Research Methods in Toxicology and Insecticide Resistance Monitoring of Rice Planthoppers

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Rice Planthopper Project
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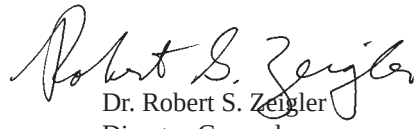
Foreword

By 2020, the world will require about 500 million tons of milled rice a year. To achieve this, production will need to average 5 tons per hectare. One constant threat for farmers to obtain this yield is losses caused by insects. When prophylactic insecticides are constantly applied, such practices will destroy ecosystem services and cause planthopper outbreaks, and will also induce rapid development of insecticide resistance. In the past 10 years, we have witnessed this type of insecticide resistance against some active ingredients. Insecticide resistance can be a major threat to the sustainability of rice production. Scientists will need to constantly monitor the development of insecticide resistance in order to design and implement strategies to manage resistance. In the field of insecticide toxicology, it is important that experimental techniques be well established and properly used to eliminate errors arising from biotic and abiotic variations in these techniques.

This book will be an important tool for scientists, professors, and students involved in insecticide toxicology and research on insecticide resistance. I would like to express our thanks to the Asian Development Bank for providing technical assistance that has made this publication possible.



Dr. Robert S. Zeigler
Director General

Preface

Rice planthoppers are developing resistance to insecticides very rapidly. Publications by several authors have reported that the brown planthopper (BPH) has acquired as much as 1,000-fold resistance to imidacloprid in some areas in China. High resistance to fipronil, fenobucarb, and buprofezin has also been reported. However, the methods used by different authors differ significantly, thereby making data comparison between different countries and sites difficult and meaningless. In order to make toxicological data across countries comparable, Matsumura and his colleagues have collected live planthoppers at sites in Asia and cultured them in the laboratory in Kyushu, where tests are carried out *ex situ*.

We have adopted an approach of developing a common standardized methodology and national training program for partners to conduct appropriate and meaningful *in situ* toxicological tests. Collaborators in a resistance monitoring network meet at least once a year to review methods, data, and interpretations. In addition to collecting standardized data sets that can be compared, this approach also develops local capacity and the skills of national scientists in insecticide toxicology.

This book describes the standardized methodology we developed with step-by-step illustrations wherever appropriate. The initial two chapters provide readers with an introduction to insect toxicology involving insect biochemistry, genetics, and physiology related to insecticide mode of action and resistance. Chapter 3 discusses quantal response data and toxicological statistics. Here, we draw heavily from the works of Finney (1977), Busvine (1971), and Robertson et al (2005) to provide readers with some basics in quantal response data and toxicological statistics. Chapter 4 describes the whole process involved in collecting specimens from the field, rearing them in an insectary, and preparing standardized test insects. In Chapter 5, preparation of a stock solution from technical-grade insecticide based on active ingredient and the test solutions used for insecticide application are discussed. Also discussed are topical application, the use of recovery cages, and posttreatment conditions for test insects.

Probit analysis was developed by Finney to estimate the median lethal dose (LD_{50}) and associated statistics to be used for comparisons. Professor Finney's program was subsequently modified and developed by Robertson and coworkers into PoloPlus©, which is now the authoritative software for probit analysis in toxicology. In Chapter 6, the step-by-step use of PoloPlus© is discussed in detail with screen-shot illustrations and interpretations.

Mixing two or more active ingredients either in a commercial product or by growers in spray tanks is a common practice in rice production in Asia. In Chapter 7, the use of PoloMix©, a program to determine whether a particular mixture has a

synergistic, antagonistic, or no added effect, is discussed. Step-by-step procedures are provided to help readers use the program.

In reporting the toxicological experiments, it is important to provide sufficient information so that other scientists can use it to make meaningful comparisons. There are several ways to document toxicological data and Chapter 8 discusses one way of presenting the data in tables and in probit line plots.

The Reference section contains the references cited in the chapters and some additional useful sources from textbooks and Internet links.

Although this book specifically describes toxicological methods for rice planthoppers, the basic principles involved in the determination of median lethal doses (LD_{50}s) are the same. The general techniques and processes can be applied to other insects.

Acknowledgments

This book would not have been possible without contributions from many people. In particular, the authors are grateful to

- The Asian Development Bank (ADB) for providing the Technical Assistance Grant RETA 13 for us to establish the insecticide resistance monitoring network, conduct training, and obtain baseline data and prepare this book. RETA 14 provided the finances to publish the book in 2011.
- Collaborating scientists from China, Thailand, Vietnam, and Malaysia for their participation and contributions in the resistance monitoring network and in collecting the baseline data. In particular, we would like to thank Ms. Wantana Sriratanasak, Ms. Maisarah Mohamad Saad, and Mr. Phan Van Tuong.
- Dr. Masaya Matsumura of Kyushu for his contributions in providing the network participants with training and equipment.

CHAPTER 1:

Introduction to insect toxicology

Toxicology (derived from two Greek words, "toxicos" = poisonous and "logos" = study) is a very broad field of study involving multidisciplinary sciences related to adverse chemical effects on living organisms—including humans. It has many definitions. Generally, toxicology can be defined as "the study of adverse, deleterious, and/or poisonous effects of chemicals on living organisms" or "the study of symptoms, mechanisms/mode of action, treatments, and detection of poisoning; and cause of resulting death."

A brief history

According to popular Chinese mythology, Shennong, "the divine farmer" (about 2696 BC), is credited for bringing agriculture to ancient China (Wu 1982). He is also known as the father of Chinese medicine for writing a treatise "On Herbal Medical Experiment Poisons." He was noted for tasting 365 herb species, from which he eventually died, probably as a result of a fatal dose.

In 399 BC, the Athenian philosopher Socrates was tried and found guilty for two charges, related to Greek gods and deities, brought against him. He was sentenced to death and executed by drinking a liquid containing hemlock, a poisonous alkaloid from the plant *Cornium maculatum* (Apiaceae), for teaching radical ideas to Athenian youths (Stone 1988). Then, in AD 50–400, the Romans used poisons to carry out many executions and assassinations.

Abu Ali Sina, also known as Avicenna (AD 980-1036), was a Persian scholar and philosopher. He wrote more than 400 treatises related to various aspects of human logic, diseases, health, pharmacology, and physiology (Nasr 2007). Two of his outstanding works were "The Canon of Medicine" and "The Book of Healing." He was responsible for limiting the spread of infectious diseases by introducing quarantine. Through his knowledge of Islamic alchemy, chemistry, and pharmacology, he was an authority on poisons and antidotes.

Moses Maimonides (AD 1200) of Jewish descent was born in Spain. He worked as a rabbi, philosopher, and physician in Spain, Morocco, and Egypt. He wrote ten medical works in Arabic, one of which was a first-aid book for poisonings titled "Treatise on Poisons and Their Antidotes." This is an early textbook dealing with medical toxicology (Rosner 2002).

Phillip von Hohenheim, better known as "Paracelsus" (1493-1541), was born a Swiss and worked in Austria as a Renaissance physician, alchemist, astrologer, and botanist. He is noted for his statement in German, "*Alle Ding' sind Gift, und nichts ohn' Gift; allein die Dosis macht, daß ein Ding kein Gift ist*" (translated as "All things are poison and nothing is without poison, only the dose permits something not to be poisonous"). He was the first to explain the dose-response relationship of toxic substances—toxicity of a poison expressed as "lethal dose" (LD). For that, he is sometimes known as "the father of toxicology" (Madea et al 2007).

Mathieu Orfila (1787-1853) was born in Spain and worked as a French chemist and toxicologist. He played a major role in forensic toxicology, and was credited with being the founder of toxicology as a distinct scientific discipline, which he established in 1815 (Bertomeu-Sánchez and Nieto-Galan 2006).

Paul Hermann Müller (1899-1965), a Swiss chemist, recognized DDT (dichlorodiphenyl-trichloroethane), which was first synthesized in 1874, as a potent insecticide. He was awarded the 1948 Nobel Prize in Physiology and Medicine for his discovery and use of DDT (Grandin 1948). Unfortunately, the indiscriminate spraying of DDT caused many undesirable environmental impacts as documented by Rachel Carson (1962) in her book *Silent Spring*. Because of much negative publicity, DDT was banned in the United States in 1972 and in many parts of the world.

Toxic chemicals or poisons

All chemicals or molecules are toxic or poisonous under the right conditions (dose dependent). Table 1 shows the approximate dosage of chemicals of very low toxicity (generally considered as nontoxic) to a very highly neurotoxic protein from the bacterium *Clostridium botulinum* that can kill a person weighing 160 pounds (approx. 73 kg).

Table 1.1 Approximate lethal doses of common chemicals (calculated for a 160-lb human based on data obtained from rats).

Chemical	Lethal dose
Sugar (sucrose)	3 quarts (2.838 L)
Ethanol (ethyl alcohol)	3 quarts (2.838 L)
Common salt (sodium chloride)	1 quart (0.946 L)
Herbicide (2,4-dichlorophenoxyacetic acid)	Half a cup (120 mL)
Arsenic (arsenic acid)	1–2 teaspoons (5–10 mL)
Nicotine	Half a teaspoon (2.5 mL)
Food poison (botulinum toxin)	70–270 nanograms (ng)

Adapted from www.iet.msu.edu/toxconcepts/toxconcepts.htm.

Many plant and animal species possess a myriad of toxic organic compounds as chemical defenses against herbivores and predators, respectively. Even cellular proteins or polypeptides from an organism can act as toxins in another organism belonging to the same or different species. Many chemicals or organic molecules may act as an allergen that causes a specific allergy that can often be fatal. Certain species of invertebrates and vertebrates may inject venom to paralyze or kill their prey during hunting.

Subdisciplines of toxicology

Toxicology involves two main fields, toxicokinetics and toxicodynamics. The former deals with how an organism handles toxic substances, such as (1) absorption; (2) distribution within its body, biotransformation, or metabolism; and (3) excretion or elimination. Toxicodynamics deals with what effects a toxic substance has on an organism such as (1) irritant, (2) corrosive, (3) teratogenic or sterilizing agent, (4) asphyxiation or suffocation, (5) carcinogen, (6) mutagen, and (7) anaesthetic or narcotic. Toxicol-

ogy can be subdivided into many subdisciplines. Almost 20 different subdisciplines are generally recognized and among them eight are well established:

(1) Aquatic toxicology
(2) Chemical toxicology
(3) Ecotoxicology
(4) Entomotoxicology (insect toxicology)
(5) Environmental toxicology
(6) Forensic toxicology
(7) Medical toxicology
(8) Toxicogenomics

Entomotoxicology or insect toxicology

Insect toxicology primarily deals with the effects of chemicals that retard insect development, growth, and metamorphosis and/or reproduction, as well as cause death in insects. It also deals with effects and mode of action of, as well as development of resistance to, insecticides. It is multidisciplinary and involves (1) entomology—anatomy, morphology, taxonomy; (2) chemistry (of inorganic and organic insecticides); (3) insect biochemistry; (4) insect ecology—chemical ecology, behavior, and population dynamics; (5) genetics (related to insecticide resistance); (6) insect physiology; (7) statistics; and (8) techniques (related to application and bioassay).

As such, to fully understand an insecticide's mode of action and resistance development requires an understanding of the basic underlying biochemical, genetic, and physiological processes involved in poisoning of certain biological systems within an insect.

Biochemical processes in energy production

Food is an important component in the survival of an insect. It is necessary to provide the energy for many physiological and behavioral processes. Three basic groups are constituents of food: carbohydrates, fats, and proteins.

For energy production, most insects generally rely on carbohydrates to be metabolized first, followed by fats during starvation or migration, whereas protein is metabolized when both carbohydrates and fat reserves are depleted. As such, we will discuss briefly the synthesis/production of high-energy molecules, especially adenosine triphosphate (ATP), from both carbohydrates and fats.

Insects, like all other invertebrates and vertebrates, store carbohydrate in the form of glycogen in the fat body (an organ that functions much like the mammalian liver). Glycogen is broken down to glucose in most vertebrates before being transported but in insects it is converted to trehalose (a disaccharide consisting of two molecules of glucose) that is then transported to muscles, especially flight muscles, where it is hydrolyzed to glucose molecules. Glucose enters the cells to be metabolized via two metabolic pathways—(1) glycolysis and (2) the Kreb's cycle—to yield usable high-energy molecules, ATP plus two cofactors, NADH (a reduced form of nicotinamide adenine dinucleotide [NAD^+]) and $FADH_2$ (a reduced form of flavin adenine dinucleotide [FAD]). NADH and $FADH_2$ yield three and two molecules of ATP, respectively, after undergoing oxidative phosphorylation in the "electron transport chain."

Glycolysis (Glycose [archaic term for glucose] + lysis [disintegration])

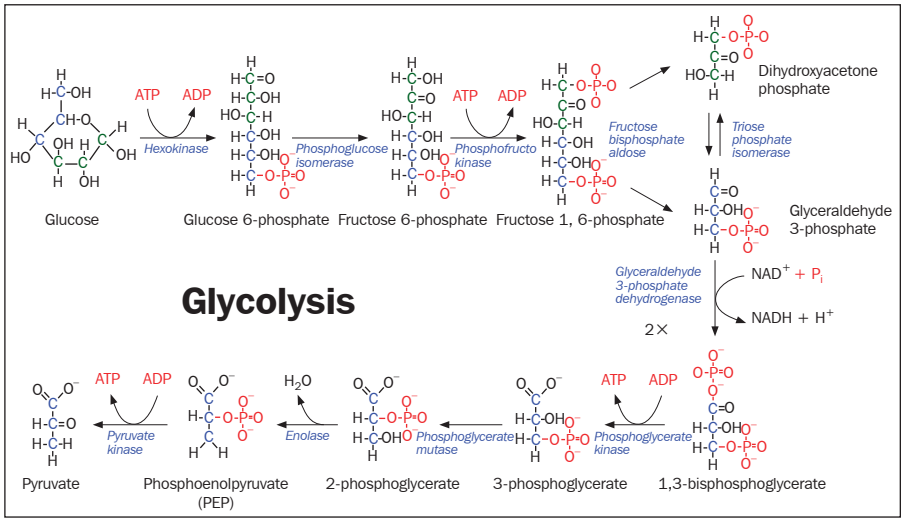


Fig. 1.1. Glycolysis pathway (it consists of 10 steps, each catalyzed by an enzyme).

This is a universal pathway (Fig. 1.1) for the breakdown of glucose (a hexose, 6C) to two molecules of triose (3C) that occurs in all types of biological cells.

Glycolysis has a 10-step biochemical pathway:

Step 1: Glucose is converted to glucose-6-phosphate catalyzed by a *hexokinase* with energy provided by an ATP.

