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Feature Review

Trends and Challenges in Pesticide Resistance Detection

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Pesticide resistance is a crucial factor to be considered when developing strategies for the minimal use of pesticides while maintaining pesticide efficacy. This goal requires monitoring the emergence and development of resistance to pesticides in crop pests. To this end, various methods for resistance diagnosis have been developed for different groups of pests. This review provides an overview of biological, biochemical, and molecular methods that are currently used to detect and quantify pesticide resistance. The agronomic, technical, and economic advantages and drawbacks of each method are considered. Emerging technologies are also described, with their associated challenges and their potential for the detection of resistance mechanisms likely to be selected by current and future plant protection methods.

Challenges of Pesticide Resistance

Since the dawn of agriculture it has been essential to protect crops from **pests** (see Glossary) to secure crop yield and safety versus pest toxins. Indeed, crop losses due to pests have been estimated at over 50% globally, depending on the crop considered [1]. The intensification of agricultural production after WWII was largely dependent on the highly effective pest control achieved with the development of chemical **pesticides** (mostly fungicides, herbicides, and insecticides) and the generalization of their use [1]. However, this progress was halted by the evolution of **resistance** against pesticides in pest populations. Such resistance has now been reported for numerous combinations of pesticides and pest species (weedscience.org/) [2,3].

Pesticide resistance is not only a textbook example of rapid adaptive evolution in response to human activities in organisms with short generation times [4], but it also has practical consequences, in that the evolution of resistance and its spread in pest populations can disrupt pest control, thereby threatening food security. This risk has been exacerbated by more stringent pesticide regulations, a direct consequence of rising public concern about the detrimental effects of pesticides on human health and ecosystems [5]. Both the development of resistance and the tightening of regulations have reduced the diversity of pesticide molecules and **modes of action** available for each crop [6,7], in turn increasing the risk of selection for resistance (e.g., [4]). Synthetic and natural pesticides have thus become a key but non-renewable resource for pest control globally. It is therefore essential to find ways to maintain their efficacy for agriculture through the implementation of integrated pest-management strategies impeding selection for resistance [4,8] (see also www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/Code/FAO_RMG_Sept_12.pdf). This requires accurate monitoring of the emergence and development of resistance in pest populations, in studies with appropriate sampling designs (e.g., reviewed in [9,10] for weeds or insects), using sensitive and reliable methods to detect and quantify the resistance.

Trends

Rational and sustainable (bio)pesticide use in a context of increasingly stringent pesticide regulations and the evolution of resistance in agricultural pests is vital to ensure global food security.

Monitoring resistance in pest populations is thus crucial and requires accurate, sensitive, and reliable methods.

Methods are reviewed for resistance detection in all major pest categories, with a view to facilitating resistance assay development and encouraging exchanges among communities working on different pests.

Resistance diagnosis assays should be sufficiently flexible to follow resistance evolution. They should also enable detection of resistant pest genotypes when their frequencies are still low enough to permit adaptation of crop protection strategies to restrict further resistance selection.

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Pesticide-resistant individuals are natural variants selected by pesticide applications because they carry genetic (i.e., nucleotide polymorphisms) or epigenetic modifications leading to biochemical, physiological, and ultimately phenotypic differences. Assays for resistance will thus focus on phenotypic, biochemical,or genetic ('molecular') modifications. The different pest categories have specific biological features, but the molecular bases of pesticide **resistance mechanisms** are otherwise most often the same across pests. Nevertheless, the scientific communities working on different categories of pests did not necessarily select and implement the same techniques to detect or quantify similar or homologous resistance mechanisms (e.g., insects [11,12], fungi [2,13], or weeds [14]). This cross-pest category review is designed to facilitate the development and implementation of diagnostic assays by providing a comprehensive overview of the approaches used by different research communities to address a common theme. It also attempts to identify new research directions and emerging techniques of particularly high relevance for the detection of pesticide resistance. Ultimately, this review is designed to foster scientific and technical exchanges between the different scientific communities.

Bioassays

Bioassays are designed to reveal differences in the phenotypic response to one or several pesticide molecules. They are based on the principle of exposing live pest clones (organisms capable of asexual reproduction or vegetative propagation), lines (organisms with sexual reproduction), populations, or isolates (all organisms) of interest to a pesticide, then comparing the consequences of this exposure with those for a **sensitive reference** through the use of appropriate descriptors. Mortality, survival, and growth are often used as descriptors, but other possibilities exist (Table 1). For the sake of simplicity, in this review we will refer to individuals, strains, clones, lines, or isolates as 'genotypes'. When setting up bioassays, sensitive references must be chosen with care so as to represent the diversity in sensitivity among pest genotypes before the use of the pesticide, if possible. The use of several reference pest populations sampled from different locations is the most common choice [2,9,11]. Bioassay methods also imply control over the exposure of the pests to the pesticides through standardization of the pesticide application procedure, taking into account the properties of the pesticide (e.g., mode of application, developmental stage targeted, penetration, expected action on pest biology, phenology, . . .) and the biological characteristics of the pest (e.g., treating genotypes all at the appropriate developmental stage). Factors determining the robustness of statistical analyses (e.g., sample size, replicates, number of pesticide doses, etc.) must also be considered.

Purposes of Bioassays

The development of bioassays is the first and compulsory step towards resistance characterization. Bioassays can be used for three main purposes (Table 1).

First, they can be used to determine the **resistance level** (RL) of the genotypes tested. This is generally achieved by analyzing dose–response curves obtained by applying a range of pesticide doses to pest genotypes and determining resistance levels on the basis of **effective concentration 50%** (EC_{50}) or **minimum inhibitory concentration** (MIC) values. This information can be used to differentiate resistant genotypes from the sensitive reference genotypes, making it possible to confirm the occurrence of resistance, to assess its relevance in the field, and/or to compare the 'strength' of resistance between genotypes.

