

## Review: Confirmation of Resistance to Herbicides and Evaluation of Resistance Levels

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As cases of resistance to herbicides escalate worldwide, there is increasing demand from growers to test for weed resistance and learn how to manage it. Scientists have developed resistance-testing protocols for numerous herbicides and weed species. Growers need immediate answers and scientists are faced with the daunting task of testing an increasingly large number of samples across a variety of species and herbicides. Quick tests have been, and continue to be, developed to address this need, although classical tests are still the norm. Newer methods involve molecular techniques. Whereas the classical whole-plant assay tests for resistance regardless of the mechanism, many quick tests are limited by specificity to an herbicide, mode of action, or mechanism of resistance. Advancing knowledge in weed biology and genomics allows for refinements in sampling and testing protocols. Thus, approaches in resistance testing continue to diversify, which can confound the less experienced. We aim to help weed science practitioners resolve questions pertaining to the testing of herbicide resistance, starting with field surveys and sampling methods, herbicide screening methods, data analysis, and, finally, interpretation. More specifically, this article discusses approaches for sampling plants for resistance confirmation assays, provides brief overviews on the biological and statistical basis for designing and analyzing dose–response tests, and discusses alternative procedures for rapid resistance confirmation, including molecular-based assays. Resistance confirmation procedures often need to be slightly modified to suit a specific situation; thus, the general requirements as well as pros and cons of quick assays and DNA-based assays are contrasted. Ultimately, weed resistance testing research, as well as resistance management decisions arising from research, needs to be practical, feasible, and grounded in science-based methods.

**Key words:** Dose–response assay, molecular-based assay, quick tests, sampling, whole-plant assay.

Resistance to herbicides is undoubtedly among the primary concerns in modern agriculture. Since the first report of resistance to 2,4-D in 1957 in wild carrot (*Daucus carota* L.) (Switzer 1957), resistance to herbicides has ballooned to include over 200 species worldwide involving at least 20 modes of action (Heap 2012). Accurate and timely diagnosis is crucial to resistance management and mitigation. Ideally, growers should be managing crop production fields to delay the onset of resistance to herbicides or avert weed population shifts that would make weed management difficult or uneconomical. In reality, growers adopt the most convenient and economical crop production practices until a critical event, such as weed resistance, forces a change in practices. Thus, close monitoring of weed populations and detection of resistance early and fast, are crucial to avert economic losses. Additionally, such monitoring efforts can enable the tracking of resistance across broad geographies. General guidelines for resistance confirmation are summarized by Moss (1999). Expanding on these guidelines will help weed science practitioners choose, modify, or design appropriate protocols for resistance testing to suit different situations. Beckie et al. (2000) presented a thorough review of resistance testing for various herbicide groups across different weed species. In the last decade, the global weed resistance database has expanded significantly (Heap 2012), and so has our collective experience in surveying, confirming resistance, and evaluating resistance

levels. DNA-based assays have been developed for target-site-based resistance and have been used for quick, high-throughput resistance confirmation. Understanding the advantages and limitations of various resistance testing approaches will help one choose the appropriate sampling and assay protocol and interpret the results properly.

### Field Surveys for Resistance Evaluation

Structured surveys are often an important component of sampling putative resistant (R) plants and collecting information to understand factors that contribute to the evolution and spread of R populations. Population sampling for resistance can be conducted with the use of various methodologies, but in-field sampling is regarded as the most precise method of gathering important management and biological information. The biology of the species is an integral part of defining the objectives of the survey. In-field surveys have been used to detect the presence of herbicide-resistant (HR) weeds ranging from several fields surrounding a single, HR seed source (Baumgartner et al. 1999; Falk et al. 2005) to many regions within a state or province (Beckie et al. 1999, 2001; Bourgeois and Morrison 1997a, 1997b; Bourgeois et al. 1997b; Davis et al. 2008; Légère et al. 2000; Llewellyn and Powles 2001; Tucker et al. 2006; Walsh et al. 2001). Beckie et al. (2000) proposed that because HR weeds can be rare, the selection of fields to survey and sample is the most critical step in determining how surveys should be interpreted. Some survey objectives are simply to find and document new cases of resistance due to reported control failures. Other surveys may be designed to estimate the frequency and distribution of previously documented R biotypes to generate risk models, which can be used to warn surrounding geographies with similar cropping systems of the potential for this biotype to evolve or migrate into new areas.

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In-field surveys can utilize systematic random procedures, nonrandom procedures, or a combination of both during selection of sampling locations (Davis et al. 2008). However, Beckie et al. (2000) recommended that samples be collected directly from where control failures are observed, in a systematic but nonrandom approach, as illustrated by Falk et al. (2005). They determined driving routes radiating from a known resistant source, and when fields with that weed species were observed, samples were collected. These data are also known as presence-only data in some ecological models. With this design it is clearly possible to confirm R biotypes among the samples collected and determine the frequency of control failures that are due to resistance. However, the data do not provide the ability to estimate the frequency of which resistance might be found in all fields, nor to determine management factors that may be contributing to resistance evolution. Surveys with those objectives require a random sampling of fields, accompanied by a survey of farming practices.

Therefore, if determining the frequency of R biotypes in a particular area is the primary goal, then sample sites should be random, but with a systematic procedure of preselecting target localities to calculate the frequency of detection, with and without the herbicide selector, appropriately (Davis et al. 2008; Owen and Powles 2009; Walsh et al. 2007). Walsh et al. (2007) and Owen and Powles (2009) utilized systematic procedures based on travel distance. Davis et al. (2008) demonstrated a systematic random sampling system that utilized Geographic Information Systems (GIS) hardware and software, in conjunction with the United States Department of Agriculture–National Agricultural Statistical Service Cropland Data Layer program. Although those materials and methods were well defined, a variety of newer hardware and software programs may be available to meet similar objectives. The primary objective of Davis et al. (2008) was to combine the resolution power of detecting herbicide resistance at low frequencies, while simultaneously generating data to calculate frequency, with the ability to model the important management parameters which predict resistance occurrence (Davis et al. 2008, 2009). With a host of new GIS technologies and current computing power, well-designed surveys can be generated based on important parameters, which may include crop rotations, tillage histories, topography and terrain, soil types, or other factors that might best define an area of interest for a given weed species. For these objectives, survey locations can be selected based on Global Positioning System (GPS) coordinates. To increase the likelihood of finding rare events, nonrandom sample data can be collected between predetermined survey locations when weed escapes are observed (Davis et al. 2008).

### Sampling Plants for Resistance Evaluation

An appropriate process to collect seeds from putative R and susceptible (S) plants for herbicide assays is critical. How to collect and how many plants will be collected should be decided carefully. There is no consensus among researchers with respect to the sample size of mother plants for resistance testing, regardless of mating behavior (Table 1). For primarily self-pollinated species like horseweed [*Conyza canadensis* (L.) Cronq.], 30 to 40 seed heads from putative R plants in a composite sample is recommended (Beckie et al. 2000; Davis

et al. 2008). Collecting a large sample size for self-pollinated species is done by other research groups (Davis et al. 2010; Weaver 2001). On the contrary, five female plants may be an appropriate sample size for an obligate outcrossing, dioecious species like waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer var. *rudis* (Sauer)] (Trucco et al. 2005). Collecting a large number of samples is unnecessary for species with outcrossing mating behavior. Where there is thorough intrapopulation genetic mixing, few plants are needed to represent the genetic diversity (and HR phenotypic diversity) within the population. Nevertheless, other groups collected 10 to 30 females per field of the dioecious species Palmer amaranth (*Amaranthus palmeri* S. Wats.) (Wise et al. 2009) and waterhemp (Legleiter and Bradley 2008). In the majority of cases, the numbers of harvested mother plants per field were not reported (Table 1) and we believe that this varied widely. Therefore, we surmise that 20 to 40 plants for self-pollinated species and 5 to 10 plants for cross-pollinated species should be sufficient to compose a bulk sample or to collect individual plant samples. Because survivors generally occur in patches, multiple bulk samples may be collected per field.

The expected resistance frequency in a field can be determined from bulk samples. However, composite samples are not appropriate if the objective is to evaluate intrapopulation diversity in resistance evolution. In such cases, individual plant samples should be collected (Hausman et al. 2011; Patzoldt et al. 2005). Care is needed to reference frequency of resistance based on previous herbicide exposure. This does not reflect the resistance frequency with respect to all plants that once were in the field prior to herbicide application. A true estimation of resistance frequency within a field would need to account for viable seed bank densities. This would require collection of soil cores. This is labor intensive and costly and impractical in many cases, but necessary for precise characterization of population dynamics.

Seed heads must be collected at a time that maximizes viable seeds for whole-plant assays, unless other assays that only require plant tissue collections are available. Weeds are generally diverse in their maturation time (Muenscher 1935), and often, weed maturation is aligned just prior to crop maturation. Therefore, the window of opportunity to collect mature inflorescences may be short due to crop harvest operations. The collection time may be even shorter if control failures are bad enough for growers to warrant preharvest herbicide applications. These time constraints must be considered during survey design and implementation.

The amount and type of extraneous data collected at each sample location must be considered based on survey objectives and weed biology. For instance, during the sample collection of a suspected new case of herbicide resistance, information regarding prior herbicide use as well as other crop management practices is critical to estimate the risk for other resistant cases to arise in similar management situations. On the other hand, if a species has been previously documented with resistance to a certain herbicide, and the survey objectives are to understand the wide-scale geography that the biotype infests, detailed historical data become less critical and understanding seed migration patterns and pollen movement potential become more important. For example, horseweed seeds are windblown, traveling long distances (Dauer et al. 2006), whereas other weed seeds may be more prone to travel

Table 1. A brief survey of recent resistance confirmation assay protocols.

