Pesticide Biochemistry and Physiology 100 (2011) 105–117

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Review

Metabolomics – A robust bioanalytical approach for the discovery of the modes-of-action of pesticides: A review

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ARTICLE INFO

Article history:
Received 16 December 2010
Accepted 18 March 2011
Available online 26 March 2011

Keywords:
Bioactive compounds
Crop protection
Functional genomics
Metabolic profiling
Natural products

ABSTRACT

The agrochemical industry is facing great undertaking that includes increasing demand for the development of new crop protection agents that are safe for the environment and the consumers, and at the same time combat the issue of the emergence of resistance pest strains. The mode-of-action (MoA) is among the features of a bioactive compound that largely determine whether the abovementioned issues are addressed or not, and subsequently whether its commercial development will be addressed. The early discovery of the MoA of bioactive compounds could accelerate pesticide research and development by reducing the required time and costs. Based on advances in synthetic and natural product chemistry, scientists have access to a vast number of compounds that could potentially be developed as crop protection agents. The screening of such compounds with respect to their MoA requires accurate and robust bioanalytical tools. Metabolomics is a powerful bioanalytical tool that will likely play a significant role in the acceleration of the discovery of MoA of bioactive compounds. In the present review, the capabilities and principles and applications of metabolomics for the study of the MoA of herbicides, insecticides, acaricides, fungicides, and antibiotics are discussed.

1. Introduction

The growing public concern about food safety and environmental health combined with the compliance of new registration requirements for pesticides [1,2] and the emergence of resistance to pesticides [3–5], have hard-pressed the agrochemical industries to discover and develop novel and improved crop protection agents. A large part of the effort focuses on the exploitation of natural sources of bioactivity, and to date, many natural products or their chemical analogues have been successfully developed as crop protection agents [6–8]. Based on the advances in synthetic chemistry and in natural product chemistry, scientists have now access to a vast number of molecules whose bioactivity could be of great value in crop protection strategies. Candidate molecules with potential to be developed as crop protection agents should share features such as, low toxicity for human and non-target organisms, efficacy even in low doses, low persistence in the environment and at the same time should combat the issue of evolved pest resistance by introducing new mode(s)-of-action (MoA). A major factor that determines such features of a bioactive molecule is its MoA. In contrast to the mechanism of action, which describes all the biochemical events that lead to the toxicity of a molecule, the MoA describes the specific biochemical interaction to which its bioactivity is mainly attributed. The existence of more than 85 different MoA of pesticides (herbicides, fungicides, and insecticides) (Tables A1, A2, A3, Fig. 1) [9–12] makes the screening for the discovery of the MoA of bioactive compounds a time-consuming and costly task. Additionally, the discovery of the MoA of bioactive molecules in the early stages of research greatly accelerates the procedure for the development of new crop protection agents by eliminating molecules and/or chemical structures that exhibit unfavorable features, for example, MoA common with already commercially developed pesticides.

Exposure of an organism to a bioactive molecule causes a general, reversible or not, disturbance of its metabolism affecting metabolic pathways and fluxes, which could finally result in its death. Thus, the comprehensive monitoring of the metabolome of an organism could provide invaluable information on its physiological status and the changes developing after exposure to bioactive

Abbreviations: ACCase, acetyl-CoA carboxylase; AHAS, acetohydroxyacid synthase; ALS, acetolactate synthase; EPSPS, 5-enolpyruvyl-shikimate-3-phosphate synthase; FT-ICR/MS, Fourier transform-ion cyclotron resonance/mass spectroscopy; GC/MS, gas chromatography/mass spectrometry; ^{1}H NMR, proton nuclear magnetic resonance spectroscopy; HPLC-ESI/MS, high performance liquid chromatography electrospray ionization/mass spectrometry; 4-HPPD, 4-hydroxyphenyl-pyruvate-dioxygenase; LC-TOF/MS, liquid chromatography time-of-flight/mass spectrometry; MoA, mode(s)-of-action; MS, mass spectrometry; PCA, principal components analysis; PDS, phytoene desaturase; PEP, phosphoenolpyruvate; PLS-DA, partial least squares-discriminant analysis; PPO, protoporphyrinogen oxidase; PSI, photosystem I; PSII, photosystem II.

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doi:10.1016/j.pestbp.2011.03.004

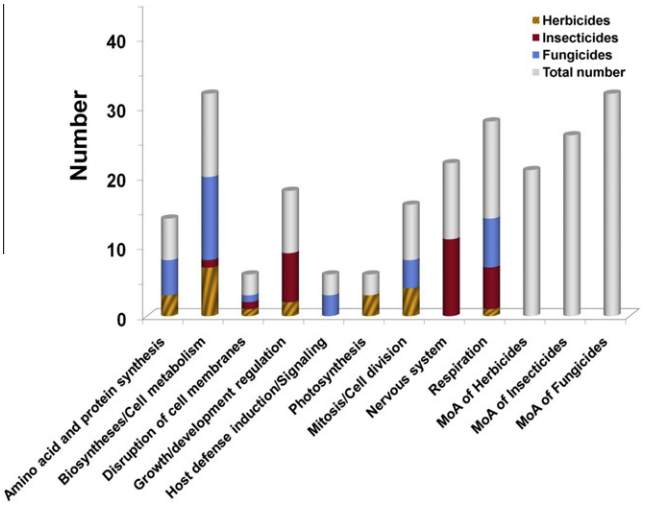

Fig. 1. Modes-of-action (MoA) of commercially developed pesticides grouped based on functional categories of targeted biochemical systems. For simplicity of discussion, MoA were categorized into one of nine functional groups. Data were retrieved from the Herbicide Resistance Action Committee (HRAC, http://www.hracglobal.com/), the Fungicide Resistance Action Committee (FRAC, http://www.frac.info/frac/index.htm), and the Insecticide Resistance Action Committee (IRAC, http://www.irac-online.org/) (Access December 2010).

compounds. Additionally, such analyses could provide information on the cause of the observed toxicity (e.g., biochemical target). This is where metabolomics is expected to significantly accelerate and assist the process of the discovery of the MoA of bioactive compounds, facilitating the robust detection of lead molecules with unique MoA. Metabolomics is a newly emerged bioanalytical tool for the study of biological systems that enables the comprehensive monitoring of global metabolite networks and their fluctuations in response to various stimuli [13,14]. Based on the central dogma of molecular biology, the genome, transcriptome, and proteome provide information on what is expected to happen in a biological system, while the metabolome provides the information on what is actually happening, thus serving as the link between genome and phenome. Presently, metabolomics is established as a powerful tool of systems biology, and its potential in the study of various biological systems has been confirmed [15–27]. Metabolomics is integrating aspects of experimental design and execution, sample preparation and chemical analyses, data processing and bioinformatics. The topics of instrument selection and data pre-processing and analyses for metabolomics are not discussed in detail in this review since they have been recently thoroughly reviewed elsewhere [15,16,28–30]. In the present review, the selection of model organisms, compounds to be applied for the development of metabolomics models, principles of metabolomics analyses, and studies on the application of metabolomics for the discovery of the MoA of herbicides, insecticides, acaricides, fungicides, and antibiotics are presented.

2. Principles of the application of metabolomics in the study of the modes-of-action of bioactive compounds

Following exposure of organisms to bioactive compounds, fluctuations in their metabolic network occur resulting in a general disturbance of their metabolism. Such alterations could be reversible or not depending on several factors such as the MoA of the applied compounds and dose rates, their metabolism, the time of exposure, the physiological condition of the organism, and environmental factors. A representative metabolomics protocol for the discovery of the MoA of bioactive compounds is displayed in Fig. 2.