Second, the use of a **discriminating dose** makes it possible for bioassays to detect resistant genotypes in pest populations, facilitating estimations of their frequency. This approach is often used to assess the spatiotemporal prevalence of resistance. The protocols used are similar to those for establishing dose–response curves, but the use of only one or a few doses of pesticide

increases the throughput of the assay, making it possible to test a large number of populations. Discriminating doses can be determined from dose–response curves. Use of the recommended field dose of pesticide is relevant only for pests where it is possible to carry out tests mimicking field conditions [2,15].

Third, bioassays can be used to provide insights into the nature of the mechanisms underlying resistance, and to distinguish between **target-site resistance** (TSR) and **non-target site resistance** (NTSR), in particular (Figure 1). This discrimination can be based on observations of the combined effect (i.e., **synergism**, **antagonism**) of the pesticide of interest and other molecules (e.g., other pesticides, inhibitors or activators of pesticides, metabolizing enzymes, inhibitors of pesticide-excreting proteins; Table 1).

When observing the resistance of a genotype to several pesticides with different modes of action, caution should be exerted before concluding that there is **cross-resistance** due to NTSR. The observed resistance profile may instead correspond to **multiple resistance**, which is common in many pests [8,16,17]. Cross- and multiple resistance should not be confused because they are inherited in different ways and have different consequences in practice (e.g., [18]).

General Features of Bioassays

The chief advantage of bioassays is their relative simplicity: they generally require basic and cheap equipment or consumables, except for whole-plant based assays which require access to a greenhouse or growth chamber facilities. Bioassays can be used to detect resistance regardless of the mechanisms involved, and preliminary identification of the resistance mechanism is not required. Different pesticides can be compared, especially for species displaying asexual reproduction or vegetative propagation. The major drawback of bioassays is that they require live pest genotypes. This requires the growth or multiplication of pests and their maintenance until the effects of the pesticide can be assessed. Bioassays are thus often labor-intensive (incurring additional costs), can be time-consuming, and require large amounts of space, although miniaturization is possible in some cases (e.g., microtiter-plate bioassays for microorganisms [19]; seed-based bioassays rather than whole-plant bioassays for weeds [20]). Bioassays can be tricky to set up, particularly regarding the crucial choice of discriminating doses. Bioassays are often specific to one species, even within a category of pests, in terms of equipment, design, pesticide exposure procedure, scoring criteria, timing, and data analysis. In addition, phenotypes are generally rated by visual assessment, thereby limiting precision and repeatability [15,21,22].

Future Prospects

The development of bioassays is the first essential step towards the detection and quantification of resistance. This approach is the only option available for identifying cases of resistance due to new, unknown, or complex mechanisms that cannot be routinely identified by biochemical or molecular assays. Developments in image analysis should drive future improvements in the technological development of bioassays. Recent developments in high-throughput plant phenotyping with automated imaging platforms and computer vision-assisted analytical tools [23,24] should greatly increase the rapidity, accuracy, and quality of phenotype scoring by making it possible to score the symptoms of diseased plants as well as weed size, color, or shape, or animal movement. The technology used by such platforms is sufficiently flexible to allow the development of tailor-made robots and miniaturized assays. This option may be cost-effective only for major pests for which very large numbers of samples must be processed for resistance monitoring or early detection purposes, in which case decreases in labor requirements should compensate for the cost of the technology [24–26].

Glossary

Antagonism: the combined effect of two or more molecules is lower than the sum of their separate effects.
Cross-resistance: resistance to several pesticides mediated by the same allele. Cross-resistance can be positive (resistance to several pesticides) or negative (resistance to some pesticides and hypersensitivity to others).
Discriminating dose: under controlled conditions, the pesticide dose killing or inhibiting the growth/development of all genotypes considered as sensitive. Any genotype displaying growth, development, or survival at this dose is considered to be resistant.
Effective concentration 50% (EC_{50}): pesticide concentration inhibiting 50% of a biological activity measured under controlled conditions; syn. ED_{50} (effective dose), IC_{50} (inhibitory concentration), LD_{50} (lethal dose).
Inhibitory concentration 50% (I_{50}): pesticide concentration inhibiting 50% of the target enzyme activity.
Minimum inhibitory concentration (MIC): lowest pesticide concentration inhibiting 100% of a biological activity measured under controlled conditions; syn. LD_{100} (lethal dose 100%)
Mode of action (MoA): the way in which the pesticide works, generally linked to a particular biochemical target.
Multiple resistance: resistance to several pesticides mediated by different alleles. May involve different mechanisms.
Non-target site resistance (NTSR): resistance caused by mechanisms other than target-site resistance (e.g., pesticide efflux, enhanced metabolism, sequestration, or reduced penetration) (Figure 1). NTSR includes multidrug resistance in fungi, that is, resistance to several pesticides mediated by toxicant efflux.
Pest: a living organism detrimental to crop production that can be an animal (arthropod, rodent, . . .), a plant (weed), or a phytopathogenic microorganism (bacterium, fungus, . . .).
Pesticide: a compound (synthetic or natural) that kills pests or inhibits their growth or development.
Resistance: (i) Natural, inheritable ability of mutant pest genotypes to

Biochemical Assays

Most pesticides act by binding to and inactivating a protein that is vital for the pest. Their efficacy depends on the number of pesticide molecules reaching their binding site (Figure 1). Biochemical assays are frequently used to characterize resistance mechanisms [27], but they can also be used to detect resistance in situations where the mechanisms of resistance have been elucidated [8,28]. Biochemical assays reveal differences related to the pesticide target (i.e., TSR) or to pesticide neutralization (i.e., NTSR) (Table 2, Figure 1).

