992) reported an increased frequency of mutant plants grown in plots with a high radioactive contamination level in the soil. Kovalchuk et al. (1998) observed a strong positive correlation of soil radioactive pollution with the percentage of chromosomal abnormalities in *Allium cepa* and *Arabidopsis thaliana*. Later, based on the analysis of 13 microsatellite loci in a population of wheat plants grown around Chernobyl, it was calculated that there was a 6-fold increase in the mutation rate over a single generation of exposure to ionizing radiation (Kovalchuk et al. 2003).

Moreover, different studies have demonstrated that common environmental stress factors like heat, cold, UV radiation, drought, flood, and salt stresses can induce DNA damage and substantial mutation accumulation in plants (Boyko et al. 2010; Yao and Kovalchuk 2011; Willing et al. 2016). Ries et al. (2000) showed that elevated solar UVB (ultraviolet B) doses increased the frequency of somatic homologous DNA

rearrangements in *Arabidopsis thaliana* and *Nicotiana tabacum* plants. Filkowski et al. (2003), using a reporter gene, showed an increase of the mutation rate by 6.7-fold in *Arabidopsis thaliana* and by 2.7-fold in *Nicotiana tabacum*, as a result of induced mutagenesis caused by UVC radiation (ultraviolet C). More recently, Yao and Kovalchuk (2011), using reporter genes in transgenic *Arabidopsis thaliana* plants, showed that exposure to abiotic stressors such as heat, cold, drought, flood, UVB and UVC lead to an increase in the frequency of homologous recombination (HRF) and point mutations, deletions, and insertions in somatic cells of exposed plants. Kiselev et al. (2018) found that somatic mutations were considerably increased when *Arabidopsis thaliana* plants were grown in soil with elevated cadmium levels, a mutagen that is known to inhibit DNA repair processes. Jiang et al. (2014) revealed that multigenerational growth of *Arabidopsis thaliana* in saline soil increases the frequency and changes the molecular mutational spectrum (the different frequencies of specific transversions and transitions) of accumulated *de novo* DNA mutations. Interestingly, saline growth conditions increased the frequency of methylation of C:G positions (CG-DMPs).

With regards to weed science, there are several reported cases of DNA damage and increased mutation frequency caused by herbicides. In the past, Plewa (1985) developed an atrazine-based system to quantify the mutation rate in maize. The frequency of starchy pollen grains was doubled by atrazine treatment on maize plants. Kovalchuk et al. (2003), using transgenic *Arabidopsis thaliana* plants, revealed an increase in homologous recombination and the number of point mutations caused by 2,4-D and dicamba herbicides. Evaluating random genetic regions (RAPD technique),

Doganlar (2012) found differences in DNA polymorphisms before and after treatment with quizalofop-p-ethyl in *Lemna minor* and *Lemna gibba* (duckweeds). Liman et al. (2014) observed DNA damage in root meristematic cells of *Allium cepa* grown in soil treated with imazethapyr. Similarly, genetic damage caused by dicamba and atrazine was detected in different varieties of South American sweetcorn (Reynoso et al. 2015).

It is not surprising that different abiotic and biotic stresses have been strongly correlated with increased mutation rates in plants. From an evolutionary survival standpoint, it seems logical that there might be an advantage if there could be more mutations when organisms need to change, e.g. a weed population “needs’’ resistance when it is sprayed with an herbicide. When a genotype is unable to cope with its environment, an increased mutation rate augments the probability of receiving a fortuitous beneficial mutation in the offspring. In other words, organisms need the enhanced random variability generated by mutations to overcome stress. Conversely, because most mutations are deleterious (or even lethal), high mutation rates have little advantage and much disadvantage when an organism is living in a non-stressful environment where random genetic variants would not increase adaptation probabilities (Gressel and Levy 2009).

In this sense, a stress condition may increase the mutation rate by directly affecting the DNA molecule (i.e. DNA damage leads to more repairing events that increase mutation probabilities), but it also may operate indirectly through the balance between mutator and anti-mutator alleles in the genome. A ‘mutator gene’ is a mutant of a normal gene that increases the mutation rate (e.g. error-prone polymerases) (Gressel and Levy 2009). Therefore, a stressful environment may select for more mutator alleles,

allowing populations to accumulate mutant varieties in the next generation, as long as a non-stressful environment may select for fewer mutator alleles to decrease the mutation rate. Moreover, a stressful situation may trigger the expression of mutator genes which were silenced during non-stressful conditions.