Step 2: Glucose-6-phosphate is isomerized to fructose-6-phosphate in the presence of *phosphoglucose isomerase*.

Step 3: Fructose-6-phosphate is converted to fructose 1, 6-bisphosphate catalyzed by *phosphofructokinase* with energy provided by a second molecule of ATP.

Step 4: Fructose 1, 6-bisphosphate is then split into two triose molecules—dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, catalyzed by a *fructose bisphosphate aldolase*.

Step 5: Dihydroxyacetone phosphate is isomerized to glyceraldehyde 3-phosphate in a reversible reaction catalyzed by *triose phosphate isomerase*—in theory, a glucose molecule can yield two 3-glyceraldehyde molecules via steps 4 and 5.

Step 6: Glyceraldehyde 3-phosphate is converted to 1,3-bisphosphoglycerate with the addition of a molecule of inorganic phosphate (P_i) catalyzed by *glyceraldehyde phosphate dehydrogenase* in the presence of a cofactor NAD^+, which is reduced to $NADH + H^+ + 2é$.

Step 7: 1,3-bisphosphoglycerate is transformed to 3-phosphoglycerate catalyzed by *phosphoglycerate kinase* with the production of a molecule of ATP from ADP.

Step 8: 3-phosphoglycerate is isomerized to 2-phosphoglycerate catalyzed by *phosphoglycerate mutase*.
Step 9: 2-phosphoglycerate is changed to phosphoenolpyruvate catalyzed by *enolase* with a release of a molecule of water.
Step 10: Phosphoenolpyruvate is finally converted to pyruvate in the presence of *pyruvate kinase* with the synthesis of a high-energy molecule (ATP) from ADP.

A molecule of glucose after undergoing glycolysis has a net yield of two molecules each of pyruvate, water, NADH + H^+ + 2é (this cofactor carrying two electrons can be used to produce three molecules of ATP—to be discussed later), and ATP. Therefore, in terms of the number of high-energy molecules produced through glycolysis, a molecule of glucose produces eight molecules of ATP.

Pyruvate, the end product of glycolysis, is used (Fig. 1.2) in (1) the process of fermentation catalyzed by pyruvate dehydrogenase in yeast and plants to produce ethanol; or (2) processes that demand quick and immediate energy in the absence of oxygen (during anaerobic activity such as vigorous exercise) in the presence of lactate dehydrogenase to form lactate—which accumulates, leading to muscular fatigue during "oxygen debt"; or (3) in most cells it enters the mitochondrion during cellular respiration, in the presence of Coenzyme A (CoA) catalyzed by pyruvate dehydrogenase complex (Mg^{++}, thiamine pyrophosphate, lipoic acid, and transacetylase) to form acetyl-CoA. Then, the acetyl-CoA enters the Kreb's cycle, in which the acetate portion of the molecule is completely metabolized to be released as water and carbon dioxide.

Kreb's cycle [citric acid/tricarboxylic acid (TCA) cycle]
This is a continuous metabolic cycle that occurs in the matrix of a mitochondrion as long as there is a constant supply of acetyl-CoA from either glucose through glycolysis or fatty acids through ß-oxidation (to be discussed later). This metabolic cycle also consists of 10 enzymic steps (Fig. 1.2):

Step 1: Acetyl-CoA first enters the cycle by combining with oxaloacetic acid in the presence of *citrate synthetase* and a molecule of water to form citric acid.
Step 2: Citric acid is transformed into *cis*-aconitic acid by the removal of a molecule of water catalyzed by *aconitase*.
Step 3: *cis*-aconitic acid is quickly changed to isocitric acid through the addition of a water molecule still in the presence of the enzyme *aconitase*.
Step 4: Isocitric acid in the presence of NAD^+ cofactor and *isocitric acid dehydrogenase* is converted to oxalosuccinic acid and yields a reduced cofactor ($NADH^+ + H^+$ + 2é).
Step 5: Oxalosuccinic acid is transformed to α-ketoglutaric acid catalyzed by *oxaloacetic acid decarboxylase* with the removal and release of a molecule of carbon dioxide.
Step 6: α-ketoglutaric acid with a removal and release of a carbon dioxide molecule catalyzed by α-*ketoglutarate dehydrogenase* combines with a CoA to form succinyl-CoA.

Step 7: Succinyl-CoA, with the addition of a water molecule and removal of CoA catalyzed by *succinyl-CoA synthetase*, is changed to succinic acid with a simultaneous synthesis of a molecule of guanosine triphosphate (GTP) from GDP (guanosine diphosphate).

Step 8: Succinic acid is transformed to fumaric acid in the presence of *succinate dehydrogenase* and cofactor FAD (flavin adenine dinucleotide), which is reduced to $FADH_2$.

Step 9: Fumaric acid with the addition of a water molecule is converted to malic acid in the presence of *fumarase*.

Step 10: Malic acid is finally oxidized, to complete the cycle, by the removal of hydrogen in the presence of NAD^+ cofactor, which is converted to its reduced form, oxaloacetic acid, which then continues in the cycle by combining with a new molecule of acetyl-CoA.

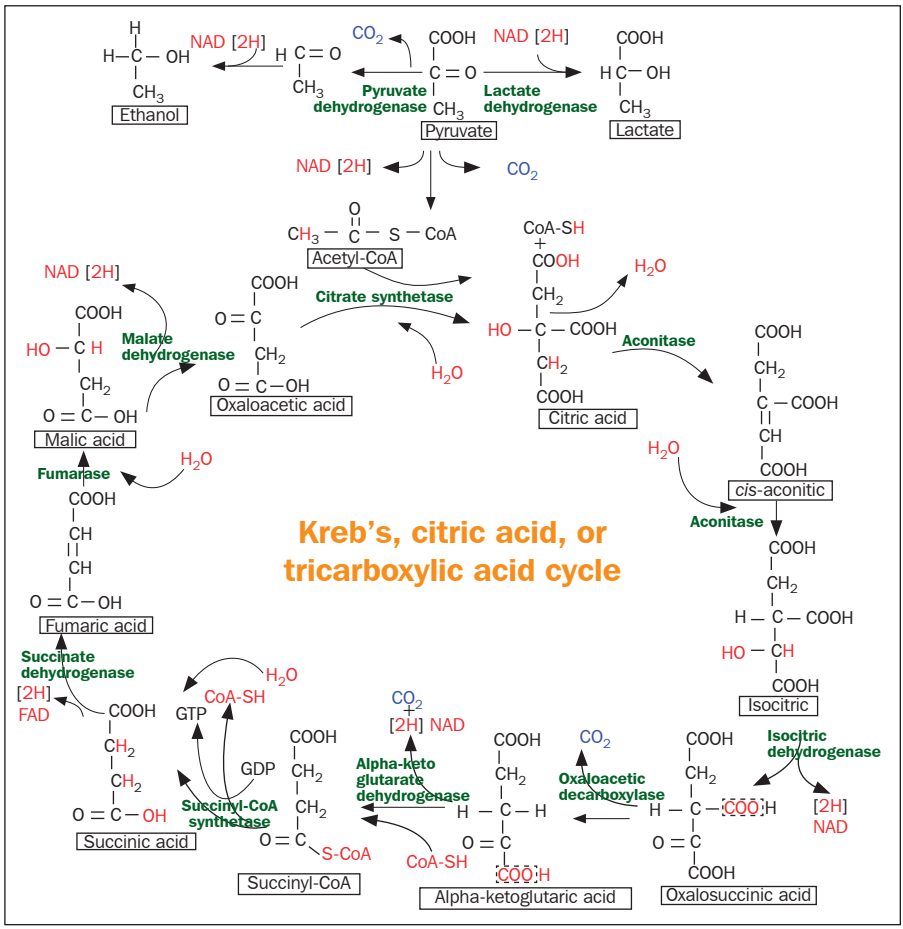


Fig. 1.2. Kreb's cycle.

Each pyruvate molecule when completely metabolized, before and after entering the Kreb's cycle, yields three molecules of water, three molecules of carbon dioxide, and, in terms of energy production, four $NADH^+$ (which subsequently yield four × three molecules of ATP), one $FADH_2$ (that eventually yields two ATP molecules), and a molecule of GTP (equivalent to an ATP). Therefore, a glucose molecule yields a net total of 38 ATP after undergoing (1) glycolysis to produce two molecules of pyruvic acid and eight ATP, and, in addition, (2) the Kreb's cycle and complete oxidation, and the two molecules of pyruvic acid produce a net of 30 (2 × 15) ATP.

ß-oxidation

This is a metabolic process responsible for the degradation of fatty acids in mitochondria and/or peroxisomes to liberate a molecule of acetyl-CoA at each turn of the metabolic cycle. Most fats are stored in the form of triglyceride, which can be broken down by *lipase* to a glycerol and three fatty acid molecules. Each fatty acid molecule must be activated in the cytosol before it can be oxidized via ß-oxidation.

Free fatty acid can cross the cell membrane into the cytosol, where it reacts with ATP to produce a reactive fatty acyl adenylate, which then combines with coenzyme A to yield a fatty acyl-CoA. The fatty acyl-CoA reacts with carnitine to yield acylcarnitine, which is then transported across the mitochondrial membrane. The activated fatty acid then undergoes ß-oxidation (a cycle of four steps—Fig. 1.3) in the mitochondria.

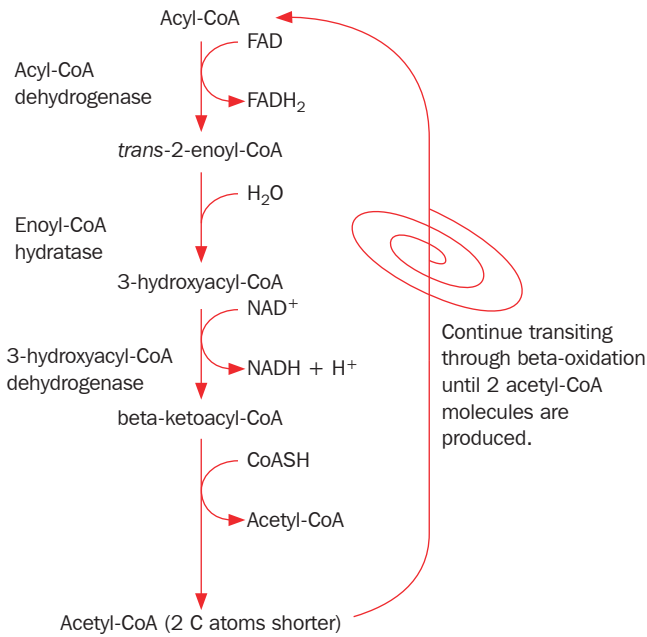


Fig. 1.3. ß-oxidation.

Step 1: Acyl-CoA with cofactor FAD is catalyzed by *acyl-CoA dehydrogenase* to produce *trans*-2-enoyl-CoA and reduced cofactor $FADH_2$.
Step 2: *trans*-2-enoyl-CoA combines with a water molecule to form 3-hydroxyacyl-CoA catalyzed by *enoyl-CoA hydratase*.
Step 3: 3-hydroxyacyl-CoA in the presence of cofactor NAD^+ catalyzed by *3-hydroxyacyl-CoA dehydrogenase* is transformed to ß-ketoacyl-CoA.
Step 4: ß-ketoacyl-CoA reacts with coenzyme A to produce a shortened acyl-CoA by the release of a molecule of acetyl-CoA.

Fatty acids with odd numbers of carbon atoms are common in plants. For such fatty acids, the end product of the last cycle of ß-oxidation is propionyl-CoA (C3) instead of acetyl-CoA. This end product will need to be transformed to succinyl-CoA to enter the Kreb's cycle.

Assuming that we start with palmitic acid representing a fatty acid (C16 fatty acid with a molecular weight of 256.2) and it is completely broken down to eight molecules of acetyl-CoA after going through seven ß-oxidation cycles, it would yield seven NADH. In terms of energy-molecule production, each palmitic acid would ultimately yield 8 × 12 ATP from 8 acetyl-CoA through the Kreb's cycle, and 7 × 5 ATP from seven turns of ß-oxidation, yielding a net total of 131 ATP molecules. When compared with glucose (molecular weight of 180), palmitic acid is 1.4 times heavier but yields 3.4-fold more ATP molecules, that is, weight for weight, fatty acid produces approximately 2.4-fold more ATP. Therefore, fats are a better form of energy reserve than carbohydrates. For this reason, insects store many more fats than glycogen as an energy reserve in the fat body. Furthermore, because of the high fat content, which can absorb, bind, and neutralize lipophilic substances, some insects are able to tolerate a higher dose of insecticide or a pesticide when compared with individuals with less fat content.

The electron transport chain

This chain takes place only in mitochondria (which supply all cellular energy) and is made up of three essential complexes of integral membrane proteins:

1. NADH dehydrogenase (Complex I),
2. Cytochrome c reductase (Complex III), and
3. Cytochrome c oxidase (Complex IV).

In addition, two diffusible molecules—ubiquinone and cytochrome c—freely shuttle electrons between specific complexes (Fig. 1.4).

Electrons in pairs, during metabolic processes such as glycolysis, the Kreb's cycle, and ß-oxidation, are transferred to either NADH or $FADH_2$. During the electron transfer along the whole chain, a ferric ion (Fe^{+++}) accepts an electron to become a ferrous ion (Fe^{++}), which in turn passes the electron to the ferric ion in the next complex/diffusible molecule, with a lower chemical potential energy, to revert back to its original ferric ion. Starting with NADH, that eventually produces three ATP:

Step 1: The pair of electrons in Complex I (NADH dehydrogenase) is released to Fe^{+++} ions to form Fe^{++} ions of ubiquinone with a proton (H^+) being pumped into the intermembrane space of a mitochondrion. The only exit of the proton

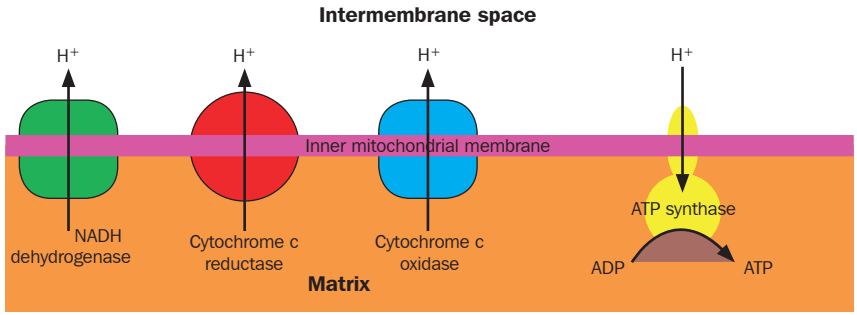


Fig. 1.4. ATP synthesis within a mitochondrion.

into the matrix of the mitochondrion is through the ATP synthase complex, at which point an ATP is produced (Fig. 1.4).