Target Enzyme Assays

TSR can result from structural changes in the pesticide target, that decrease the affinity for pesticide binding, or from target overproduction (Figure 1). Decreases in pesticide binding affinity can be detected by directly monitoring the effect of the pesticide on substrate conversion by the target enzyme through the measurement of absorbance or fluorescence [29–31]. Resistance can be diagnosed, by *in vitro* or *in vivo* assays, by a higher **inhibitory concentration 50%** (I_{50}) value of the target enzyme in the presence of the pesticide in resistant versus sensitive genotypes (Table 2) [29,30,32,33]. Target overproduction (due to overexpression of the corresponding gene; Figure 1) leads to an increase in enzyme activity with no change in pesticide affinity [34]. It can be differentiated from structural changes in the target by measuring the **specific activity of** the enzyme [35,36]. Target enzyme assays can be used for routine or high-throughput analysis if the substrate or suitable analogs are readily available. For this purpose, miniaturized assays on crude protein extracts can be performed in microtiter plates because enzyme activity is generally sufficiently high for most plant and insect target enzymes [37].

Metabolic Enzyme Assays

Pesticide neutralization by enzymes involved in metabolism pathways (Figure 1) is widespread and has been frequently reported in arthropods [38] and weeds [8]. It has been observed only rarely as the principal resistance mechanism in phytopathogenic fungi, despite the capacity of soil fungi for such neutralization, as demonstrated by their use in xenobiotic bioremediation (e.g., [39]). The main enzyme families involved in pesticide catabolism and/or sequestration are cytochrome P450-dependent mixed-function oxidases, glutathione-*S*-transferases, glycosyltransferases, and carboxylesterases [11,40–42]. In resistant genotypes, pesticide-degrading enzymes are generally overproduced (gene amplification or upregulation of expression) [43] or modified, resulting in more efficient pesticide degradation as a result of an increase in specific activity [44]. However, cytochrome P450s or esterases involved in pro-pesticide activation [45–47] may be repressed in resistant genotypes. Assays based on colorimetric or fluorimetric detection can be used to reveal variations in the activity of pesticide-degrading enzymes via the use of specific substrates if commercially available [48]. Alternatively, ELISA-based techniques may be used if appropriate antibodies are commercially available [49–51].

Other assays can be developed if enzyme assays are not relevant or technically feasible. The most common technique involves the use of radiolabeled pesticides for the *in vivo* monitoring of pesticide penetration, translocation or excretion [52,53], degradation (via pesticide metabolite detection (e.g., [51,54]), or binding [33,55]. Such assays require access to radiolabeled pesticides and costly hi-tech laboratory equipment (LC-MS, HPLC-MS). They are therefore rarely used to monitor resistance in pest populations [55].

Finally, some 'unusual' biochemical assays specific for a particular pest category and type of pesticide have been developed for resistance monitoring. Examples include tests assessing blood coagulation in rodents [56] or based on isothermal calorimetry and FT-Raman spectroscopy for the detection of NTSR in weeds [57] (Table 2). The feasibility of adapting these techniques to other pests or pesticides remains to be evaluated.

survive pesticide concentrations that kill or inhibit the development of wild-type genotypes of the same species (sensitive genotypes); (ii) outcome of the adaptive evolution of pests as a result of selection for the least pesticide-sensitive genotypes under intense pesticide selective pressure.

Resistance level (RL) or resistance ratio (RR): the ratio of the pesticide concentrations required to obtain the same efficacy against resistant genotypes as for reference sensitive genotypes. The RL is usually expressed as the ratio (EC_{50} resistant/EC_{50} sensitive), determined by exposing these genotypes to an appropriate range of concentrations (under identical, controlled conditions).

Resistance mechanism: any mechanism allowing a resistant genotype to survive at a pesticide concentration that kills or inhibits the growth or development of sensitive genotypes.

Sensitive (susceptible) reference or reference population: a population expected to contain only pesticide-sensitive genotypes that should be representative of the pest population before pesticide use.

Specific activity: the amount of product generated by an enzyme per unit of time and per unit quantity of protein.

Synergism: the combined effect of two or more compounds is greater than the sum of their separate effects.

Target-site resistance (TSR): resistance caused by genetic modifications affecting the pesticide target protein (target protein modification, gene amplification, or gene expression changes; Figure 1).

Table 1. Bioassays Used To Detect or Quantify Resistance in Pest Populations

Method	Descriptor Observed	Advantages	Drawbacks	Purpose of the Bioassay (Selected References)		
				Dose–Response, Discriminating Dose Establishment	Detection or Quantification	Indications About Resistance Mechanism
Weeds						
Whole-plant bioassay	Mortality Biomass	Similar to in-field conditions	The relevance of dose–response is questionable for genetically heterogeneous populations. Space-consuming, requires reliable spraying equipment. Tricky for soil-applied herbicides	[9,15]	[15]	
Seed bioassay	Mortality Root or shoot elongation Shoot bleaching	Miniaturized (in soil, agar, blotting paper, or microtiter plate). Faster than whole-plant bioassay	Relevance of dose–response questionable for genetically heterogeneous populations. Non-dormant seeds are required: end-of-season test only. Consistency with whole-plant sensitivity must be checked, especially for leaf-applied herbicides	[20,103]	[20,103]	
Use of pesticide-degrading enzyme inhibitors	Restoration of sensitivity	Indicates NTSR	No conclusion can be drawn in the absence of sensitivity restoration			[104]
Use of poorly metabolizable herbicides	Sensitivity of individuals resistant to highly metabolizable herbicides Resistance of individuals resistant to highly metabolizable herbicides	Indicates NTSR Indicates TSR	Absence of TSR not demonstrated (not all TSR alleles confer resistance to all herbicides)			[105]
Use of herbicide safeners[a]	Increase in genotype resistance level/frequency of resistant genotypes	Indicates NTSR	May not reveal all NTSR mechanisms May not work on dicotyledonous weeds			[62]