The occurrence of stress-induced mutagenesis (SIM) is well studied in bacteria. In *Escherichia coli*, more than 25 genes were recognized to be involved in the elevation of mutation rates when cells were poorly adapted to their environment (Horst et al. 1999; Al Mamun et al. 2012). In eukaryotes, mutants of the *mutS* and *mutL* genes were identified in yeast and human cancer cells (Modrich and Lahue 1996; Metzgar and Wills 2000). Furthermore, although error-prone polymerases are the best-documented mutators in most organisms, mutator genes may also be related to proteins involved in repair pathways (Lynch et al 2016). For example, superoxide dismutases detoxify DNA-damaging reactive oxygen species (ROS). In addition, mutator mechanisms can be related to other non-gene-based mutagenic elements, like transposons or epigenetic changes.

Also, there are well-documented cases of mutators in plants. In *Arabidopsis thaliana*, a nuclear-encoded mutator (*chm1*) was found to increase the mutation load in the plastome and in the mitochondrial DNA (Redei and Plurad 1973). Similarly, the *AtMSH2* gene was linked to mutator effects in the plastome, and the gene *iojap* was identified as a plastome mutator in maize (Byrne and Taylor 1996). Impressively, a transposon was proposed to be an inducible chloroplast mutator in barley (Prina et al. 1996). More recently, a mutator-like transposon in *Arabidopsis thaliana* was found to be activated by temperature stress (Young et al. 2005).

Because weeds seem to be more adaptive than other plant species since they can evolve very quickly to different perturbations, it was speculated that they may have an enhanced mutation rate driven by mutators (Gressel 2011). For example, the high-frequency of atrazine-resistant mutants in wild-type populations of lambsquarters (*Chenopodium album*) was attributed to a factor altering random mutation occurrence (i.e. mutator) (Darmency and Gasquez 1990).

3.1.2 Herbicide-resistance evolution driven by low-rate herbicide applications

Although herbicides—when applied at the rates and plant growth stage prescribed in the label— cause high mortality of target weed plants, applications at sub-lethal doses are common in farming situations. In the first place, applications are not uniform across the target population (Gressel 2011). In a field, weeds are not regularly distributed, and even one plant can shade another, preventing spray contact. Furthermore, if herbicide treatment is applied to plants of bigger size than the recommended height, the rate is effectively diminished (Manalil et al. 2011). The spray itself may not be evenly applied depending on the equipment and the operator accuracy (e.g. spraying during windy weather may increase spray drift). And finally, environmental variability may decrease the effective rate received by the target plants (e.g. a rain may wash the product off of weed leaves; residual activity may vary upon soil conditions). These variables combine to reduce the effective rate applied to target weed populations and so labels attempt to compensate for this by recommending rates that are several-fold overkill. Unfortunately, the registered labeled rate of a particular herbicide compound may have great variation between brands, countries, and even regions within countries (Manalil et al. 2011). For example, registered use rates in

Australia are lower than in the United States. Moreover, because the optimum economic return in some farming situations is reached at low herbicide rates, farmers often apply rates lower than the label-recommended level.

Early models of evolution to pesticides predict that the use of high rates—i.e. high selection pressure—would lead to the selection of one or two major genes conferring high levels of resistance (most likely target-site resistance), while lower doses—i.e. low selection pressure—would delay the evolution of major resistance traits (Gressel 2011). Whereas that remains true, it was later reported that the sequential application of low doses may select for minor resistance traits, increasing the resistance mean of the population in each generation (Neve 2007; Busi et al. 2013). Along these lines, some studies have demonstrated that the recurrent selection for survival of low-dose applications of diclofop in populations of rigid ryegrass (*Lolium rigidum*) leads to the rapid evolution of herbicide resistance under both laboratory and field conditions (Neve and Powles 2005, Manalil et al. 2011). Similarly, recurrent selection of a susceptible *Lolium rigidum* population with low rates of glyphosate resulted in the evolution of a modest level of glyphosate resistance (Busi and Powles 2009). The likeliest explanation is that these low rates selected for low-level resistance polygenes which, when accumulated through cross-pollination and recurrent selection, are capable of conferring a noticeable resistance level (Gressel 2011). Importantly, this kind of polygenic resistance is often metabolic, and thus produces cross-resistance to other herbicides beyond the selecting agent.

