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Experimental Methods for Estimation of Plant Fitness Costs Associated with Herbicide-Resistance Genes

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Herbicide Resistance Genes and Plant Fitness

Since the beginning of agriculture, crops have been exposed to recurrent invasion by weeds that can impose severe reductions in crop quality and yield. There have been continuing efforts to reduce the impacts of weeds on production. More than 40 yr ago, overreliance on herbicide technology to reduce weed infestations resulted in the selection of adaptive traits that enabled weed survival and reproduction under herbicide treatments (Délye et al. 2007; Powles and Yu 2010; Vila-Aiub et al. 2008). As a result, herbicide resistance in > 200 weed species has evolved worldwide (Heap 2013; Powles 2008).

Resistant weeds are able to withstand the toxicity of herbicides because of the presence of resistance alleles originating from random DNA mutations (Powles and Yu 2010). These resistance alleles regulate a number of highly efficient, constitutive defense mechanisms that prevent herbicides from inhibiting key metabolic pathways. A set of defense mechanisms are involved in a reduction in herbicide that reaches the herbicide target protein (nontarget-site resistance) (Powles and Yu 2010). Mechanisms that (1) impair herbicide leaf uptake or translocation within plants via vacuolar sequestration or reduced cellular uptake, or (2) change the chemical properties of herbicides via herbicide-enhanced metabolism (detoxification) are included in this group (Ge et al. 2012; Nandula et al. 2008; Preston and Wakelin 2008; Preston et al. 1996; Sammons et al. 2010; Vila-Aiub et al. 2012; Wakelin et al. 2004).

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Another type of defense mechanism against herbicides involves a structural modification, via changes in the amino acid sequence, of the herbicide target protein, which minimizes herbicide binding (target-site resistance) (Powles and Yu 2010). Gene overexpression, resulting in the synthesis of excessive herbicide sensitive target protein because of promoter changes or gene amplification, is also regarded a target-site resistance defense mechanism (Dinelli et al. 2006; Gaines et al. 2010).

Target and nontarget-site herbicide resistance alleles protect plants from fatal damage caused by herbicides. In other words, the presence of resistance alleles minimizes the plant fitness reduction expected from herbicide activity. Fitness can be defined as the average success in producing offspring contributing to the next generation by a particular phenotype relative to another phenotype (Crawley 1997; Primack and Hyesoon 1989; Scott et al. 2006). A simple way to represent plant fitness (W) is given in Futuyma (2013):

$$W = P_{(S-R)}N \quad [1]$$

where P is the probability of the fraction of plants that survive from seed (S) dispersal to reproduction (R), and N equals the amount of offspring produced by adult plants.

An accurate estimation of the probability of survival to the age of reproduction ($P_{[S\text{-}R]}$) requires the estimation of many sequential processes of growth, development, and phenology. During these processes, many fitness components or traits, such as germination rate, growth rate, competitive ability, tolerance to pests, and seed dispersal, are likely to be correlated and interact with the environment (Roff 2002; Stearns 1989). Consequently, assessment of a single or a few fitness traits may lead to incorrect estimations of plant fitness.

Equation 1 highlights two important aspects of plant fitness. Fitness is a phenotypic response resulting from the combination of evolved life-history traits, and as such, it is significantly influenced by environmental and genetic variation

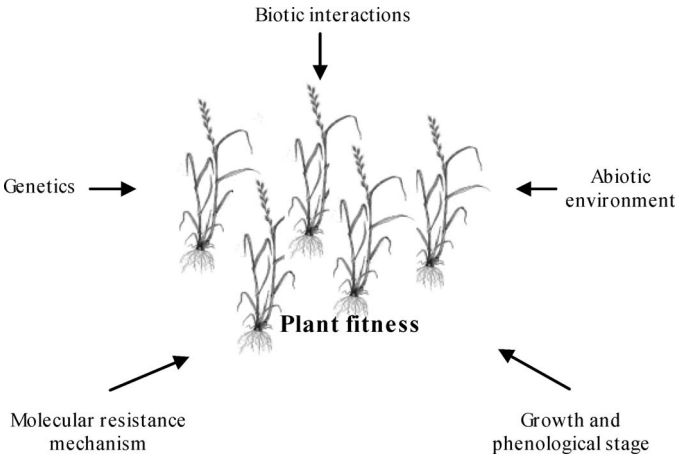

Figure 1. Plant fitness is the ultimate phenotypic expression resulting from the interaction of plant genetics (genetic background), molecular basis of resistance (resistance mechanism/gene/allele; allelic interactions and number of resistance alleles), and biotic and abiotic environment. Plant fitness is likely to change during particular growth and phenological stages.

(Figure 1) (Crawley 1997; Primack and Hyesoon 1989). Secondly, assuming the phenotype that produces the greatest number of offspring is the fittest could be erroneous because other traits, such as poor germination and establishment or decreased competitive ability or higher herbivory or some combination of these traits, may compensate or even overcompensate by a large production of seed (Primack and Hyesoon 1989).