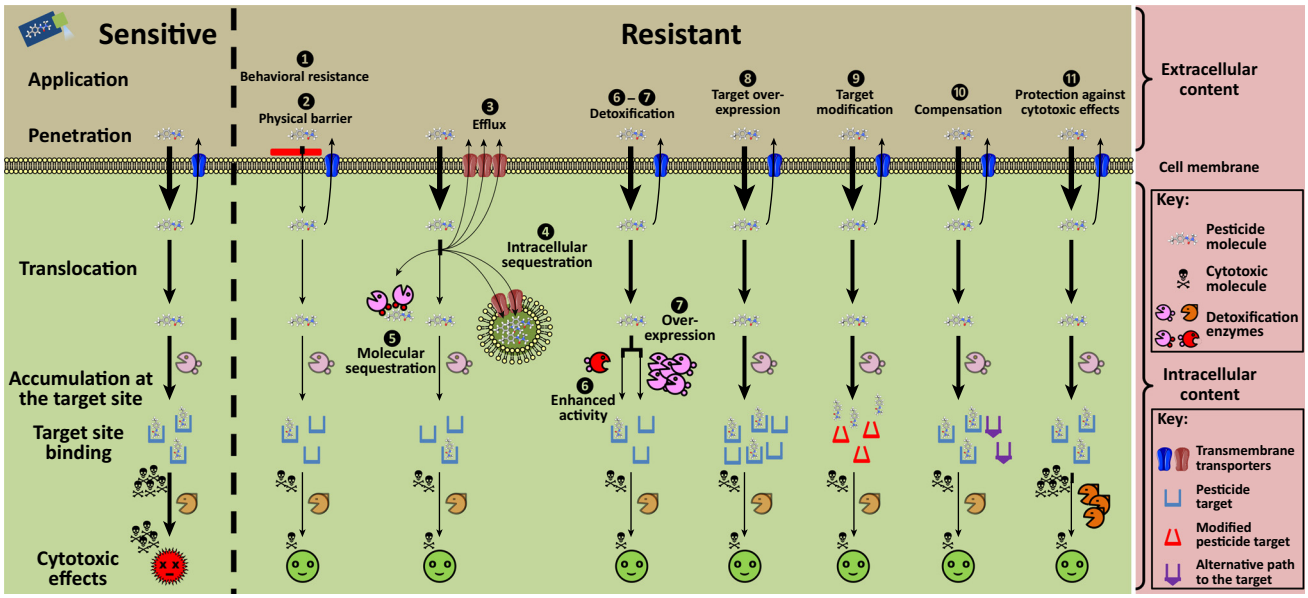
Table 1. (continued)

Method	Descriptor Observed	Advantages	Drawbacks	Purpose of the Bioassay (Selected References)		
				Dose–Response, Discriminating Dose Establishment	Detection or Quantification	Indications About Resistance Mechanism
Insects and Mites						
Ingestion bioassay	Mortality	Artificial medium as a food source, possibly in microtiter plate. Useful for most modes of action	Uses insects that are feeding, usually F1 neonates May need population amplification via sexual reproduction	[28,106]	[107]	
Bioassay of contact with and/or exposure to vapor	Mortality	Tarsal or topic exposure, in vials, by dipping insects, or with sticky cards	Population heterogeneity Acceptable confidence interval	[108]	[107,109]	
In planta bioassay (plantlet or detached organ in Petri dishes or microtiter plates)	Mortality	Leaf spraying or dipping	Quantification of exposure dose	[110–112]	[48]	
Insecticide + inhibitor of pesticide-degrading enzymes	Restoration of sensitivity	Indicates NTSR	Needs many individuals			[48,113]
Behavior bioassay	Avoidance of insecticide exposure	Contact or ingestion	Scoring			[106,114]
Fungi and Bacteria						
In vitro (Petri dishes) bioassay on individuals	Germination, germ tube elongation, mycelial growth or sporulation	Comparison of several criteria possible, according to MoA	Standardized artificial conditions Needs purification of strains	[115,116]	[117]	
In vitro (microtiter plates) bioassay on individuals	Mycelial growth	Miniaturized	Standardized artificial conditions Not suitable for modes of action requiring early scoring Needs purification of strains	[115,118]		
In vitro (Petri dishes or microtiter plates) bioassay on bulk individuals	Germination Germ tube elongation	Fast, no need for strain purification High level of representativeness of the population	Expert scoring Scoring complicated by environmental contaminants		[71]	

Table 1. (continued)

Method	Descriptor Observed	Advantages	Drawbacks	Purpose of the Bioassay (Selected References)		
				Dose–Response, Discriminating Dose Establishment	Detection or Quantification	Indications About Resistance Mechanism
In planta (plantlet or detached organs in Petri dishes or microtiter plates) bioassay on individuals	Germination Germ tube elongation Disease symptoms on plantlets	Mimics field conditions Suitable for use with biotrophs	High variability Scoring subjectivity Needs purification of strains	[2,119]	[2,119]	
Fungicide + modulator of membrane transporter	Restoration of sensitivity	Suggests MDR if transporter modulators restore sensitivity	The mode of action of modulators is not always known			[53]
Cross-resistance bioassay	Cross-resistance between specific fungicides with different MoA	Suggests MDR in positive cross-resistance	May be confused with multiple resistance if inappropriate fungicides are used			[120,121]
Synergy/ antagonism bioassay	Change in additional sensitivity	Excludes TSR	No conclusion can be drawn in the absence of synergy/antagonism			[122,123]
Rodents						
Ingestion bioassay	Lethal feeding period	Simple	Possible additional behavioral resistance		[124]	

[a]Safener, a herbicide metabolism enhancer initially intended for selective crop protection.