2.1. Selection of the model biological systems

The central step in the development of metabolomics for the discovery of the MoA of bioactive compounds is the selection of the model biological system (Fig. 2). The bioactivity of the compounds being tested will mainly determine the selection of the model organism. Species that can be easily grown under controlled laboratory conditions requiring limited handling and also producing uniform populations are ideal for the development of metabolomics models. Metabolomics as a functional genomics approach could be employed for the study of genome regulation and thus, species with sequenced genomes would greatly facilitate the development of metabolomics models within the context of systems biology and should be included in such studies. A listing of biological systems that have been used for the development of metabolomics models in studies on the MoA of pesticides is displayed in Table 1.

Several plant species have been used for the study of the MoA of synthetic and natural herbicidal compounds applying metabolomics. These include monocots, such as maize (*Zea mays* L.) [31,32], wild oat (*Avena sterilis* L.) [33], and the aquatic microphyte duckweed (*Lemna minor* L.) [34], and dicots such as Arabidopsis (*Arabidopsis thaliana* L.) [35].

Organisms with nervous system such as earthworms (*Eisenia* spp. and *Lumbricus* spp.) have been extensively used in environmental metabolomics [18,36], however, they are underexploited as model biological systems for the study of the MoA of pesticides. Aquatic organisms such as the crustaceous *Daphnia* sp. seem to have a great potential for the study of the toxicity of bioactive compounds [37,38]. On the other hand, insects, and more specifically the fruit fly *Drosophila* [39] and the mosquito *Anopheles gambiae*, whose genomes are sequenced [40,41], have features that make them ideal organisms for the development of metabolomics models for the study of the MoA of insecticidal compounds. Until now, metabolomics have been successfully applied for the study of the physiology of *Drosophila melanogaster* related to hypoxia [42], heat stress [43,44], and mutations [45], making it a suitable biological system model for the study of the MoA of insecticides.

Cell cultures could be an alternative biological system for MoA studies applying metabolomics [46]. However, their potential in such application is yet to be validated. Cell cultures can grow under limited laboratory space providing a uniform material for experiments. Additionally, they enable bioactive molecules to exert their bioactivity directly on the primary site of action avoiding misinterpretation that could plausibly be caused by factors affecting absorption, translocation, or metabolism in the targeted organism.

In studies related to the discovery of the MoA of antifungal and antibiotic compounds applying metabolomics, the yeast (*Saccharomyces cerevisiae*) [47] and the pathogenic bacterium *Staphylococcus aureus* [48,49] have been used as model organisms.

Taken together, it is evident that the numbers of model biological systems that have been used in metabolomics studies for the study of the MoA of bioactive compounds is limited to few and that metabolomics approaches are largely unexploited. There are many more suitable organisms that could be successfully used as models for the development of robust metabolomics models for the discovery of unknown MoA of pesticidal compounds, and the detection of novel biomarkers of toxicity.

2.2. Selection of bioactive compounds

Following the selection of the model biological system, appropriate bioactive compounds should be chosen for the development of metabolomics models (Fig. 2). The first criterion for the selection of compounds is their MoA. Selected compounds should at least cover the most common MoA of pesticides (Tables A1, A2, A3), thus

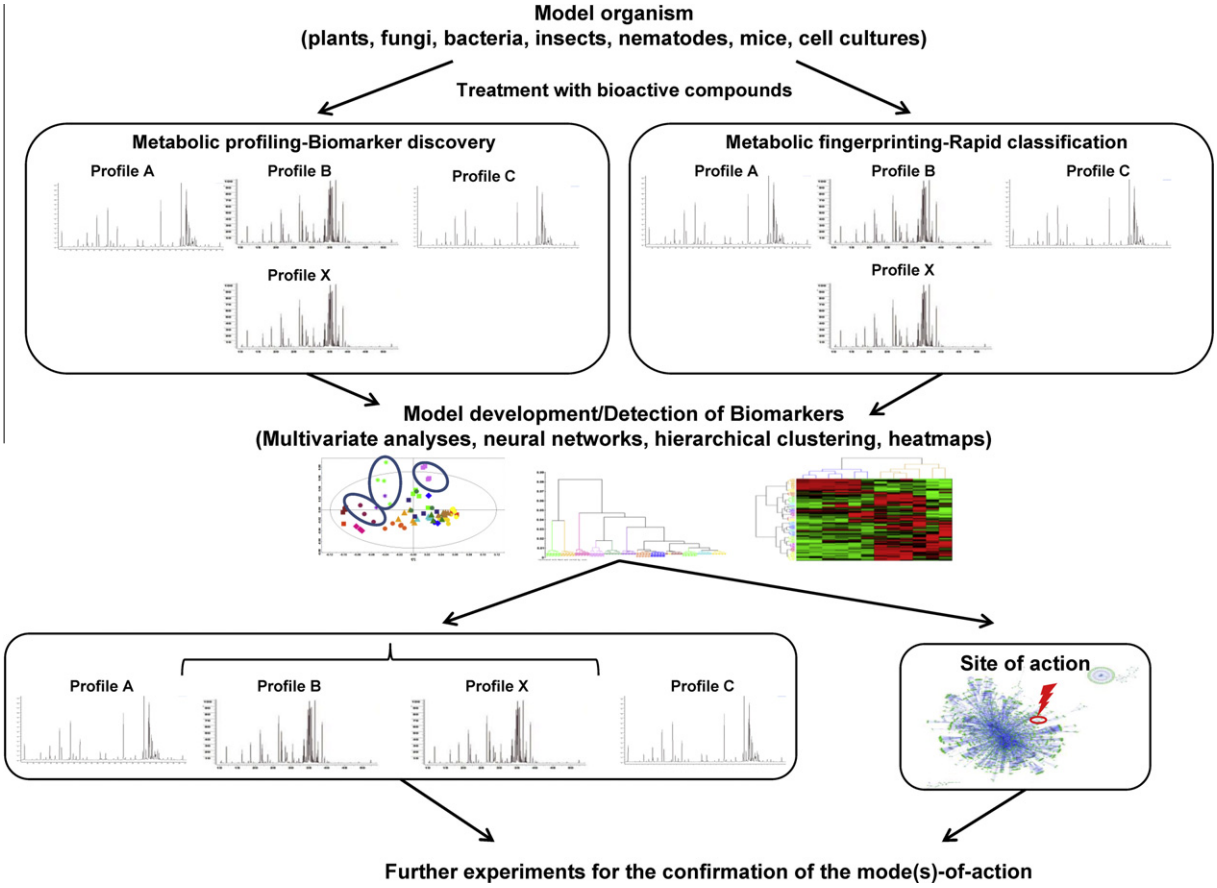

Fig. 2. Diagram showing the pipeline discovery of an unknown mode-of-action (MoA X) of a bioactive compound applying metabolic profiling, metabolic fingerprinting, or metabolic footprinting. The selected model organism is treated with bioactive compounds with known MoA and the resulting metabolic profiles are compared to those after treatments with the compound under testing (*X*) applying statistical modeling. Analyses reveal similarities between the analyzed metabolic profiles as well as biomarkers for the different MoA. Complementary experiments might be required for the confirmation of the unknown MoA.

increasing the possibilities of a "positive matching" of their MoA with the unknown MoA of the compound being studied. Furthermore, different compounds with identical MoA should be included in these studies in order to test the metabolomics protocols and the validity of the developed predictive models. The second criterion is the physicochemical properties of the compounds. It is preferable to select water-soluble compounds in order to avoid undesirable effects of organic solvents on the treated tissues that could lead to misinterpretation of results. However, in case of water insoluble compounds, organic solvents should be used but appropriate controls as well as a thorough study of their effects on the physiology of the treated organisms or tissues should be performed prior to metabolomics models development. Additionally, for highly bioactive compounds their translocation towards the primary sites of action and reactions with various components of the cells should be investigated. After the selection of the compounds, experiments are required to study dose–response relationships under the specified experimental conditions and determine the time of sampling. For metabolomics analyses it is recommended that compounds be applied at sub-lethal doses thus enabling the detection of their primary effects on the metabolism of the biological system, while excluding undesirable secondary effects.