Step 2: The two electrons are then shuttled by Fe^{++} ions of ubiquinone (CoQ) to Fe^{+++} ions of Complex III.

Step 3: Then the two electrons are transferred from Fe^{++} ions of Complex III to Fe^{+++} ions of cytochrome c and a proton is simultaneously pumped out and subsequently leads to the production of an ATP as described in Step 1.

Step 4: Cytochrome c shuttles the pair of electrons finally to Complex IV's Fe^{+++} ions, which revert back to Fe^{++} ions by releasing the electrons to the ATP synthase complex (sometimes known as Complex V) to be used in the production of ATP, for which they are used in the reaction between oxygen and hydrogen to form a water molecule via "oxidative phosphorylation." At the same time, a hydrogen proton is pumped into the intermembrane space, where it will exit to the mitochondrial matrix to form an ATP as in step 1 (Fig. 1.4).

As for $FADH_2$, it enters the electron transport chain by transferring a pair of electrons to ubiquinone via an electron donor Complex II (succinate dehydrogenase). So, it ultimately produces only two ATP instead of three via "oxidative phosphorylation" by following steps 2–4 in the electron transport chain.

Degradation/detoxification/metabolic enzymes in the fat body

The fat body of an insect is the organ for food storage/reserve as well as a site for most metabolism and detoxification, equivalent to the mammalian liver. There are numerous different types of enzymes responsible for all the metabolic processes in the fat body. In insect toxicology, three main groups of enzymes play a major role in the detoxification of insecticides/pesticides: (1) cytochrome P450 mono-oxygenases, (2) esterases, and (3) transferases.

i) *Cytochrome P450 (CYP) oxygenases*

These belong to a diverse and large group of enzymes that specifically catalyze the oxidation of organic compounds. Their substrates include many metabolic intermediates, such as fats/lipids, plus numerous xenobiotic compounds, for example, plant defense substances and drugs.

The most common detoxifying reaction encountered is that catalyzed by CYP mono-oxygenase, in which an organic substrate (RH) is oxidized to an alcohol by insertion of an atom of oxygen while the other atom of an oxygen molecule is reduced to form water:

$$RH + O_2 + 2H^+ + 2é \longrightarrow ROH + H_2O$$

The alcoholic product of this reaction can be easily eliminated or excreted.

The CYP mono-oxygenases are also responsible for the oxidation of many toxic compounds such as the active ingredient of an insecticide/pesticide as well as the breakdown of peroxides.

ii) *Esterases*

These are a very diverse and large group of enzymes belonging to *hydrolases* (EC 3.1). They are responsible for the breakdown of an ester via hydrolysis in the presence of water into an acid and alcohol:

$$\underset{\text{“ester”}}{R_1O\text{-}CO\text{-}R_2} + \underset{\text{“water”}}{H_2O} \longrightarrow \underset{\text{“alcohol”}}{R_1OH} + \underset{\text{“organic acid”}}{R_2COOH}$$

Among the diverse classes of *esterases*, the following classes are either inhibited/affected by or able to hydrolyze/detoxify certain insecticides, particularly organophosphorus insecticides (OPs):

1. A-/*aryl-esterases* (EC 3.1.1.2) hydrolyze aromatic esters and OPs.
2. B-/*carboxyl-esterases* (EC 3.1.1.1) hydrolyze esters of carboxylic acid and are progressively inhibited by OPs.
3. C-/*acetyl-esterases* (EC 3.1.1.6) remove acetyl groups from acetyl esters; they are resistant to and do not hydrolyze OPs.
4. *Acetylcholine esterases* (EC 3.1.1.7) inactivate neurotransmitter acetylcholine, which is split into acetic acid and choline; they are inhibited by carbamates and OPs.
5. Phosphatases (EC 3.1.3.x) hydrolyze phosphoric esters into a phosphate and alcohol.
6. Phosphotriesterases (EC 3.1.8.1) hydrolyze OPs.

iii) *Transferases*

Glutathione S-transferases (GSTs) (EC 2.5.1.18) can be divided into eight distinct classes. But, all of them are catalysts for the detoxification of electron-loving compounds (“B” in the reaction below), such as carcinogens, drugs, products of oxidative stress (including highly reactive oxygen ion and other “free radicals”), toxins, many insecticides/pesticides, and xenobiotic substances. The conjugation reaction basically involves the transfer and binding of the glutathione that contains a sulfur atom to the toxic compound, that is, via the transfer of sulfur (S) representing glutathione as shown by the following reaction:

$$A - S + B \rightarrow A + B - S$$

Mitochondria

A mitochondrion is a membrane-enclosed organelle present in most cells. It is composed of several compartments, each with specialized function(s)—from outside inward—the outer membrane, the intermembrane space, inner membrane, cristae, and matrix. The number of mitochondria in a cell varies tremendously (from 1 to several thousand) depending on the tissue type as well as species of the organism. Mitochondria are known as "cellular power plants" because they generate most of the chemical energy in the form of ATP through glycolysis, the Kreb's cycle, ß-oxidation, and the electron transport chain (described previously). Besides those processes, mitochondria are involved in other cellular processes such as the cellular cycle, death, differentiation, growth, and signals.

Another unique character of a mitochondrion is that it has its own mitochondrial genome in the form of a circular DNA molecule (2–10/mitochondrion) of approximately 16 kilobases. The latter encodes the genes responsible for subunits of respiratory complexes in the electron transport chain as well as for mitochondrial transfer-RNA and ribosomal RNA required for protein synthesis.

Insect cuticle—growth and development

Most insects have a stiff and hard outer skeleton (exoskeleton) that comprises the epicuticle, which is composed of a thin waxy and water-resistant outer layer without any chitin, and a thick inner layer of procuticle. The procuticle consists of a hard and tough layer of exocuticle (consisting of sclerotin—hard and dark—formed by a reaction via cross linkages between artropodin and quinone and/or N-acetyl dopamine that diffuses inward after being secreted from dermal glands) and a tough and flexible endocuticle (composed of numerous layers of chitin and protein-artropodin).

Chitin is an important component in insect cuticle. It is a polymer of N-acetylglucosamine, which is derived from uridine diphosphate-N-acetylglucosamine-1-phosphate catalyzed by *chitin synthase*, and can be inhibited by certain "insect growth regulators" that are urea-based compounds.

Because of the hard and impermeable exoskeleton, any growth and development can occur only after an insect sheds its cuticle through molting (ecdysis). Molting is controlled by neurohormone and hormone (Fig. 1.5).

Insect development, metamorphosis (change of form), and reproduction are regulated by the neuroendocrine system, which consists of the neurosecretory cells in the brain, a pair of corpus cardiacum, a pair of corpus allatum (in the head capsule), and the prothroracic gland in the thorax (Triplehorn and Johnson 2005).

The lateral group of neurosecretory cells in the brain produces juvenile hormone (JH), which is stored in the corpora allata and then released to regulate larval development or, in adults, to regulate egg production for reproduction.

The medial group of neurosecretory cells in the brain produces a neurohormone prothoracicotropic hormone (PTTH)**,** temporarily stored in the corpora cardiaca and then released prior to molting to stimulate the prothoracic gland to secrete ecdysone or molting hormone (MH) that induces and enhances epidermis cellular division via mitosis as well as initiates ecdysis.

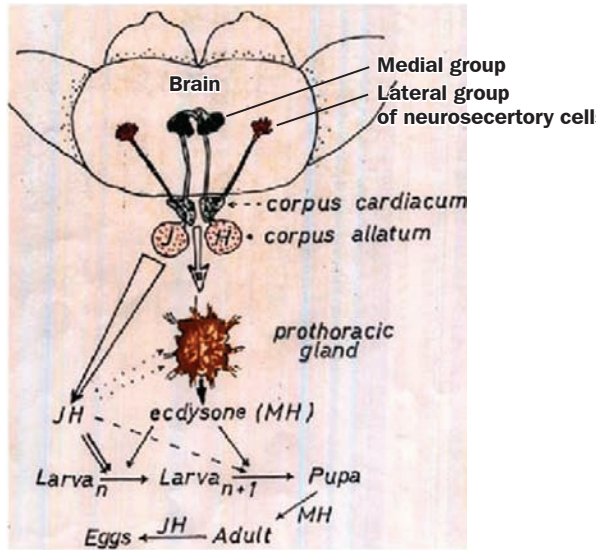


Fig. 1.5. The insect neuroendocrine system that regulates development.

Insect development and metamorphosis are regulated by the interplay of the two hormones MH and JH. MH induces molting while JH determines development—at high concentration, a larva will change to the next stage of larval development; at low concentration, a larva will change into a pupa; and in its absence the adult stage is attained (Fig. 1.5). During development, if a larva receives a dose of anti-JH (e.g., precocene I and II), it will transform into a precocious adult that does not reproduce. However, if a female adult receives a dose of anti-JH, it will not produce eggs and will become sterile.

The nervous system

The insect nervous system of a primitive insect such as a cockroach or bristle tail consists of a brain, three thoracic ganglia, and eight abdominal ganglia connected by two nerve cords. The numbers of thoracic and abdominal ganglia can vary depending on the species. In the most advanced insects such as the housefly, all thoracic and abdominal ganglia combine into one. The nervous system is made up of mono-, di-, and multipolar neurons. An impulse is generated at a point, normally from a receptor, the brain, or a ganglion, and is transmitted to a muscle. During this process, the impulse has to be transmitted from neuron to neuron.

Initiation of a nerve impulse. A nerve impulse plays a central role in neuron-to-neuron communications and is transmitted by changes in relative ionic charges and action potential along the membrane of an axon. During rest, the relative ionic charges of the neuron membrane are positive on the outer side of the membrane and negative on the inner side. As such, the resting potential is approximately –70 mV. An impulse is initiated when there is a temporary change in the resting potential caused by an opening

of sodium channels allowing sodium ions to flow into the axon so that the charges at that point in the inner membrane become positive. When the resting potential reaches the threshold potential (–55 mV), more sodium channels open, thus allowing a gush of sodium ions into the axon, causing a depolarization of the membrane. This allows the membrane potential to attain almost +35 mV, shown as a spike in Figure 1.6. At the peak of the spike, the sodium channels close and simultaneously potassium channels open to allow potassium ions to rush out of the axon during repolarization until the membrane potential falls to below the resting potential to cause a hyperpolarization before returning to the membrane resting potential (when all channels are shut) during the refractory period. Therefore, the action potential is made up of membrane potential changes during depolarization and repolarization within two milliseconds (Fig. 1.6). Further, an impulse can travel along an axon in only one direction because of the refractory period.

Action potential is a very short-lasting occurrence. Besides its occurrence in neurons, it can occur in several types of excitable cells such as endocrine and muscle cells. There are two types of action potential: the first type is generated by a voltage-gated sodium channel, which is very short lasting as described above, and the second type is generated by a voltage-gated calcium channel lasting 100 milliseconds or longer—a calcium spike produces a muscular contraction.

Impulse transmission between neurons at a synapse. An electrical impulse (action potential) cannot cross a very narrow gap (synapse) between two neurons. As such, when an impulse reaches the presynaptic end of an axon, the opening of the calcium channel allows calcium ions to enter the axon. The calcium ions then stimulate the

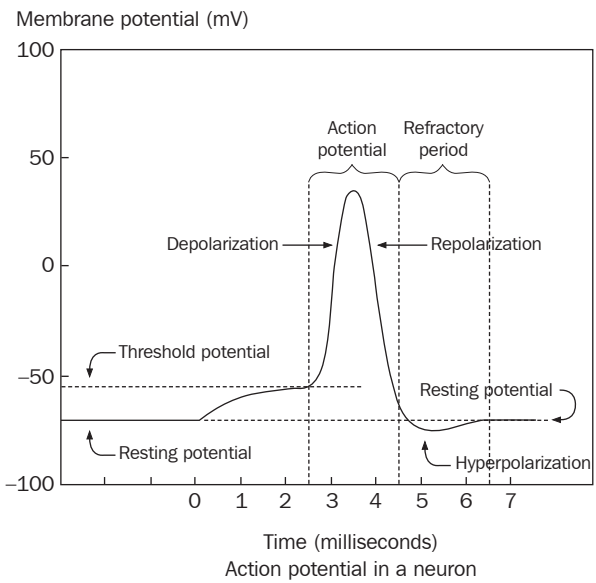


Fig. 1.6. Initiation of a nerve impulse in a neuron.

release of acetylcholine (a common neurotransmitter found in insects) from insect vesicles into the synaptic space. The acetylcholine then quickly diffuses and reaches channel receptors at the postsynaptic axonic membrane, where it binds to receptor sites to open and close the sodium and potassium channels. As such, an action potential is created at the postsynaptic axonic membrane with the simultaneous hydrolysis of acetylcholine (deactivation of the neurotransmitter) into acetic acid and choline catalyzed by *acetylcholine esterase* (reaction below). This enzyme is the common target of most organophosphate and carbamate insecticides.

There are two types of postsynaptic acetylcholine receptors in animals: (1) nicotinic acetylcholine receptors and (2) muscarinic acetylcholine receptors. The former predominates in insects, the latter in mammals. Owing to this important factor, a new group of neonicotinoid insecticide that targets only nicotinic acetylcholine receptors, with much lower toxicity to mammals, has been developed.

Genetics: gene regulation in cells

Most insecticides act via inhibition of either target enzymes or receptors, all of which are proteinaceous in nature. As such, it is pertinent to understand how genes are regulated in the production of the necessary proteins to act as either enzymes or receptors. Basically, there are two types of gene regulation, negative and positive. In negative gene regulation, a repressor that binds and suppresses the promoter of a gene requires the binding of an activator to form a complex. This allows a dissociation of the repressor-activator complex from the promoter, which then combines with *RNA polymerase* enzyme, allowing the expression of the gene by transcribing a messenger RNA (mRNA). In positive gene regulation, an inactive activator binds with an activator that then sits on the promoter, enabling the binding of *RNA polymerase*, resulting in the transcription of mRNA.