In many cases, low-dose applications of herbicide do not kill all the individuals of the target population (i.e. sub-lethality). Although not killed, surviving plants are

expected to be highly stressed and prone to the accumulation of mutations. In fact, it was recently argued that the exposure of weeds to sub-lethal herbicide treatments could elevate mutation rates in a similar way to how bacteria respond to stressful environments (Gressel and Levy 2009). For example, atrazine is used for weed control in corn because maize naturally detoxifies the herbicide. However, while atrazine is metabolized by the maize plant, it consumes glutathione, an antioxidant which plays a role in free radical detoxification, and also momentarily blocks photosynthesis, leading to free radical accumulation and energy depletion (Gressel 2011). So, atrazine may not be lethal to the crop, but it is stressful.

Going a step further, if low herbicide rates increase the mutation rate, it may be inferred that it also increases the probability of generating new genetic variants conferring resistance. As stated previously, it is known that herbicides are capable of damaging DNA, which may potentially lead to new mutations. Alternatively, it was also proposed that herbicide-mediated stress may select for mutators and even induce mutator activity within genomes. In that hypothetical case, mutators can be selected only when plants under stress survive (e.g. a sub-lethal herbicidal dose). In addition, not only mutators can be inherited, but also the rest of the potential changes occurring in plants during stressful conditions may be passed on the next generation, leading to adaptive evolution (Migicovsky and Kovalchuk 2012). Epigenetic changes can be maintained for several generations in the absence of the original stress (Molinier et al. 2006; Bruce et al. 2007). Also, a high homologous recombination frequency was observed in the somatic tissue of non-stressed progeny derived from stress-exposed plants (Yao and Kovalchuk 2011). This may imply that the use of herbicides at low

doses in one season can affect resistance evolution in the following seasons, regardless of rates applied or even the mix of products.

3.2 Objective

In addition to the determination of the mutation rate conferring ALS resistance described in Chapter 2, we aimed to check if the mutation rate increases with a low-dose treatment of herbicide. Specifically, the objective of this experiment was to determine the frequency of ALS-resistant mutants in the offspring of a sensitive plant population treated with a sub-lethal dose of atrazine. Then, resistant plants would be evaluated for mutations in the ALS gene to calculate any change in the mutation frequency induced by the herbicide treatment. Such a change should reflect the occurrence of mutations resulting from spontaneous replication mistakes, plus those generated by the repair of DNA damage.

3.3 Materials and methods

3.3.1 Plant material

Because the results of this experiment were compared with the mutation rate determination experiment outlined in Chapter 2, the plant population in this study corresponds to a batch of grain amaranth plants with identical characteristics as that of the previous study. Therefore, all the cultivation procedures are the same as described above. Labor dates are laid out in Table 3.1.

3.3.2 Sub-lethal herbicide treatment

The selected herbicide treatment aimed to increase the mutation rate without reducing the seed output. In this sense, the application time was determined by the

following reasoning. If applied in the early plant stages (prior to flower formation), a mutation would likely remain in the vegetative tissue without reaching the reproductive structures and may increase plant branching which may affect seed output. If applied after embryo formation, because embryo cells are not dividing actively, the scarcity of DNA replication events would decrease the mutation rate. As a result, the time window to apply the mutagen is from flower initiation to embryo formation. However, if the herbicide is applied directly to the panicle, it may sterilize the flowers decreasing seed output. The herbicide was applied, therefore, 5 days before floral meristem appearance in most plants (Table 3.1). At that time, many meiosis events should have already started, thus the mutation rate could be significantly increased, but the reproductive tissue is not directly affected as it is still covered by the stem. Plant height at this point ranged from 15 to 25 cm between individuals.

For the herbicide stress, we chose atrazine (AAtrex® Nine-O®, 0.882 gr a.i. /gr; Syngenta Crop Protection, Greensboro, NC) for several reasons. By attaching to the Qb binding site in the photosystem-ll D1 protein, atrazine blocks photosynthesis and leads to the accumulation of free radicals that damage the DNA, thus potentially increasing the mutation rate. It is more commonly applied in pre-emergence to the soil but also may be applied in a post-application over the plants if needed. Furthermore, unlike most other contact burners which are non-systemic, atrazine moves upstream through the xylem, ensuring DNA damage in the meristem, however, it does not accumulate in the meristem as truly systemic herbicides do (which move by both xylem and phloem) so that seed output is not deeply affected. Additionally, with grain amaranth as an

indeterminate growth crop, atrazine's residual activity in the soil prolongs plant exposure to the potential mutagen.