Theoretical Considerations of Fitness Costs

In theory, herbicide resistance is expected to come with a fitness cost (*FC*). A fitness cost (sometimes named resistance cost) can be defined as the reduction of plant fitness in a herbicide-free environment caused by negative pleiotropic effects of resistance alleles on either or both fitness components (Equation 1). The reason for this is the generally low frequencies of herbicide-resistance traits that occur in plant populations in the absence of selection with herbicides (Jasieniuk et al. 1996; Preston and Powles 2002). There is considerable interest in fitness costs for herbicide resistance because these may lead to lower frequencies of resistant genotypes in weed populations when the herbicide selection is discontinued and may identify strategies for managing resistant weeds (Vila-Aiub et al. 2009b). The expectation of fitness cost associated with herbicide resistance genes are conceived from a fundamental, evolutionary "growth–defense" trade-off that contends that plant resource use is defined by two competing and mutually exclusive evolved functions: defense or growth and reproduction (Chapin et al. 1993; Herms and Mattson 1992; Purrington 2000). Plant strategies to adapt to environments differing in productivity and disturbance have resulted in clear resource-use trade-offs that ensure rapid growth and reproduction vs. survival and stress resistance (Grime 1977). According to this paradigm, a trade-off between traits promoting growth/fecundity and those promoting survival constrains the evolution of resistance because herbicide-resistant weeds would use resources for defense against herbicides that might otherwise be used for growth and reproduction and, consequently, will display a fitness cost, which will be more evident when herbicides are not used. In simple terms, if this growth–defense trade-off did not exist, then, all plants should be herbicide resistant. From an evolutionary perspective, a plant fitness cost associated with evolved herbicide resistance is seen as an "adaptation cost" to the herbicide (Vila-Aiub et al. 2011).

It is possible to speculate, then, that fitness costs can derive from pleiotropic effects of herbicide-resistance genes involving an extraenergetic expense, leading to higher costs of plant development and resource allocation that might otherwise be available to growth functions (Uyenoyama 1986; Vila-Aiub et al. 2009b). For example, this would likely be the case with constitutively enhanced–herbicide metabolism that involves the regulation of higher enzymatic activity (cytochrome [CYP]-450, glutathione *S*-transferases [GSTs]) (Reade et al. 2004; Siminszky 2006) and gene amplification, overexpression, or both, resulting in higher synthesis of messenger RNA (mRNA) and herbicide target protein (Gaines et al. 2010). Fitness costs associated with monooxygenase CYP-450 activity have been reported in herbicide-resistant plants and insecticide-resistant insects (Dasgupta et al. 2011; Hardstone et al. 2009; Vila-Aiub et al. 2005a, 2009a), although no costs associated with the amplification of the glyphosate target *EPSPS* gene have been identified (Giacomini et al. 2014; Vila-Aiub et al. 2014; Wang et al. 2013).

Fitness costs that originate from amino acid substitutions in herbicide-resistant target enzymes are more difficult to predict within the context of the plant resource–allocation theory. However, target-site herbicide-resistance mutations are also thought to have negative pleiotropic effects on normal plant metabolism (Preston et al. 2006; Vila-Aiub et al. 2009b). Many mutations within the target-site protein are expected to reduce normal

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substrate binding and/or alter enzyme activity and/or kinetic parameters (substrate affinity [K_m], feedback inhibition, maximal velocity [V_{max}]) that translate into shortage, excess, or imbalance of products or substrates or both; therefore, fitness costs are likely to manifest at the plant level (Menchari et al. 2008; Tardif et al. 2006; Vila-Aiub et al. 2009b; Yu et al. 2010).

When fitness costs are driven by ecological interactions, such as, for instance, plant competition or predation, they are often termed ecological fitness costs (Vila-Aiub et al. 2009b). This would be the case when, compared with plants carrying herbicide susceptible alleles, resistant plants display a reduction in fitness because they attract either less pollinators or more pests (Gassmann and Futuyma 2005), or they exhibit a reduction in the ability to compete for resources (Vila-Aiub et al. 2009a).

Despite the anticipated evolutionary constraint, no universality in the expression of fitness costs associated with herbicide resistance alleles has been found. Fitness costs have been shown to exist depending on (1) the particular herbicide-resistance defense mechanism (Vila-Aiub et al. 2005a) and resistance allele (Menchari et al. 2008), (2) the pleiotropic effects on the kinetics of herbicide target proteins (Ashigh and Tardif 2007; Purrington and Bergelson 1999; Yu et al. 2010), (3) the dominance of the fitness cost (Roux et al. 2004), (4) the genetic background (Paris et al. 2008), and (5) the environmental abiotic and biotic conditions. Moreover, and in clear contradiction with the growth–defense trade-off prediction, a plant-fitness advantage associated with particular herbicide-resistance genes and alleles have been reported (Wang et al. 2010, 2013).

Herbicide Resistance Evolution and Role of Fitness Costs

Two conditions must be met for herbicide resistance to evolve: an adaptive trait must endow a fitness (W) advantage under herbicide selection (the so-called *resistance benefit* [RB]), and this advantage should exceed any fitness cost (FC) associated with the resistance-defense trait (Beckie et al. 2000; Simms and Rausher 1987; Vila-Aiub et al. 2009b). Provided a positive, net fitness difference ($RB > FC$) exists, rapid herbicide-resistance evolution is possible, especially if the resistance traits endow a substantial fitness benefit under herbicide selection and show no or negligible fitness costs. Conversely, resistance traits or resistance-defense mechanisms in weeds endowing only a marginal resistance benefit when exposed to herbicides and showing a significant fitness cost ($RB < FC$) are likely to evolve more slowly (Gressel and Segel 1990; Jasieniuk et al. 1996; Maxwell and Mortimer 1994). As a result, it is evident that fitness costs have a fundamental role in predicting the spread of herbicide-resistance alleles and determining their population equilibrium frequencies under a range of environmental conditions (see reviews by Bergelson and Purrington 1996; Vila-Aiub et al. 2009b, 2011).