2.3. Experimental design

The development of metabolomics models with high predictive abilities requires that a large number of replications be included in the analyses for achieving the maximum possible variation. A crucial factor for successful metabolomics analyses is ensuring that experimental and analytical conditions for all treatments and samples are identical.

The latest developments in analytical chemistry with the construction of powerful analyzers along with the design of software for high-throughput analyses of bioanalytical data [50,51], have facilitated the comprehensive monitoring and modeling of fluctuations in the metabolome of organisms in response to various stimuli and the detection of corresponding biomarkers of effect. MS analyzers (i.e., GC/MS, LC/MS, FT-ICR/MS, and Orbitrap MS) and NMR spectrometers are the most common analytical platforms employed in metabolomics [16,27,29,30,52–54]. Nonetheless, the integration of data derived from more than one analytical platform provides richer and more reliable information on the composition of the analyzed samples, and subsequently a wider coverage of the metabolome than using a single analytical platform [55–58]. Therefore, when available, the use of more than one analytical platform with analytical capabilities that complement each other is preferred for high-throughput metabolomic studies. Recorded spectra are pre-processed prior to statistical analyses [59] following guidelines that have been established for the standardization of metabolomics data processing and reporting [60,61].

The approach to discovery an unknown MoA of a bioactive compound applying metabolomics is displayed in Fig. 2. In principle, metabolomics models are developed for the association of the different MoA with the metabolic changes caused in the metabolomes of the model biological systems, followed by the detection of signatory metabolites (biomarkers). The unique capabilities of metabolomics are highly advantageous for pesticide research and

Table 1
Metabolomics models that have been developed for the study of the mode(s)-of-action (MoA) of bioactive compounds and/or the discovery of biomarkers of toxicity.

MoA (target)[a]	Chemical groups[b]	Bioactive compounds[c]	Model biological systems[d]	Analytical platforms[e]	Methodologies[f]	References
Amino acid, protein and nucleic acid synthesis[g]						
AHAS (ALS)	Imidazolinones	Imazamethabenz (H)	*Z. mays*	^{1}H NMR	NN	[31,32]
		Imazamox (H)	*A. thaliana*	FT-ICR/MS	MVA (PCA)	[35]
		Imazapyr (H)	*Z. mays*	^{1}H NMR	NN	[31,32]
		Imazethapyr (H)	*Z. mays*	^{1}H NMR	NN	[31,32]
	Sulfonylureas	Chlorimuron ethyl (H)	*S. cerevisiae*	LC-TOF/MS	MVA (PCA, DFA, HCA)	[47]
		Chlorsulfuron (H)	*Z. mays*	^{1}H NMR	NN	[31,32]
		Halosulfuron (H)	*A. thaliana*	FT-ICR/MS	MVA (PCA)	[35]
		Pyrazosulfuron (H)	*A. thaliana*	FT-ICR/MS	MVA (PCA)	[35]
		Sulfometuron (H)	*Z. mays*	^{1}H NMR	NN	[31,32]
DHP	Carbamates	Asulam (H)	*Z. mays*	^{1}H NMR	NN	[32]
EPSPS	Glycines	Glyphosate (H)	*A. sterilis*	^{1}H NMR	MVA (PCA, PLS-DA, SIMCA)	[33]
		Glyphosate (H)	*A. thaliana*	FT-ICR/MS	MVA (PCA)	[35]
		Glyphosate (H)	*L. minor* L.	^{1}H NMR	MVA (PCA, PLS-DA, HCA)	[34]
		Glyphosate (H)	*Z. mays*	^{1}H NMR	NN	[31,32]
Unknown (EPSPS?)	Macrolides	Pyrenophorol (H)	*A. sterilis*	^{1}H NMR	MVA (PCA, PLS-DA, SIMCA)	[33]
GS	Phosphinic acids	Bialaphos (H)	*Z. mays*	^{1}H NMR	NN	[32]
Protein synthesis	Amides	Ampicillin (A)	*S. aureus*	GC/MS	MVA	[91]
		Ceftriaxone (A)	*S. aureus*	GC/MS	MVA	[91]
		Oxacillin (A)	*S. aureus*	GC/MS	MVA	[91]
		Penicillin (A)	*S. aureus*	GC/MS	MVA	[91]
	Glycoconjugates	Vancomycin (A)	*S. aureus*	GC/MS	MVA	[91]
	Glycosides	Amikacin (A)	*S. aureus*	GC/MS	MVA	[91]
		Gentamicin (A)	*S. aureus*	GC/MS	MVA	[91]
	Macrolides	Erythromycin (A)	*S. aureus*	HPLC-ESI/MS	MVA (PCA)	[48,49]
		Erythromycin (A)	*S. aureus*	GC/MS	MVA	[91]
	Nitrobenzenes	Chloromycetin (A)	*S. aureus*	GC/MS	MVA	[91]
		Chloromycetin (A)	*S. aureus*	HPLC-ESI/MS	MVA (PCA)	[48,49]
	Polycyclic hydrocarbons (aromatic)	Tetracycline (A)	*S. aureus*	GC/MS	MVA	[91]
	Pyrrolidines	Clindamycin (A)	*S. aureus*	GC/MS	MVA	[91]
		Lincomycin (A)	*S. aureus*	HPLC-ESI/MS	MVA (PCA)	[48,49]
	Quinolones	Ciprofloxacin (A)	*S. aureus*	GC/MS	MVA	[91]
	Tetracyclines	Achromycin (A)	*S. aureus*	HPLC-ESI/MS	MVA (PCA)	[48,49]
	Thiazines	Cefoxitin (A)	*S. aureus*	GC/MS	MVA	[91]
DNA replication/transcription	Quinolones	Norfloxacin (A)	*S. aureus*	HPLC-ESI/MS	MVA (PCA)	[48,49]
Transcription	Glucosides	Streptomycin (A)	*S. aureus*	HPLC-ESI/MS	MVA (PCA)	[48,49]
	Lactams, Macrocyclic	Rifampicin (A)	*S. aureus*	HPLC-ESI/MS	MVA (PCA)	[48,49]
Biosynthesis/cell metabolism[g]						
ACCase	Aryloxyphenoxypropionates	Quizalofop (H)	*A. thaliana*	FT-ICR/MS	MVA (PCA)	[35]
		Cyhalofop (H)	*A. thaliana*	FT-ICR/MS	MVA (PCA)	[35]
	Cyclohexanediones	Alloxydim (H)	*A. thaliana*	FT-ICR/MS	MVA (PCA)	[35]
		Clethodim (H)	*A. thaliana*	FT-ICR/MS	MVA (PCA)	[35]
		Sethoxydim (H)	*Z. mays*	^{1}H NMR	NN	[31,32]
Carotenoid biosynthesis (unknown)	Triazoles	Amitrole (H)	*Z. mays*	^{1}H NMR	NN	[32]
Ergosterol biosynthesis		Epoxiconazole (F)	*S. cerevisiae*	LC-TOF/MS	MVA (PCA, DFA, HCA)	[47]
		Fluquinconazole (F)	*S. cerevisiae*	LC-TOF/MS	MVA (PCA, DFA, HCA)	[47]
		Triadimenol (F)	*S. cerevisiae*	LC-TOF/MS	MVA (PCA, DFA, HCA)	[47]
	Morpholines	Fenpropimorph (F)	*S. cerevisiae*	LC-TOF/MS	MVA (PCA, DFA, HCA)	[47]
4-HPPD	Cyclohexanones	Sulcotrione (H)	*Z. mays*	^{1}H NMR	NN	[32]
		Mesotrione (H)	*A. sterilis*	^{1}H NMR	MVA (PCA, PLS-DA, SIMCA)	[33]
		Mesotrione (H)	*L. minor*	^{1}H NMR	MVA (PCA, PLS-DA, HCA)	[34]
	Pyrazoles	Pyrazoxyfen (H)	*A. thaliana*	FT-ICR/MS	MVA (PCA)	[35]
		Pyrazolate (H)	*A. thaliana*	FT-ICR/MS	MVA (PCA)	[35]
PDS	Pyridazines	Norflurazon (H)	*Z. mays*	^{1}H NMR	NN	[32]
		Norflurazon (H)	*A. sterilis*	^{1}H NMR	MVA (PCA, PLS-DA, SIMCA)	[33]
		Norflurazon (H)	*L. minor*	^{1}H NMR	MVA (PCA, PLS-DA, HCA)	[34]
Peptidoglycan biosynthesis	Glycopeptides	Vancomycin (A)	*S. aureus*	HPLC-ESI/MS	MVA (PCA)	[48,49]
	Thiazines	Cefotaxime (A)	*S. aureus*	HPLC-ESI/MS	MVA (PCA)	[48,49]