Species	MOA <sup>a</sup>	Herbicides tested <sup>b</sup>	Plants per replication <sup>c</sup>	Dose	Replication	Repeated	Growth stage treated	Response variable <sup>d</sup>	Comment on populations (POP)	Reference
<i>Alopecurus myosuroides</i>	ALS	Sulfometuron, mesosulfuron + iodosulfuron	6	1 ×	5	No	Three leaf	Fresh weight, injury at 28 DAT	S standard no prior exposure	Marshall and Moss (2008)
<i>Amaranthus palmeri</i>	ALS	Imazapic	ns; seeded in flats	70 and 700 g	1	Yes	5–10 cm	Injury at 14 and 21 DAT	30 females/ POP, mixed; 10 selected R POPs;	Wise et al. (2009)
<i>Amaranthus rudis</i>	EPSPS	Various MOAs Glyphosate; various MOAs	ns 20	1 × 2 ×	4	Yes	15 cm	Dry weight, 14 DAT Injury, fresh weight, survivor at 21 DAT	10–20 females/POP, mixed; S from different cropping system	Legleiter and Bradley (2008)
<i>Amaranthus tuberculatus</i>	HPPD	Triketones	1	1 ×	4	Yes	10–12 cm	Injury 7–21 DAT	Each female kept separate	Hausman et al. (2011)
<i>Bromus rigidus</i>	PPO, ALS, PSII Various MOAs	PPOs; ALSs; atrazine	ns 50	1 × 1 ×	2	No	10–12 cm Two–three leaf	Injury, frequency of R progenies Mortality, 21 DAT; > 95% survivor = R	Each female kept separate	Patzoldt et al. (2005)
<i>Coryza canadensis</i>	ALS	Cloransulam	100 from R; 21 from S	1 ×	1	No	2.4–5-cm-diam rosettes	Injury, dry weight at 28 DAT; R frequency	S from different locality	Owen et al. (2012)
<i>Hordeum leptorhizon</i>	ALS	Sulfosulfuron, sulfometuron	ns	1 ×	2	No	Two–three leaf	Mortality, 21 DAT	S standard from different island	Zheng et al. (2011)
<i>Leptochloa chinensis</i>	ACCcase	APP, CHD	6	4 doses	6	No	Seedlings	Dry weight, 21 DAT; agar plates	S standard from different province	Yu et al. (2007)
<i>Leptochloa chinensis</i>	ALS	Chlorsulfuron, imazamox	40–50 seeds	1 ×	ns	No	Three leaf	Injury, 21 DAT	S standard no prior exposure	Maneechote et al. (2005)
<i>Lolium perenne ssp. multiflorum</i>	ACCcase	Sethoxydim, pinoxaden	50 seeds	1 ×	3	No	Two leaf	Injury, 21 DAT	S standard, commercial	Yu et al. (2012)
<i>Papaver rhoeas</i>	ACCcase; EPSPS	Pinoxaden, diclofop; glyphosate	40	1 ×	2	yes	Three–four leaf	Injury, 21 DAT; mortality, 28 DAT	S standard, commercial	Kaundun (2010)
<i>Papaver rhoeas</i>	EPSPS	Glyphosate	80	2 ×	1	No	Tillering	Mortality, 28 DAT	S standard, commercial	Dickson et al. (2011)
<i>Papaver rhoeas</i>	ALS	Various SUs; other ALS families	15	1, 4, and 8 ×	4	No	Six–eight leaf	Fresh weight, injury at 28 DAT	Clone; detect R and S from same POP	Dickson et al. (2011)
<i>Phalaris minor</i>	ACCcase	Tribenuron APPs	15	1 × 1 ×	4 3	No No	Four–six leaf Three–four leaf	Fresh weight	Roadside survey; S standard no prior exposure	Kaloumenos et al. (2011)
<i>Sorghum halepense</i>	ACCcase	APPs	5	S = 1 ×; R = 1, 2, 4 ×	4	Yes	Rhizomes, four–five leaf; seedlings, four–five leaf	Injury, 14 and 28 DAT	R and S populations from different regions	Gherekhloo et al. (2011)

<sup>a</sup> MOA = mode of action; ALS = acetolactate synthase inhibitor; ACCcase = acetyl coenzyme-A carboxylase; EPSPS = 5-enolpyruvylshikimate-3-phosphate synthase inhibitor; HPPD = 4-hydroxyphenyl pyruvate dioxygenase inhibitor; PPO = protoporphyrinogen oxidase inhibitor; PSII = photosystem II inhibitor.

<sup>b</sup> APP = aryloxyphenoxypropanoate; CHD = cyclohexenedione; SU = sulfonlyurea.

<sup>c</sup> ns = not specified; R = resistant; S = susceptible.

<sup>d</sup> DAT = days after treatment.

via farm implements (Rew and Cussans 1997), particularly harvesting equipment.

### Classical Approach to Resistance Confirmation

The classical approach (classical assay) of confirming resistance is to collect bulk seeds from surviving plants in suspected fields, plant these in pots, and apply either PRE or POST herbicides. To represent the problem areas, seeds from multiple plants need to be collected (Moss 1999), but the number of plants used to constitute a bulk varies widely. The goal is to collect enough good-quality seeds to conduct various tests (see previous section on sampling). From the field to the laboratory, care should be taken to prevent exposure of collected seeds to unfavorable conditions (e.g., hot, moist conditions) that would trigger seed deterioration or secondary dormancy. Prior to using these seeds for bioassay, it may be necessary to break seed dormancy to obtain uniform germination. Recalcitrant seeds may have to be pregerminated and then transplanted to the assay medium (Burke et al. 2006; Délye et al. 2002a; Huan et al. 2011; Xu et al. 2010). For PRE herbicides, field soil must be used to obtain a realistic herbicide activity, whereas commercial potting media are sufficient for POST herbicides. To test resistance of a species for the first time, conducting a dose–response curve, relative to a susceptible (S) standard, is better than using a single dose, as this will show the magnitude of resistance and the discriminating dose. In subsequent tests of other populations of the same species, a single dose can be used. The majority of researchers use the recommended field dose in pot assays to screen a large number of putative R samples, and the response compared with that of a chosen S standard and respective nontreated checks (Table 1). If space and manpower allow, including more than one dose in the screening test is beneficial because it gives some indication of resistance level among populations. Thus, in some cases, two to four doses had been used in resistance confirmation assays (Kaloumenos et al. 2011; Manechote et al. 2005; Wise et al. 2009). Where there are few (i.e., < 5) populations to test, one may opt to conduct a dose–response assay instead to confirm resistance and determine the resistance level in one experiment. In Petri plate assays, putative R and S populations are first tested with a wide range of doses to determine the discriminatory dose before conducting the large-scale resistance testing (Bourgeois et al. 1997a; Kaundun et al. 2011b). Where an R population has already been identified, an R standard may also be included. An S standard should be included in every run of a resistance assay.

The selection of an S standard has been discussed at great lengths in many venues. What matters to growers is resistance to the recommended field dose. For scientists, knowing whether a population is gaining the capability to survive the recommended dose helps in promoting mitigation measures soonest. Putative herbicide-S plants need to be collected from the same agricultural region to confirm resistance and conduct the herbicide dose assays, but within reasonable distance from the problem field. Plants in areas adjacent to the source field may be contaminated with the resistance trait because of gene flow. In this case, a true wild type should be collected at a far enough distance from the source field. Plants adjacent to the source field are also exposed to low doses of the selector herbicide because of drift from spraying field edges. It is documented in rigid ryegrass (*Lolium rigidum* Gaud.) and

Palmer amaranth (Busi and Powles 2009; Neve and Powles 2005a, 2005b; Norsworthy 2012) that resistance, specifically polygenic, is gradually selected by iterative exposure of a weed population to sublethal doses of a herbicide. Therefore, populations in the immediate vicinity of a source field would most likely exhibit reduced sensitivity to the herbicide than populations with no prior exposure to it. In most cases, researchers use an S standard with no prior exposure to the herbicide or collect from a different field in the same locality, state, or region (Table 1). It is common for research laboratories worldwide to use the same S standard population for testing the resistance of multiple populations across a large region. Under these situations, utilizing a common S population is appropriate to compare levels of resistance between different populations and between different experiments.

When a species is being investigated for resistance to a herbicide with a previously undocumented mechanism of action, a putative S population should be collected from a relatively close distance if possible. This is important because genetic diversity among weed species may be greatly influenced by different climate and geographical conditions; S and R populations from within the same locality should be similar in extraneous genetic characteristics that could impact response to herbicides.

Comparison between populations is most commonly done by determining the effective dose that causes 50% inhibition (GR<sub>50</sub>) of growth noted by biomass reduction and/or visual ratings or the dose needed to kill 50% of the plants (LD<sub>50</sub>) through rate titration experiments. The procedure and calculations to determine the GR<sub>50</sub> and LD<sub>50</sub> are explained in more detail in the next section. Determining the value of the S population with an appropriate representation of the expected normal wild-type population is just as critical as determining the value of the putative R population because the GR<sub>50</sub> or LD<sub>50</sub> value for S sets the resistance index. Ideally, one should compare responses of multiple S populations, and thereby obtain baseline herbicide sensitivity data as well as an indication of the natural variability of the species. An average S population should be used. GR<sub>50</sub> or LD<sub>50</sub> values should not be compared between experiments using S populations with different sensitivity levels to the herbicide. Furthermore, in some screening experiments multiple R standards may be needed because different resistance mechanisms could result in different levels of herbicide tolerance.