The goal of this contribution is to provide a conceptual and methodological framework for a correct understanding and estimation of fitness costs associated with herbicide-resistance genes.

Establishing and Testing a Hypothesis

There are inherent difficulties in measuring the expression and magnitude of fitness costs associated with herbicide-resistance genes. Reviews of fitness-cost studies have concluded that many studies assessing fitness costs are fundamentally flawed, and as a result, their conclusions are ambiguous and difficult to interpret (Bergelson and Purrington 1996; Vila-Aiub et al. 2009b). A correct evaluation of fitness costs relies on the correct understanding of plant fitness and its inherently dynamic process (Mills and Beatty 1979; Orr 2009).

A common failing of fitness studies on herbicide resistance is the failure to establish and test an appropriate hypothesis. There are numerous examples of studies where the growth of a resistant population is compared with a susceptible population with or without competition and claims made about fitness of the resistance trait. Such a study compares the relative growth (and competitiveness) of two populations and provides little information regarding the resistance allele. To test the fitness associated with a resistance trait, the resistance allele has to be separated from all other alleles that differ between the resistant and susceptible populations. How to achieve this is discussed below.

A second common failing is the assumption that early biomass production is reflective of the true cost of a fitness allele. Although biomass is generally correlated with seed production (Weiner et al. 2009), many other factors can contribute to fitness. For example, there may be differences in seed size, resulting in fewer seed (Pedersen et al. 2007); differences in seed dormancy (Dyer et al. 1993); differences in plant maturity (Purba et al. 1996); or differences in pollen production, which may

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contribute to overall fitness. To unambiguously measure fitness of a resistance allele, a multigenerational study measuring the frequency of resistance alleles in the population is required. This is discussed in more detail below.

A third assumption made is that measurements of the biomass of plants in the absence of competition under glasshouse conditions are representative of what will happen in the field. Controlled conditions are useful for measurements of components of fitness, but fitness components measured under controlled conditions may be swamped by other factors in the field. Given the environmental, abiotic changes driven by weed–weed and weed–crop competitive interactions, resource competition is thought to trigger or magnify negligible fitness costs expressed in the absence of plant competition (Jordan 1999). Measuring fitness in the absence of intraspecific or interspecific competitive interactions may underestimate the size of any fitness cost.

Fitness Tip 1. Establish a hypothesis, and select methods that directly test the hypothesis.

Managing for Genetic Background Effects

A fitness cost is estimated as the difference in fitness (*W*) between a herbicide-resistant (*R*) and -susceptible (*S*) genotype. Fitness costs are estimated in herbicide-free environments because any herbicide treatment would confound the outcome because of the large fitness benefit endowed by the resistance trait under herbicide selection, masking the potential expression of fitness costs. The absence of herbicide selection when estimating a fitness cost makes the fitness assessment as complex as it needs, as shown in Equation 1, to encompass different life-history traits to determine survival probabilities and growth preceding and following the plant reproductive stage (Vila-Aiub et al. 2009b).

Before any empirical assessment of fitness costs (see below for methodological options), a fundamental experimental requirement needs to be fulfilled. Herbicide resistant and susceptible individuals or genotypes must share the same genome except for that gene or genes endowing resistance. The control of genetic background in fitness-cost studies has been largely overlooked (Bergelson and Purrington 1996; Vila-Aiub et al. 2009b); therefore, attribution of many observed fitness results to the herbicide resistance–endowing gene or genes is equivocal. Extensive discussion on methods for genetic background control can be found elsewhere (Strauss et al. 2002; Vila-Aiub et al. 2011) but will be briefly summarized here.

The ideal way of controlling for background genetic effects is through the creation of near-isogenic lines (Gressel and Bensinai 1985; Hart et al. 1992). Near-isogenic lines create two populations that differ by only a few alleles, including the resistance allele. Therefore, any difference identified during the fitness study is more likely to result from the effect of the resistance allele. Creation of near-isogenic lines is time consuming, taking five to eight generations and is often difficult in weed species. The creation of near-isogenic lines requires the ability to easily cross individuals of the species. The resistant plant needs to be crossed with the susceptible plant, and then, a series of backcrosses, usually to the susceptible genetic background, is performed. In each generation, 50% of the alleles from the resistant parent will be removed. After seven generations of backcrosses, the two populations should differ at $< 0.5\%$ of alleles. However, linkage groups will tend to remain intact, so alleles located close to the resistance allele on the genome will be inherited along with the resistance allele.

Isogenic lines may also be made using the transgenic approach, which involves the introduction of a resistance gene via a vector (of bacterial origin in most cases) into a susceptible genetic background. The gene is introduced into a background in which it was absent or not functional to obtain plants whose genomes differ solely because of the presence of the resistance gene. Any fitness differences between susceptible and transgenic resistant lines may not, however, be entirely due to the effect of the resistance gene. The introduction of foreign DNA may modify the expression of the genome near the site of insertion, and that may affect the expression of the resistance gene. Thus, several, independent transgenic lines have to be generated to account for those positional effects (Bergelson et al. 1996). Inclusion of marker genes, such as antibiotic resistance, as part of the transgene makes it possible to identify plants that have been successfully transformed. However, pleiotropic effects of those linked marker genes may affect the adaptive value associated with a resistance gene. Transgenic lines are usually backcrossed with the herbicide susceptible wild type genotype to obtain homozygous lines with and without the transgene. A few published studies have demonstrated the enormous potential of this technique for assessing fitness costs in a completely controlled genetic

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background (Al-Ahmad and Gressel 2006; Al-Ahmad et al. 2005, 2006; Bergelson et al. 1996; Burke and Rieseberg 2003; Jackson et al. 2004; Purrington and Bergelson 1997, 1999).