Table 1 (*continued*)

MoA (target)[a]	Chemical groups[b]	Bioactive compounds[c]	Model biological systems[d]	Analytical platforms[e]	Methodologies[f]	References
Growth/development regulation[g]						
Auxin-like	Quinolinecarboxylic acids	Quinclorac (H)	*Z. mays*	^{1}H NMR	NN	[32]
Auxin transport	Phthalamates	Naptalam (H)	*Z. mays*	^{1}H NMR	NN	[32]
Photosynthesis[g]						
PPO	Diphenyl ethers	Acifluorfen (H)	*Z. mays*	^{1}H NMR	NN	[32]
	Oxadiazoles	Oxadiazon (H)	*A. sterilis*	^{1}H NMR	MVA (PCA, PLS-DA, SIMCA)	[33]
		Oxadiazon (H)	*L. minor*	^{1}H NMR	MVA (PCA, PLS-DA, HCA)	[34]
PSI	Bipyridyliums	Paraquat (H)	*Z. mays*	^{1}H NMR	NN	[32]
		Paraquat (H)	*A. sterilis*	^{1}H NMR	MVA (PCA, PLS-DA, SIMCA)	[33]
		Paraquat (H)	*L. minor*	^{1}H NMR	MVA (PCA, PLS-DA, HCA)	[34]
PSII	Ureas	Diuron (H)	*A. sterilis*	^{1}H NMR	MVA (PCA, PLS-DA, SIMCA)	[33]
		Diuron (H)	*L. minor*	^{1}H NMR	MVA (PCA, PLS-DA, HCA)	[34]
		Diuron (H)	*Z. mays*	^{1}H NMR	NN	[31,32]
		Lenacil (H)	*Z. mays*	^{1}H NMR	NN	[32]
	Nitriles	Bromoxynil (H)	*Z. mays*	^{1}H NMR	NN	[32]
	Triazinones	Atrazine (H)	*L. rubellus*	^{1}H NMR	MVA (PCA, PLS-DA, SIMCA)	[79]
Mitosis/cell division[g]						
VLCFAs (cell division)	Chloroacetamides	Acetochlor (H)	*Z. mays*	^{1}H NMR	NN	[32]
Microtubule assembly	Dinitroanilines	Oryzalin (H)	*Z. mays*	^{1}H NMR	NN	[32]
Mitosis/microtubule organization	Carbamates	Propham (H)	*Z. mays*	^{1}H NMR	NN	[32]
Nervous system[g]						
CNS stimulant (various MoA)	Alkaloids	Caffeine	Rat brain tissue cultures	HPLC/MS	MVA (PCA)	[46]
GABA-gated chloride channels	Cyclodienes	Endosulfan (I)	*E. fetida*	^{1}H NMR and GC/MS	MVA (PCA)	[80]
Neurotoxic (various)	Heavy metals	Cadmium	*D. magna*	FT-ICR/MS	MVA (PCA, PLS-DA)	[38]
		Methyl mercury chloride	Rat brain tissue cultures	HPLC/MS	MVA (PCA)	[46]
		Cadmium chloride	*L. rubellus*	^{1}H NMR	MVA (PCA, PLS-DA, SIMCA)	[79]
	Hydrocarbons	Fluoranthene	*L. rubellus*	^{1}H NMR	MVA (PCA, PLS-DA, SIMCA)	[79]
Sodium channel	Hydrocarbons (chlorinated)	DDT (I)	*E. fetida*	^{1}H NMR and GC/MS	MVA (PCA)	[80]
Respiration[g]						
Mitochondrial electron transport (complex II)	Amides	Carboxin (F)	*S. cerevisiae*	LC-TOF/MS	MVA (PCA, DFA, HCA)	[47]
Mitochondrial electron transport (complex III)	Acrylates	Azoxystrobin (F)	*S. cerevisiae*	LC-TOF/MS	MVA (PCA, DFA, HCA)	[47]
		Kresoxim methyl (F)	*S. cerevisiae*	LC-TOF/MS	MVA (PCA, DFA, HCA)	[47]
Oxidative phosphorylation	Dinitrophenols	Dinitrophenol (H, I)	*D. magna*	FT-ICR/MS	MVA (PCA, PLS-DA)	[38]
		Dinoseb (H, I)	*Z. mays*	^{1}H NMR	NN	[32]
	Pyridines	Fluazinam (F)	*S. cerevisiae*	LC-TOF/MS	MVA (PCA, DFA, HCA)	[47]
Various[g]						
Contact and stomach action	Pyrethrins	Fenvalerate (I)	*D. magna*	FT-ICR/MS	MVA (PCA, PLS-DA)	[38]
Membrane destabilization (unknown in non-target organisms)	Alcohols	Propranolol	*D. magna*	FT-ICR/MS	MVA (PCA, PLS-DA)	[38]

[a] ACCase, acetyl CoA carboxylase; AHAS, acetohydroxyacid synthase; ALS, acetolactate synthase; CNS, central nervous system; DHP, dihydropteroate synthase; PPO, protoporphyrinogen oxidase; PSI, photosystem I; PSII, photosystem II; PDS, phytoene desaturase; 4-HPPD, 4-hydroxyphenyl-pyruvate-dioxygenase.

[b] Data were retrieved from the databases Chemspider (http://www.chemspider.com) and PubChem (http://pubchem.ncbi.nlm.nih.gov/).

[c] A, antibiotic; F, fungicide; H, herbicide; I, insecticide.

[d] *A. sterilis*, *Avena sterilis* (wild oat); *D. magna*, *Daphnia magna*; *E. Fetida*, *Eisenia fetida* (earthworm); *L. rubellus*, *Lumbricus rubellus* (earthworm); *S. aureus*, *Staphylococcus aureus*; *S. cerevisiae*, *Saccharomyces cerevisiae* (yeast); *Z. mays*, *Zea mays* (maize).

[e] GC/MS, gas chromatography/mass spectrometry; FT-ICR/MS, Fourier transform-ion cyclotron resonance/mass spectroscopy; HPLC-ESI/MS, high performance liquid chromatography electrospray ionization/mass spectrometry; LC-TOF/MS, Liquid chromatography time-of-flight/mass spectrometry; ^{1}H NMR, proton nuclear magnetic resonance spectroscopy.

[f] DFA, discriminant function analysis; HCA, hierarchical cluster analysis; MVA, multivariate analyses; NN, neural networks; PCA, principal-component analysis; PLS-DA, partial least squares-discriminant analysis; SIMCA, soft independent modeling of class analogy.

[g] Functional categories of targeted biochemical systems.

development, where the high-throughput screening and discovery of the MoA of a vast number of candidate bioactive molecules is required, without at the same time any loss of information and false assessment. Metabolomics data are multivariate thus, the discrimination and classification of samples and the detection of biomarkers is mainly based on multivariate analyses [62]. The application of such statistical methods enables the indirect association of the MoA of the compounds under study to the changes caused in the metabolome of the model organism and thus, compounds that cause identical metabolic alterations in the model organism is expected to have the same MoA.