Trends in Plant Science

Figure 1. Resistance Mechanisms Evolved by Pests Against Pesticides. Resistant genotypes have mechanisms that are absent or regulated differentially from sensitive genotypes, and that interfere with pesticide action, allowing them to survive. Resistance mechanisms can interfere with all steps of pesticide action, from pesticide contact with the pest after application to the triggering of cytotoxic effects following pesticide binding to its target site. Behavioral resistance (1) reduces the exposure of the pest to a pesticide. This includes increases in repulsion or irritancy when exposed to the pesticides, modification of the pest's habitat preferences towards untreated habitats, and modification of the life cycle resulting in an absence of the pest developmental stage targeted by the pesticide at the treatment date. Pesticide penetration can be limited by modifications to the physicochemical properties of the pest cuticle, epidermis, or digestive tract (2). Several types of mechanism can reduce the accumulation of the pesticide at its target site: excretion by transporters (enhanced efflux, 3), intracellular compartmentalization (4) or sequestration by molecular binding (5), enhanced detoxification due to isoforms more active against the pesticide (6), or the overproduction of pesticide-neutralizing enzymes (7). Other possibilities include compensation for the inhibition of the pesticide target site by an alternative pathway or enzyme (10) or by neutralization of cytotoxic molecules generated by pesticide action (11). All these mechanisms pertain to non-target site resistance. Target-site resistance mechanisms involve an increase in the concentration of the intracellular target protein [target overexpression (8)] or structural modifications decreasing pesticide binding (9). These resistance mechanisms are not mutually exclusive and can be combined within the same genotype. The reader is referred to specific reviews for more detailed information about resistance mechanisms [2,12,40].

General Features of Biochemical Assays

One major advantage of biochemical assays is that they generally require only basic laboratory equipment. This, and the potential for miniaturization, makes relatively cheap high-throughput analyses possible (e.g., with a microtiter plate reader [50]). In metabolic enzyme assays, preliminary pesticide application may not be necessary. However, on the down-side, biochemical assays have several constraints in common with bioassays: the results must generally be considered in comparison to sensitive references that must be chosen with care (see above); a threshold value for a specific activity or pesticide I_{50} must also be defined to make it possible to distinguish between resistant and sensitive genotypes [32,58]; and most biochemical assays are performed on living material that must be maintained under artificial or controlled conditions, which can in some cases affect the level of enzyme activity compared to in-field conditions (e.g., [59,60]). This material must be treated with pesticides in many cases to detect pesticide-induced enzymes. An additional disadvantage of most enzyme or protein assays is that specific pest organs, tissues (e.g., insect midgut), or subcellular fractions (e.g., membranes) must be analyzed, necessitating fastidious dissection steps (animals), subcellular extract preparation (plants and fungi), or enzyme purification before the activity assays. Dissection may not be possible for tiny organisms, and biochemical analysis of whole-organism homogenates may fail to detect enzyme activities restricted to specific tissues or may face interference from enzyme inhibitors present in other tissues (e.g., [61]). Proteins may not withstand freezing and must therefore often be extracted immediately before the assay. The pigments present in plants, such as chlorophyll

Table 2. Biochemical Assays Used To Detect or Quantify Resistance in Pest Populations

Method	Mechanism of Resistance Identified	Starting material (Techniques)	Advantages	Disadvantages	Refs
Target Enzyme Assays					
Quantification of target enzyme activity and inhibition	TSR	Fresh tissue or protein extracts (fluorimetry, colorimetry)	High-throughput possible (microplates)	Requires living material Substrate may not be readily available	[11,32,56,58]
		Mitochondrial or microsomal extract		Time-consuming, requires hi-tech equipment, may require labeled pesticides for binding studies	[11,27,33,125]
Metabolic Enzyme Assays					
Detection/quantification of enzyme activity (cytochromes P450, glutathione-*S*-transferases, glycosyltransferases, hydrolases, esterases)	Non-activation, detoxification, sequestration, protection against oxidative stress, excretion	Fresh tissue or protein extracts (fluorimetry, colorimetry) Microsomal extracts (cytochromes P450)	High-throughput possible (microplates) Diagnosis of constitutive resistance does not require pesticide application	Relatively time-consuming. Does not identify the isoform involved in resistance Pesticide application required to detect pesticide-induced resistance May require radiolabeled substrates	[11,28,47,50, 104,126]
Detection/quantification of proteins involved in metabolism by antibody-based methods (all metabolic enzymes)	Non-activation, detoxification, sequestration, protection against oxidative stress, excretion	Protein extracts	High-throughput possible (microplates) or directly in field (lateral flow stick) Specific for one class of proteins/one isoform Possibility of freezing (−80 °C) to facilitate batch analyses	Requires specific antibodies Pesticide application is necessary to detect pesticide-induced resistance	[28,49–51]
Other Assays					
Monitoring of pesticide penetration, accumulation, and translocation	Altered uptake (e.g., energy-dependent efflux) or translocation	Whole organism, fresh tissues		Requires labeled pesticides and expensive hi-tech equipment Highly time-consuming	[53,127,128]

Table 2. (continued)

Method	Mechanism of Resistance Identified	Starting material (Techniques)	Advantages	Disadvantages	Refs
Chlorophyll fluorescence imaging	TSR and NTSR	Whole organism (fluorimetry)	High-throughput possible (microplates)	Requires pesticide application	[63,129]
Metabolite-based assay for resistance detection	Potentially any mechanism modifying pest metabolism	Whole organism, fresh tissues	No need to know the resistance mechanism	Requires pesticide application, accuracy to be checked, identifying relevant metabolites can be tricky	[54]
Blood clotting test	Anticoagulant resistance in rodents	Live rodents		Live rodents for intraperitoneal test solution injection	[56]
Future Prospects					
Near-infrared spectroscopy (NIRS) FTIR spectroscopy	Potentially any mechanism	Whole organism	Non-destructive for living organisms, not time consuming, cheap, could be used in the field	Protocol to be developed according to the organism Needs large updated databases specific to each pest organism	[65]
Proteomic profile (i.e., 2D gel electrophoresis, identification of differential protein spots by mass spectrometry)	Potentially any mechanism	Protein extract	Quantitative proteomics	Requires costly laboratory equipment Highly time-consuming; more suitable for R&D purposes	[62,130]

and phenolic compounds, can distort colorimetric or fluorimetric measurements. Lastly, the amount of starting material required for some biochemical assays may imply pooling tissues from several genotypes, which reduces assay sensitivity and makes determining the frequency of resistant genotypes impossible.