An alternative approach is to select resistant and susceptible individuals from within a population (Vila-Aiub et al. 2005b). This approach assumes that the background genetics of the susceptible individuals in the population is the same as that of the resistant individuals. This assumption needs to be tested. The assumption is unlikely to be true for self-pollinated species and may not be true for outcrossing species. Although selecting resistant and susceptible individuals from the same population is a relatively quick and simple approach to the creation of populations, it should not be adopted without an understanding of the diversity of background genetics in the population (Vila-Aiub et al. 2011).

A third approach is to use artificially created, segregating F_2 populations (Giacomini et al. 2014; Preston et al. 2009; Roux et al. 2004). This approach distributes the background genetics among the various resistance genotypes. This is useful when a single allele confers resistance but breaks down when resistance is polygenic. This approach can be time consuming because it requires two generations to create a segregating population, but it is not as time consuming as, and is simpler than, creating near-isogenic lines. However, it is likely to increase the variability associated with measurements, and so, small fitness costs may be missed.

Another approach to reduce the effect of differences in genetic background is the exploration of resistance costs by comparison of many herbicide resistant and susceptible populations (Cousens et al. 1997; Strauss et al. 2002). This experimental design assumes that a significant difference in the mean value between compared *R* and *S* accessions increases the probability that those differences are caused by fitness costs associated with resistance alleles, minimizing the chances for a type I error. An elegant study has identified a significant fitness cost associated with the Trp-574-Leu *ALS* gene mutation in Powell amaranth (*Amaranthus powellii* S. Wats.) after comparing a set ($n = 6$) of field-collected, resistant and susceptible populations (Tardif et al. 2006). For a proper comparison to be made, this method requires all resistant genotypes to have identical resistance alleles (i.e., Trp-574-Leu) because different alleles may be associated with quantitatively different magnitudes of fitness costs (Roux et al. 2004; Vila-Aiub et al. 2009b). The multiple population procedure does not minimize the differences in gene pools of the resistant and susceptible lines compared, but one of its advantages is that helps determine the importance of different genetic backgrounds in influencing the fitness of the resistance genes (Paris et al. 2008). This experimental protocol has been one of the most-employed protocols in the literature to estimate fitness costs (Vila-Aiub et al. 2011).

Fitness Tip 2. Create a population where background genetic effects on fitness are minimized.

Understanding the Molecular Biology and Biochemistry of Resistance

The identification of resistance genes and the characterization of physiological bases of herbicide resistance is important to the interpretation and estimation of fitness costs. More than one mechanism may endow resistance to the same herbicide, and individual plants may possess more than one resistance mechanism to a particular herbicide. For example, either or both CYP-450 or GST-based, enhanced herbicide metabolism and target-site mutations in the *ACCase* gene may endow resistance to acetyl-coenzyme A carboxylase (ACCase)-inhibiting herbicides in blackgrass (*Alopecurus myosuroides* Huds.) and rigid ryegrass (*Lolium rigidum* Gaudin) populations (Cocker et al. 1999; Preston et al. 1996; Tardif and Powles 1994). Moreover, target-site resistance to ACCase-inhibiting herbicides may be conferred by different point mutations in the *ACCase* gene, each translating into different resistance codons at the protein level (i.e., different alleles) (Délye 2005; Powles and Yu 2010; Tranel and Wright 2002; Yu et al. 2007). Another example of diversity of resistance mechanisms to a particular herbicide is given by a *EPSPS* gene target-site mutation and amplification and by vacuolar sequestration, all conferring resistance to glyphosate (Ge et al. 2010; Preston et al. 2009; Wakelin and Preston 2006). Empirical studies have shown that, although some resistance mechanisms exhibit negligible fitness costs, others are associated with significant costs (Vila-Aiub et al. 2009b). Studies with field-evolved weeds and laboratory model species have reported that different resistance mutations in the same gene may differ in their effects on fitness (Menchari et al. 2008; Roux et al. 2004; MM Vila-Aiub et al., unpublished data). The

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ultimate expression of a fitness cost has also been shown to depend on the number of mutated alleles (Menchari et al. 2008; Roux et al. 2004). In diploid species, for example, costs associated with a particular resistance gene may be evident in homozygous, but not heterozygous, resistant plants (i.e., recessive fitness cost) (Roux and Reboud 2005; Roux et al. 2004). This is important because a resistance allele that is dominant ($RR = RS > SS$) in the presence of the commercial herbicide dose and recessive ($SS = RS > RR$) for a fitness cost is likely to increase in frequency over a short period.

For studies estimating fitness costs, characterization of the molecular and biochemistry bases of resistance can be helpful. This will enable any fitness cost to be ascribed to a particular resistance mechanism and, if known, the specific allele. Conversely, when this information is unknown, there is a risk that fitness-costs estimations could represent the combined effects of dissimilar resistance mechanisms, genes, and alleles on plant fitness. This may be the case when evaluating fitness costs in several resistant populations, each displaying a different resistance mechanism and allele (Powles and Yu, 2010), or in a multiple herbicide-resistant population in which two or more resistance alleles are present (Délye et al. 2013). In neither case would it be possible to attribute the expression of fitness costs to a particular resistance mechanism or allele.