The detection of biomarkers of toxicity could provide a confirmation for the MoA of the bioactive compound that is being tested. Based on this principle and in combination with the development of powerful analyzers, metabolomics could facilitate the detection and exploration of novel MoA. However, in cases where a large number of biomarkers are identified, advanced bioinformatics software and databases such as the software Cytoscape [63], the databases and tools of BioCyc [64], PathVisio [65], and Kegg [66], are required for the biological interpretation of metabolomics results. Reviews on available software and databases for metabolomics data analyses have been recently published [27,67,68].

3. Investigating the modes-of-action of pesticides applying metabolomics

3.1. Phytotoxic compounds

Plants are complex organisms with a variety of biochemical systems that are composed of a vast number of metabolites with diverse physicochemical properties. The majority of phytotoxic compounds target the plant's essential functions required for their survival and development. Major MoA of compounds that are commercially developed as herbicides are listed in Table A1. From a crop protection perspective, the presence of distinct biochemical systems such as the photosystem in non-desirable plants is advantageous for achieving selectivity for non-target organisms (e.g., insects), but the conundrum is achieving selectivity in fields in which weeds co-exist with crops. In this case, the selectivity is determined by several factors that include the MoA (e.g., sensitivity of the biochemical system to the bioactive molecule), anatomic and physiological characteristics of the plants that will determine the concentration of the molecule in the plant cells, and the time and method of the applications of herbicides.

There are currently over twenty established MoA for numerous commercially developed phytotoxic compounds (Table A1, Fig. 1). Considering the complexity of the plant's metabolism and the size of the plant's metabolome, only a small number of potential biochemical sites are targets for existing herbicides, which is indicative that there are biochemical targets for herbicides yet to be exploited. Studies on the MoA of phytotoxic compounds applying metabolomics along with the methodology and analyses are listed in Table 1.

Many phytotoxic compounds act on primary as well as secondary biochemical targets. Comprehensive information for such bioactivity is vital for the development of novel bioactive compounds, and metabolomics could play a crucial role in uncovering hidden effects of chemical compounds on complex physiological processes. Aranibar et al. [31] were the first to report on the classification and identification of herbicidal MoA against maize (*Z. mays* L.) applying ^{1}H NMR spectroscopy and artificial neural networks. A discrimination between the metabolomes of the plants following treatments with inhibitors of acetohydroxy acid synthase (AHAS, also known as acetolactate synthase-ALS), acetyl-CoA carboxylase (ACCase), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), and photosystem II (PSII) was achieved, leading to a satisfactory correlation of the changes in plant's metabolome with the MoA of the four applied herbicides. Based on a similar protocol and using maize as the model plant, herbicides representing nineteen different MoA were used for the construction of artificial neural networks applying ^{1}H NMR spectroscopy [32]. Results confirmed that artificial neural networks could be used for the classification of the nineteen MoA that were studied based on the automated pattern recognition analyses of the metabolome that is embodied in the ^{1}H NMR spectrum and plausibly for the discovery of new MoA.

In another study, Aliferis and Chrysayi-Tokousbalides [33] were the first to develop a ^{1}H NMR metabolomics protocol based on multivariate analyses for the discovery of the MoA of secondary phytotoxic fungal metabolites. The isomer (5*S*, 8*R*, 13*S*, 16*R*)-(−)-pyrenophorol was isolated from fungal cultures of a *Drechslera avenae* pathotype with host specificity for wild oat (*A. sterilis*) and found to be selectively phytotoxic to *A. sterilis* [69]. Such selectivity at the plant level combined with the observation that the structure of the metabolite is not shared with commercial herbicides is indicative of an interesting structure–activity relationship. Applying PLS-DA, the fluctuations in the metabolic fingerprints of *A. sterilis* in response to pyrenophorol were compared to those caused by inhibitors of phytoene desaturase (PDS), protoporphyrinogen oxidase (PPO), EPSPS, 4-hydroxyphenyl-pyruvate-dioxygenase (4-HPPD), PSII and photosystem I (PSI) electron diverters. The analyses showed that pyrenophorol has a MoA different than those of the herbicides that were tested. Such an observation makes pyrenophorol an interesting molecule for potential consideration *per se* or as lead molecule in the context of crop protection. Using ^{1}H NMR fingerprinting on the model plant *L. minor*, Aliferis and coworkers [34] showed that pyrenophorol causes alterations in plant's metabolome that resemble those caused by glyphosate (EPSPS inhibitor). These findings suggest the operation of a similar MoA for both compounds. The potential of MS analytical platforms for the discovery of the MoA of herbicides was exploited by Oikawa et al. [35]. A metabolic phenotyping scheme on the basis of FT-ICR/MS analyses coupled with computational data tools was developed and applied to metabolic phenotyping of the model plant *A. thaliana* treated with different herbicides for pathway-specific inhibitions. The results revealed distinct metabolome clusters among treatments, and detailed comparison of the metabolomes led to detection of specific metabolite accumulation following herbicide treatments. However, specific biomarkers of the MoA were detected only in plants treated with glyphosate, in which accumulation of shikimate phosphate (shikimate 3P) was observed.

3.2. Insecticidal compounds

To date, more than twenty-six different MoA for insecticides have been reported with the vast majority of the commercially developed insecticides targeting the functionality of the nervous system of insects (Table A2, Fig. 1). The presence of distinct systems in insects such as the nervous and hormone systems is a great advantage for avoiding toxic effects to crops following application of insecticides. However, it is a disadvantage for achieving selectivity between pest-insects and non-target organisms with nervous systems. With respect to insect populations in the agro-ecosystem, the elimination of beneficial insects that are parasites or predators could have a negative impact resulting to increased populations of pest-insects due to the lack of their natural enemies. A good example of selectivity of insecticidal agents is the various strains of the bacterium *Bacillus thuringiensis* (BT) [70,71] which exhibit toxicity against herbivore insects (e.g., Lepidoptera, Coleoptera, Diptera). Such toxicity is attributed to the ingestion of crystalline (Cry) and cytolytic (Cyt) proteins (Table A2) and consecutive action of

midgut proteases resulting in the production of δ-endotoxins. The potential of *B. thuringiensis* has been exploited within the context of integrated pest management (IPM) with the development of genetically modified (GM) plants (*Bt*-plants) expressing *B. thuringiensis* encoding genes for toxins [72,73]. However, the increasing use of GM crops has raised concerns over their safety. One of the challenges that require serious consideration is to understand whether alterations in GM plant's genome cause changes in its metabolome that are potentially harmful to human, animal, as well as to non-target organisms. The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) recognized the potential of metabolomics as a functional genomics tool for the risk assessment of GM plants and the estimation of unintended effects, and included metabolic profiling as a complementary methodology to the already existing ones for the risk assessment of GM crops [74].

In a first view, metabolomics seems to be of limited application for the study of the fluctuations in insects' metabolome following exposure to neurotoxic compounds. However, it could provide important information and insights on the effects of insecticides acting on the nervous system of pests [e.g., acetylcholinesterase (AChE) inhibitors] when applied at sub-lethal doses. Findings of such studies could be exploited in developing IPM strategies based not exclusively on direct mortality. Furthermore, new insecticides act by disturbing the insects growth and development (e.g., juvenile hormone mimic, chitin biosynthesis inhibition, and moulting disruption) (Table A2, Fig. 1) causing deleterious effects, and undoubtedly, metabolomics could assist in the discovery and development of analogous crop protection agents, and the in-depth study of the metabolic effects associated with their MoA. On the other hand, metabolomics could provide insights into the bioactivation of insecticides in plants or insects that in many cases determines their toxicity and selectivity [75–77] and which could be misinterpreted by performing experiments using solely the targeted organisms.