Treatments should be replicated and the test repeated, to verify the results. Generally, three to four replications are used (Table 1) with a few cases using five to six replications (Manechote et al. 2005; Marshall and Moss 2008). The goal is to test a large enough number of plants per population to increase the power of resistance detection. In cases where the resistance test was not replicated, a large number of seeds were planted in flats and 80 to > 100 plants per population were treated (Dickson et al. 2011; Wise et al. 2009; Zheng et al. 2011). Where only two replications were prepared, 40 to 50 plants were tested per replication (Dickson et al. 2011; Owen et al. 2012); the test by Dickson et al. (2011) was repeated in time. If a replicate consists of a single plant, at least 10 replicates should be used. The number of plants to include per replicate is determined based on the objectives of the screening, plant size, and growth characteristic, concerns of herbicide coverage (for POST treatments), greenhouse space availability, and other factors. Although the majority of resistance confirmation tests are not repeated (Table 1), we

recommend repeating in time. This increases the chances of detecting rare events and also verifies the test results from the first run. Although it is obvious that appropriate plant growth conditions for the weed species of interest must be maintained, it is worth noting nonetheless. Typically, herbicides will be most effective on healthy, rapidly growing plants in the early vegetative growth phase.

In the academic system, test results are immediately conveyed to the growers via extension personnel. Private testing companies employ different channels to send feedback. To provide growers with immediate options for resistance management, a test for sensitivity to other herbicides is also generally conducted. Best management programs for resistance management that integrate cultural and chemical options are then formulated and disseminated to growers.

### Evaluation of Resistance Level

A dose–response experiment is often conducted to determine the level of resistance and obtain a glimpse of potential resistance mechanism. Resistance beyond the recommended dose is no longer important to the grower, but it is relevant to researchers because resistance level provides clues to resistance mechanism(s), the understanding of which helps in designing management strategies. Researchers have used a wide range of doses, from 4 to 15 with 3 to about 200 total plants per dose (Table 2). Researchers oftentimes repeat their dose–response assays, but not the confirmation assays. This is probably because of the need to refine the dose–response curve; the first run being an exploration of the dose range. POST herbicides are applied at the recommended growth stage, generally two to four leaf, with the recommended surfactant or additives. Lately, glyphosate assays have been conducted with the use of the plain glyphosate acid formulation; then the prescribed surfactant is added separately at a constant concentration (Dickson et al. 2011; Legleiter and Bradley 2008; Norsworthy et al. 2008). This is because when a formulation with a built-in surfactant system is used, plant response to glyphosate may be confounded with the increasing surfactant concentration as the dose is increased. The response variables evaluated are either fresh weight or dry weight of shoot tissues, with or without visual injury assessments. Either of these responses could be used to estimate resistance levels or amounts of herbicides that would cause a certain level of growth reduction or control. Plant response is evaluated within 1 to 4 wk of herbicide application depending on the mode of action. For POST herbicides, 1 wk after treatment (WAT) is appropriate for those with contact action, whereas slower-acting herbicides are best evaluated at 2 to 4 WAT. To evaluate regrowth after initial necrosis of aboveground tissues, a follow-up rating is done at 4 WAT. Tests are completed in 1 to 2 mo and results are used for decision making in the next cropping season.

**Biological Consideration.** Whatever the method of investigation, dose–response curves are instrumental in quantifying the magnitude of resistance relative to a predefined S population with the ratio R/S, which is similar to the general relative potency among herbicides. The R/S answers the question “How much more of a herbicide must I use to get the same effect as I had before the evolution of R populations?” Most agree upon measuring the R/S at the

GR<sub>50</sub> level. The relative position on the dose axis and shape of the curves may differ (Figure 1). In Figure 1A, the comparison is straightforward, the two curves having similar shape and the relative displacement on the dose axis of the R population and the R/S is invariant of the response levels. In Figure 1B, R/S is more ambiguous because the curves have slightly different shapes; consequently R/S is only defined at the response level (here GR<sub>50</sub>) under which it is estimated (Ritz et al. 2006; Ritz 2010).

When comparing accessions we cannot assume they all have the same growth as the nontreated control. Figure 1C illustrates some difference between the upper limit of the two populations, clearly illustrating that GR<sub>50</sub> is a relative term based upon the upper (*d*) and lower limit (*c*) of the log-logistic curve (Equation 1). The parameter *b* is the relative slope around the GR<sub>50</sub>:

$$y = c + \frac{d - c}{1 + \left(\frac{x}{ED_{50}}\right)^b} = c + \frac{d - c}{1 + \exp(b \cdot (\log(x) + \log(ED_{50})))} \cdot [1]$$

The most common way of dealing with differences in growth between populations is to calculate responses as percentage of the nontreated control, although this standardization prior to curve fitting is not statistically sound. Another drawback is that one should omit the nontreated control, always being 100, from the analysis. Various other sigmoid regression models are available and have been described elsewhere (Ritz 2010). The best way to fit the curves is to use raw data and calculate R/S from the GR<sub>50</sub>. If one feels it will be more illustrative to show the relative scale, it can be done after curve fitting by using the estimated parameters of the upper *d* and lower *c* limits to scale the raw data (Streibig et al. 1995).

In many cases, we also want to answer the more specific question of “How much herbicide is needed to kill 50 or 90% of the population?” The survival rate of a population is useful in predicting potential seed deposit into the soil seed bank or potential patch expansion of R plants. To answer this question, mortality data are collected and lethal doses (i.e., LD<sub>50</sub> or LD<sub>90</sub>) are estimated for the population in question from a dose–response experiment. The analysis is a logistic regression of binomial data (McCullagh and Nelder 1989), which almost corresponds to logit on a log scale of the dose axis. Whereas the GR<sub>50</sub> of the same plants could change significantly because of environmental conditions, the LD<sub>50</sub> would be less affected.

To generate reproducible dose responses the doses should cover the whole response range, from virtually no effect around the nontreated control to 100% control at large doses. Some highly resistant species are no longer killed by the highest possible herbicide concentration in the spray mixture, in which case the dose range should extend to a point where no further response is observed. This applies to all regression analyses, but compared with the linear models, nonlinear models are particularly vulnerable to nonuniform distribution of responses. For the log-logistic curves the distribution of doses should be based upon preliminary experiments, so GR<sub>50</sub> is roughly known and thus doses can be distributed around GR<sub>50</sub> to make sure that most of the response range is covered.

Figure 2A shows an example with well-distributed responses for both populations, and Figure 2B illustrates a poor distribution of the R population. In Figure 2A, the R/S is 8 with a lower and upper 95% confidence limit of 1.8 to

3.9. Consequently, we can be confident that the R/S is significantly different from 1. Whether this statistically different R/S from 1.0 is large enough is a biological question, not a statistical one. Because of natural intrapopulation variation in sensitivity to herbicides, a plant may have R/S = 10 relative to the most susceptible plant, but is still sensitive to the full dose of the herbicide. In Figure 2B the R/S also is 8, but now the upper and lower limits are 0.7 to 14.6, respectively; and this huge span between upper and lower limit is definitely due to the poor distribution of the response range for one of the curves in Figure 2B. A fit with a log-logistic dose–response model shows that the R/S on the GR<sub>50</sub> levels is not significantly different from 1, probably because responses for the R population are displaced to the lower part of the curve. Even though the R/S ratios were rather similar in the two cases, the uncertainty was much greater because of the poorly distributed responses. For the R/S on the GR<sub>90</sub> levels the confidence interval does not cover 1.0 and thus the R/S was significantly different whatever the distribution of responses. On the basis of the above, the R/S ratios should always be given with their associated standard error.

Another issue is to run the assay curves simultaneously in order to get the best precision, because an assay in a greenhouse in the spring and in the autumn may make a huge difference of the GR<sub>50</sub> and perhaps also of R/S. Often a bioassay comprises numerous accessions, and running the whole assay one time might be difficult. It is imperative that when running in sequence there should be one or more internal standards to detect any drift of R/S.

One of the ensuing discussions is the number of replications. If experience shows that the log-logistic regression model describes the variation within acceptable limits, then it is better to use more doses than more replicates. For example, if your limit is a total of 24 experimental units, then it will be more advisable to use 8 or 12 doses and 3 or 2 replications rather than 6 doses and 4 replicates. Simulation has shown that the precision of GR<sub>50</sub> increases dramatically when we substitute replications with more doses. The GR<sub>50</sub> is not an agronomically sound control level, GR<sub>90</sub> or GR<sub>80</sub> would be more realistic. However, it is important to note that the farther away from the midpoint GR<sub>50</sub> the less precise the estimates of GR<sub>80</sub> or GR<sub>90</sub> become (see Figure 2).

One of the questions rarely addressed is how large would the R/S be in the field before the farmer realizes an imminent problem with resistant weeds. For biochemical and physiological response variables, we see a colossal difference with R/S of several hundred or even thousands (Taylor et al. 2002; Trainer et al. 2005). In the field, farmers seem to realize the problem when about 35% of the population of a weed species has acquired resistance (Gressel and Segel 1978, 1990). Recently, Mennan et al. (2011) showed that farmers in Europe already complain when the R/S ratio is about two. When farmers in the southern United States started complaining about Italian ryegrass escaping glyphosate burn-down treatments, the escapes were in patches of up to 0.25 ha (K. L. Smith, Extension Weed Specialist, personal communication) and the resistance index of sampled plants was within two- and fourfold (Dickson et al. 2011; Nandula et al. 2007). There are no data on what percentage of survivors would prompt growers to notify technical advisors, but generally southern U.S. farmers initiate communicating the problem after the third season of observing the increasing number of survivors (K. L. Smith, personal communication).

One must be aware that any obtained R/S level will be a function of the absolute magnitude of resistance expressed by the R biotype and the susceptibility of the S biotype, regardless of resistance frequency within the R population. Additionally, more than one R biotype may be present within a population (i.e., different plants may have different resistance mechanisms), and plants may be heterozygous or homozygous for the resistance allele(s). Thus, it is difficult to discuss the relevance of R/S ratios and compare them among populations without having some knowledge of the uniformity within the populations.