The following flow chart shows the various activities or processing of DNA, RNA, and protein that occur within the nucleus and cytosol:

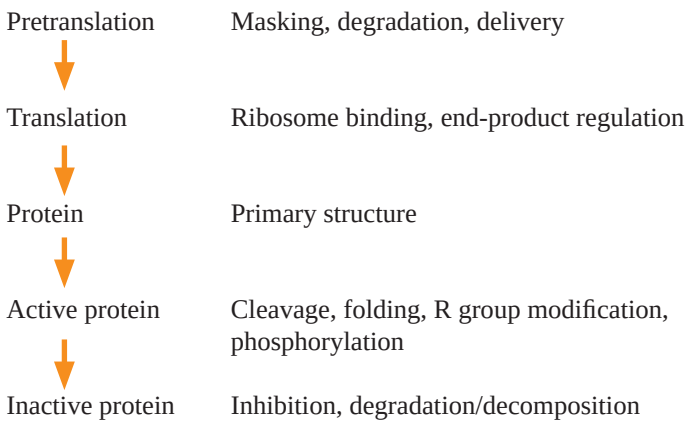
a) Nucleus

Packing, methylation, amplification, rearrangements, X-inactivation, heterochromatin, DNA organization

Promoters, enhancers, transcription factors, binding proteins, repressors

Capping, polyA tail, splicing, variable splicing

b) Cytosol



In the production of an active enzyme or receptor site from a set of genes, three important processes are involved—transcription, translation, and activation. Certain toxic compounds could interfere with any one of these processes.

When selection pressure is high, such as with the extensive and intensive spraying of an insecticide, the gene(s) responsible for the targeted enzyme or receptor sites may be selected to adapt or mutate (usually by a single-point mutation) so as to induce the development of insecticide resistance in an insect population. The modified gene(s) will naturally produce a modified enzyme or receptor site that is insensitive to the insecticide that caused the eventual development of resistance. It is a fact that insects can develop resistance to all kinds of insecticides, even to their own hormone when used as a pest control measure.

CHAPTER 2:

Insecticide toxicology

An insecticide is a pesticide used to kill or eliminate insect pests in agriculture, households, and industries. Judicious use of insecticides may be a factor in the increase of agricultural productivity. But, by their nature of having high toxicity to nontarget organisms and capability to develop resistance through widespread use, most insecticides have high potential to significantly affect and alter ecosystems. Many are toxic to humans and animals (both domestic and wildlife), and can accumulate as concentrates in the food chain and water resources, giving rise to serious environmental contamination and pollution.

Toxicity of a chemical is usually expressed in relative toxicity. All chemicals, even those generally considered nontoxic, can become toxic depending on the dosage given to an organism. As such, even a common consumable substance such as water has an LD_{50} of just over 80 g/kg, sugar (sucrose) an LD_{50} of 30 g/kg, and alcohol (ethanol) an LD_{50} of 13.7 g/kg, and these can be toxic above a certain dosage. Therefore, most insecticides, like other toxic chemicals, have varying degrees of toxicity. Toxic chemicals with relative toxicity of 50 mg/kg and below are considered highly toxic and those within the 50–500 mg/kg range are generally considered moderately toxic. Some examples follow:

Highly toxic chemicals (0–50 mg/kg)	
Botulinum toxin	0.00001 (= 10 ng)
Dioxin	0.1
Parathion	13.0
Strychnine	30.0
Nicotine	50.0

Moderately toxic chemicals (50–500 mg/kg)	
Paraquat	95
Caffeine	200
Carbaryl	270
Malathion	370
2,4-dichlorophenoxyacetic acid	375

Brief history of insecticide usage in pest control

A brief history of insecticide usage in the control of insect pests appears in Table 2.1. It should be pointed out that, up to 1950, the dominant insecticide used was arsenic-based. With the discovery of DDT as a potent insecticide after World War II, organochlorines were mainly used for insect control until they were replaced by organophosphates and carbamates by 1975. Pyrethrins extracted from plants were effective insecticides but were quickly degraded by UV (ultraviolet) light in the field and thus were ineffective as agricultural insecticides. Based on the pyrethrin molecule, a pyrethroid, permethrin (stable under UV light), was discovered and synthetized specifically for use in agriculture in the late 1970s. In the early 1980s, several pyrethroids began to be used widely.

Because of the widespread use of organochlorines, organophosphates, carbamates, and pyrethroids, insecticide resistance (cross- and multiple-resistance) developed in many species of insect pests. Insecticide resistance renders many insecticides ineffective as a control measure. Consequently, many chemical companies involved in the manufacturing of insecticides have been replacing them with new and less toxic chemicals.

Table 2.1. History of insecticide usage and insect control methods.

Period	Insecticide usage and insect control methods
Approx. 4,500 years ago	Sulfur dusting was used in ancient Mesopotamia.
15th century	Toxic inorganic chemicals, for example, arsenic, lead, and mercury, were applied to crops.
17th century	Nicotine sulfate extracted from tobacco was used as an insecticide.
1940-41	Methyl bromide was used as a fumigant against stored product pests.
Mid-1940s	DDT and other organochlorines with a wide spectrum of toxicity, that were inexpensive and had a persistent residual effect, eventually gave rise to serious environmental problems. Organophosphates (OPs) with high toxicity acting on the nervous system were introduced in 1944.
1950s	Carbamates were first introduced in 1956. They had high insecticidal toxicity, were less toxic to humans, and had relatively faster breakdown.
1960s	Pyrethrins (botanical insecticides) and male annihilation (combining a lure/attractant and an insecticide—usually OPs).
1970s	Juvenile hormone analogs/mimics (insect growth regulator, IGR) and insect pheromones/semiochemicals ("attract and kill").
1980s	Synthetic pyrethroids, insect growth regulators (inhibitor of chitin synthesis), biological control, and integrated pest management (IPM).
1990s	Neonicotinoids, area-wide male annihilation, sterile insect technique.
2000s	Phenyl pyrazoles, IPM.

Some of the new insecticides with their respective sites or modes of action are listed below:

a) Neonicotinoids (syn. neonicotinyls, chloronicotines, and chloronicotinyls) Block nicotinic acetylcholine receptors
b) Fipronil Blocker of GABA-gated chloride channels
c) Chlorfenapyr Inhibits oxidative phosphorylation
d) Sulfluramid Disrupts energy metabolism
e) Spinosads Increase excitability of acetylcholine receptors
f) Buprofezin Inhibits chitin synthesis
g) Diafenthiuron Inhibits mitochondrial *ATPase*
h) Indoxacarb Blocks sodium channels in nerve axon
i) Metaflumizone Blocks sodium channels in nerve axon
j) Pymetrozine Inhibits feeding in sucking insects

Classification of insecticides

Insecticides can be classified according to

1. Target insect stage of development, for example, ovicides, larvicides, and adulticides kill insect eggs, larvae, and adults, respectively.

2. Application technique, for example, dusting, fumigant, spray, residual, and topical.
3. Modes of action.
4. Active group in the insecticide, for example, carbamate, organochlorine, organophosphate.
5. Chemical nature.

Common insecticides are usually classified on the basis of their chemical nature:

1. **Arsenical insecticides** based on inorganic arsenite, e.g., calcium arsenate, potassium/sodium arsenite, copper acetoarsenite/lead arsenate.
2. **Botanical insecticides**, e.g., anabasine, azadirachtin, d-limonene, nicotine, pyrethrins, cinerins, jasmolin, quassia, rotenone, ryania, sabadilla, veratrum alkaloids.
3. **Antibiotic/microbial insecticides**, e.g., allosamidin, thuringiensin.
   - **Macrocyclic lactone insecticides**, e.g., avermectin insecticides—abamectin, doramectin, emamectin, and eprinomectin.
     a. Milbemycin insecticides—lepimectin, milbemectin, milbemycin oxime.
     b. Spinosyn insecticides—spinetoram, spinosad.
4. **Organochlorine insecticides**, e.g., DDT, HCH, γ-HCH (lindane), pentachlorophenol.
5. **Organophosphorus insecticides**, e.g., dichorvos, naled, TEPP, malathion, chlorpyrifos, diazinon, etc.
6. **Carbamate insecticides**, e.g., carbaryl, carbofuran, methomyl, propoxur, etc.
7. **Fluorine insecticides**, e.g., cryolite, sodium fluoride, sulfluramid.
8. **Oxadiazine insecticides**, e.g., indoxacarb.
9. **Pyrrole insecticides**, e.g., chlorfenapyr.
10. **Pyrazole insecticides**, e.g., chlorantraniliprole, dimetilan, tolfenpyrad.
    - **Phenylpyrazole insecticides**, e.g., acetoprole, fipronil, pyraclofos, pyriprole.
11. **Pyrethroid insecticides**, e.g., allethrin, barthrin, cypermethrin, deltamethrin, fenvalerate, permethrin, resmethrin, tetramethrin, transfluthrin.
12. **Nicotinoid insecticides**, e.g., flonicamid.
    - **Neonicotinoids** (pyridylmethylamine insecticides), e.g., acetamiprid, imidacloprid, nitenpyram, thiacloprid, thiamethoxam.
13. **Insect growth regulators (IGR)**
    i) **Chitin synthesis inhibitors**, e.g., bistrifluron, buprofezin, chlorfluazuron, teflubenzuron.
    ii) **Juvenoids/juvenile hormone mimics**, e.g., epofenonane, fenoxycarb, hydroprene, methoprene, pyriproxyfen.
    iii) **Anti-JH/precocenes**, e.g., precocene I, II, and III.
    iv) **Molting hormone agonists**, e.g., chromafenozide, halofenozide, methoxyfenozide, tebufenozide.
    v) **Prothoracicotropic hormone (PTTH) antagonists**, e.g., azadirachtin.
14. **Thiourea insecticides**, e.g., diafenthiuron.

Where do insecticides act in the insect body?

Most fast-acting insecticides act by inhibiting the transmission of nerve impulses and/or activity of neurotransmitters in the insect nervous system. The slower acting insecticides inhibit or block specific enzymes in cells or the electron transport chain in mitochondria. Slow-acting insecticides such as insect growth regulators (IGRs) disrupt hormonal action or chitin synthesis in the insect body.

Insecticide modes of action

From the onset, some terms in toxicology need to be clarified. First, a "ligand" is any substance, for example, a drug, hormone, and insecticide functional group, that binds reversibly to another chemical group/entity to form a larger complex compound. A ligand may function as an "agonist" or "antagonist." Second, the two terms, namely, "agonist" and "antagonist," sometimes wrongly used interchangeably, need to be clearly differentiated. An agonist is a chemical, often a mimic of a natural compound, for example, a hormone that binds to a receptor of a cell to produce an action. An antagonist, on the other hand, is a chemical that blocks or acts against an action.

Basically, insecticides have five very broad modes of action:

A. Physical poisons—dusts, fumigants, and oils. These poisons kill insects by asphyxiation, that is, blocking the flow of oxygen through the insect tracheal (respiratory) system.
B. Protoplasmic poisons are inorganic chemicals that physically destroy cells.
C. Metabolic inhibitors either interfere with metabolic pathways or inhibit certain enzymes.
D. Neuroactive agents affect the transmission of nerve impulses or the neurotransmitter.
E. Insect growth inhibitors disrupt growth and the development or malformation of cuticle.

Under these five broad modes of action, more than 20 different specific modes of action are found in insecticides. For the purpose of this manual, only 11 specific modes of action encountered by commonly used insecticides will be discussed.

1. Blocks deactivation of acetylcholine esterase in nerve synapse

Carbamates and organophosphates inhibit *acetylcholine esterase* by binding to the hydroxyl group of serine (an amino acid) at the active site of the enzyme. Therefore, the neurotransmitter, acetylcholine, after its release into the synapse, is not deactivated. This leads to a continuous and persistent stimulation of the postsynaptic membrane in neurons, giving rise to immediate hyperactivity, paralysis, and eventual death of the affected insect.

2. Action of insecticides on synaptic receptors

The nervous system has different types of synaptic receptors:

(a) Nicotinic acetylcholine receptors (nAChRs) are the most common and domi-

nant in insects. In the neurons, the nAChRs binding sites for acetylcholine are formed from amino acid residues of both α and ß subunits. Only when an agonist, such as acetyl cholinesterase, binds to the sites, all subunits undergo changes leading to the opening of a channel having a pore of approximately 0.65 nm in diameter. The nAChRs are blocked by irreversible binding of neonicotinoids, for example, imidacloprid, resulting in nontransmission of nerve impulses.

(b) GABA (gamma-aminobutyric acid) receptors are activated by avermectin, phenylpyrazole, organochlorine, and pyrethroid insecticides, leading to the opening of chloride channels. As such, inhibitory postsynaptic potential is created, thus blocking action potential that gives rise to a nerve impulse.

(c) Octopamine receptors. Amitraz (a member of the amidine class; is an insecticide and acaricide mostly used against mites, leaf miners, aphids, and scale insects) and its metabolites are agonists to octopamine receptors, especially alpha-adrenoreceptors, by inhibiting the enzyme *monoamino-oxidase*.

3. Noncompetitive blocking of GABA-gated chloride channels

Under normal conditions, nerve axons allow chloride ions to flow freely inward. However, the active ingredient of an insecticide, such as fipronil (a phenyl-pyrazole), avermectins, lindane, and cyclodienes (organochlorines) and pyrethroids/pyrethrins, blocks the flow of chloride ions through the GABA receptor as well as glutamate-gated chloride channels, and both components are present in the central nervous system.

4. Blocking of sodium channels in nerve axon

Indoxacarb (an oxadiazine compound) insecticide blocks the sodium channels in a nerve axon. This will prevent the initiation of an electrical spike; thereby, no nerve impulse occurs and, eventually, this inhibits any propagation of nerve impulse/potential.

5. Affecting voltage-dependent sodium channels (sodium channel modulators)

This mode of action is different from that of blocking sodium channels as previously described. Here, the insecticide directly affects membrane voltage, which prolongs the current flowing through sodium channels by slowing the closing of the channels. This leads to a large increase in neurotransmitters from nerve terminals.

This mode of action is shown by certain botanical insecticides, such as Sabadilla—a seed extract from genus *Schoenocaulon* (Melanthiaceae); veratrum alkaloids from a plant genus, *Veratrum* (Melanthiaceae); and pyrethroids/pyrethrins such as allethrin, cypermethrin cyhalothrin, deltamethrin, fenvalerate, fluvalinate, and permethrin. Regarding pyrethroids, Type 1 compounds (without α-cyano moiety), for example, permethrin, induce multiple spike discharges in the peripheral sensory and motor nerves, while Type 2 compounds (with α-cyano moiety), for example, cypermethrin, reduce the amplitude of the action potential, which eventually leads to a loss of electrical excitability of neurons.

6. Inhibiting the transfer of electrons in the electron transport chain

Rotenone, a botanical insecticide with moderately high toxicity, is able to block the transfer of electrons from Complex I to ubiquinone during oxidative phosphorylation that occurs in most cells, thereby interfering with the electron transport chain in mitochondria. This action primarily prevents the NADH cofactor from being processed to yield energy in the form of ATP.