Earthworms (*Lumbricus* spp. and *Eisenia* spp.), which have a simple and sensitive nervous system, are the most extensively used model organisms in environmental metabolomics for the study of xenobiotics toxicity and the assessment of environmental health [18,78]. However their use for the study of the MoA of bioactive compounds is limited. Metabolic profiles of *Lumbricus rubellus* following exposure to sub-lethal concentrations of the heavy metal chloride $CdCl_2$, the herbicide atrazine, and the polycyclic aromatic hydrocarbon (PAH) fluoranthene were recorded applying ^{1}H NMR spectroscopy [79] (Table 1). In conjunction with multivariate analyses, the applied metabolomics approach succeeded in detecting individual metabolite biomarkers for atrazine (fumarate and β-hydroxybutyrate), $CdCl_2$ (succinate and nicotinic acid), and fluoranthene (lactate and various unidentified metabolites) toxicities. In another study, *Eisenia fetida* was used as the model organism for the study of the toxicity of the insecticides dichlorodiphenyltrichloroethane (DDT) (sodium channel modulator) and endosulfan (GABA-gated chloride channel antagonist) applying ^{1}H NMR and GC/MS metabolomics (Table 1) [80]. Results showed that the alanine to glycine ratio could serve as a biomarker for DDT and endosulfan toxicity on *E. fetida*. Such studies clearly show that metabolomics approaches are capable of discriminating between MoA of bioactive compounds.

In addition to earthworms, the water flea *Daphnia magna*, a minute freshwater crustacean, is another organism that is extensively used in ecotoxicological risk assessment studies [81,82]. The suitability of *D. magna* for the development of robust metabolomics models was recently established. Taylor et al. [38] applied mass spectrometry-based metabolomics for toxicity testing in *D. magna*. Specifically, they utilized direct infusion (DI) nanoelectrospray FT-ICR/MS to study the toxicity of four bioactive compounds (cadmium, fenvalerate, dinitrophenol, and propranolol) with different MoA (Table 1). Multivariate analyses suggested the underlying mechanisms of toxicity and the applicability of the developed protocol for the study of the MoA of bioactive compounds using *D. magna* as the model biological system.

The high potential of mammalian cell cultures for the study of the effects of bioactive compounds applying metabolomics was also recently exploited. Using rat brain cell cultures as the model system, toxicity responses of methyl mercury chloride (neurotoxicant with various MoA) and caffeine [central-nervous-system (CNS) stimulant-various MoA] applying HPLC/MS metabolomics (Table 1) were determined [46]. The compounds were applied at sub-cytotoxic concentrations and corresponding biomarkers for methyl mercury chloride (GABA, choline, creatine, and spermine) and caffeine (creatine) toxicity were detected applying principal component analysis (PCA).

3.3. Fungicidal and antibiotic compounds

According to the Fungicide Resistance Action Committee (FRAC, http://www.frac.info), more than forty MoA of fungicidal/bactericidal compounds have been discovered, whereas that of several others is yet to be elucidated (Table A3, Fig. 1). The use of sulfur as a fumigant dates back to Mycenaean times (1600 BC-1100 BC), and amazingly, until now, sulfur and copper are components of crop protection strategies due to their non-specific MoA, which inhibits the evolution of resistant pathogenic strains. As a rule of thumb, the number of sub-cellular biochemical targets of a bioactive compound is negatively correlated with the risk for evolved target site resistance. The vast majority of the commercially developed fungicides interfere with biochemical systems that are essential for the survival of microorganisms (Table A3). Thus, metabolomics could serve as a suitable tool for the study of the perturbations of the metabolism of microorganisms following treatments with fungicides/antibiotics and subsequently the correlation of those perturbations to the MoA of the applied compounds. Additionally, based on the advances of metabolomics in the study of plant physiology [15,83], it could assist in the discovery of compounds that induce plant defense mechanisms against fungal or bacterial plant pathogens (Table A3).

Although microorganisms exhibit features such as, growth under limited laboratory space and production of relatively uniform populations under controlled conditions, which make them an ideal model for metabolomics, their growth habits and requirements make their metabolomics analyses challenging. For a comprehensive monitoring of the physiology of a microorganism, fluctuations in the intracellular metabolites (endo-metabolome) as well as extracellular metabolites released into their nutritional substrates (exo-metabolome) should be simultaneously monitored [84].

Until now, metabolomics has been exploited in the study of yeast [85–88], the physiology of fungal structures such as the sclerotia [57], and metabolite profiling of fungi [89,90]. Nonetheless, the application of metabolomics for the discovery of the MoA of antifungal and antibiotic compounds is still largely unexploited. Yeast, and more specifically the species *S. cerevisiae*, is the most extensively studied microorganism applying metabolomics. Allen et al. [47] developed a liquid chromatography time-of-flight/mass spectrometry (LC-TOF/MS) model based on the metabolic footprints of *S. cerevisiae* cultures (Table 1). Multivariate analyses revealed a strong discrimination between the footprints of the cultures after treatments with fungicides that act on ergosterol biosynthesis, amino acid biosynthesis, or respiratory inhibitors, leading to a satisfactory correlation between changes in *S. cerevisiae* exo-metabolome and the MoA of the applied fungicides. These findings suggest that metabolic footprinting could be used as a

reliable indicator of disturbances in the metabolism of fungi caused by fungicidal compounds and subsequently the discovery of their MoA.

Metabolomics studies that focus on the discovery of the MoA of antibiotics against plant pathogenic bacteria do not yet exist, however, there are some studies using human and animal pathogens as model organisms that highlight the potential of metabolomics (Table 1). Yu et al. [49] developed a methodology for the discovery of the MoA of natural antibiotics based on high performance liquid chromatography electrospray ionization/mass spectrometry HPLC-ESI/MS metabolic profiling. Using the human and animal pathogen, *S. aureus* as the model organism, metabolic profiles after treatment with antibiotics with known MoA were compared to those obtained from treatments with the anti-bacterial rhizome extracts of the plant *Tinospora capillipes* Gagnep (Table 1). PCA analyses revealed that the MoA of the rhizome extract resembles that of rifampicin (i.e., inhibition of RNA polymerase) and norfloxacin (i.e., inhibition of cell division). Similar studies using *S. aureus* as the model organism, Yi et al. [48] and Liu et al. [91] developed metabolomics models for the discovery of the MoA of natural and synthetic antibiotics (Table 1).

4. Conclusion

Although, metabolomics is not a universal solution, its development as a new tool of systems biology could greatly assist pesticide research and development by enabling the robust and high-throughput screening of a vast number of molecules regarding their MoA, which is a key factor for their further development as crop protection agents. Thus, the overall procedure is accelerated and becomes cost effective by screening out in the early stages of research bioactive molecules on the basis of their MoA. Of great value is also the detection of metabolites that could serve as biomarkers for a given MoA applying metabolomics. Although the discovery of biomarkers for different MoA applying metabolomics is in its infancy, the use of advanced analyzers with superior analytical capabilities in metabolomics is expected to facilitate the discovery and detection of biomarkers for various MoA. Moreover, the application of metabolomics could enable the study of the bioactivation of molecules, which represent a significant component of their MoA. Based on the studies presented in this review, it is evident that the potential of metabolomics in the study of the MoA of insecticides and fungicides is still largely unexploited. Within a systems biology approach, metabolomics should be integrated with other "omics" data for a firm conclusion on the MoA of a bioactive compound. The genome sequencing of organisms that could potentially be used as model biological systems is expected to greatly assist the development of metabolomics models for the study of the MoA of bioactive compounds.

Acknowledgments

The authors acknowledge the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant to S. Jabaji for funding this review.

Appendix A

See Tables A1–A3.

Table A1
Mode(s)-of-action (MoA) of commercially developed phytotoxic compounds. Data were retrieved from the site of the Herbicide Resistance Action Committee (HRAC, http://www.hracglobal.com/, access December 2010).