Dose level was determined by selecting a rate that not only was non-lethal, but also did not decrease the seed output by more than 10%, compared to a non-treated plant. In this batch, plants were divided into two treatments: 110 plants treated at 0.2 kg/ha and 110 plants treated at 0.1 kg/ha. Also, an untreated group of 20 plants was included as a control. Herbicide applications were performed in the research spray chamber calibrated with the same parameters as described in Chapter 2. The nozzle was maintained approximately 45 cm above the plants. Plants were visually affected as proof of the herbicide activity (Figure 3.1). Because atrazine requires high light levels to work effectively, natural sunlight was supplemented with 1000 watt metal halide lamps.

3.3.3 Screening for ALS resistance

Seedlings were screened with for ALS-inhibitor resistance following the same procedures described in Chapter 2.

3.4 Results and Discussion

Although visually affected, plants recovered well from atrazine treatment, with growth and development similar to the non-treated group. The yield per plant did not differ significantly between treatments. Moreover, yield parameters of the whole batch of plants did not differ from the untreated batches described in Chapter 2. From treated plants, more than 14 million seeds were obtained (Table 3.2). According to the germination rate (0.84), more than 11,000,000 seedlings were screened for ALS-inhibitor resistance (Table 3.3). No resistant mutants were found.

Many reasons may account for the non-identification of resistant mutants. First of all, we do not know the lower limit of the mutation rate conferring ALS-inhibitor resistance, so we do not know exactly how many screened individuals are needed to catch a resistant mutant. In this sense, a sub-lethal dose of atrazine may lead to an increase in the frequency of ALS-resistant progeny, but we may need to screen a larger population to catch it. Furthermore, the treatment may have generated ALS-inhibitor-resistant mutants that our screening procedure did not select. As discussed in Chapter 2, a dose of 10 times the labeled rate of imazethapyr may be too high to select for some of the mutations known to provide resistance to the herbicide. Moreover, the atrazine treatment may have increased the mutation load in the offspring, even in the ALS gene, but these mutations do not confer resistance to imazethapyr.

On the other hand, it may be that the herbicide treatment utilized in this experiment is not effective in generating mutant offspring. The utilized atrazine rates may be too low to induce mutations, or herbicide activity in the plant could be decreased. For example, the intensity of the photosynthetically active radiation intercepted by the plants in the greenhouse may be not as high as required by atrazine to generate large quantities of ROS. Even though it may be inferred that the applied doses of atrazine were harmful to DNA as plants were visually affected, that does not necessarily lead to the rise of new mutations. Additionally, mutations, if created in the soma, may not have reached the gametes in parental plants, and so would not be present in the following generation.

Additionally, it can be argued that a hypothetical increase in the mutation rate under stress that is mediated by mutators, that may not be addressed in this

experiment. When theory assigns the rapid evolution towards herbicide resistance to the role of mutators, it is implied that the evolutionary history of weeds has strongly selected for these mutators. For example, a weed population that evolved resistance to an herbicide had to adapt to other perturbations in the past where an elevated mutation rate could be advantageous (e.g. other herbicide or tillage). Following this reasoning, a potential activation of a mutator during the stress provoked by a sub-lethal dose of herbicide can occur only if that mutator was selected before. However, the plant population used in the present study is not completely a weed or at least it has not evolved as a weed in the recent past. Moreover, in favor of genetic homogeneity, it is feasible that many sources of genetic variability have been purged out from the genetic background of this cultivar during its domestication process. Therefore, it is expected that the utilized plant population is less likely to evolve herbicide resistance as a product of mutator-based genetic modulation compared to weeds.

Although we did not find empirical evidence supporting it, the hypothetical stress-mediated increase of mutation rates leading to herbicide resistance remains biologically logical. In case this theory is eventually corroborated as true, resistance-mitigation strategies should be re-thought.

3.5 Tables and figures

Table 3.1. Labor dates in atrazine-treated batch. In previous grain amaranth batches (Chapter 2), the first plant to show a flowering meristem (flowering date) occurred typically one week before most plants started flowering. Therefore, the date of the first flowering plant (3/19/2017) was used as an indicator of the treatment timing (3/20/2017), which was established to be 5 days before most plants start flowering (3/26/2017).