Fitness Tip 3. Where possible, understand the genetic mutations and biochemical basis of resistance in the population. This will allow you to attribute the fitness cost to a particular resistance mechanism or allele. Identifying a specific mutation will allow tracking of the frequency of that mutation across generations.

Estimating Fitness Costs through Changes in Herbicide Resistance Frequency over Time

According to the definition of fitness, the contribution of genes to the next generation in a given environment depends on the rates of plant survival and reproduction. We consider a herbicide resistance (R) and susceptible (S) allele in a population of a diploid weed species whose frequencies are, respectively, denoted as p and q. Under the condition of random mating among individuals carrying those alleles (RR, RS, and SS) and absence of any evolutionary force such as migration, genetic drift and selection (equal fitness between plants carrying R and S alleles), the equilibrium frequency of the R allele (assuming complete dominance of resistance) should equal $p^2 + pq$. After one generation, the frequencies of both the R and S ($q = 1 - p$) alleles should remain invariant over time as long as the conditions mentioned above continue (Hardy-Weinberg equilibrium).

The starting hypothesis is that plants carrying R alleles (RR and RS) exhibit fitness (W_{RR} and W_{RS}) less than (e.g., 80%) than plants carrying two S alleles (W_{SS}). This is, herbicide-resistant plants, both RR and RS, will exhibit reduced survival or reproduction or both, compared with herbicide-susceptible plants (SS), which can be denoted as $W_{RR}/W_{SS} = W_{RS}/W_{SS} = 0.8$. This means that RR and RS plants will exhibit a selection coefficient (s) of 0.2, which represents a decrease in fitness associated with the resistance allele (i.e., 20% fitness cost). Under the conditions of constant fitness cost over generations and no herbicide selection (but rather, natural selection), the ratio of the different genotypes (RR, RS, and SS) is determined by both their frequencies and their fitness. The new equilibrium accounting for the frequency of the herbicide R allele (p_{n+1}) expressing a fitness cost will equal $p\{[p(1 - s) + q]/(1 - 2pqs - p^2s)\}$. As a result, R and S alleles will become relatively less and more common in the population, respectively. Then, the single-generation rate of frequency decrease of the herbicide R allele will be equal to $\Delta p = (p_{n+1} - p = spq^2)/(1 - 2pqs - p^2s)$, which will show a decline in the phenotypic resistance frequency over time (Gillespie 1998; Orr 2009).

Based on the theoretical considerations mentioned above, the most robust methodological protocol to estimate fitness costs is the study of herbicide-resistance allelic, genotypic, or phenotypic frequencies (or some combination) in isolated or interconnected populations over many generations (Vila-Aiub et al. 2011). A costly resistance allele will decrease in frequency over time, and significant deviations of the observed resistance frequencies from the expected resistance frequencies (Hardy-Weinberg equilibrium) in the absence of herbicide selection should provide clear evidence for the presence of fitness costs. Experiments set at diverse agroecological conditions imposing weed–crop (competition) and weed–pest (herbivory, parasitism) interactions and contrasting resource limitations

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(nutrients, irradiance) is a useful approach to estimate changes in the frequency of the resistance allele over time. The validity of the results obtained from this experimental approach depends on proper control of genetic background in large populations (to overrule potential genetic drift) with a known initial frequency of the resistance allele or genotype or phenotype. After each generation, genotyping (if the molecular genetic basis of resistance is known) or phenotyping of several hundred individuals is required to ascertain changes in resistance frequency. Despite the power of this method to estimate costs, it does not enable the identification of the particular plant traits or fitness components involved in the expression of costs. Only a few studies have employed this approach to assess potential fitness costs associated with alleles involved in target- and nontarget-site resistance (Preston et al. 2009; Roux et al. 2005). For polyploid species with multiple *R* and *S* alleles, it is also possible to account for any deviation from the expected equilibrium allelic frequencies due to expression of fitness costs associated with resistance.

The same methodological principle applies when the molecular bases of resistance are unknown (e.g., CYP-450 herbicide metabolism). In this case, the change in the phenotypic resistance frequency over several generations, instead of the genotypic frequency, from a known initial frequency is the parameter evaluated (Vila-Aiub et al. 2009b).

Some experimental issues need to be considered when assessing fitness costs through this multigenerational study. When assessing the changes in allele frequencies due to differential fitness costs attributed to *R* and *S* alleles, it is also necessary to account for allele frequency deviations resulting from random genetic drift, which may occur in populations of finite size. Thus, allele frequency deviation arising from directional selection on fitness differences need to be compared with the expected variance in allele frequencies from genetic drift alone (Falconer and Mackay 1996). The initial population size is an important experimental parameter that will affect the chances for drift to occur. The chance that allele frequency changes are due to drift is slower in populations with larger effective sizes (N_e) than it is in those with smaller effective sizes (Wright 1931). Similarly, a large number of replicates and plant generations will ensure a higher probability of identifying relatively low fitness costs. For example, changes in resistance allele frequencies were estimated in a population size of 120 individuals in the monoecious species mouse-ear cress [*Arabidopsis thaliana* (L.) Heynh.], assuming an outcrossing rate of 2% with no overlapping generations, with 12 experimental replicates for three to seven plant generations (Roux et al. 2005). In an obligate, cross-pollinated, monoecious species (blackgrass), changes in allele frequencies associated with CYP-P450-enhanced herbicide metabolism were observed in three replicated populations of 100 individuals after three generations (MM Vila-Aiub et al., unpublished data).