Rotenone is extracted from plant species *Deris elliptica, D. involuta, D. walchii, Lonchocarpus nicou, L. utilis, L. urucu, Mundulea sericea, Piscidia piscipula, Tephrosia virginiana,* and *Verbascum thapsus.* Besides being an insecticide, it is also very toxic to fish. Therefore, its use is very limited in an aquatic environment, especially in rice fields. It causes an irritating action in humans, leading to nausea.

7. Uncoupling of oxidative phosphorylation

This mode of action is shown by pyrrole insecticides such as chlorfenapyr. Chlorfenapyr by itself is not toxic to an insect but is toxic when it is biotransformed to an active metabolite by oxidative removal of an N-ethoxymethyl group catalyzed by mixed-function oxidases. The active metabolite works by disrupting the production of ATP after uncoupling oxidative phosphorylation in the mitochondria. The disruption of ATP production subsequently leads to cell death and ultimately kills the insect.

Sulfluramid is a flourine insecticide, and by itself also does not uncouple metabolite oxidative phosphorylation. But, after its ethyl component is removed in a reaction catalyzed by cytochrome P450 oxidases to form a de-ethylated metabolite, this is a very potent uncoupler of phosphorylation during mitochondrial respiration.

8. Inhibition of *adenosine triphosphatase (ATPase)*

This enzyme has a function opposite that of *ATP synthase*, which is responsible for the synthesis of ATP. *ATPase*, however, catalyzes the decomposition of ATP to form ADP and a free phosphate ion with free energy liberated for biochemical processes catalyzed by certain enzymes, especially *kinases*. This reaction of dephosphorylation releases all the essential energy requirements for most cellular processes.

Diafenthiuron (a thiourea insecticide/acaricide) is metabolically activated to its carbodiimide with the dissociation of its urea derivative. The carbodiimide metabolite is the actual compound responsible for the inhibition of *ATPase* in the mitochondria. Diafenthiuron also blocks the use of ATP as a source of energy.

9. Juvenile hormone and its mimics (juvenoid-IGR insecticide)

Prior to molting of a larva/nymph, if juvenile hormone (JH) is present in high concentration in the body, it will molt into the next larval/nymphal stage. The main role of JH in development and metamorphosis is to retain the juvenile characters of an insect. Therefore, at a critical stage of development, that is, just before the last larval stage becomes a pupa or the pupa becomes an adult insect, if a juvenoid insecticide is applied, the treated individual will change to an intermediate form, that is, larva-pupa or pupa-adult intermediate. This intermediate will eventually die.

Juvenile hormone II present in most insects is also found in *Cyperus* (*C. iria*) plants. Juvenoid insecticides are generally not toxic, for example, methoprene, LD_{50}

>30 g/kg, and fenoxycarb, LD_{50} 16.8 g/kg, when compared with other nerve-acting insecticides. It should be noted that this group of IGRs is not very suitable for agricultural insect pests, as it tends to promote supernumerary molt, especially in lepidopteran insects.

10. Inhibitors of chitin synthesis (chitin inhibitor–IGR insecticide)

Normal insect cuticle is made up of layers of chitin along with structural protein, artropodin. The enzyme responsible for the production of N-acetyl-glucosamine, an important building block for the chitin polymer, is chitin synthase, which can be inhibited by phenylureas belonging to the group of benzoylphenyl ureas. In this group of insecticides, dimilin and diflubenzuron were the early compounds introduced for commercial use. Subsequently, more products such as buprofezin, chlorfluazuron, polyoxin C, and nikkomycin Z, which have extremely low water solubility (< 1 ppm) and mammalian toxicity, became available.

11. Inhibition of prothoracicotropic hormone (PTTH) (PTTH inhibitor–IGR insecticide)

This hormone from the insect brain stimulates the prothoracic gland to secrete the molting hormone that induces insect molting. Insecticide azadirachtin, derived from the neem tree (*Azadirachta indica*), disrupts the synthesis and thus production of PTTH and ultimately kills the insect. Azadirachtin is also a potent antifeedant (feeding deterrent).

At this point, it is beneficial to note that (a) an insecticide may have more than one mode of action and (b) all IGRs directly affect only insect hormones, growth, and development. Insect development and metamorphosis are entirely dependent on the interactions of two hormones, JH and molting hormone, which are totally different and unrelated to those of higher animals. For this reason, IGR insecticides generally have very low toxicity to vertebrates.

Classification based on mode of action

It should be noted that the Insecticide Resistance Action Committee (IRAC[1]) has been advocating the use of mode of action for classification of insecticides and acaricides (Fishel 2008). To develop insecticide management strategies, it is important to know which type(s) of resistance is existing in a pest population within a region or cultivated area. Some pests are known to have cross-resistance, which means they have acquired resistance to one insecticide and that has rendered them resistant to another that has the same mode of action. For instance, imidacloprid resistance in the brown planthopper is directly related to thiomethoxam resistance because the two insecticides

[1] IRAC, the Insecticide Resistance Action Committee (IRAC), formed in 1984, is a technical group of the industry association CropLife to provide a coordinated industry response to prevent or delay the development of resistance in insect and mite pests. The main goals are to facilitate communication and education on insecticide resistance and promote the development of insecticide resistance management strategies to maintain efficacy and support sustainable agriculture and improved public health. Details are available at www.irac-online.org/.

from different chemical classes have the same mode of action (Matsumura et al 2008). Classifying insecticides by their modes of action will enable better development of insecticide introduction and mix strategies. For instance, most organophosphate and carbamate insecticides have the same mode of action, as aforementioned, by acetylcholinesterase inhibition; thus, introducing a new carbamate into an area to control a pest population with high resistance to an organophosphate, or vice versa in terms of insecticides, would not be a wise strategy.

Multiple-resistance is the development of resistance to insecticides based on more than one mode of action by an insect population, such as the situation found in most populations of the diamond-back moth, *Plutella xylostella* (Yu and Nguyen 1992). When multiple-resistance has developed in a particular pest population, the pest can become very difficult to manage.

Synergism

An insecticide synergist is a chemical that on its own does not possess inherent insecticide activity, but enhances or increases the effectiveness of an insecticide when combined. Currently, piperonyl butoxide (PBO) is the most used synergist for several classes of insecticides, such as avermectins, carbamates, organophosphates, pyrethroids, and certain insect growth regulators. Methylenedioxybenzene derivatives can also act as synergists for the same classes of insecticides as those of PBO.

A majority of the other known synergists, including (1) DEF (S,S,S-tributyl phosphorotrithioate), (2) DEM (diethyl maleate), (3) IBP (S-benzyl diisopropyl phosphorothiolate, (4) K1 (2-phenyl-4H-1,3,2-benzodioxaphosphorothiolate), (5) K2 (2-phenoxy-4H-1,3,2-benzodioxaphosphorin 2-oxide), (6) sesamex (5-[1-[2-(2-ethoxyethoxy) ethoxy]ethoxy]-1,3-benzodioxole), (7) SV-1 (O,O-diethyl-O-phenyl phosphorothiolate), and (8) TPP (triphenyl phosphate), also have a similar mode of action by blocking the metabolic processes that break down insecticide molecules, such as disrupting the detoxification catalyzed by mono-oxygenases and/or hydrolyzing enzymes, especially esterases.

This has been well documented. Combining certain insecticides (within and between classes) may have a synergistic effect against certain insect pest species. Examples follow:

1. Mixtures of N-methyl- and N-phenyl-carbamates increased mortality by at least twofold when applied as a mixture compared with the respective individual compounds against resistant (to aryl N-methylcarbamates) strains of green rice leafhopper *Nephotettix cincticeps* (Takahashi et al 1977).
2. Mixtures of a pyrethroid with either a carbamate or an organophosphate induce much higher toxicity than either the insecticide alone against an insecticide-resistant strain of *N. cincticeps.* Tested mixtures were fenvalerate with malathion, diazinon, or MPMC (3,4-xylyl N-methylcarbamate) and phenothrin with MTMC (3-methyl-phenyl-N-methyl-carbamate) or BPMC (2-sec-butylphenyl N-methylcarbamate) (Ozaki et al 1984).
3. Synergism between permethrin (a pyrethroid) and propoxur caused a drastic increase in acetylcholine concentration in synapses, thereby causing a nega-

tive feedback of acetylcholine release in the American cockroach, *Periplaneta americana*. Nonetheless, atropine—a muscarinic receptor antagonist—completely reversed the effect of the insecticide mixture in the cockroach (Corbel et al 2006).

4. Several patents related to mixtures of insecticides, for example, a neonicotinoid with pyrazole or pyrrole insecticides, against the brown planthopper are pending approval.

Insecticide resistance

This is the ability of an insect population to withstand or tolerate the adverse effects of an insecticide, that is, to survive a lethal dose of an insecticide that would have killed most normal/susceptible individuals of the same species, via adaptation, mutation, and/or natural selection.

With the introduction plus extensive and frequent spraying of initially effective organic synthetic insecticides, such as DDT, against insect pests in the 1940s, resistance to DDT was first detected and confirmed in housefly, *Musca domestica*, by 1947. Resistance to OPs and carbamates was detected 14 and 7 years after their introduction, respectively (Brattsten 1990). Since then, numerous cases of resistance have been confirmed for every new class of insecticides introduced, starting from cyclodienes of organochlorines, formamidines, pyrethroids, thuringiensis (*Bt*), spinosyns, and insect growth regulators to neonicotinoids, after 2–20 years of use.

The speed at which resistance can develop in an insect pest population is dependent on four important factors:

1. Intensity of selection pressure—frequency of applications of an insecticide in an area;
2. The frequency of resistance genes present in a field population of the pest species (very low initially);
3. Characteristics of resistance genes (dominant or recessive, and single or multiple); and
4. Reproductive dynamics and potential of an insect pest population, for example, the number of generations per year.

In all insect pest species, satisfactory control can be obtained when an insecticide is first applied because the number of insects having resistance genes is extremely low. However, with increased frequency of application of the same insecticide, the number of individuals with resistance genes increases, leading to occasional crop losses. In other words, frequent and continued use of an insecticide, especially through indiscriminate, extensive, intensive, and/or prophylactic applications over time, provides an extremely high selection pressure for a pest population to adapt and evolve resistance. Unfortunately, when resistance has developed, agricultural producers and farmers become more desperate to stop pest resurgence and/or emergence of secondary pests (Heong et al 2009). This usually leads to desperate and extreme measures, such as further intensive applications with much higher dosages, to be taken. As a result, the pest population will increase exponentially, resulting in outbreaks of pests. This

phenomenon is known as the "pesticide treadmill," and it will actually enhance insect adaptation and evolution to survive by developing inheritable traits that specifically resist very high selection pressure of an insecticide. This is currently exhibited in many Asian countries, especially for the brown planthopper, which has given rise to unprecedented, serious, and widespread outbreaks resulting in huge losses plus serious social and economic problems among producers and farmers as well as within their respective communities.

Mechanisms of resistance to insecticides

Understanding resistance mechanisms is a very important component of an effective resistance management strategy.

Mechanisms of resistance can be divided into four categories:

1. Reduce penetration of an insecticide through the cuticle, resulting in very low resistance.
2. Behavioral resistance—through avoidance or due to an insecticide acting as a repellant.
3. Metabolic resistance—through detoxification by increased activity of specific enzymes.
4. Genetic resistance—through mutation of a gene in receptors or active sites of enzymes.

Most cases of insecticide resistance detected and confirmed (see summary in Table 2.1) are due to metabolic or genetic resistance mechanisms or a combination of both. Li et al (2007) provided an excellent review on the metabolic resistance to synthetic and natural xenobiotics, especially in relation to insecticides. In this chapter, we will limit discussion to rice planthoppers as far as possible.

Table 2.1. Mechanisms of resistance to major insecticide groups.

Mechanism(s)	Insecticide group to which resistance evolved
Detoxication by	
a) *Carboxylesterases*	Carbamates, organophosphates (OPs), pyrethroids
b) *Cytochrome P450/mixed-function oxidases*	Carbamates, OPs, pyrethroids, neonicotinoids
c) *Glutathione S-transferases*	Organochlorines, OPs, pyrethroids
d) *DDT dehydrochlorinases*	DDT
Disruption of GABA-gated chloride channels	Avermectin, cyclodiene, phenylpyrazoles, spinosyn
Disruption of sodium ion channels	Organochlorines, pyrethroids
Inhibition of adenosine *triphosphatase*	Thiourea insecticide/acaricide
Inhibition of chitin synthase	Phenylurea-insect growth regulators
Insensitive *acetylcholinesterases*	Carbamates, OPs
Insensitive nicotinic acetylcholine receptors	Neonicotinoids
Uncoupling oxidative phosphorylation	Pyrroles, fluorine-based insecticides

Metabolic resistance via detoxification

Most insecticides can be detoxified or inactivated by three main groups of enzymes: (1) esterases, (2) cytochrome P450 mono-oxygenases, and (3) glutathione S-transferases.

a) *Esterases*

As early as the early 1970s, hydrolases, especially the subgroup of *esterases*, were implicated in insecticide resistance (see review by Sudderudin and Tan 1973). Most detoxifications in insects are attributed to *carboxylesterases*, with a few rare cases catalyzed by *arylesterases* (aromatic *esterases*). Detoxification can be caused by increased *esterase* activity or amplification of a gene encoding the *esterase* gene. This is shown by examples of rice hoppers.

i) In the green rice leafhopper, *Nephotettix cincticeps*, five strains found in Japan had increased *carboxylesterase* activity and two of them also had decreased *cholinesterase* susceptibility (Miyata and Saito 1976). Strains with field resistance to carbamates showed very high *carboxylesterase* activity in comparison with susceptible strains (Lim and Tan 1995).

ii) In the brown planthopper, *Nilaparvata lugens*, malathion and MTMC-selected resistant strains showed high degradation of malathion induced by high *aliesterase* (*carboxylesterase*) activity. It was also shown that the other detoxification enzyme, glutathione S-*transferase*, was not involved in the detoxification of malathion when compared with susceptible strains (Endo et al 1988). The resistance in a BPH strain from Sri Lanka was due to one elevated *esterase* band, which was responsible for sequestration of OPs. The resistance mechanism is therefore not due to metabolism of OP insecticides (Karunaratne et al 1999).