Planting in inserts	Days inserts	Transplant to 10.1 cm pots	Days in 10.1 cm pots	Transplant to 9.4 L pots	Days in 9.4 L pots	Atrazine treatment	Flowering date	Harvest	Total days
1/27/2017	12	2/8/2017	40	3/20/2017	117	3/20/2017	3/19/2017	7/15/2017	169

Control

Atrazine 0.1 kg/ha

Atrazine 0.2 kg/ha

Figure 3.1. Plants 15 days after atrazine treatment. Treated plants showed clear symptoms of herbicide injury. Plants treated at the highest rate (0.2 kg/ha) were more affected in average than plants treated at the lowest rate (0.1 kg/ha). Many plants re-grew from axillar buds.

Table 3.2. Yield information per atrazine-treatment group.

Atrazine treatment	Number of plants	Yield (g)	Yield/plant (g)	Ave. 100 seeds (g)	Yield (seeds)	Seeds/Plant
control	19	454	24	0.0651	697060	36687
0.1 kg/ha	110	5149	47	0.0651	7907034	71882
0.2 kg/ha	108	4064	38	0.0651	6240479	57782

Table 3.3. Calculated number of screened plants per atrazine-treatment group.

Atrazine treatment	Yield (seeds)	Germination rate (%)	Screened plants
control	697060	0.84	584833
0.1 kg/ha	7907034	0.84	6634002
0.2 kg/ha	6240479	0.84	5235762

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CHAPTER 4

4.1 Concluding remarks

Recognizing herbicide resistance as an evolutionary process makes it necessary to understand the evolutionary forces underpinning the phenomenon to design effective resistance mitigation strategies (Neve 2007). Herbicides act as selective agents that, over time, increase the frequency of initially rare resistance alleles (Heap 2014). These alleles may come from the standing genetic variation within the population, from a resistant population via pollen or seeds, or arise spontaneously by *de novo* mutations (Jasieniuk et al. 1996; Délye et al. 2013). The relative importance of each of these three sources deeply affects what strategies should be applied to counteract herbicide resistance evolution, however, empirical determinations of these parameters are scarce.

Here, we aimed to calculate the *de novo* mutation rate generating herbicide-resistant individuals. To achieve this goal, we performed a method to effectively select spontaneous herbicide-resistant mutants by screening millions of plants, using grain amaranth and resistance to ALS-inhibiting herbicides as a model system. After screening 70,000,000 plants, no spontaneous resistant genotypes were detected, determining the probability to find a spontaneous ALS-resistant mutant in a given sensitive plant population to be lower than 2×10^{-8}. Based on theoretical calculations, the probability of an ALS-resistant mutant discovery in this experiment should range between 1.71×10^{-9} and 7.9×10^{-8}. Thus, the population size of this experiment may not have been big enough for effective resistant selection. To identify the mutation rate conferring spontaneous ALS-resistant variants in a plant population, it is necessary to scale up this experiment.

In addition, addressing the idea that the exposure of weeds to sub-lethal herbicide treatments could elevate mutation rates, we determined the frequency of ALS-resistant mutants in the offspring of a sensitive plant population treated with a sub-lethal dose of atrazine (Gressel and Levy 2009; Gressel 2011). After screening more than 11,000,000 seedlings, no ALS-inhibitor resistant mutants were found.

To sum up, this study establishes a higher limit of 2×10^{-8} for the occurrence of spontaneous ALS-resistant mutants in an *Amaranthus* sensitive population, implying that spontaneous mutations conferring herbicide resistance do not arise at high frequency in plant populations. This contrasts the speculation that weeds are more adaptive than other plant species because they have an enhanced mutation rate (Gressel 2011). Furthermore, we found no evidence that low doses of herbicides may lead to herbicide-resistant mutants' generation. In this sense, the design of resistance-mitigation strategies should focus more on herbicide resistance evolving from the standing genetic variation of weed populations. However, it should be considered that plant populations in field conditions may have a higher mutation rate than in a greenhouse, accounting for an underestimation of the mutation rate impact measured by this study. Therefore, future work looking into herbicide-resistance mutation rate determination should consider using a field setting.

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APPENDIX: Mutation rates across the three of life

Table A.1. Mutation rates determined by whole-genome sequencing. Direct calculations of the mutation rate are performed by sequencing mutation accumulation (MA) lines or by sequencing parents and their offspring (PO). **u*: mutation rate per site per haploid genome per generation.