Ideally, a 1 : 1 ratio of resistance : susceptible alleles (or phenotypes) represents the genotypic (or phenotypic) frequency at the initial generation. For those cases in which resistance is controlled by a single, major, known gene, the ratio of resistance : susceptible alleles is achieved after estimation of the frequency of *RR*, *RS*, and *SS* genotypes in the weed population.

A number of diverse agroecological conditions should be simulated in vegetation-free experimental plots to identify environmentally induced fitness costs: both intraspecific competitive interactions between plants carrying either the resistance or susceptible allele, and interspecific competition among herbicide-resistant and -susceptible individuals and a crop species. Variations in the level of resources via applied specific changes of, for instance, nitrogen or water, or indirectly through changes in weed–crop plant densities, are potential scenarios that are useful for magnifying the expression of fitness costs (Vila-Aiub et al. 2009b). The choice of the crop and its density should be established according to the specific weed species and their natural coexistence in agroecosystems. Similarly, to determine the size of the plots, the size and growth habit of the weed and crop species should be taken into account, which will, thus, minimize any border effect and magnify the biotic interactions among plants.

After the timely seeding (or transplanting) of both the resistant and susceptible weed genotypes (or phenotypes) and crop, in which the weed genotypes are placed randomly between the crop rows, emergence of seedlings from other invasive weed species should be eliminated. Ideally, all seeds produced by the resistant and susceptible weed genotypes (or phenotypes) should be collected and mixed at the end of each generation. A proportion of those collected seeds (e.g., 500 to 1,000) should be kept at laboratory conditions for further genotyping (or phenotyping), whereas the remaining seeds should be randomly dispersed back into the corresponding experimental plot.

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The management of the crop should include annual sowing (for those annual crops) and all agronomic practices (fertilization, irrigation, etc.) usually applied for the specific crop, except for the application of herbicides for which the resistance allele endows protection. Consequently, a significant increase in density of the weed genotypes (or phenotypes) is often observed after the first generation. Because this weed-density increase may jeopardize the crop growth, a reduction in the number of weed seeds, collected randomly from several samples across the field plots, returned to the field after each generation may be required. The number of generations necessary to observe any genotypic deviations from the initial frequency will depend on the magnitude of the fitness cost. For example, within the first three generations (i.e., years), significant declines in the resistance frequency associated with glyphosate vacuolar sequestration and enhanced CYP-450 herbicide metabolism has been observed in rigid ryegrass (Preston and Wakelin 2008; MM Vila-Aiub et al., unpublished data).

Unless the experimental protocol involves the assessment of fitness costs in interconnected populations where a resistance cline is established through the balance of migration–selection processes (Roux et al. 2006), gene flow (pollen, seeds) among experimental plots should be minimized. This is especially true for cross-pollinated species. Enclosures or live crop borders in each plot will minimize the pollen movement among the experimental units and ensure that changes in *R* and *S* allele frequencies result from selection process within each experimental treatment.

A decline in resistance frequency over time represents the ultimate goal of any herbicide-resistance management program, and a number of potential, reactive agronomic strategies may be applied to exploit the fitness cost of resistance genes. For example, the inclusion of pasture phases in crop rotations, increasing crop seeding rates or tactics that reduce seed set may provide situations where the fitness costs can be exploited to reduce the frequency of resistance alleles in populations (Powles and Holtum 1994; Vila-Aiub et al. 2005b, 2009a).

Fitness Tip 4. A multigenerational study in which the frequency of herbicide resistance alleles is measured across generations will provide the most accurate measure of a fitness cost.

Managing for Environmental Effects

Variations in fitness costs are expected under a gradient of ecological interactions (competition, herbivory, disease) and abiotic conditions (light, temperature, nutrients) and, thus, should be considered when estimating fitness costs. For example, competitive interactions, insect herbivory, and light intensity have been shown to amplify the magnitude of fitness costs associated with enhanced CYP-450 herbicide metabolism, chloroplastic *psbA* gene in the photosystem II D1 protein, and *ALS* gene mutation, respectively (Ashigh and Tardif 2009; Gassmann 2005; Vila-Aiub et al. 2009a). In other cases, resistance genes have been shown to have significant pleiotropic effects on germination traits under particular thermal and light regimes (Délye et al. 2013; Dyer et al. 1993; Vila-Aiub et al. 2005b). As mentioned above, pleiotropic effects of resistance alleles on traits at particular life or phenological stages are difficult to translate into effective fitness-cost expression until the effect of those resistance alleles throughout life history processes are evaluated, which is especially evident in the presence of trade-offs between different life history stages, and hence, changes in one component of plant fitness may involve compromises in other fitness traits (Harper 1977). Nevertheless, identification of the effects of resistance alleles on individual fitness traits is likely to provide knowledge for the adoption of agronomic tools to manage herbicide-resistant weed populations.

The experimental conditions under which fitness of *R* and *S* phenotypes is evaluated are likely to influence the result. Evaluation of fitness traits associated with herbicide-resistance traits in isolated plants under controlled conditions (laboratory, growth-chamber, or greenhouse) are valuable in determining underlying physiological mechanisms associated with fitness costs (i.e., physiological costs). For example, reduced photosynthesis rates evaluated under controlled environmental conditions are evident when a target-site mutation (Ser-264–Gly) in the *psbA* gene is present in various weed species (Holt and Thill 1994). Similarly, impaired relative growth rates (RGR) associated with CYP-450-enhanced herbicide metabolism are revealed in rigid ryegrass plants growing under controlled conditions (Vila-Aiub et al. 2005a). However, the true extent of the fitness cost may only be evident when plants are exposed to the full range of environmental interactions. Whereas some interactions may be expressed under experimental

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controlled conditions (resource competition), others, such as predation or parasitism, are more likely to be expressed under natural or field conditions (i.e., ecological costs). When plants with either the Ser-264–Gly point mutation or CYP-P450-enhanced herbicide metabolism were grown under competitive interactions, higher susceptibility to pathogens and impaired competitive ability, respectively, were observed (Salzmann et al. 2008; Vila-Aiub et al. 2009a). Both physiological and ecological fitness costs merit consideration for the design of experiments to assess fitness costs associated with herbicide-resistance alleles.