In situations in which the resistance mechanism is unknown, proteomic analysis of resistant individuals may identify the protein(s) involved, making it possible to develop biochemical assays, but proteomic analysis requires hi-tech equipment [62].

Future Prospects

Pesticide resistance mechanisms, particularly NTSR mechanisms, can result in differences in pest metabolism. Determinations of metabolites or cell components can thus be useful for detecting some types of resistance. Several studies have reported a strong correlation in weeds between pesticide resistance and endogenous concentrations of metabolites not directly involved in resistance, such as sugars or anthocyanins [63,64]. These correlations could be used as the basis for colorimetric or spectroscopic resistance assays. Near-infrared spectroscopy (NIRS) is a non-destructive technique that can reveal differences in the chemical composition of any target organism. This approach is principally used in clinical practice, where it has multiple applications, but it has also been shown to detect fungicide resistance rapidly and with high sensitivity [65]. Because enhanced metabolic enzyme activities involved in cuticle hydrocarbon composition in insects have been linked to resistance, NIRS may also be used to detect insecticide resistance (H. Ranson, personal communication). The broader use of this technique in the detection of resistance in multiple pests would require the establishment of specific protocols and reference spectra.

Molecular Assays

Molecular, nucleic acid-based assays detect genes or mutations involved in resistance. The starting material is living or dead tissue, either from one genotype or from a bulk genotypes (i.e., a population). Sufficient DNA or RNA of suitable quality must be extracted for downstream analyses. The different types of nucleic acid-based assays described to date for pests are described in Table 3, in increasing order of technical complexity.

Molecular assays can be classified into two groups on the basis of the nature of the technology used. 'Rugged' and/or low-throughput assays make use of basic techniques to detect a few mutations in a limited number of samples, and are potentially suitable for use in the field. 'Hi-tech' and/or high-throughput assays require more elaborate technologies and equipment, and have considerable potential for use in the simultaneous high-throughput detection of multiple resistance mutations in large samples. However, such assays are still largely underused for pests.

Genotyping Assays

Many assays are based on the genotyping of known resistance mutations. 'Rugged' assays can be used for genotyping after DNA amplification or in a ligation assay. DNA amplification is generally based on PCR, requires basic molecular biology equipment, and can be performed on crude DNA extracts [66]. Non-PCR-based amplification technologies have the potential for applications in the field involving the use of dedicated, cheap, and robust instruments (e.g., loop-mediated isothermal amplification, LAMP [67]; recombinase polymerase amplification, RPA [68]). 'Hi-tech' genotyping assays have a higher throughput capacity but involve elaborate methods requiring costly equipment and staff with a higher degree of technical skill. They generally provide the most accurate and sensitive detection and quantification of mutations (e.g., [69]). The principal advantage of quantitative molecular-resistance diagnosis assays over all other types of assay is their very low detection threshold (reviewed for insecticide resistance in [70]). They may allow the detection of resistant genotypes in a pest population sufficiently early

Table 3. Molecular Assays Used To Detect or Quantify Resistance in Pest Populations

Method	Purpose[a]	Detection	Advantages	Limitations	Examples of Techniques[b]	Refs
Genotyping (Known) Mutations by DNA Amplification or Ligation Detection						
'Low-tech' PCR-derived mutation genotyping	D/Q	SNPs, indels	Cheap, simple, basic technical requirements	Adaptation to high-throughput requires equipment	PCR-RFLP/CAPS, PIRA-PCR/ dCAPS Allele-specific PCR	[131–136] [92,137,138]
'Hi-tech' PCR-derived mutation genotyping	D/Q	SNPs, indels	High-throughput, allows sample pooling	Requires costly equipment and reagents, calibration for quantification	MALDI-TOF/Sequenom® MassARRAY HRM, SimpleProbe® melting curve analysis SNuPE (SNaPshot™) ASPPAA, KASPar-qPCR™, ARMS/Scorpion®, TaqMan®	[139–141] [142–144] [145,146] [147–153]
Oligonucleotide ligation Assay	D/Q	SNPs, indels	Cheap, rapid, high-throughput possible	Adaptation to high-throughput requires equipment	OLA, HOLA, SOTLA	[154,155]
Isothermal amplification	D/Q	SNPs, indels	Cheap and rugged, high-throughput possible	Adaptation to high-throughput or quantification requires equipment; complex assay design	LAMP	[156–158]

Table 3. (continued)