Widespread resistance to OPs in the BPH is due to the elevation of a cDNA-encoded *carboxylesterase, Nl-EST1*, which has a 547 amino acid protein also present in nonresistant strains (Small and Hemingway 2000). The *esterase* gene was amplified 3–7-fold, contributing to the increase in *esterase* activity of 8–10-fold higher in resistant (to OPs and carbamates) strains than in susceptible strains.

iii) In the small brown planthopper, *Laodelphax striatellus*, two isozymes of *aliesterase* (*carboxyesterase*) showed high activity in the malathion-resistant strain when compared with a susceptible strain. The two isozymes were shown to be able to hydrolyze aliphatic esters and malathion, and they were controlled by a single codominant autosomal factor (Sakata and Miyata 1994).

iv) In the whitebacked planthopper, *Sogatella furcifera*, a large increase in *esterase* activity, in conjunction with *oxidases*, was detected in a field population resistant to fipronil. PBO synergist inhibited both *esterase* and *P450 oxygenase* activity but TPP inhibited only *esterase* activity (Tang et al 2009).

b) *Cytochrome P450 mono-oxygenases*

These enzymes are a key metabolic system responsible in the detoxification of xenobiotics, and therefore a major mechanism by which an insect species evolves insecticide resistance. Some examples related to rice pests follow.

The resistance of BPH to imidacloprid was reported to be attributed to the detoxification caused by enhancement of *P450 mono-oxygenases* (Wen et al 2009). Sequence analysis of nicotinic receptor α1 subunit from two field-collected strains of BPH resistant to imidacloprid did not show the point mutation previously assumed as the resistance mechanism involved. Nonetheless, there was about a 5-fold increase in *oxidase* activity, suggesting that imidacloprid was metabolized by increased *cytochrome mono-oxygenase* activity as the major resistance mechanism against the neonicotinoid (Pulnean et al 2010).

In the small brown planthopper, *Laodelphax striatellus*, biochemical analysis showed that the increase in *cytochrome P450 mono-oxygenase* and *esterase* plus *acetylcholinesterase* insensitivity may be the mechanisms involved in multiple resistance (to imidacloprid, two OPs—chlorpyrifos and acephate—and deltamethrin) found in strains collected from Jiangsu Province in China (Gao et al 2008).

In the whitebacked planthopper, *Sogatella furcifera*, the field population resistant (5–50-fold) to fipronil showed a considerable increase in *mono-oxygenase* activity (Tang et al 2009).

c) *Glutathione S-transferases (GSTs)*

A laboratory colony of BPH was selected for pyrethroid resistance using permethrin and λ-cyhalothrin, which, besides their neurotoxic properties, induce oxidative stress and peroxidation of lipids (fats). Increased *GSTs* in the resistant strains reduced pyrethroid-induced lipid peroxidation and mortality. The elevated *GSTs* provided a major mechanism for pyrethroid resistance in BPH (Vontas et al 2001). Further, molecular analyses indicated that the *NIGSTD1* gene, through gene amplification, conferred pyrethroid resistance in BPH (Vontas et al 2002).

Genetics of resistance

Genetic inheritance of traits through mutation resulting in genomic changes that lead to amplification, overexpression, and/or altered coding sequence of major groups of genes for the three pertinent enzymes mentioned previously, responsible for developing resistance to a group of insecticides, is the sole cause of genetic resistance. Point mutations are generally accepted to be the major cause of increased insensitivity of enzymes or receptors to an insecticide. The understanding of the evolution of insecticide resistance mostly comes from target site mutations in many species of insects involving genes/regulatory elements (Plapp 1986), such as the following:

1. *AChE-R*—altered *AChE* gene; different alleles confer a different level of resistance.
2. *ace* (*acetylcholinesterase* gene)—three-point mutations identified in *Batrocera dorsalis* (the oriental fruit fly) gene.
3. *dld-r*—a recessive gene that confers resistance in cyclodienes by changing the target site of insecticide.
4. *kdr*—a recessive knockdown gene resistant to DDT and pyrethroids, it modifies the target site; low-level (*kdr*) and high-level (super *kdr*) alleles have been reported.

5. *pen*—a recessive gene that decreases uptake of an insecticide. By itself, it confers little resistance, but it acts as a modifier of other resistance genes by doubling resistance levels.
6. Mutated codon (single amino acid mutation) of llS6 membrane-spanning region of the sodium channel gene. It confers target-site DDT-pyrethroid resistance in insects; a single-point mutation in *ace-1* is responsible for propoxur resistance in mosquitoes—in which GGC (glycine) codon at position 119 is replaced by an AGC (serine) codon in resistant mosquitoes (mutation G119S) (Weill et al 2003).
7. Regulatory element "Barbie Box"—it allows induction of insecticide-detoxifying oxidase and esterase resistance genes. Several mutations leading to amino-acid substitutions have been detected in the *P450 mono-oxygenases* gene CYP6A2 of a resistant strain in *Drosophila melanogaster* (Berge et al 1998).
8. *Esterase* A2-B2 amplicon (a family of amplified esterase genes) is found within the same amplification unit. More than a hundred copies of this amplicon may be present in a single insect.

Insensitive enzyme target site

Because of gene mutation, usually "single-point mutation" of an enzymic target site, the active site of an enzyme may alter with one or more amino-acid changes. As a result, the modified enzyme may show a varying degree of insensitivity toward the insecticide that interferes with or blocks the unmodified enzyme activity.

Insensitive *acetylcholinesterase*

Insensitivity of *acetylcholinesterase* (*AChE*) as an insecticide resistance mechanism was first detected about 40 years ago. An *AChE* insensitive to carbamates was shown in highly resistant strains of BPH selected in the laboratory against carbofuran and fenobucarb. After 30 generations of selection, LD_{50} values increased 93–101-fold for fenobucarb and 51–68-fold for carbofuran. At the same time, *AChE* sensitivity to both insecticides decreased greatly in the resistant strains (Yoo et al 2002).

In the green leafhopper, the most resistant field population against vamidothion in Taiwan had *AChE* sensitivity reduced by 4-fold when compared with a susceptible population. In addition, electrophoretic analysis revealed an extra band with strong *carboxylesterase* activity and moderate *AChE* activity in another resistant field population (Sun et al 1980). Additionally, a modified *cholinesterase* that is insensitive to a carbamate was revealed in the resistant strain. This is the insensitive *AChE* modified from the original enzyme found in a susceptible strain (Hama 1976).

In other insects, such as *Schiazaphis graminum* and *Anopheles gambiae*, an *AChE* paralogous to *Ace* (*acetyl cholinesterase* gene) with various amino-acid substitutions was found corresponding to different biochemical properties of *AchE* insensitivity (Kono and Tomita 2006).

Insensitive chitin synthase

In a field population of BPH after 65 generations, of which 56 were selected against buprofezin, the colony developed a 3,599-fold resistance to buprofezin. Tests using SV1, PBO, and DEM synergists against the resistant strain increased buprofezin toxicity by only 1.5–1.6-fold when compared with the susceptible strain. This suggested that detoxification of *esterases*, *P450 mono-oxygenases*, and *glutathione S-transferases* was not responsible for the extremely high buprofezin resistance in BPH. Further investigation to understand the actual resistance mechanism in *N. lugens* was suggested (Wang et al 2008). Since buprofezin is a specific *chitin synthase* inhibitor and it is not detoxified by the three major groups of detoxifying enzymes in the resistant strain of BPH, mutation causing modification/changes of amino-acid composition in the enzyme target site is probably one of the major resistance mechanisms resulting in a resistance factor of 3,600-fold against buprofezin, though this may be speculative at this stage.

Insensitive cytochrome P450 mono-oxygenases

In the housefly resistant strain NG98, which had resistance of 3,700-fold against permethrin, resistance was caused by *kdr* on autosome 3 and *mono-oxygenase*-mediated resistance on autosomes 1, 2, and 5. Resistance mediated by *mono-oxygenases* seemed to have evolved using different *P450 oxygenases* and likely different regulatory signaling to control *P450 oxygenase* expression (Scott and Kasai 2004).

Insensitive GABA-gated chloride channel subunit

A mechanism of resistance to cyclodiene insecticides in several insect species is due only to the same single mutation in the GABA-gated sodium channel subunit. Replacement of a single amino acid (alanine 302) in the chloride ion channel pore of the protein is responsible for the resistance. Replacement of alanine 302, besides directly affecting the binding site, also destabilizes the preferred conformation of the receptor (French-Constant et al 2000).

Nicotinic acetylcholine receptor mutation

To understand the molecular basis of imidacloprid resistance in BPH, five nicotinic acetylcholine receptor (nAChR) subunits (Nlα1–Nlα4 and Nlβ1) were cloned. When comparing the nAChR subunit genes from imidacloprid-susceptible and imidacloprid-resistant strains, a single-point mutation at a conserved position (Y151S) in two nAChR subunits, Nlα1 and Nlα3, was identified (Liu et al 2005). Therefore, the mechanism of resistance was shown to be the Y151S point mutation for the observed high level of resistance to imidacloprid. This was demonstrated by Liu and his colleagues by providing evidence that the mutation in the receptor target site was responsible for a significant reduction in the binding of imidacloprid.

Possible methods to avoid or delay insecticide resistance

There are several ways to delay or even avoid insecticide resistance by using the following methods:

1. Use of an appropriate synergist, for example, piperonyl butoxide, DEM, and S,S,S-tributyl phosphotrithioate, to increase the effectiveness of an insecticide without increasing the dosage of the insecticide in use. Nonetheless, it must be remembered that the constant or frequent use of an insecticide over time will encourage resistance development.
2. Overcoming metabolic resistance using insecticide composed of two or more isomers of the active insecticidal ingredient.
3. In *N. cincticeps*, a mixture of N-propyl and N-methyl carbamates—the former inhibits altered *ACh-esterase* in the resistant strain, while the latter inhibits the enzyme in the susceptible strain.
4. A change to using a different class of insecticides with different modes of action. This is one of the obvious methods to delay resistance built up for any one class of insecticide. Rotation of two or more appropriate classes of insecticides with entirely different modes of action will go a long way to delaying resistance to any one of the insecticides used. However, it should be cautioned that getting involved in the "pesticide treadmill" should be discouraged. This is because the best way to avoid resistance is to avoid the use or total dependence on insecticides for insect pest control.
5. Avoid intensive spraying or reduce/avoid the use of insecticides and practice a good area-wide IPM program. This practice of implementing sound area-wide IPM should be the pillar of support for ecofriendly insect pest management through the judicious use (only when it is absolutely necessary) of an effective insecticide within the context of a reliable "economic threshold."

Conclusions

Frequently, among pest control agencies or farmers, there is a belief or assumption that the discovery and/or marketing of new insecticides will always be way ahead of resistance development. Nonetheless, the ever-increasing cost of research and development for a new insecticide and, more importantly, the number of insect pest species or strains resistant to even recently introduced insecticide—such as imidacloprid and buprofezin against the BPH—demand the implementation of pest control strategies within a proper IPM (integrated pest management) program to delay or avoid resistance.

The development of insecticide resistance is an inevitable event when an insecticide is used over a period of time with frequent and indiscriminate or extensive and intensive applications. With the understanding of the mode of action as well as the mechanism of resistance to insecticides, management of insecticide resistance in the control of insect pests can be better planned, developed, and implemented, thereby enhancing the involvement of insect toxicology in the proper management of insecticide resistance within a well-planned and well-executed insect pest management program. It is also important to note that the main defense against the development of insecticide resistance is tight and regular surveillance, without any slipshod approach, of the susceptibility of insecticide(s) in use within the targeted field area so as to enhance an insect pest management program.

CHAPTER 3:

Quantal response data and toxicological statistics

Insecticide research generally involves comparing the level of toxicity of different compounds or comparing the susceptibility of different insect species or the same species from different environments. A useful way to make comparisons is to determine doses that have equal toxicity and there are three general ways to bioassay compounds to obtain the critical doses (Finney 1964). First is through direct assaying to measure the exact doses necessary to kill individual animals by gradually increasing the doses up to the critical point. For insects, these methods are not practical. The other two ways involve indirect assaying and this is performed by exposing batches of individuals to standard doses and recording the responses, which may be death, knockdown, deformity, or discoloration, depending on the expected effects of the compound on the insect species. Bioassays may be based on quantitative responses, such as time of survival, but there are technical difficulties in determining survival times and thus this method is not useful for testing insecticides. The third method is to use quantal response bioassays. The binary quantal response with one explanatory variable is the simplest and most common bioassay test used in insecticide research. In such dose-response or concentration-response bioassays, the explanatory variable is a range of dosages or concentrations and the response is an all-or-nothing observation, such as dead or alive, knocked down or remaining standing, deformed or not deformed, and discolored or not discolored. The other two quantal response bioassays are more complex, time-consuming, and less frequently used. Details can be found in Robertson et al (2007).

In experiments based on quantal response, the data needed are the proportions of each batch responding to the compound in a particular way. The purpose is to estimate the dose level that is just sufficient to produce death (or a particular response) within the given proportion of insects and to use the estimate to make comparisons. It is generally easiest to estimate the median (50%) response level of the population.

The median lethal dose is a quantitative expression of tolerance of a particular species under a given condition or location. It is a definitive biological characteristic and depends on other physiological and physical characteristics such as age, sex, rearing conditions, and temperature. In the older literature, it is often abbreviated as MLD, but this can be confused with the "minimum lethal dose." Usually, the abbreviation LD_{50} is used for a 50% lethal dose. The other levels are abbreviated LD_{90} or LD_{95} to refer to 90% and 95% lethal doses, respectively. For other dosage variables, the abbreviations are LC_{50} for concentrations, LT_{50} for lethal time exposures, KD_{50} for knockdown dosages, and ED_{50} for effective doses. LD_{50} and other measures provide estimates of the toxicity of the insecticide used and are expressions of the tolerance of the insect. The higher the LD_{50} value, the lower the toxicity.

Bioassays

Quantal response data are obtained using bioassays and each unit in the bioassay is the entity that receives the treatment. In assays in which each insect is individually treated, the unit is the individual insect. When a group of insects are treated by spray or fed a treated diet, the group (not individuals) is the unit. ***For experimental precision, each unit must be a constant***, for instance, the insects are obtained from the

same place, and have the same age, stage, sex, nutrition, and rearing conditions. The rearing and preparation of standardized insects or experimental units are discussed in Chapter 4.

In the bioassay, batches of insects are exposed to a range of doses of the poison. The size of each batch is often determined by practical considerations. The larger number per batch will have more accuracy. However, there is little advantage in exceeding 30 to 50 per batch unless the population is very heterogeneous (Busvine 1971). For rice planthoppers, experimental batches of 10 to 15 in 4 or 5 batches of a total of 40 to 65 standardized units will often suffice. Selection of insect units for each batch is best done in a randomized manner. In selecting the doses or concentrations of the poison for the experiment, it is best to space them evenly over the mortality range. Since toxicity is related to the logarithm of dose, a dose range in a geometric series is preferred, such as 2, 4, 8, 16, 32 or 1, 3, 9, 27. The control batches are exposed to the same treatments, except for the inclusion of the poison, which means that control insects need to be treated with the solvent used to dilute the solutions. Replications are best done on different days within a short period assuming that day-to-day variability is not a source of error. Within each replicate, the order in which treatment doses are used should be from the lowest to the highest.