*u**	Species	Reference	Approach
7.6E-12	*Tetrahymena thermophila*	Long et al. (2016)	MA
1.9E-11	*Paramecium tetraurelia*	Sung et al. (2012)	MA
2.9E-11	*Dictyostelium discoideum*	Saxer et al. (2012)	MA
6.8E-11	*Chlamydomonas reinhardtii*	Sung et al. (2012)	MA
7.9E-11	*Pseudomonas aeruginosa*	Dettman et al. (2016)	MA
1.3E-10	*Burkholderia cenocepacia*	Dillon et al. (2015)	MA
1.4E-10	*Ruegeria pomeroyi*	Sun et al. (2017)	MA
1.7E-10	*Saccharomyces cerevisiae*	Zhu et al. (2014)	MA
1.7E-10	*Schizosaccharomyces pombe*	Behringer and Hall (2016)	MA
1.9E-10	*Rhodosporidium toruloides*	Long et al. (2016)	MA
2.1E-10	*Chlamydomonas reinhardtii*	Ness et al. (2012)	MA
2.1E-10	*Schizosaccharomyces pombe*	Farlow et al. (2015)	MA
2.2E-10	*Escherichia coli*	Lee et al. (2012)	MA
2.9E-10	*Saccharomyces cerevisiae*	Nishant et al. (2010)	MA
3.0E-10	*Escherichia coli*	Foster et al. (2015)	MA
3.3E-10	*Saccharomyces cerevisiae*	Lynch et al. (2008)	MA
5.0E-10	*Deinococcus radiodurans*	Long et al. (2015)	MA
5.3E-10	*Mycobacterium smegmatis*	Kucukyildirim et al. (2015)	MA
6.7E-10	*Caenorhabditis elegans*	Meier et al. (2014)	MA
9.6E-10	*Chlamydomonas reinhardtii*	Kraemer et al. (2016)	MA
1.3E-09	*Caenorhabditis briggsae*	Denver et al. (2012)	MA
1.5E-09	*Caenorhabditis elegans*	Denver et al. (2012)	MA
2.0E-09	*Pristionchus pacificus*	Weller et al. (2014)	MA
2.3E-09	*Daphnia pulex*	Flynn et al. (2016)	MA
2.7E-09	*Caenorhabditis elegans*	Denver et al. (2009)	MA
2.8E-09	*Drosophila melanogaster*	Keightley et al. (2014)	P-O
2.9E-09	*Heliconius melpomene*	Keightley et al. (2015)	P-O
3.2E-09	*Oryza sativa*	Yang et al. (2015)	P-O
3.5E-09	*Drosophila melanogaster*	Keightley et al. (2009)	MA
3.6E-09	*Bumbus terrestris*	Liu et al. (2016)	MA
4.3E-09	*Daphnia pulex*	Keith et al. (2016)	MA
5.2E-09	*Arabidopsis thaliana*	Jiang et al. (2014)	MA
5.4E-09	*Mus musculus*	Uchimura et al. (2015)	MA

Table A.1. (cont.)

5.5E-09	*Drosophila melanogaster*	Schrider et al. (2013)	MA
6.5E-09	*Arabidopsis thaliana*	Ossowski et al. (2010)	MA
6.8E-09	*Aphis mellifera*	Yang et al. (2015)	P-O
7.4E-09	*Arabidopsis thaliana*	Yang et al. (2015)	P-O
7.8E-09	*Prunus persica*	Xie et al. (2016)	P-O
9.8E-09	*Mesoplasma florum*	Sung et al. (2012)	MA
1.0E-08	*Daphnia pulex*	Keith et al. (2016)	MA
1.1E-08	*Homo Sapiens*	Roach et al. (2010)	P-O
1.1E-08	*Homo Sapiens*	Conrad et al. (2010)	P-O
1.2E-08	*Homo Sapiens*	Kong et al. (2012)	P-O
1.2E-08	*Homo Sapiens*	Campbell et al. (2012)	P-O
1.2E-08	*Pan troglodytes*	Venn et al. (2014)	P-O
1.5E-08	*Pan troglodytes*	Tatsumoto et al. (2017)	P-O
1.6E-08	*Homo Sapiens*	Wang and Zhu (2014)	P-O
2.0E-08	*Salmonella typhimurium*	Lind and Andersson (2008)	MA
3.3E-08	*Bacillus subtilis*	Sung et al. (2015)	MA

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