Method	Purpose[a]	Detection	Advantages	Limitations	Examples of Techniques[b]	Refs
Genotyping Mutations by Sequencing						
PCR + Sanger sequencing	D	SNPs, indels	Detection of unknown resistance mutations	Not suitable for large samples, requires informatics analysis	PCR followed by Sanger sequencing	
PCR + next-generation sequencing (NGS)	D/Q	SNPs, indels	High-throughput; detection of numerous resistance mutations; allows sample pooling	Relevant only for large samplings; requires costly equipment or subcontracting, downstream bioinformatics analysis Quantification must take the ploidy of the species and sequencing error rate into account	PCR followed by pyrosequencing, 454, or Illumina sequencing	[66,159,160]
Gene Copy and Transcript Quantification						
Quantitative PCR	D	Gene amplification	Can be adapted to high-throughput	Requires costly equipment and reagents, calibration for quantification	qPCR	[161–163]
Reverse-transcription + quantitative PCR	D	Differences in gene expression	Detects differences in expression when the causal mutations are unknown	RNA as starting material; requires costly equipment and reagents Threshold for resistance diagnosis to be set Relevant if only a few genes are involved in resistance	RT-qPCR	[164]
Future Prospects						
New PCR-based genotyping technologies	D/Q	SNPs, indels	Potentially cheap (fewer reagents); fast; potential for high-throughput	Requires costly equipment and reagents, calibration important	Bead-based HRM qHRM	[165] [144]
Whole-transcriptome sequencing	D	Differences in gene expression	Detects differences in the expression of numerous genes when the causal mutations are unknown	RNA as the starting material; requires very costly equipment or subcontracting and downstream bioinformatics analysis Expression threshold of the targeted genes to be set for resistance diagnosis	RNA-Seq, (miRNA-Seq)	[87,166]
Isothermal amplification	D	SNPs, indels	Fast, rugged, cheap	Complex assay design, specific equipment	Recombinase polymerase amplification (RPA), helicase-dependent isothermal amplification	[67,167,168]
Third generation sequencing	D/Q	SNPs, indels, gene amplification	High-throughput; detection of numerous resistance mutations; allows sample pooling	Only relevant for large samples; requires very costly equipment or subcontracting and downstream bioinformatics analysis Quantification must take into account the ploidy of the species and sequencing error rate	PacBio Nanopore, MinION Nanopore, SMRT®	[169] [76,83,170,171]

[a]D, detection of mutation(s); Q, quantification of mutation frequency within the analyzed sample. When Q is possible, samples or populations can be pooled beforehand.
[b]Abbreviations: ARMS PCR, amplification refractory mutation system PCR; OLA, oligonucleotide ligation assay; ASPPAA, allele-specific probe and primer amplification assay; CAPS, cleaved amplified polymorphic sequence; dCAPS, derived cleaved amplified polymorphic sequence; HOLA, heated oligonucleotide ligation assay; HRM, high-resolution melting analysis; KASPAR-qPCR, competitive allele-specific assay reagent-quantitative PCR; LAMP, loop-mediated isothermal amplification; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight mass spectrometry; miRNA-Seq, micro-RNA sequencing; PCR-RFLP, PCR restriction fragment length polymorphism; PIRA-PCR, primer-induced restriction analysis PCR; qHRM, quantitative HRM; qPCR, quantitative PCR; RNA-Seq, RNA sequencing; RT-qPCR, reverse transcription-quantitative PCR; SMRT®, single-molecule real-time DNA sequencing; SNuPE, single-nucleotide primer extension assay; SOTLA, short oligonucleotide tandem ligation assay.

during the selection of resistance to allow the pest control program to be adapted to eliminate or control the evolution of these genotypes [8,38,71]. 'Rugged' assays remain a reasonable alternative if only a few samples are to be analyzed or if cost or equipment is an issue.

Sequencing Assays

Genotyping by sequencing is an alternative to mutation genotyping. The detection of mutations in a gene by genotyping methods generally involves the development of a set of assays. By contrast, sequencing captures the full spectrum of nucleotide variation within a region of interest, making it possible to detect and identify all mutations at positions crucial for pesticide susceptibility, in addition to identifying new mutations of potential interest. DNA sequencing has long been based on the Sanger method [72]. The throughput of this approach has increased over the years, but the sequencing of numerous samples remains time-consuming and expensive. Sanger sequencing should thus be considered a flexible and reliable approach for the analysis of small samples. The advent of high-throughput sequencing (also called next-generation sequencing, NGS) technologies (described in [73]) has opened up many new possibilities for the accurate detection and quantification of mutations conferring pesticide resistance [74] by enabling the sequencing of one or several amplicon(s) of interest in numerous genotypes. Several batches of genotypes can be analyzed in a single NGS run using adequate amplicon tagging (e.g., [66]). However, the use of these techniques for this purpose is still in its infancy in crop protection [66].

NGS-based approaches to resistance diagnosis remain cost-effective only for large-scale experiments with vary large numbers of samples. Another limitation is the high error rate of NGS technologies, rendering the choice of mutation detection threshold a crucial issue [66], with downstream bioinformatics analysis being necessary to filter out sequencing errors [75]. The new generation of NGS technologies (referred to as third-generation sequencing technologies in Table 3) should resolve both these problems [76].

Gene Copy Number or the Quantification of Expression

Variations in gene copy-number or expression levels leading to pesticide resistance can be detected by qPCR-based techniques. RT-qPCR is relevant if a set of a few known genes is involved in resistance (e.g., target site overexpression, upregulation of genes involved in pesticide degradation and/or efflux). Novel NGS-based approaches, such as RNA-Seq, are gradually replacing microarray hybridization [77] and can detect larger sets of differentially regulated genes. However, their cost and the complexity of the required downstream bioinformatics analyses make this approach potentially useful only when resistance is driven by differences in the expression of many genes, as currently suspected for non-target site-based resistance to herbicides or insecticides (e.g., [78]).

General Features of Nucleic Acid-Based Assays

Current nucleic acid-based assays are fast, accurate, and can be adapted to the analysis of numerous samples. They do not require living material, which is a major advantage for biotrophic or slow-growing pests. Their major limitations are the need to identify the genetic variants involved in pesticide resistance and their associated resistance patterns before the development of detection assays. For polyploid species, assays must also be gene- and genome-specific, unless a mutation at one of the homologous loci confers resistance (e.g., [66,79,80]). Furthermore, the need to develop new assays for each newly identified genetic variant makes these tests most useful for the detection of major resistance alleles (although this may not apply to sequencing-based assays). As the dominant resistance mechanisms are expected to change over time in pest populations [2,8], nucleic acid-based assays must be regularly reassessed to ensure that they remain relevant for resistance detection.