**Statistic Consideration.** To utilize the R/S factors and compare them among species and published articles, it is important that the statistic assumptions behind the parameter estimates are correct, viz., the assumption of normality of residuals and homogeneous variance of responses (Ritz and Streibig 2009). In our experience, the most important one is that of homogeneous variance. If we have large differences between maximum and minimum responses, there is a probability of variance heterogeneity and it has to be dealt with by using transform-both-sides techniques or weighed regression (Ritz and Streibig 2009). Plots of residuals will usually reveal major violations. The overused/abused R<sup>2</sup> does not say much about the test for lack of fit and in the nonlinear case its use should be discouraged, particularly when there are replications, so a proper test for lack of fit against an ANOVA could be used.

### Quick Assays for Testing Resistance

The classical whole-plant assay requires a large amount of bench space, takes up to 2 mo to obtain results, and is not amenable for large-scale testing. Attempts have been made to develop resistance assays that will allow growers to use the resistance information to make real-time decisions regarding management of current weed control failures. Some of these relatively quick assays have already been adopted, but no single assay fits all situations; some are more widely applicable than others; all need further testing for adaptation to other species and herbicides. Few of these assays have been commercialized [Herbicide Resistance Action Committee (HRAC) 2012]. Quick assays using whole plants, seedlings in Petri dishes, or leaf discs are independent of resistance mechanisms. The majority of these assays have good potential for commercialization.

**Rapid Whole-Plant Assay for POST-Applied Herbicides.** A Syngenta Quick test was developed for grass species, specifically rigid ryegrass and blackgrass (*Alopecurus myosuroides* Huds.), collected from suspect fields (Boutsalis 2001). Vegetative tillers are separated then the shoots are cut and potted. The regenerated cuttings can be sprayed with foliar herbicides after at least 1 wk from transplanting. Chlorsulfuron [acetolactase synthase (ALS) inhibitor]; diclofop, fenoxaprop, fluazifop, haloxyfop, and sethoxydim [acetyl coenzyme-A carboxylase (ACCase) inhibitors]; and isoproturon [photosystem II (PSII) inhibitor] have been tested on cuttings of either blackgrass or rigid ryegrass and produced robust confirmation of resistance to these herbicides. Field-collected plants can survive several days of transport. This approach has been adapted to confirm resistance to glyphosate and to conduct a glyphosate-dose

Table 2. A brief survey of recent herbicide dose response protocols.

Species	MOA <sup>a</sup>	Herbicides tested <sup>b</sup>	No. of doses	Reps	Plant/ rep	Repeat	Growth stage treated	Response variable <sup>c</sup>	Comment on populations <sup>d</sup>	Reference
<i>Alopecurus myosuroides</i>	ACCcase	APP, CHD, PS II	6; various ranges	12 to 20	1	No	Two–three leaf	Fresh weight, 21–28 DAT	S standard from different region	Moss et al. (2003)
	ALS	Sulfometuron	8; 3.125–400 g	16	1	No	Three leaf	Fresh weight, injury at 28 DAT	S standard no prior exposure	Marshall and Moss (2008)
<i>Amaranthus palmeri</i>	ALS	Imazapic	5; 0.019×–40×	4	5	Yes	5–10 cm	Dry weight, 14 DAT	10 selected R POP;	Wise et al. (2009)
	ALS	Imazaquin	6; 1.25×–24×	3	2	Yes	Five–six leaf	Dry weight, 14 DAT	S standard from different county	Burgos et al. (2001)
	EPSPS	Glyphosate ac + separate surfactant	11; 1/48×–2.7×	8	1	No	Five–seven leaf	Survivor, 28 DAT	S from different state; 4 females/POP	Norsworthy et al. (2008)
<i>Amaranthus rudis</i>	EPSPS	Glyphosate ac + separate surfactant	5; 0.5×–8×	4	20	Yes	15 cm	Injury, fresh weight, survivor at 21 DAT	1–20 females/POP, mixed; S from different county, corn–RR soy rotation	Legleiter and Bradley (2008)
<i>Amaranthus tuberculatus</i>	HPPD, PSII, ALS, phenoxy	Mesotrione, atrazine, thifensulfuron, 2,4-D	5; various ranges	8	ns	Yes	3–4 cm	Injury 11 DAT;	S POP no prior exposure	McMullan and Green (2011)
	HPPD	Mesotrione, tembotrione	11; various ranges	6	1	Yes		Dry weight; 21 DAT	Females kept separate; dose range log-based	Hausman et al. (2011)
	PPO, ALS, PSII	Six PPOs; two ALS; atrazine	6; log scale	6	1	Yes	10–12 cm	Dry weight, 14 DAT	Females kept separate	Patzoldt et al. (2005)
<i>Apera spica-venti</i>	ALS and PSII	Five SUs	7–8	4	10	Yes	Three leaf, one tiller	Injury, dry wt, 28 DAT;	S POP from organic farm; used field soil	Hamouzová et al. (2011)
<i>Avena fatua</i>	ACCcase	Three families	4–5	3	4	No	Three–four leaf	Fresh weight	R and S POPs from different regions	Cruz-Hipolito et al., (2011)
<i>Avena sterilis</i>	ACCcase	APP	9; 0.125×–64×;	6	6	No	Two–three leaf	Dry weight, 28 DAT;	S standard no prior exposure	Maneeshote et al. (1997)
<i>Bromus rigidus</i>	ALS	SU and IMI	4 to 6	3	20	Yes	Two leaf	Dry weight, 28 DAT	S standard from different locality	Owen et al. (2012)
<i>Capsella bursa-pastoris</i>	ALS	tribenuron	4; 0.1–1000 g	4	10	Yes		Dry weight, 21 DAT	S standard no prior exposure	Gui-qi et al. (2011)
<i>Conyza canadensis</i>	ALS	All ALS chemical families	9; various ranges	10	1	Yes	2.4–5-cm-diam. rosettes	Dry weight, 28 DAT	S standard from different locality	Zheng et al. (2011)
<i>Cyperus difformis</i>	ALS	All chemical families	6; 0.125×–4×	6	2	Yes	Four leaf	Fresh weight, 23 DAT	S standard no prior exposure	Merotto et al. (2009)
<i>Descurainia sophia</i>	ALS	Tribenuron	7; 0.15×–80×	ns	5	No	Three–four leaf	Fresh weight, 21 DAT	S standard no prior exposure, China	Xu et al. (2010)
<i>Digitaria ischaemum</i>	ACCcase	APP, CHD	4–12	5	1	No	Two tillers	Dry weight, 14 DAT	S standard no prior exposure	Kuk et al. (1999)
	Growth regulator ACCcase	Quinclorac	6; 0.5×–32×	5	4	No	Three leaf	Fresh weight, 14 DAT	S standard, commercial	Abdallah et al. (2006)
<i>Echinochloa crus-galli</i>	ACCcase	Quizalofop	5; 0.48–4,800 g	3	10	No	Three–four leaf	Fresh shoot weight, 14 DAT	S standard no prior exposure; used field soil	Huan et al. (2011)
<i>Lepochloa chinensis</i>	ACCcase	APP, CHD	6–7	4	100 seeds	No	Seedlings	Dry weight, 21 DAT;	S standard from different region	Maneeshote et al. (2005)
<i>Lolium perenne</i>	EPSPS	Glyphosate	5–6	4–5	10–15	No	Four leaf	Injury, 21 DAT	S standard - commercial	Dickson et al. (2011)
<i>sp. multiflorum</i>	EPSPS	Glyphosate	15; 1/96–42×	4	1	No	Two leaf	Mortality and fresh weight, 28 DAT	S standard - commercial	Dickson et al. (2011)
		Glyphosate	7; 0.25×–16×	3	1	No	Tiller, clone	Injury, 28 DAT	R and S plants from same POP	Salas et al. (2012)
	ACCcase, ALS	Diclofop, mesosulfuron	11–12; 0.016×–32×	3	3	Yes	Two–three leaf	Dry weight, 14 DAT	S standard from same region	Kuk and Burgos (2007)

Table 2. Continued.

Species	MOA <sup>a</sup>	Herbicides tested <sup>b</sup>	No. of doses	Reps	Plant/ rep	Repeat	Growth stage treated	Response variable <sup>c</sup>	Comment on populations <sup>d</sup>	Reference
<i>Phalaris minor</i>	ACCcase	APP	8; 0.25×–32×	3	4	No	Three–four leaf	Fresh weight	R and S POPs from different regions	Gherekhlou et al. (2011)
	ALS	SU, TP, IMI	6; 1–32×	4	10	No	Two–three leaf	Dry weight, mortality at 21 DAT	S standard no prior exposure	Han et al. (2012)
<i>Phalaris paradava</i>	ACCcase	All chemical families	4–5	3	4	No	Three–four leaf	Fresh weight	R and S POPs from different regions	Cruz-Hipolito et al. (2012)
<i>Rottboellia cochinchinensis</i>	ACCcase	APP, CHD	7; various ranges	4	5	No	Three–four leaf	Fresh weight, 14 DAT	S standard from different region	Avila et al. (2007)
<i>Setaria viridis</i>	ALS	Imazethapyr, nicosulfuron, pyriithiobac	7; various ranges	4	4	Yes	Two–four leaf	Dry weight, 10 DAT	15–50 seed heads/POP; S standard from different region	Laplante et al. (2009)
<i>Sorghum halepense</i>	ACCcase	APPs	6; 1–32×	3	5	Yes	Rhizomes, seedlings, four–five leaf	Fresh weight, 28 DAT	S standard from different region, no prior exposure	Kaloumenos and Eleftherohorinos (2009)

<sup>a</sup> MOA = mechanism of action; ALS = acetolactate synthase inhibitor; ACCcase = acetyl coenzyme-A carboxylase; EPSPS = 5-enolpyruvylshikimate-3-phosphate synthase inhibitor; HPPD = 4-hydroxyphenyl pyruvate dioxygenase inhibitor; PPO = protoporphyrinogen oxidase inhibitor; PSII = photosystem II inhibitor.