MoA[a]	Representative active ingredients	Chemical groups[b]
Amino acid biosynthesis[c]		
Inhibition of AHAS (ALS)	Chlorsulfuron, metsulfuron-methyl	Sulfonylureas
	Diclosulam, florasulam	Triazolopyrimidines
	Flucarbazone-Na, propoxycarbazone-Na	Sulfonylaminocarbonyl-triazolinones
	Imazapyr, imazethapyr	Imidazolinones
	Pyribenzoxim, pyrithiobac-Na	Pyrimidinyl(thio)benzoates
Inhibition of EPSPS	Glyphosate, sulfosate	Glycines
Inhibition of GS	Glufosinate-ammonium, bialaphos	Phosphinic acids
Biosyntheses/cell metabolism[c]		
Inhibition of DHP	Asulam	Carbamates
Inhibition of DOXP synthase	Clomazone	Azoles
Fatty acid biosynthesis		
Inhibition of ACCase	Alloxydim, sethoxydim	Cyclohexanediones (DIMs)
	Fenoxaprop-P-ethyl, fluazifop-P-butyl	Aryloxyphenoxy-propionates (FOPs)
	Pinoxaden	Phenylpyrazolines (DEN)
Inhibition of lipid biosynthesis (not ACCase)	Benfuresate, ethofumesate	Benzofurans
	Bensulide	Phosphorodithioates
	Cycloate, molinate	Thiocarbamates
	TCA, dalapon	Chloro-Carbonic-acids
Pigment biosynthesis		
Inhibition of 4-HPPD	Benzobicyclon	Benzoylcyclohexanediones
	Isoxachlortole, isoxaflutole	Isoxazoles
	Mesotrione, sulcotrione	Triketones
	Pyrazolynate, pyrazoxyfen	Pyrazoles
Inhibition of PDS	Diflufenican, picolinafen	Pyridinecarboxamides
	Norflurazon	Pyridazinones
Inhibition of carotenoid biosynthesis (unknown MoA)	Aclonifen	Diphenylethers
	Amitrole (*in vivo* inhibition of lycopene cyclase)	Triazoles
	Clomazone	Isoxazolidinones
	Fluometuron	Ureas
Growth/development regulation[c]		
Indoleacetic acid-like action	2,4-D, 2,4-DB	Phenoxy-carboxylic acids
	Clopyralid, picloram	Pyridine carboxylic acids
	Dicamba, TBA	Benzoic acids
	Quinclorac, quinmerac	Quinoline carboxylic acids
Inhibition of auxin transport	Naptalam, diflufenzopyr-Na	Phthalamates, Semicarbazones
Inhibition of cellulose biosynthesis	Dichlobenil, chlorthiamid	Nitriles
	Flupoxam	Triazolocarboxamides
	Isoxaben	Benzamides
	Quinclorac	Quinoline carboxylic acids
Mitosis/cell division[c]		
Inhibition of microtubule assembly	Amiprophos-methyl, butamiphos	Phosphoroamidates
	DCPA	Benzoic acids
	Dithiopyr, thiazopyr	Pyridines
	Pendimethalin, trifluralin	Dinitroanilines
	Propyzamide, tebutam	Benzamides
Inhibition of mitosis/microtubule organization	Chlorpropham, carbetamide	Carbamates
Inhibition of VLCFAs (cell division)	Alachlor, butachlor	Chloroacetamides
	Dimethachlor, metolachlor	
	Propachlor, propisochlor	
	Diphenamid, napropamide	Acetamides
	Flufenacet, mefenacet	Oxyacetamides
	Fentrazamide	Tetrazolinones
Photosynthesis[c]		
Inhibition of PPO	Azafenidin, sulfentrazone	Triazolinones
	Benzfendizone, butafenacil	Pyrimidindiones
	Bifenox, oxyfluorfen	Diphenylethers
	Cinidon-ethyl, flumioxazin	N-phenylphthalimides
	Fluazolate, pyraflufen-ethyl	Phenylpyrazoles
	Fluthiacet-methyl, thidiazimin	Thiadiazoles
	Oxadiazon, oxadiargyl	Oxadiazoles
	Pentoxazone	Oxazolidinediones
Inhibition of PSII	Amicarbazone	Triazolinones
	Atrazine, simazine	Triazines
	Chloridazon	Pyridazinones
	Desmedipham, phenmedipham	Phenyl-carbamates
	Lenacil, terbacil	Uracils
	Metamitron, metribuzin	Triazinones
Inhibition of PSII	Diuron, linuron	Ureas
	Propanil	Amides
Inhibition of PSII	Bentazon	Benzothiadiazinones

(*continued on next page*)

Table A1 (*continued*)

MoA[a]	Representative active ingredients	Chemical groups[b]
	Bromoxynil, ioxynil	Nitriles
	Pyridate, pyridafol	Phenyl-pyridazines
PSI-electron diversion	Diquat, paraquat	Bipyridyliums
Respiration[c]		
Uncoupling of oxidative phosphorylation (membrane disruption)	DNOC, dinoseb	Dinitrophenols

[a] ACCase, acetyl CoA carboxylase; AHAS, acetohydroxyacid synthase; ALS, acetolactate synthase; DHP, dihydropteroate synthase; DOXP, 1-deoxy-d-xylulose-5-phosphate; 4-HPPD, 4-hydroxyphenyl-pyruvate-dioxygenase; PDS, phytoene desaturase; PPO, protoporphyrinogen oxidase; PSI, photosystem I; PSII, photosystem II; VLCFAs, very long chain fatty acids.
[b] Data were retrieved from the databases Chemspider (http://www.chemspider.com) and PubChem (http://pubchem.ncbi.nlm.nih.gov/).
[c] Functional categories of targeted biochemical systems.

Table A2
Mode(s)-of-action (MoA) of commercially developed insecticides/acaricides compounds. Data were retrieved from the site of the Insecticide Resistance Action Committee (http://www.irac-online.org/, access December 2010).

MoA[a]	Representative active ingredients	Chemical groups[b]
Growth/development regulation[c]		
Ecdysone receptor agonists	Chromafenozide, halofenozide	Hydrazines
Inhibition of chitin biosynthesis		
Type 0	Diflubenzuron, teflubenzuron	Benzoylureas
Type 1	Buprofezin	Thiodiazines
Juvenile hormone mimics	Hydroprene, methoprene	Fatty acids (juvenile hormone analogues)
	Fenoxycarb	Carbamates
	Pyriproxyfen	Pyridines
Mite growth inhibition	Clofentezine	Hydrocarbons (halogenated)
	Hexythiazox	Thiazolidines
Moulting disruption (Dipteran)	Cyromazine	Triazines
Nervous system[c]		
Activation of nAChR allosteric	Spinetoram, spinosad	Spinosyns
Feeding blockers (Homopteran)	Pymetrozine	Triazines
	Flonicamid	Pyridines
GABA-gated chloride channels	Chlordane, endosulfan	Cyclodiene organochlorines
	Ethiprole, fipronil	Phenylpyrazoles
Inhibition of AChE	Aldicarb, carbofuran	Carbamates
	Chlorpyrifos, dimethoate	Organophosphates
nAChR agonists	Acetamiprid, imidacloprid	Neonicotinoids
	Nicotine	Alkaloids
nAChR channel blockers	Bensultap, thiocyclam	Nereistoxin analogues
Octopamine receptor agonists	Amitraz	Benzene Derivatives
Sodium channels modulators	Cypermethrin, deltamethrin	Pyrethroids
	DDT, methoxychlor	Hydrocarbons (chlorinated)
Voltage-dependent Na channel blockers	Indoxacarb	Oxazines
	Metaflumizone	Semicarbazones
Respiration[c]		
Inhibition of mitochondrial ATPase	Diafenthiuron	Thioureas
	Propargite	Cyclohexanes
Inhibition of mitochondrial electron transport		
Complex I	Rotenone	Flavonoids
	Fenazaquin	Quinazolines
	Fenpyroximate	Benzoic Acid
Complex II	Cyenopyrafen	Pyrazoles
Complex III	Hydramethylnon	Pyrimidines
	Acequinocyl	Naphthalenes
Complex IV	Aluminum phosphide, calcium phosphide	Phosphines
Uncoupling of oxidative phosphorylation	DNOC	Phenols
	Sulfluramid	Hydrocarbons (halogenated)
Various[c]		
Activation of chloride channels (nerve and muscles)	Abamectin, milbemectin	Avermectins, Milbemycins
Disruption of insect midgut membranes	*Bacillus thuringiensis*, *B. sphaericus*	Bacterium
Inhibition of ACCase	Spirodiclofen, spiromesifen	Polycyclic hydrocarbons (aromatic)
	Cyanide	Nitriles
Multiple targets	Methyl bromide, chloropicrin	Hydrocarbons (halogenated)
Ryanodine receptor modulators (nerve and muscles)	Chlorantraniliprole, flubendiamide	Benzoic acids
Unknown	Azadirachtin	Terpenes
	Dicofol	Hydrocarbons (halogenated)

[a] ACCase, acetyl CoA carboxylase; AChE, acetylcholinesterase; GABA, γ-Aminobutyric acid; ATPase, adenosine triphosphatase; nAChR; nicotinic acetyl-choline receptor.
[b] Data were retrieved from the databases Chemspider (http://www.chemspider.com) and PubChem (http://pubchem.ncbi.nlm.nih.gov/).
[c] Functional categories of targeted biochemical systems.