Correction for control mortality—the Abbott formula

In bioassays, it is common to expect a proportion of the insects in the control batches to die during the experiment due to natural causes or the control treatment with the solvent. To correct for this, the Abbott formula is often used. The formula attributed to Abbott (1925) had in fact been used earlier by Tattersfield and Morris (1924) and is usually in the form

$$P = \frac{Po - Pc}{100 - Pc} \times 100$$

where P is the corrected mortality, Po is the observed mortality, and Pc is the control mortality, all expressed in percentages.

Probit analysis—a statistical method in bioassays

The statistical theory and techniques using probit analysis for analyzing data from dose-quantal response experiments were developed by D.J. Finney (1971) and details are discussed in Finney (1978) and Robertson et al (2007).

Data obtained from bioassays are generally in percent response (mortality or affected) at the corresponding doses (or concentrations). When the percent responses are plotted against the doses, an S-shape curve is obtained. This is because toxicity is better related to the logarithm of the dose; thus, in the analysis, the dose variable is normally transformed into the logarithmic scale. The usual way to estimate LD_{50} is from a regression line relating log dose to a transformed percentage response (Busvine 1971) and the usual transformation used is probits. Transformation of percent

response to probits is available in Appendix A and can also be calculated by using a microcomputer (Krejcie 1991).

Critical LD_{50} values can be estimated from probits and log doses in several ways. The simplest is by graphical methods. Another is by using standard computation with a calculator (Finney 1971, Heong 1981). Step-by-step calculations are also available in Busvine (1971). A faster and more accurate way is using a computer program or software. Several statistical packages such as SAS and SPSS have probit analysis options. In this book, we focus our attention on using POLO software (Russell et al 1977), further refined by LeOra software (2002). Details on the use of PoloPlus© are discussed in Chapter 6.

Relative potency

The toxicities of two or more insecticides are compared on the basis of potency or the reciprocal of an equitoxic dose (Busvine 1971). ***For valid comparison, the dose-mortality lines for the insecticides should be parallel***. Otherwise, the relative potency will vary with the mortality used. If two regression lines are written as

$$Y_1 = a_1 + bx_1$$
$$Y_2 = a_2 + bx_2$$

when the slopes are similar, b is common and at the equitoxic dose

$$Y_1 = Y_2$$

and, hence, $a_1 + bx_1 = a_2 + bx_2$

$$x_1 - x_2 = \frac{a_2 - a_1}{b} = M$$

M is thus the difference in position of the two slopes and its anti-logarithm is the potency ratio. PoloPlus computes the potency ratio and its fiducial limits (at $P = 0.95$). The detailed output is in Chapter 6.

CHAPTER 4:

Rearing and preparation of test insects

As discussed in Chapter 3, for experimental precision, each unit or insect must be a constant. Insect populations need to be collected from the same location and reared in the same nutrition and environmental regimes. At the same time, there is a need to provide an adequate supply of test insects for the bioassays. This chapter will discuss the procedures and techniques used to rear and prepare standardized test materials.

The insects used are planthoppers but the methods can be easily adapted for use with any insect species.

Collection of insects

A suitable rice field is identified and its location noted, preferably with the name and geographic position. About 50 healthy unparasitized adult females or about 100 nymphs are collected from the study fields. Planthopper adults, preferably short-winged, are collected from the base of the rice plants using an aspirator (by mouth or suction bulb) and placed into test tubes covered with nylon mesh (Fig. 4.1A). Alternatively, insects can be collected from the field using a sweep net (Fig. 4.1B).

The collected planthoppers are transferred immediately onto clean potted plants enclosed with circular or rectangular mylar cages. These plants and cages should be prepared in the research center before going on the collection trip. Alternatively, collected insects can be transferred to rearing cages with clean potted plants. In China and Japan, the collected insects are also kept in test tubes with seedlings and small boxes with seedlings. Collection cages (Fig. 4.2) are then labeled with the respective collection dates, location names, and geographic positions.

The insects collected are brought back to the research center and reared in a greenhouse or insectary maintained with a temperature of 27 $\pm$ 2 °C and 12 hours of light.

Fig. 4.1. Collection equipment: (A) a mouth aspirator and test tube covered with nylon mesh; (B) a sweep net.

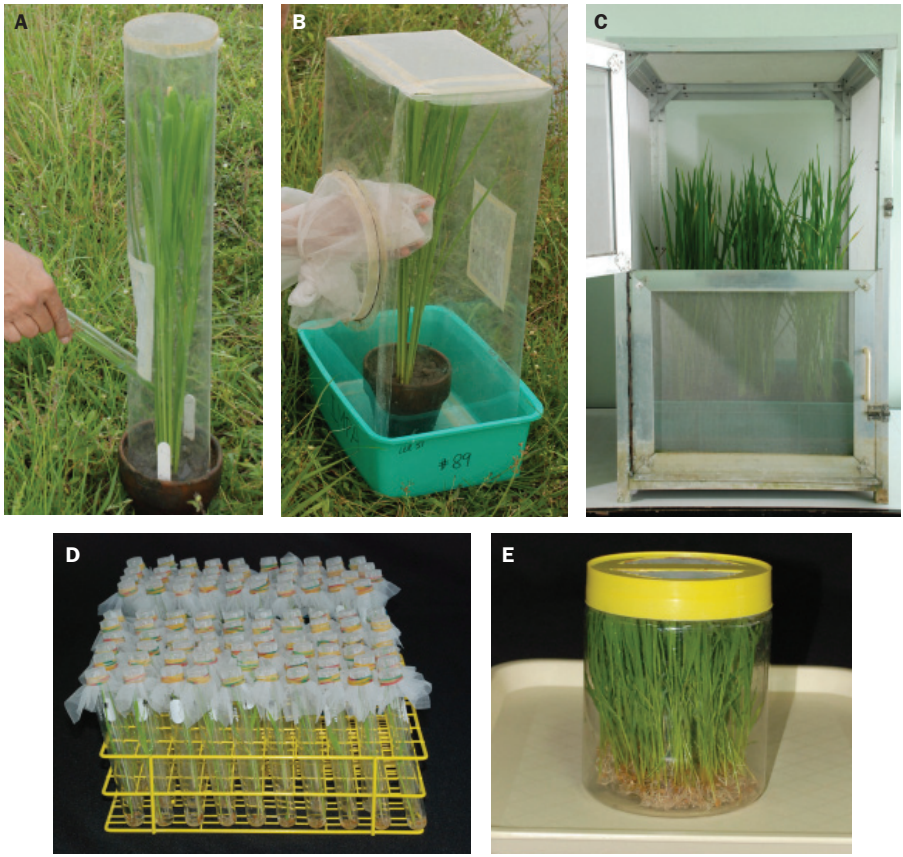


Fig. 4.2. Collection cages: (A) potted plant with circular mylar cage, 61 cm high and 10.5 cm in diameter; (B) rectangular mylar cage, measuring 29 cm × 21.5 cm × 56.5 cm, with a potted plant; (C) aluminum rearing cage, measuring 56.5 cm × 56.5 cm × 91.5 cm, with potted plants; (D) test tubes with rice seedlings; (E) box with rice seedlings.

Rearing methods

Rice planthoppers are commonly reared in two ways. Susceptible varieties should be used.

1. Aluminum cages

One way of rearing planthoppers is using aluminum cages (Fig. 4.3). One month before the collection date, 10-day-old seedlings are planted in clay pots 10 cm in diameter. Fifteen days after transplanting, 2 g of ammonium sulfate fertilizer are applied per pot. One week prior to planthopper collection, the potted plants are cleaned and the outer leaf sheaths and/or infested tillers are removed. The potted plants are covered with mylar cages or kept inside the greenhouse to avoid further infestation by other insect pests.

Fig. 4.3. Aluminum rearing/oviposition cage (56.5 cm × 56.5 cm × 91.5 cm) with aluminum wire mesh on three sides, top, and doors for ventilation.
Fig. 4.4. Flexi-glass cage (30 cm × 25 cm × 30 cm) with seedling mat.

In the greenhouse or insectary, the adult males and females (at 1:1 ratio) are transferred into the oviposition (egg-laying) aluminum cages and labeled with the respective collection dates and locations. The oviposition cages are provided with 35-day-old clean potted plants, which are replaced daily to have uniform populations. For insecticide testing, the preparation of standardized insects is discussed later.

Adult hoppers are removed from the oviposition cage and the nymphs are allowed to emerge. Each rearing cage can accommodate six to eight potted plants that can sustain 600–800 hoppers. The standard test insects (1- to 2-day-old female adults) are collected from these daily rearing cages. The plants are replaced every 2 days (or as needed) and transferred to new rearing cages (labeled with egg collection dates and locations).

2. Flexi-glass cages

Another rearing method to mass-rear planthoppers in the insectary is the use of seedlings in a transparent flexi-glass cage (Fig. 4.4). The three sides of the cage are provided with fine-mesh nylon cloth for ventilation. Insects are reared on rice seedling mats (measuring about 22 cm x 28 cm) grown in nutrient solution, adapted from Yoshida et al (1976). Approximately 12 g of seeds can be sown per seedling mat.

The procedures for the preparation of a seedling mat are as follows:

1. Select clean and healthy seeds of any susceptible variety.
2. To minimize fungal growth, soak seeds in hot water (70 °C) for 10 minutes.

3. After the hot water has been drained, soak seeds for another 2–3 days in a glass container and cover them with a paper towel.
4. After soaking, thoroughly wash the germinated seeds (3 to 4 times) with filtered drinking water to avoid any further contamination.
5. Then, line the flexi-glass trays with two layers of moistened gauze to keep the seeds in place.
6. Place a flexi-glass guide on top of the gauze and sow the seeds in rows.
7. Remove the guide and add enough rice nutrient solution to cover the seeds.
8. Cover the prepared seedling mat with flexi-glass to protect the seeds from infestation by other pests.
9. Water the seedling mats daily or as needed with filtered drinking water to maintain enough moisture and keep the seedling mats wet.
10. Add nutrient solution again after 3–4 days or when the seedlings are yellowish in color.

When the seedlings have grown (about 5–7 days after sowing) and the roots are entangled in the gauze, the mats can be transferred into a rearing cage. Fifty adult insects are introduced for oviposition and removed after 1 day. The cage can be inverted and another seedling mat put into the cage as nymphs emerge. A seedling mat can accommodate about 1,000 late-instar nymphs.

Instead of gauze, peat moss can be used for seedling mats. About 20 g of seed for one seedling mat are pregerminated for 3–4 days and sown on moistened peat moss on flexi-glass trays. The mats (Fig. 4.5) are covered with rectangular flexi-glass and are also watered as needed to keep them wet.

One week after seeding, the mats can be transferred into a rearing cage. Adult insects (100–200 pairs) are introduced for oviposition and removed after 1–2 days. The nymphs are allowed to emerge and the seedling mat is replaced weekly until the planthoppers become adults.

Likewise, the rearing cages are placed in an insectary with a controlled temperature of 27 $\pm$ 2 °C and 12 hours of light.

A schematic diagram for collecting and rearing planthoppers is shown in Figure 4.6.

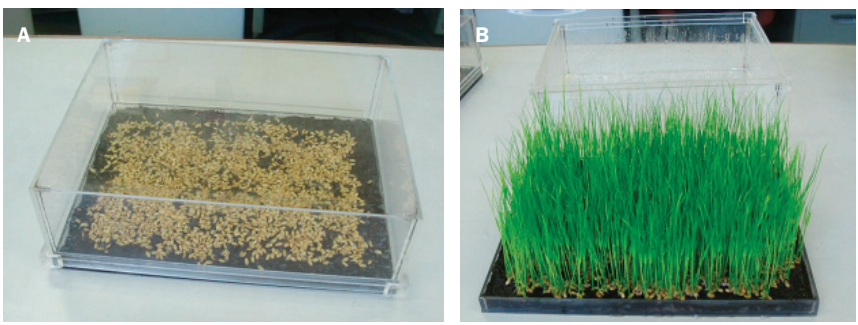


Fig. 4.5. A seedling mat (A) with germinated seeds and covered with rectangular flexi-glass; (B) a 1-week-old seedling mat.

Preparation of standardized test insects

Insecticide bioassays in the laboratory need to have consistent and accurate results. This requires standardization of the test insects to be used for each treatment. Age, sex, and physiological condition of the insects affect their susceptibility to insecticides. After field collection, bioassays can be done using planthoppers from the second generation up to the fifth generation.

Newly emerged adult insects are generally more susceptible. Thus, 1-day-old to 2-day-old adults should be used in insecticide bioassays. To have approximately the same insect age, adults of the same sizes are collected from daily oviposition cages.

Either brachypterous or macropterous adult female planthoppers can be used for insecticide treatments but they should not be mixed in one set of treatments.

In addition to standardization, the preparation and rearing of planthoppers and plant materials to be used for the bioassays can be planned based on the life cycle to

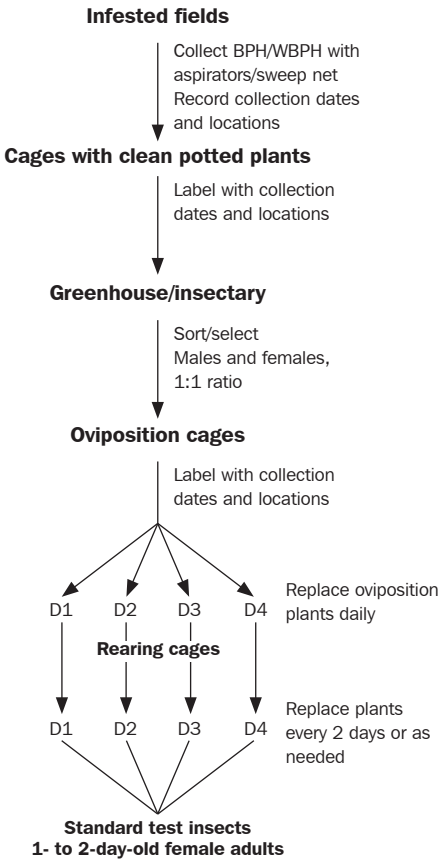


Fig. 4.6. Schematic diagram of collecting and rearing planthoppers for insecticide bioassays.

synchronize their availability. The example schematic diagram for BPH (Fig. 4.7) can be used as a guide to when to plant the needed seedlings to coincide with the peak of the planthopper population needed for the bioassays.