<sup>b</sup> APP = aryloxyphenoxypropanoate; CHD = cyclohexenedione; SU = sulfonylurea.

<sup>c</sup> DAT = days after treatment.

<sup>d</sup> R = resistant; S = susceptible.

response assay and other experiments on the same set of Italian ryegrass (*Lolium perenne* ssp. *multiflorum* L.) plants (Salas et al. 2012). This assay can be used on some broadleaf weeds (Walsh et al. 2001), but needs to be pretested for applicability to other species.

Using regenerated cuttings has many advantages. It has broad applicability to grass species and POST-applied herbicides. There is no need to wait for maturation of surviving plants to produce seeds. Clones of confirmed resistant tillering plants can be used in dose–response assays and other experiments. This also allows for detailed intrapopulation studies. If plants are sampled early in the cropping season, results can be used to recommend potential remedial herbicide application in the same season. Resistance detection is not affected by age of plants as regenerated plants respond similar to seedlings (Boutsalis 2001). On the other hand, this test does not offer any space-saving advantage relative to the classical whole-plant seedling assays conducted in pots in the greenhouse. Although its applicability is limited to foliar-applied herbicides, it has the best prospect for commercialization because it is most tightly correlated with the classical whole-plant assay.

Similarly, tillering annual bluegrass (*Poa annua* L.) was tested for resistance to mitotic inhibitors, pendimethalin and prodiamine, in a hydroponics assay (Cutulle et al. 2009). In this case, the tillers were separated, the roots cut at a uniform length, and the clones cultured in Hoagland's solution containing the desired herbicide concentrations. In 10 d, root lengths were measured. Setting up the culture tubes takes some time, but the tubes can be reused. Preparation of the culture solution does not require special skills once the protocol is set; however, this can be expensive for large-scale testing.

**Seed Germination Assays.** A Petri dish assay was used to determine cross-resistance patterns of a large collection (> 80) of wild oat (*Avena fatua* L.) accessions to ACCase inhibitors (Bourgeois et al. 1997a). Wild oat seeds are germinated in agar medium with several concentrations of clodinafop and clethodim to determine the discriminatory dose. The level of resistance is then assessed based on elongation of coleoptiles and radicles of treated seeds relative to the nontreated seeds. This assay detects target-site and metabolism-based resistance. It is simple, faster, and requires less space than the whole-plant assays described previously. However, this still requires harvesting seeds from escaped plants, so results are utilized for the next growing season.

A popular technique for a quick resistance test is to incubate pregerminated seeds in Petri dishes containing various concentrations of the herbicide in question, under optimum conditions for each species. Coleoptile length is used as indicator for resistance after 3 to 7 d of incubation. This has been used to test resistance to ACCase inhibitors in barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.] (Huan et al. 2011), green foxtail (*Setaria viridis* L.) (Délye et al. 2002b), and johnsongrass [*Sorghum halepense* (L.) Pers.] (Burke et al. 2006) and resistance to ALS inhibitors in flixweed [*Descurainia sophia* (L.) Webb ex Prantl] (Xu et al. 2010). Dose–response curves have been generated with the use of this technique.

Resistance to glyphosate in ryegrass can also be detected by pregerminating seeds in Petri dishes and transferring germinated seeds to ELISA plates, filled with glyphosate



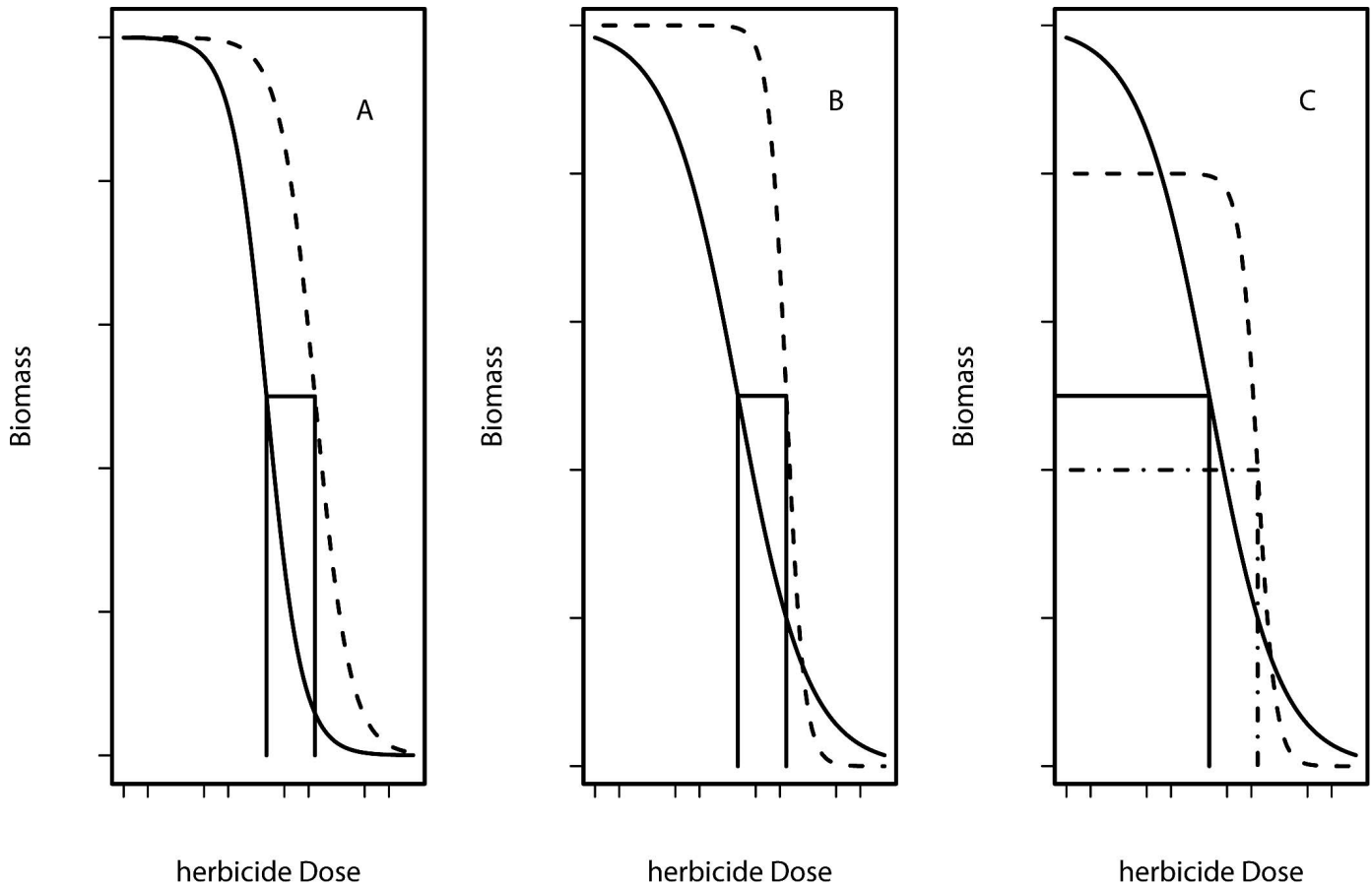


Figure 1. Various scenarios for comparing response curves and calculating the R/S at the ED<sub>50</sub> levels. The curves in (A) are similar, (B) curves have different slopes, and (C) curves with different upper limits. The herbicide dose is on a logarithmic scale.

solution of various concentrations, incubated at 15 C for 7 d, and the coleoptile lengths measured (Ballot et al. 2009). The assay is completed in 16 d total and does not use much space or specialized equipment. Results are tightly correlated ( $R^2 = 0.95$ ) with those of the classical assay. For quick resistance confirmation of multiple samples, a discriminating concentration and optimum number of seedlings to test per sample need to be determined.

A variation of the above assay is germinating seeds in pots filled with perlite medium and watering the pots daily with nutrient solution with or without herbicide (Breccia et al. 2011). Pots are placed either in the greenhouse or growth chamber under conditions favorable for the species tested. This method was developed to test sunflower (*Helianthus annuus* L.) resistance to imidazolinone herbicides, specifically imazapyr, at a dose range of up to 10  $\mu$ M. Seedlings are allowed to grow for up to 2 wk, after which roots and shoots are measured. This assay works with either soil- or foliar-active herbicides, but the daily watering with nutrient solution could be a deterrent for commercialization.

**Agar-Based Seedling Assays.** The continued quest for a simple assay that will yield information for use in the same crop growing season has ushered in the first in-season, agar-based seedling assay for testing blackgrass resistance to ACCase-, ALS-, and PSII-inhibiting herbicides in Europe (Claude et al. 2004). Field-collected seedlings are transplanted to agar plates, placed in the growth chamber or greenhouse

until new roots and shoots develop, sprayed with discriminating doses of herbicides, and evaluated 14 d after treatment. The main differences between this and the Syngenta Quick whole-plant assay are the use of field-collected seedlings prior to any POST-applied herbicide and the growth medium. It still takes about 1 mo to finish the assay, but it requires less space than the whole-plant assay. The major disadvantage is it requires constant maintenance to keep the agar from desiccating over the 4-wk period. Therefore, this assay is not amenable to commercialization.