In agricultural systems, there is likely to be as much competition from the crop as from other genotypes of weeds. Therefore, to identify the full ecological impact of fitness costs, it is essential that fitness experiments be conducted in an agricultural environment. This means sowing the various genotypes or segregating populations into a crop or pasture situation (Jordan 1999; Williams et al. 1995). If it is desirable to understand the fitness cost in a different environment, then the genotypes or segregating population have to be grown in that environment.

Fitness Tip 5. Environment can have an effect on the expression of fitness costs. The appropriate environmental conditions need to be chosen for the hypothesis being tested.

Estimating Fitness through Identification of Modified Plant Traits

Another major methodological approach to estimate fitness costs associated with herbicide-resistance genes consists of identifying negative pleiotropic effects on plant traits that affect whole-plant fitness. The assessment of fitness costs may be performed through the comparison of fitness traits between discrete resistant and susceptible phenotypes (or genotypes). This will be possible when resistance is controlled by a single major gene, and thus, it is possible to identify, segregate, and select discrete resistant and susceptible lines that share a common genetic background, except for the resistance gene/allele. There is an extensive number of experimental protocols that enable the identification and selection of herbicide resistant and susceptible discrete lines in weed populations (Vila-Aiub et al. 2011).

The determination of a fitness cost associated with a given herbicide-resistance gene must encompass the study of phenotypic fitness traits at all plant stages at the population level. This requirement becomes evident because assessment of plant fitness is only possible after estimation of plant survival and growth rates and reproductive traits (Equation 1). It is possible to establish the different and successive life stages that determine the population structure (age- or size-structured). In herbaceous plants, the establishment of stages based on age is difficult; therefore, stages are usually based on size. For simplicity, consider an annual life cycle that consists of the following stages: seed, seedling, young adult or nonreproductive adult, and reproductive adult. Only a proportion of individuals progress through the successive stages, and this is given by the transition rate (e.g., seed/seedlings; seedlings/young adult plants, young adult plants/reproductive adults; reproductive adults/seed). The two main components of fitness are survival (involving the first three transitions) and reproduction, which is the size of the progeny (e.g., seed number) from adult reproductive plants.

Fitness Tip 6. When assessing plant traits to identify fitness costs, measure transition rates.

Variation in seed-related traits (i.e., viability, longevity, vigor, dormancy, size, etc.) has major implications for population demography in general and for resistance evolution of weeds in particular. The importance of seed dynamics in the soil depends on whether the species in a specific environment forms a persistent (i.e., seed dormancy) or transient seed bank. If persistent, the soil seed bank may serve as reservoir of *R* or *S* alleles and as a strategy for spreading seedling recruitment in time. Seed dormancy associated with *R* alleles may offset the expression of associated fitness costs over time (Owen et al. 2011). Differences in seed dispersal, dormancy and germination may be due to differences in seed size, dispersal structures, or seed coat thickness. Larger seeds may provide benefits to seedlings against competition, herbivory, and nutrient limitation but be more susceptible to predation and show low dispersal rates (Gómez 2004; Jakobsson and Eriksson 2000; Jurado and Westoby 1992; Reader 1993; Westoby et al. 1996). On the other hand, small seeds, usually associated with low germination rates and poor growth in highly competitive communities, may experience less

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predation and high dispersal rates (Gómez 2004; Reader 1993; Westoby et al. 1996). This suggests that the overall fitness consequence of selection of a particular fitness trait at a particular developmental or phenological stage or a particular environment that may be seen as advantageous may show a trade-off at a different phenological stage or environment. For this reason, an accurate estimation of a fitness cost should consider the estimation of several fitness traits during the entire life cycle of the weed species in natural conditions in which ecological interactions may take place.

Seeds may differ in longevity (the ability to maintain viability) and the relative importance of this trait will depend on whether seeds are buried or germinate on the soil surface (tillage vs. nontillage systems) (Chauhan et al. 2006; Gundel et al. 2008). The resistance trait in a population may also be associated with the overall level of dormancy or a specific requirement of environmental stimuli for germination (Délye et al. 2013; Dyer et al. 1993; Gundel et al. 2008; Owen et al. 2011; Vila-Aiub et al. 2005b). For example, rigid ryegrass plants carrying the Ile-1781–Leu *ACCase* gene mutation exhibit a strong requirement of light and fluctuating temperatures to germinate and emerge from soil burial (Vila-Aiub et al. 2005b). These are well-known traits with adaptive value by which seeds may adjust their germination timing to take advantage of safe sites to emerge (at least in terms of competition). Taken together, variability in these traits may create small differences at the beginning of the life cycle that, in combination with particular ecological scenarios, may trigger differential fitness of resistant and susceptible populations (Weiner 1990; Weiner and Thomas 1986).