Future Prospects

Emerging technologies should make it possible to develop two types of assays: first, flexible, high-throughput assays for the quantification of resistance by well-equipped facilities or subcontractors; second, cheap, 'rugged', and rapid assays for 'in-field' detection of resistance. This can be achieved by taking advantage of the increasing availability of 'grower-friendly' molecular biology equipment and/or of miniaturized nucleic acid amplification systems comparable to those developed for point-of-care diagnostics [81–83]. The type of assay to be developed will depend on the following issues: the cost of the analysis, the number of mutations to be sought, the number and type of samples to be analyzed (genotypes vs pools of genotypes), the purpose (detection or quantification), the need to reconstruct haplotypes for resistance detection, and the detection threshold for quantitative assays (Table 3).

Because future molecular assays will also target mutations, they will be subject to the same limitations as the techniques currently in widespread use: they will only be able to reveal mechanisms of resistance already identified and characterized. Future assays should also facilitate the detection of new types of resistance mechanisms that are gradually being elucidated. Most such mechanisms confer quantitative resistance via differential gene regulation mediated by partial genome duplications (e.g., aneuploidy [84]), by epigenetic changes such as DNA methylation [85,86], or by noncoding RNAs [87–89]. Indeed, little is currently known about these mechanisms, although these may play an important role in quantitative resistance. Current and future NGS technologies should play a major role in forthcoming assays targeting these types of mechanisms [90].

Concluding Remarks and Future Perspectives

Intensive agriculture is highly dependent on the efficiency of pesticide-based pest control [1], and resistance diagnosis is the key in sustaining this efficiency, particularly in the current global context of decreasing pesticide use (e.g., Directive 2009/128/EC; USA Conventional Reduced Risk Pesticide Program). The current need for resistance assays constitutes an admission of failure in the way pesticides have all too often been used. A more proactive use of resistance diagnosis assays should help to prolong pesticide efficiency. One option is to carry out analyses with tools and sampling adapted to allow the early detection of very low frequencies of resistant genotypes in pest populations, such that pest control strategies can be adapted before resistance evolution has become unavoidable [4,8] (see Outstanding Questions). Another option is to make use of biological material from accelerated pesticide-resistance selection experiments (e.g., [91,92]) to identify the potential resistance mechanisms before they evolve in the field. Proactive resistance detection should make it possible to nip the resistance in the bud by restricting resistance gene flow, adapting pest control programs, or assessing the efficiency of anti-resistance strategies [4,13,71].

It is technically possible to diagnose almost any type of resistance in any pest, using the methods outlined in this review. Biochemical and molecular technologies for assessing the evolution of resistance require a preliminary characterization of resistance mechanisms, whereas such characterization is not necessary for bioassays. It is relatively straightforward to develop diagnostic assays for TSR, but much more challenging for NTSR, although technological developments should facilitate the identification of resistance determinants [40,53]. Instead of simply using the technique best mastered in one's own laboratory, the choice of technique for resistance diagnosis should be carefully selected, taking into account the pest, the purpose of the test (detection vs quantification), the resistance mechanism (known vs unknown), the number of samples expected, and several additional parameters, as summarized in the Table S1 in the supplemental information online.

Outstanding Questions

Rapid and efficient detection of very low frequencies of resistant genotypes in pest populations is crucial for successfully implementing pest-control strategies and efficiently hampering resistance emergence. What would best guarantee the success of early resistance detection? Assay throughput and/or detection threshold, but also sampling design, need to be optimized for this purpose.

Which option should be preferred for efficient resistance monitoring? Costly high-technology assays (implying centralized facilities), or cheap and rugged 'grower-friendly' methods?

How can new technologies enhance pesticide resistance detection throughput while reducing space and labor requirements? Highly promising emerging technologies for this purpose include miniaturized robotized bioassays, near-infrared spectroscopy, and nanopore next-generation sequencing.

Is the precise knowledge of the mechanism of resistance to a pesticide essential for resistance diagnosis? While resistance detection does not necessarily involve the identification of resistance mechanisms (e.g., bioassays), knowing the resistance mechanism and its consequences (cross-resistance pattern, possible deleterious pleiotropic effects, ...) facilitates the design and the efficient implementation of resistance management strategies. Identifying the mechanisms at play in quantitative non-target-site-based resistances is of major importance for this purpose.

Will current and emerging detection technologies be adequate to address resistance to the future crop protection methods, in other words biocontrol, plant defense response inducers, RNAi, and CRISPR-designed crops? These methods will undoubtedly lead to the selection of novel resistance mechanisms and start a new round in the arms race between pests and crop protection.

Resistance is an evolutionary process. Under continuous pesticide selection pressure, resistance should continue to evolve towards more efficient mechanisms with very few if any deleterious pleiotropic effects [3,8,93,94]. The determinants of resistance in a pest species are therefore expected to change during the development of diagnostic assays, and diagnosis is thus always likely to lag behind evolution in the domain of resistance. Massive or 'hi-tech' assays must therefore be continually updated. For this purpose, bioassays, which are not the most 'glamorous' of resistance assays, are and will probably remain absolutely necessary for the detection of new types of resistance emerging from the tremendous adaptive potential present in pest populations.

There is currently growing interest in 'biopesticides' or in 'biotech-pesticides' based on plant defense-inducers [95], RNAi [96], or CRISPR-engineered plants [97–99]. Unfortunately, these compounds or agents are unlikely to provide a lasting solution to resistance. Biopesticides are also prone to the evolution of resistance [38,100,101], and the observation of variation in the response to RNAi-based pesticides [102] leaves little doubt that this also applies to biotech-pesticides. These future crop protection methods are more flexible and diverse than classical pesticides. However, if they are to live up to expectations and alleviate the resistance problems currently undermining pest control globally, lessons must be learnt from past errors in the management of chemical pesticides. Failing this, resistance assays will remain essential for many years to come.

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Supplemental Information

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