Recently, a resistance in-season quick (RISQ) test was developed to test resistance to ACCase and ALS inhibitors among grass species, including blackgrass, green foxtail, phalaris (*Phalaris paradoxa* L.), ryegrass, and wild oat (*Avena fatua* L.), in agar medium (Kaundun et al. 2011b). The herbicides tested were clodinafop-propargyl, pinoxaden, and iodo-mesosulfuron. This assay requires at least a benchtop under grow lights, Petri plates, agarose or agar, and other basic materials. One- to three-leaf seedlings are placed horizontally on the agar medium containing a discriminating dose of herbicide, with the roots in full contact with the agar. The Petri plates are then incubated at prescribed conditions for 10 to 14 d. Resistant plants develop new leaves and new roots at the discriminating dose, but S plants do not. Tests for Italian ryegrass resistance to pinoxaden in the United States consistently showed development of new roots in the R plants, but none in the S plants (Burgos et al. unpublished data). The Petri plates can be incubated under a wide range of

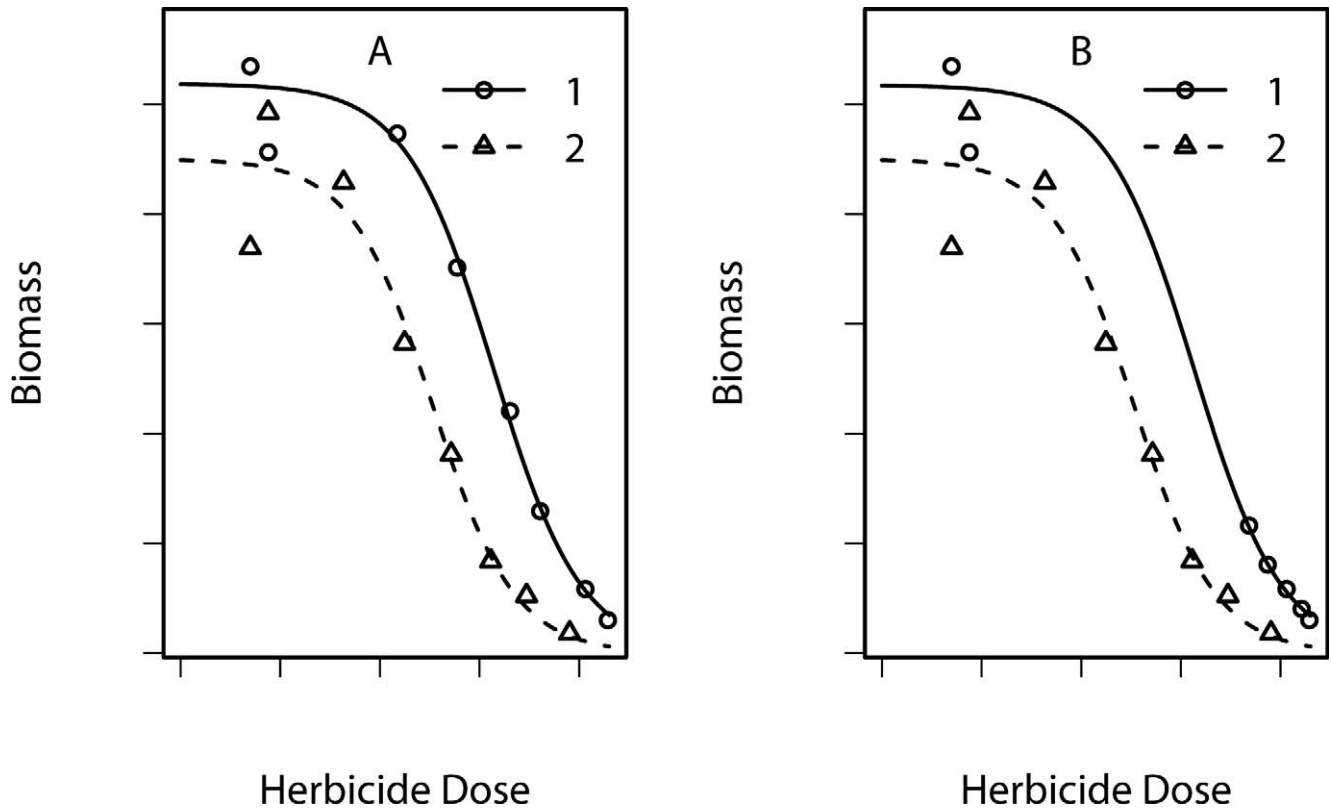


Figure 2. Scenarios for the distribution of responses and the estimation of R/S with 95% confidence intervals for the very same curves, but with different distribution of responses in the resistant biotype. The herbicide dose is on a logarithmic scale.

light ( $50$  to  $180 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and temperature ( $25$  to  $35 \text{ C}$ ) conditions. To conserve bench space, the plates can be stacked up to five deep in the glasshouse; changing the plate placement periodically during the incubation period will minimize bias in plant response. The grower can use this information to make an in-season weed management decision. This is faster than the quick whole-plant assay described previously, offers the additional advantage of space saving, and also detects resistance regardless of mechanism. One disadvantage of this assay is that the preparation of seedlings for incubation in the agar medium is labor intensive. The RISQ assay is adaptable to several species that can be grown in agar medium for a short period of time (Kaundun et al. 2011b). As with other quick assays, it needs to be tested with other herbicides to expand its utility, including soil-active herbicides. This assay can be commercialized.

**Leaf Disc Assays.** Various leaf disc assays have been developed over the last 30 yr to screen weed populations for resistance to herbicides with different mechanisms of action. Leaf disc assays have the advantages of being rapid, mechanism-of-action specific, and nondestructive. The major assays that have been developed and literature citations are shown in Table 3. Although many of these leaf disc assays can be used to understand the mechanism of herbicide resistance, the assays can also be used as an initial screen for detecting R populations in the field.

One must use leaf disc assays properly. It is critical that the appropriate tissue is selected for screening and that the plants are healthy and vigorously growing. Leaf disc assays rely on living tissue. The ALS inhibitor and shikimate accumulation assays absolutely require using young, rapidly expanding

tissue. ALS and EPSPS are most active in meristematic tissues and enzyme activities decrease rapidly as leaves mature (Gerwick et al. 1993; Shaner et al. 2005). In addition, the plants need to be growing vigorously and not under any kind of stress. Stressed plants have reduced enzyme activities. In many cases the assay depends on the leaf actively photosynthesizing, so the assay needs to be done under light. Bacterial contamination is also a concern. If the discs are not excised with a sharp instrument, the crushed cells at the cut surfaces can release cytoplasm. This provides a rich broth for bacterial growth and, unless the assay is done under sterile conditions, the bacteria can interfere with the assay by giving a response that confounds the plant's response to the herbicide. If assay conditions are not met, false negatives will be rampant.

In the shikimate assay, one should always include a high concentration of glyphosate ( $1$  to  $10 \text{ mM}$ ) in the assay. All of the glyphosate-resistant populations tested to date will accumulate shikimate at very high concentrations of glyphosate (Singh and Shaner 2008). The discriminating dose will be approximately  $100 \mu\text{M}$  where the S plants will accumulate shikimate and the R plants will not.

Each new species will require some optimization of the leaf disc assay. These assays may not work for all species because of differences in metabolite accumulation, tissue type, and other factors. Each leaf disc assay has its quirks that need to be taken into consideration to interpret the results properly. It is also necessary to have a known S population to use as a standard for the assay so that one can determine if the assay is working properly.

The strength of leaf disc assays is the rapid turnover. In many cases, one can make an initial determination of resistance within 24 to 48 h. The microtiter plate assays do

Table 3. Petri plate and microtiter plate assays for detecting herbicide-resistant ecotypes.

Mechanism of action <sup>a</sup>	Measurement	Plant tissue	Incubation conditions	Time	Caveats	Response		
						Susceptible	Resistant	References
ALS inhibitors	Accumulation of acetolactate	Leaf discs, stem segments, flowers, roots, etc.	Incubate under continuous light or supplement with sucrose	24–48 h	Tissue age, bacterial contamination; effectiveness of KAR1 inhibitor	No accumulation of acetolactate	Accumulation of acetolactate	Gerwick et al. (1993); Kuk et al. (2003); Lamego et al. (2009); Kuk and Burgos (2007); Burgos et al. (2001)
						Accumulation of shikimate	No accumulation of shikimate at low concentrations of glyphosate	Cromarie and Polge (2000); Koger et al. (2005)
EPSP synthase inhibitor	Accumulation of shikimate	Leaf discs, stem segments, flowers, roots, etc.	Incubate under continuous light or supplement with sucrose	24–72	Tissue age	Accumulation of shikimate at low concentrations of glyphosate	No accumulation of shikimate except at very high concentrations of glyphosate	Shaner et al. (2005); Fierst et al. (1985); Lehoczki et al. (1992)
PS I inhibitors	Chlorophyll fluorescence	Green leaf tissue	Incubate under continuous light	6–24		High fluorescence	Low fluorescence	Fierst et al. (1985); Lehoczki et al. (1992)
PS II inhibitors	Floating leaf discs	Green leaf tissue	Incubate under continuous light	24		Leaf discs sink	Leaf discs float	Truelove et al. (1974); Hensley, (1981)
	Chlorophyll fluorescence	Green leaf tissue	Incubate under continuous light	24		Low fluorescence	High fluorescence	Gronwald, (1994); Ahrens et al. (1981); Korres et al. (2003); Norsworthy et al. (1999)
PPO inhibitors	Chlorophyll fluorescence, for detection of resistance to triazinones	Green, intact leaf (three leaves per plant)	Incubate under natural light in herbicide solution, then dark adaptation for 30 min	4	First, validate the protocol with reference population	PS yield relative to nontreated check < 90%	PS yield relative to nontreated check $\geq$ 90%	Mechant et al. (2010)
						Cell membrane leakage	Leaf discs do not bleach	D. Shaner (unpublished data)
PPO inhibitors	Cell membrane leakage	Green leaf tissue	Incubate under continuous light with paraquat	16	Incubate in darkness and then light	Leaf discs rapidly bleach when moved to light. High electrolyte leakage.	Leaf discs do not bleach rapidly and limited electrolyte leakage.	Becceril and Duke (1989); Falk et al. (2006)
						Pollen germination	No pollen germination	Letouze and Gasquez, (2000)
ACCase inhibitors	Pollen germination	Viable pollen	Incubate on agar with herbicide	48–72	Pollen that will germinate on agar	Pollen germination	Letouze and Gasquez, (2000)	
Multiple MOAs	Seed germination	Seed	Incubate on agar with herbicide	72–144	Viable seed source	No germination	Germination	Bourgeois et al. (1997b)

<sup>a</sup> MOA = mechanism of action; ALS = acetolactate synthase inhibitor; ACCase = acetyl coenzyme-A carboxylase; EPSP = 5-enolpyruvylshikimate-3-phosphate synthase inhibitor; HPPD = 4-hydroxyphenyl pyruvate dioxygenase inhibitor; PPO = protoporphyrinogen oxidase inhibitor; PSI = photosystem I inhibitor; PSII = photosystem II inhibitor.