The seedling stage is a key developmental period determining the rate of transition to "young, nonreproductive adults." This key event, with significant implications for population dynamics and demography, involves the change from the heterotrophic to the autotrophic state, which coincides with the highest ratio between leaf area and biomass. At the seedling stage, individuals, particularly herbicide-resistant seedlings, may show higher susceptibility to pathogens and pests (Gassmann 2005; Gassmann and Futuyma 2005; Salzmann et al. 2008); exhibit impaired growth, assimilation rates, and resource competitive ability; and resource competition from larger individuals (Holt and Thill 1994; Menchari et al. 2008; Vila-Aiub et al. 2005a, 2009a). The phenotypic variability on the relative growth rate of seedlings and young adults from resistance alleles may affect the transition rate from one stage to the next. These linked traits are likely to affect the relative performance and fitness of *R* and *S* phenotypes (genotypes) in absence of herbicide selection.

Assuming similar survival, transition rates of herbicide resistant and susceptible phenotypes (or genotypes) in the absence of herbicide selection (the first parameter in Equation 1), progeny size (i.e. seed number) may be regarded as the ultimate trait defining potential differences in fitness associated with herbicide resistance and susceptible alleles. However, in assessing seed number, there is a risk of accounting for female reproductive fitness only. In an outcrossing species, where coexisting resistant and susceptible individuals are allowed to cross freely, estimation of seed production will only account for resistant and susceptible female reproductive fitness. This is evident if, for example, susceptible plants provide most of the pollen. In such a situation, measuring and comparing only the seed number of coexisting resistant and susceptible plants will overestimate the reproductive fitness of the resistance allele. To avoid this confounding effect, estimation of production, viability, growth, and competition of resistance and susceptible pollen will be necessary (Delph et al. 1998; Song et al. 2002). Assessment of resistance vs. susceptible pollen discounting in weed species with both selfing and outcrossing reproductive systems will also ensure a better estimation of potential fitness costs (Chang and Rausher 1998).

Alternatively, estimation of seed number and mass should be conducted in isolated stands of discrete herbicide resistant and susceptible phenotypes (or genotypes). However, this may not identify differences in pollen competition between resistant and susceptible plants and thus mask fitness costs of the resistance allele. Finally, genotypic determination of seeds produced in mixed resistant and susceptible populations can be used to estimate pollen fitness effects.

Fitness Tip 7. Consideration needs to be given to the effect of male fitness as well as female fitness in seed production. One way to do this is to measure the frequency of resistance alleles in progeny.

Differences in the relative allocation of resources to seeds (harvest index) as well as the trade-off between seed number and seed mass can also be

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modified by the evolution of herbicide resistance (Vila-Aiub et al. 2009a). Likewise, small differences in the relative allocation to sexes, that is, to ovules and pollen production in monoecious or dioecious plants, may have important implications on fitness. Pollen discounting (Harder and Wilson 1998), apomixis (Ribeiro et al. 2013), and protandry or protogyny associated with herbicide resistance alleles are potential biological processes that may affect the dynamics of resistance genes in the agricultural landscape. Once the fitness traits defining plant survival probabilities or reproductive ability of discrete resistant and susceptible genotypes comprising a population in a particular environmental condition are evaluated, then, the magnitude (%) of a fitness cost (*FC*) may be quantified as follows:

$$FC = 1 - (W_R / W_S) \times 100 \quad [2]$$

where *W* denote fitness, in the sense of Equation 1, of resistant (*R*) and susceptible (*S*) weed genotypes.

The identification and selection of discrete resistant and susceptible plants to quantify and compare fitness traits becomes difficult and less accurate (if not impossible) when resistance segregates as a phenotypically continuous quantitative variation. This would be the case for resistance controlled by multiple additive genes (Busi et al. 2013), by single major genes in polyploid species (Yu et al. 2012), or by single genes with different levels of amplification or expression within individuals (Gaines et al. 2010). Hence, the recognition of modified plant-fitness traits associated with these resistance alleles requires either the identification of seed families or single individuals in a range of increasing levels of resistance (Baucom and Mauricio 2004; Cockerton 2013; Vila-Aiub et al. 2011). For example, whereas increasing amplification of the *EPSPS* gene in Palmer amaranth (*Amaranthus palmeri* S. Wats.) has been shown to endow higher glyphosate resistance, no evidence of pleiotropic effects on various fitness traits has been found (Giacomini et al. 2014; Vila-Aiub et al. 2014).

Concluding Remarks

In seeking to address fitness costs associated with herbicide resistance genes and alleles, it is important to start with the correct plant material. Much of the literature on the topic is of little value in addressing fitness costs because it compares populations from different locations that differ by more than the resistance alleles. Two populations from adjacent fields may have different cultural histories as well as different herbicide histories that select for different traits. Minimizing the differences between genotypes by using near-isogenic lines or by averaging the background genetics across genotypes allows the effect of the resistance trait on fitness to be more accurately identified.

It is also important to match the experimental protocol to the hypothesis being tested. Frequently, fitness cost studies estimate ecological fitness by measuring biomass under glasshouse conditions. Such an experimental approach does not measure ecological fitness. Measurements of ecological fitness require the genotypes to be grown under the conditions in which they would normally grow. If the hypothesis is to address some of the components of fitness, such as biomass accumulation, then that can be done in the glasshouse. Tracking the frequency of herbicide resistance alleles over several generations provides the most accurate measure of total fitness costs. However, that approach is rarely useful in identifying the factors that contribute to the fitness cost and has to be supplemented by experiments under more-defined conditions.

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