Table A3
Mode(s)-of-action (MoA) of commercially developed fungicides/bactericides. Data were retrieved from the site of the Fungicicide Resistance Action Committee (FRAC, http://www.frac.info/frac/index.htm, access December 2010).

MoA[a]	Representative active ingredients	Chemical group[b]
Amino acid, protein, and nucleic acid synthesis[c]		
DNA/RNA synthesis	Hymexazol	Isoxazoles
	Octhilinone	Isothiazolones
DNA supercoiling (DNA topoisomerase type II gyrase)	Oxolinic acid (bactericide)	Carboxylic acids
Methionine biosynthesis	Cyprodinil, pyrimethanil	Pyrimidines
Protein synthesis	Blasticidin-S	Glycosides–nucleosides
	Kasugamycin	Glycosides–aminoglycosides
	Streptomycin (bactericide)	Glycosides–aminoglycosides
	Oxytetracycline (bactericide)	Tetracyclines
Purine metabolism (adenosine-deaminase)	Ethirimol	Pyrimidines
	Bupirimate	Sulfonic acids
RNA synthesis (RNA polymerase I)	Metalaxyl, benalaxyl	Acylalanines
	Ofurace, oxadixyl	Acetamides
Biosyntheses/cell metabolism[c]		
Lipid and membrane synthesis		
Phospholipid biosynthesis (methyltransferase)	Pyrazophos, iprobenfos	Phosphorothiolates
	Iso-prothiolane	Dithiolanes
Lipid peroxidation	Dicloran, quintozene (pcnb)	Nitrobenzenes
	Biphenyl, chloroneb	Benzenes
	Tolclofos-methyl	Thiophosphate
	Etridiazole	Thiadiazoles
Cell membrane permeability, fatty acids	Propamocarb, prothiocarb	Carbamates
Phospholipid biosynthesis and cell wall deposition	Dimethomorph, flumorph	Cinnamic acid amides
	Mandipropamid	Mandelic acid amides
	Iprovalicarb, benthiavalicarb	Valinamide carbamates
Melanin biosynthesis in cell wall		
Dehydratase in melanin biosynthesis	Carpropamid	Cyclopropane carboxamides
	Diclocymet	Carboxamides
	Fenoxanil	Propionamides
Reductase in melanin biosynthesis	Fthalide	Isobenzofuranones
	Pyroquilon	Pyrroloquinoliones
	Tricyclazole	Triazolobenzothiazoles
Sterol biosynthesis in membranes		
SBI class I: DMI fungicides (C14 demethylase-erg11/cyp51)	Fenarimol, nuarimol	Pyrimidines
	Imazalil, prochloraz	Imidazoles
	Pyrifenox	Pyridines
	Tebuconazole, bitertanol	Triazoles
	Triforine	Piperazines
SBI class II: Δ^{14} reductase (erg24) and $\Delta^{8} \rightarrow \Delta^{7}$ isomerase (erg2)	Aldimorph, fenpropimorph	Morpholines
	Fenpropidin, piperalin	Piperidines
	Spiroxamine	Spiroketal-amines
SBI class III: k 3-keto reductase in C4-demethylation	Fenhexamid	Hydroxyanilides
SBI class IV: squalene epoxidase	Naftifine, terbinafine (antimycotic)	Allylamines
Trehalase and inositol biosynthesis	Validamycin	Sugar alcohols
Chitin synthase	Polyoxin B	Polyoxins
Disruption of pathogen cell membranes	Bacillus subtilis strain qst 713	Bacterium
Host defense induction[c]		
Host defence induction (unknown)	Probenazole	Thiazoles
	Laminarin	Polysaccharides
Salicylic pathway	Acibenzolar-S-methyl	Thiazoles
Mitosis/cell division[c]		
Cell division	Pencycuron	Ureas
Delocalisation of spectrin-like proteins	Fluopicolide	Benzamides
β-Tubulin assembly in mitosis	Carbendazim, thiabendazole	Benzimidazoles
	Thiophanate, thiophanate-methyl	Thiophanates
β-Tubulin assembly in mitosis	Zoxamide	Amides
Respiration[c]		
ATP production	Silthiofam	Amides
Inhibition of mitochondrial electron transport		
Complex I (NADH Oxido-reductase)	Diflumetorim	
Complex II (succinate dehydrogenase)	Bixafen, penthiopyrad	Pyrazole carboxamides
	Boscalid	Pyridine carboxamides
	Carboxin, oxycarboxin	Oxathiin carboxamides
	Fenfuram	Furan carboxamides
	Fluopyram	Pyridinyl-ethyl benzamides
	Mepronil, benodanil	Phenyl-benzamides
	Thifluzamide	Thiazole carboxamides

(*continued on next page*)

Table A3 (*continued*)

MoA[a]	Representative active ingredients	Chemical group[b]
Complex III [cytochrome bc1 (ubiquinone reductase)-Qi site]	Cyazofamid, amisulbrom	Sulfonamides
Complex III [cytochrome bc1 (ubiquinone reductase)-unknown Q site]	Ametoctradin	Triazolopyrimidines
Complex III [cytochrome bc1 (ubiquinol oxidase)-Qo site (cyt b gene)]	Azoxystrobin, picoxystrobin	Methoxy-acrylates
	Famoxadone	Oxazolidine-diones
	Fenamidone	Imidazolinones
	Fluoxastrobin	Dihydro-dioxazines
	Kresoxim-methyl, trifloxystrobin	Oximino-acetates
	Orysastrobin, dimoxystrobin	Oximino-acetamides
	Pyraclostrobin	Methoxy-carbamates
	Pyribencarb	Benzylcarbamates
Inhibition of oxidative phosphorylation (ATP synthase)	Fentin acetate, fentin chloride	Organotin Compounds
Uncoupling of oxidative phosphorylation	Fluazinam	Aminopyridines
	Meptyldinocap	Dinitrophenols
Signal transduction[c]		
Osmotic transduction (MAP/histidine-kinase, Os-2, HOG1)	Fenpiclonil, fludioxonil	Phenylpyrroles
Osmotic transduction (MAP/histidine-kinase, Os-1, Daf1)	Iprodione, vinclozolin	Azoles
Signal transduction (unknown)	Proquinazid	Quinazolinone
	Quinoxyfen	Quinolines
Multi-site action[c]		
	Anilazine	Triazines
	Captan, folpet	Phthalimides
	Chlorothalonil	Chloronitriles
	Dithianon	Anthraquinones
	Iminoctadine, guazatine	Guanidines
	Maneb, zineb	Dithiocarbamates
	Sulfur, copper	Inorganics

[a] SBI, sterol biosynthesis inhibitors; cyp51, sterol 14α-demethylase; DMI, demethylation inhibitors; MAP, mitogen-activated protein.
[b] Data were retrieved from the databases Chemspider (http://www.chemspider.com) and PubChem (http://pubchem.ncbi.nlm.nih.gov/).
[c] Functional categories of targeted biochemical systems.

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