Table 4. Molecular polymorphisms identified in weeds and generally accepted as conferring herbicide resistance.

Target site	Polymorphism(s)	Reference(s)
D1 protein ALS	Val <sub>219</sub> Ile, Ala <sub>251</sub> Val, Phe <sub>255</sub> Ile, Ser <sub>264</sub> Gly/Thr, Asn <sub>266</sub> Thr Ala <sub>122</sub> Thr/Tyr/Val, Pro <sub>197</sub> XXX, <sup>a</sup> Ala <sub>205</sub> Val, Asp <sub>376</sub> Glu, Arg <sub>377</sub> His, Trp <sub>574</sub> Leu, Ser <sub>653</sub> Thr/Asn/Ile, Gly <sub>654</sub> Asp	Powles and Yu (2010) Tranel et al. (2012); Tranel and Wright (2002)
ACCase	Ile <sub>1781</sub> Leu/Val, Trp <sub>1999</sub> Cys, Trp <sub>2027</sub> Cys, Ile <sub>2041</sub> Asn/Val, Asp <sub>2078</sub> Gly, Cys <sub>2088</sub> Arg, Gly <sub>2096</sub> Ala	Collavo et al. (2011); Délye (2005); Powles and Yu (2010)
EPSPS	Pro <sub>106</sub> Ser/Thr/Ala/Leu, EPSPS amplification	Gaines et al. (2010); Kaundun et al. (2011a); Powles and Yu (2010); Salas et al. (2012)
Tubulin	Leu <sub>136</sub> Phe, Thr <sub>239</sub> Ile, Met <sub>268</sub> Thr	Powles and Yu (2010)
PPO	Gly <sub>210</sub> deletion	Patzoldt et al. (2006)
PDS	Arg <sub>304</sub> Ser/Cys/His	Michel et al. (2004)

<sup>a</sup> At least nine different amino acid substitutions of Pro<sub>197</sub> have been reported to confer resistance.

not require much space or material and can be done relatively inexpensively. However, leaf disc assays can be very labor intensive. The tester needs a certain level of skill and experience to know which tissue will be best and how to avoid some of the potential problems with the assay, such as bacterial contamination. Although these types of assays have been used to screen populations in the field (Hanson et al. 2009), the interpretation of results need to be done very cautiously. These types of assays are good for initial screening, which allows immediate feedback to the grower. However, a follow-up with more robust assay is needed to determine the scope of resistance within a weed population accurately. Another weakness of a leaf disc assay is that it needs to be optimized for each new weed species. This requires time and patience by the tester. These assays are not definitive enough to be used as the only method to identify a resistant population. Leaf disc assays should be part of a system that includes whole-plant screening to confirm resistance.

**Pollen Germination Test.** This assay uses pollen germinated in agar medium containing the desired herbicide concentration (Letouzé and Gasquez, 2000). Resistance is determined by evaluating pollen germination under a microscope wherein > 50% germination is expected of the R biotypes and < 10% germination for the S biotypes. The test is fast (Table 3), but detects only target-site-based resistance and is severely limited by the difficulty of germinating pollen. This is not commercially viable and has very limited applicability.

#### DNA-Based Assays for Weed Resistance to Herbicides.

Now that many cases of weed resistance have been elucidated at the DNA level, it is becoming increasingly common for DNA-based tests to be used in resistance detection. The primary advantage of DNA-based tests over other methods is their speed; a typical herbicide-resistance test based on the polymerase chain reaction (PCR) can provide a yes/no answer within a day. Furthermore, they typically can be easily scaled for high-throughput analysis, allowing a single lab to run hundreds of samples a day with minimal space requirements.

Despite these advantages, DNA-based tests have some major disadvantages that one must consider before using them. Firstly, they are obviously limited to only those resistances in which the mechanism has been elucidated at the DNA level. Thus, DNA assays currently are limited to diagnosing only target-site-based resistance and for only the following herbicide targets: D1 protein, ALS, tubulin, ACCase, EPSPS, phytoene desaturase (PDS), and PPO

(Table 4). Second, because they test for a specific mechanism, one must be very careful in interpreting a negative test result: false negatives will occur any time a plant is resistant, but by a different mechanism (e.g., enhanced herbicide detoxification or a different target-site mutation) than that for which the test is designed. False-positive results are less likely, but also may occur as a result of, e.g., DNA contamination, PCR artifacts, or the presence of a nonfunctional or pseudogene carrying the mutation. One should also keep in mind that, even if a particular mutation is responsible for resistance in a given biotype, there is no guarantee that that is the only resistance mechanism present (Kaundun et al. 2011a). If one is only interested in a yes/no confirmation of resistance, then the presence of additional mechanisms is irrelevant. However, the presence of more than one resistance mechanism within a plant or population could impact, for example, the cross resistance or multiple resistance profile to other herbicides. Third, DNA tests typically must be designed and optimized for each resistance mutation/species combination. However, recent efforts have developed universal DNA tests for some herbicide resistances (see below).

**DNA Sequencing.** Nearly all known mutations conferring herbicide resistance in weeds are single nucleotide polymorphisms (SNPs) that result in the substitution of one amino acid for another (Table 4). One way to detect such a polymorphism is to sequence the gene of interest. Identification of an inferred amino acid change previously demonstrated to confer resistance can be taken as strong confirmation that the biotype is indeed resistant. In addition to mutations shown in Table 4, other mutations suspected or known to confer herbicide resistance have been identified via intentional selection (i.e., they did not evolve in field weed populations) in plant and/or nonplant organisms. For example, many more resistance-conferring ALS mutations are known than the ones that have thus far been identified in weeds (Tranel and Wright 2002). The patent literature also is a source of mutations conferring herbicide resistance that may or may not have yet been identified in weeds. For example, although target-site resistance to 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors has not yet been documented in weeds, HPPD mutations that confer resistance have been identified (Boudec et al. 2001; Busch et al. 2011).

If, after DNA sequencing, the only amino acid polymorphism(s) (relative to a sensitive biotype) identified is different from those previously demonstrated to confer resistance, then further research is needed to verify the significance of the polymorphism. Such research might entail overexpressing and

purifying variants of the enzyme with and without the suspect mutation, and comparing enzyme activities in the presence of herbicide, as was done when PDS mutations were first identified (Michel et al. 2004). Alternatively, one could compare herbicide sensitivity among organisms bearing transgenes that differ only in the presence/absence of the suspect polymorphism. For example, Whaley et al. (2007) utilized this approach with the easily transformed model plant *Arabidopsis thaliana* to demonstrate that the Asp<sub>376</sub>Glu ALS mutation identified in smooth pigweed (*Amaranthus hybridus* L.) was sufficient to confer resistance. In some cases, even more facile transgenic systems that rely on nonplant organisms (such as bacteria or yeast) have been utilized. For example, Baerson et al. (2002) and Patzoldt et al. (2006) used *Escherichia coli* mutants (which were deficient for EPSPS and PPO, respectively) to confirm target-site mutations suspected of conferring resistance to either glyphosate or PPO inhibitors.

**SNP Assays.** Although DNA sequencing can be used for herbicide-resistance diagnosis as just described, less expensive and faster assays are often desired. These assays focus on specific SNPs already demonstrated to confer resistance. For herbicide-resistance diagnosis, the most commonly used SNP assays utilize either restriction fragment-length polymorphism after PCR (PCR-RFLP) or PCR amplification of specific alleles (PASA; also commonly called allele-specific PCR or ASPCR) (Corbett and Tardif 2006).

The RFLP method utilizes a PCR step to amplify a region of the gene containing the SNP, followed by a restriction digest step, in which the PCR product is or is not digested, depending on what nucleotide is present at the SNP site. Gel electrophoresis is then used to visualize the lengths of the resulting DNA fragments and thereby determine if the PCR product was digested. The PCR-RFLP technique has been used widely in herbicide-resistance research. For example, it has been used to detect resistance mutations in *ALS* for at least a couple of decades (Guttieri et al. 1992). Two primary advantages of this approach include: (1) it yields a codominant marker and, thus, heterozygous and homozygous individuals can be identified; and (2) it generally is more robust than PASA (see below), because the diagnostic step (the restriction digest) occurs after the PCR reaction.

If the SNP conferring resistance does not result in the gain or loss of a restriction site, a variation of a PCR-RFLP marker, called a derived cleaved amplified polymorphic sequence (dCAPS) marker, potentially can be used (Neff et al. 1998). In this case, one of the PCR primers is designed such that it anneals adjacent to the SNP site and introduces one or more mutations, thereby creating a new restriction site that discriminates at the SNP site (Figure 3). Computer software is available to assist in designing primers for dCAPS markers (Neff et al. 2002).

PASA is an alternative approach to identify SNPs and is not dependent on the presence of a natural or derived RFLP. In this approach, one of the primers is designed to anneal only to the wild type or only to the resistance allele. Thus, the presence or absence of a PCR product is diagnostic for the SNP. Typically, the primer is made selective by a single nucleotide mismatch (corresponding to the SNP) at its 3' end. Greater selectivity (i.e., better discrimination between wild type and resistance alleles) sometimes can be achieved by

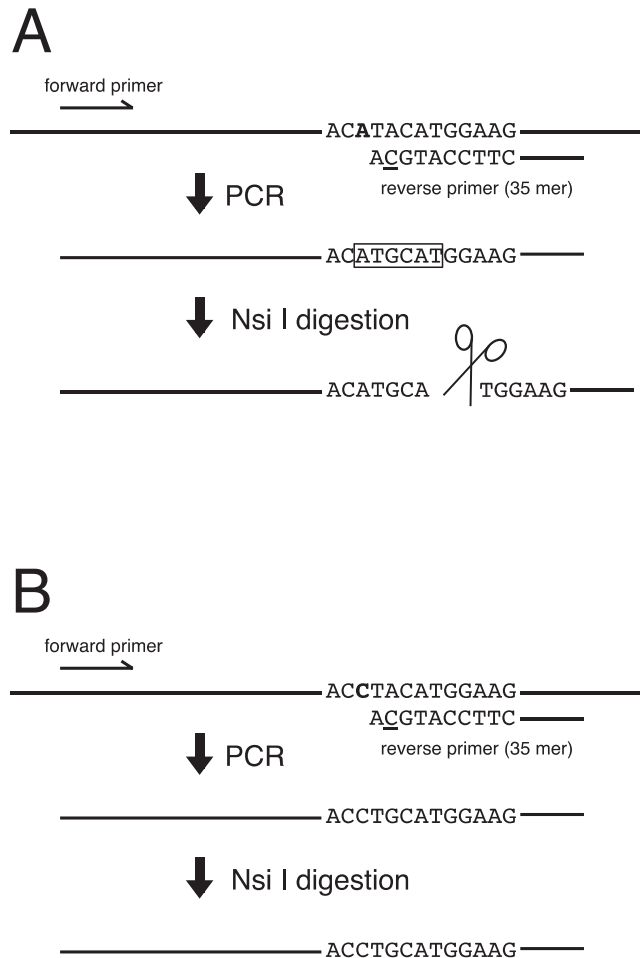


Figure 3. Example of the dCAPS technique. The reverse PCR primer was designed to anneal adjacent to the SNP of interest (A or C), and introduced a mutation (C) that resulted in the creation of an *Nsi* I restriction site (boxed) for the wild type allele (Panel A). For the resistance allele (Panel B), an *Nsi* I restriction site is not introduced. Thus, the final DNA products after digestion differ between the wild type and resistance alleles by about the length of the reverse primer and can be distinguished by gel electrophoresis. This example was taken from Kaundun and Windass (2006), who utilized it to detect resistance-conferring mutations at Ile<sub>1781</sub> of ACCase.

adding to the primer another nucleotide mismatch adjacent to or near its 3' end. Guidelines for designing primers that will be allele specific are available (Kwok et al. 1990; Pettersson et al. 2003). A disadvantage of the PASA approach is that the diagnostic step occurs during the PCR reaction, the robustness of which is influenced by quality and quantity of the DNA template. Thus, two non-allele-specific primers are often used in the PCR to generate a product that serves as a positive control for the reaction (Corbett and Tardif 2006). Additionally, a single PASA marker is not codominant. Thus, two separate PASAs (one each with a primer specific for the wild type or mutant allele) were utilized to detect, for example, plants that were homozygous or heterozygous for the Trp<sub>574</sub>Leu ALS mutation (Zheng et al. 2005). Délye et al. (2002a) utilized a similar approach for detection of Ile<sub>1781</sub>Leu ACCase mutation, but combined bidirectional allele-specific primers, which resulted in different-sized amplicons, in a single reaction. The combination of different allele-specific primers in a single reaction also can be used to enable simultaneous detection of different resistance mutations. For example, Corbett and Tardif (2008) developed such a

Table 5. General process flow for testing herbicide-resistant weeds.

Step	Description	Comment
1	Investigating a suspect field for resistance problem	Look for telltale signs of an evolving resistant population (Burgos et al. 2006; Moss 1999)
1a	Conducting field surveys	Structured surveys and field sampling are needed to determine resistance spread, frequency of resistance, or species distribution across a landscape. Considerations: Geographical coverage, biology of target species; survey information to collect—field sampling structure, number of fields to sample, time of year.
2	Collecting plant or seed samples	In general, one bulk seed sample is collected per field. Detailed information about resistance frequency or intrapopulation diversity will require separate plant samples per field. Considerations: Biology of target species, representation of field population, quantity and quality of seeds, quantity of separate plants to sample, sampling pattern.
3	Seed storage	Avoid conditions that will induce secondary seed dormancy or cause loss of seed viability. Considerations: Transport conditions from the field to the laboratory, drying conditions, preassay storage conditions.
4	Pregermination	To obtain uniform-size plants for testing, seeds may need to be pregerminated, then transplanted. Considerations: Seed dormancy-breaking treatments may eliminate the need for pregermination, hasten germination, and improve the uniformity of germination.
5	Choice of assay and designing the experiment	Depending on the objective and the number of samples to test, one can use either whole plants, seeds, leaf discs, molecular techniques, or any combinations of these. New investigators should consult a statistician. Considerations: Facility, equipment, or instrumentation needs of the assay; level of technical skills required; cost of materials; labor requirement; assay duration.
6	Choice of standard populations	A susceptible (S) standard must be included in every assay. Considerations: If possible, use a population with no prior exposure or minimal exposure to the herbicide; S standard from the same region as putative R population, but the same standard for large-scale tests; multiple S populations to represent the average species response.
7	Data analysis and interpretation of results	New investigators should consult a statistician. Considerations: Resistance level could be definite or just hovering around the recommended field dose, which signals a brewing problem (Moss 1999). Molecular-based tests and some quick tests need careful interpretation.

multiplex PASA assay that enabled detection of four different ALS resistance mutations from a single PCR.

DNA-based approaches also have been used to detect DNA changes other than SNPs that confer herbicide resistance. Resistance to PPO-inhibiting herbicides in waterhemp was conferred not by an SNP in the target-site gene, but rather by a codon deletion (Patzoldt et al. 2006). A PASA marker was developed for the detection of this mutation (Lee et al. 2008). In Palmer amaranth, resistance to glyphosate is due to several-fold amplification of the EPSPS gene (Gaines et al. 2010). A quantitative PCR (qPCR) assay was thus developed that enabled quantification of EPSPS gene copy levels relative to a control gene. Subsequently, Tranel et al. (2011) used this same approach, qPCR of the EPSPS gene, to screen waterhemp samples for resistance to glyphosate.

For high-throughput analysis, herbicide-resistance SNP assays can be developed that take advantage of real-time PCR (RT-PCR) systems (Giancola et al. 2006; Kaundun et al. 2006; Warwick et al. 2008). An RT-PCR assay utilizes fluorescence-based detection of the PCR product as it is produced, thereby eliminating the labor-intensive and time-consuming step of gel electrophoresis for PCR product detection. In addition to facilitating analysis of numerous individual samples, a high-throughput approach can be utilized to determine the frequency of resistance alleles in pooled samples. For example, Kaundun et al. (2006) developed a RT-qPCR assay capable of detecting one resistant (heterozygous) individual among 1,000 plants.

Towards facilitating the use of DNA-based assays as routine screening tools for herbicide resistance, Délye et al. (2011) recently reported dCAPs markers that should be widely applicable for the detection of target-site-based resistance to ACCase and ALS inhibitors in grass weeds. This suite of nine dCAPS markers was ingeniously designed to be universal both in terms of working across multiple grass species and in revealing the presence of a resistance allele regardless of the particular amino acid substitution. Consequently, dCAPs

markers now exist that can be used in grass species for all currently known ACCase and two of the most common ALS mutations found in these species.

## Summary

Weed science practitioners are inadvertently confronted with the need to test for known cases of resistance or evaluate new cases of resistance involving a different mechanism of action or different species. A steady flow of scientific publications and educational materials in print and electronic media had significantly increased resistance awareness and our knowledge about resistance testing approaches. As we continue to learn about the various aspects of weed resistance, we also continue to refine and diversify the techniques for surveying, sampling, and testing for weed resistance. Proper field surveys, plant or seed sampling, seed storage, choice of assays, use of reference populations, and analysis of test results are critical for planning resistance management actions or mitigation strategies. The general steps for resistance testing are the same (Table 5); however, details of each step vary depending on several factors, primarily the herbicide mode of action, weed species, timing of application, and the test objectives. There is no general consensus about sample size of plants to represent a population. Thus, we recommend a large sample size (20 to 40 mother plants) for self-pollinated species and small sample size (5 to 10 mother plants) for cross-pollinated species. The number of offspring tested per population varies widely. A large number is needed to detect rare events, but one should consult a statistician to determine the minimum number to test for resistance screening. Resistance confirmation is done either with a range of doses or a single, commercial dose for whole-plant assay, or a discriminating dose for soil-less assay. To estimate resistance levels, a wide range of doses should be used. It is generally accepted that large-scale resistance testing can be done with a discriminating dose involving either seedlings or seeds, or

using high-throughput DNA-based assays as appropriate. Whole-plant assays generate results that are closest to plant response in the field and are independent of mode of action. Results of quick assays are rarely compared with those of pot assays. In fact, soilless assays should be compared with pot assays to validate the reliability of the assay. Only resistance categories (high, intermediate, low) translate from soilless assays to the field. Discriminating doses in Petri plate assays are much lower than field recommendations. Many quick assays are specific to the mode of action or weed species; DNA-based assays are target-site specific. Thus, choosing the appropriate assay is critical. Reliability, efficiency, and cost of the assay are major considerations for commercialization; only few of the published protocols are used on a commercial scale. Some commercial resistance testing facilities are operating in Australia, Canada, the United States, and the United Kingdom, as surveyed by the Herbicide Resistance Action Committee (HRAC, 2012). Most growers rely on free testing services offered by academic, extension service, or public research institutions.

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