When rearing planthoppers using a seedling box, the sowing of seedling mats starts from the beginning of the egg caging. The sowing interval could be daily, every other day, or weekly depending on the size of the planthopper populations to be maintained.

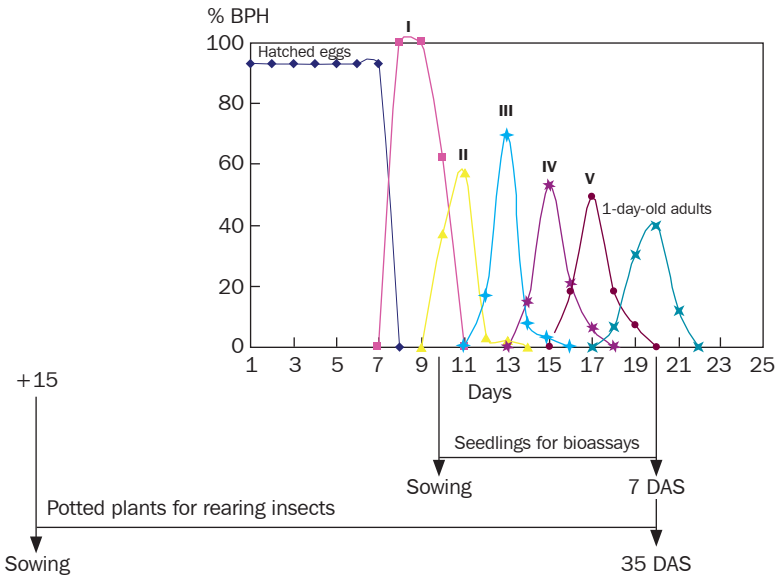


Fig. 4.7. Schematic diagram of BPH life cycle in a controlled room (26 °C) and the preparation of plant materials for the BPH cultures and bioassays.

CHAPTER 5:

Preparation of test solutions and estimating the median lethal dose (LD_{50})

The median lethal dose (LD_{50}) of insecticides is an accurate assessment of the comparative toxicity of the insecticides. The lower the insecticide estimated LD_{50} value, the higher the toxicity or potency of the insecticide. This value also quantifies the tolerance of an insect population to an insecticide treatment. In order to accurately estimate and compare toxicities, there is a need to ensure that the insecticide active ingredients are diluted in a standardized manner.

Preparation of stock solutions

Technical-grade (95–99% pure) insecticides are used for laboratory tests. The active ingredient (a.i.) of the insecticides varies so a 100% stock solution (SS) is prepared using the correction factor (CF) as below:

$$CF = 100\%/\%\ \text{a.i. of the insecticides}$$

For a technical insecticide with 99.5% a.i., CF = 100%/99.5% = 1.005.

Given the CF, the weight of the technical insecticide needed can be computed and the desired volume and concentration can be prepared using the formula

$$\text{Concentration of insecticides} \times \text{volume} \times CF$$

To prepare 2.5 mL of 10,000 µg/mL SS, the weight of insecticide needed will be 10,000 µg/mL × 2.5 mL × 1.005 = 25,125 µg = 25.125 mg = 0.025 g.

First, 0.025 g of technical-grade insecticide is weighed in a 6-mL screw cap vial using an analytical weighing balance (Fig. 5.1). Some 2.5 mL of technical-grade acetone is added as a solvent to obtain the 100% stock solution.

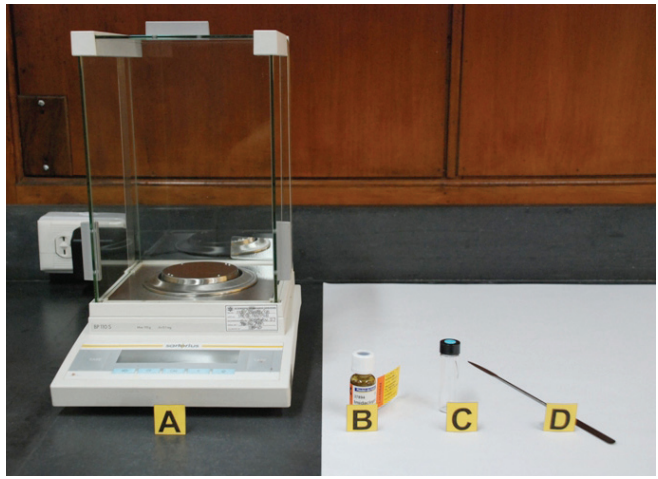


Fig. 5.1. Materials for the preparation of stock solution: (A) analytical weighing balance, (B) technical-grade insecticide, (C) 6-mL screw cap vial, (D) spatula.

Preparation of insecticide concentrations for tests

The insecticide concentrations (at least 5) with a range of 15–85% insect mortality based on a preliminary test are prepared from the stock solution (SS). Serial dilution starts from the highest to the lowest concentration. The materials needed are shown in Figure 5.2.

From the SS, serial dilutions are prepared using the equation $C_1V_1 = C_2V_2$, where C_1 = initial concentration, V_1 = initial volume, C_2 = final concentration, and V_2 = final volume.

To prepare 2 mL of 5,000 µg/mL from 10,000 µg/mL SS, the volume needed using the formula above will be (10,000 µg/mL) (x) = (5,000 µg/mL) (2 mL) = 10,000x = 10,000, where x = 1 mL SS + 1 mL acetone.

Serial dilution is continued using the above equation or a 1:1 dilution for the next 10–12 concentrations is done consecutively from the highest to lowest concentration. The cap of the vial is secured with parafilm to minimize evaporation. The prepared insecticide dilutions are stored in a refrigerator (4 °C) or freezer (preferably –20 °C). After preparation of an insecticide, the pipettor tips are replaced and disposed of properly.

Preparation of recovery cages with seedlings

Seven-day-old rice seedlings (at least 15) of any local susceptible variety can be used for the recovery cages. The roots of the seedlings are wrapped in a half paper towel folded into three and placed in a container with enough water to avoid drying of the seedlings. Before insecticide treatment, the prepared seedlings are placed inside clear tumbler cages. The recovery cages are labeled with the insecticide treatment, doses, and replications (Fig. 5.3).

Fig. 5.2. Materials for preparation of serial dilutions: (A) stock solution, (B) 6-mL screw cap vials with label, (C) technical-grade acetone, (D) pipettor tips, (E) pipettor, (F) parafilm strips, (G) disposable nitrile gloves, (H) disposable mask.

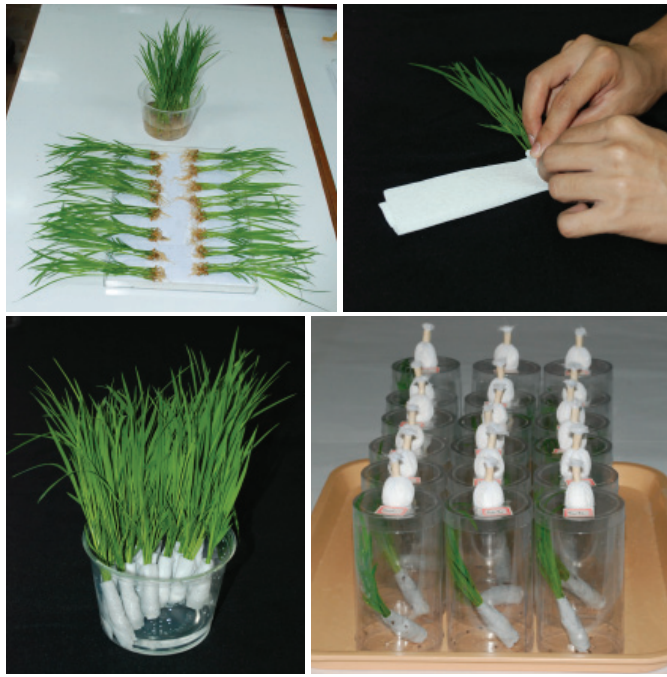


Fig. 5.3. Recovery cage preparation: (A) 7- to 10-day-old rice seedlings of any local susceptible variety, (B) wrapping seedling roots with paper towel, (C) 15 rice seedlings wrapped with paper towel and soaked in water, (D) clear tumbler cages (12.5 cm tall, 7 m in diam.) with seedlings and cotton stopper.

Topical application

The final treatment also used at least five concentrations and a minimum of three replications with 20 insects per replication.

For planthoppers reared in the greenhouse, the daily rearing cages with 5th-instar nymphs can be transferred to the testing room 2 days before insecticide treatment. This is done to acclimatize the planthoppers to be used for the bioassays.

The treatment starts with all the control insects treated with analytical reagent acetone, followed by the insecticide treatment from the lowest to the highest concentration.

Prior to treatment, the planthoppers, 1- to 2-day-old female adults (BPH or WBPH), are collected from the culture cages using an aspirator. They are confined into a vial with a wire-mesh screen. Ten insects are collected per vial and anaesthesized with carbon dioxide (CO_2) for 10–30 seconds to facilitate handling during treatment (Fig. 5.4).

The anaesthetized insects are transferred on a watchglass wrapped with gauze secured by a rubber band. Insecticide is applied topically with a Hamilton Repeating Dispenser plus a 10-µL microsyringe (Fig. 5.5). Some 0.2 µL of the insecticide is applied on the thoracic region of each test insect.

Fig. 5.4. Preparation of test insects for topical application: (A) collection of 10 female adult planthoppers (1 to 2 days old) to be placed in a vial with a wire-mesh screen cap; (B) anaesthetization with CO_2.

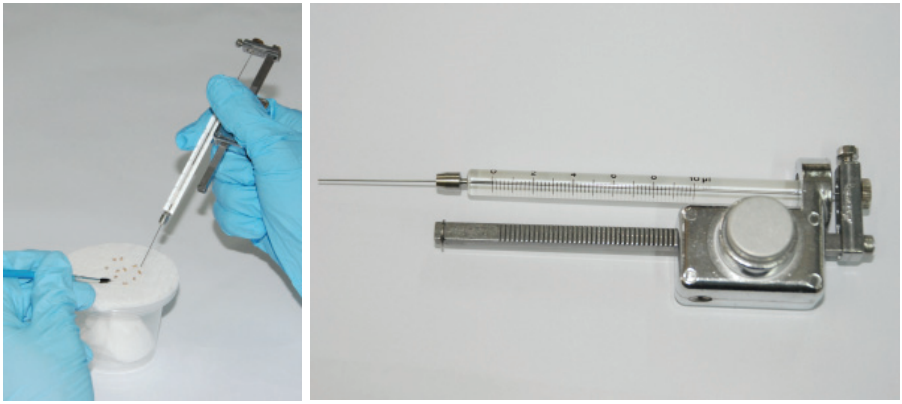


Fig. 5.5. Topical application: (A) anaesthetized planthoppers on watchglass wrapped with gauze; (B) Hamilton Repeating Dispenser and microsyringe.

The treated insects are transferred in clear tumbler cages through a funnel with the aid of a small camel-hair brush to minimize mechanical damage (Fig. 5.6). The cages with treated insects are placed in a controlled room with a temperature range of 25 to 30 °C and 12 hours of light.

After an insecticide treatment, either the gauze or the whole watchglass covered with gauze is replaced to avoid contamination of new batches of test insects with the previous insecticide.

Fig. 5.6. Treated planthoppers are transferred into clear tumbler cages through a funnel.

Twenty-four hours after treatment, insect mortality is recorded. A convenient way is to use Excel to create a data sheet as illustrated in Appendix B. Moribund insects are considered dead. The mortality count is continued up to 48 hours after treatment in some insecticide groups such as insect growth regulators.

From these data, LD_{50} values are estimated using the PoloPlus© probit program (to be described in Chapter 6) and will be recorded in ng/g body weight of the insect.

CHAPTER 6:

Analyzing quantal response data with PoloPlus©

In Chapter 3, we discussed probit analysis for analyzing dose-quantal response data. The standard computation used involves many steps. Today, various software is available to perform the computation.

PoloPlus© (LeOra Software 2002) is a user-friendly software developed by LeOra Software to do computations described in Finney's probit analysis (Finney 1971). To enhance the program's use in toxicological analyses, PoloPlus© has several useful features:

1. It provides estimates of median lethal dose of specified mortality levels (such as LD_{50}, LD_{90}, and LD_{95}) that can be used for statistical comparison of each preparation with a standard.
2. It calculates standardized residuals and maps out a fitted response curve for each preparation.
3. It presents the residuals in plots for the identification of sources of lack-of-fit to the probit or logit model.
4. It tests equality and parallelism.
5. It computes relative potencies and fiducial limits of two or more insecticides.

Details of the software, including other features, are further explained in the book by Robertson et al (2007).

Installation

PoloPlus© is usable in a microcomputer with Windows 95 and above. The software comes in a CD packaged with PoloEncore©, PoloPlus©, PoloDose©, and PoloMix©. This book focuses on PoloPlus©. The installation procedure is as follows:

1. Insert the CD in the drive and click My Computer.
2. Click the PoloPlus file to open the folder.
3. Choose SetupPoloPlus.exe.
4. Select No-Questions-Asked Installation.
5. Click Thanks to end the installation.

Data format for PoloPlus©

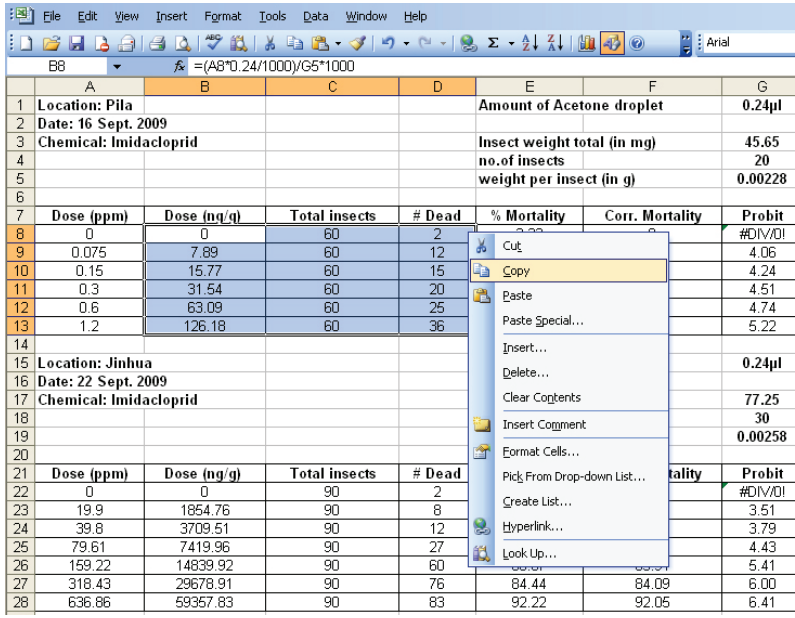
In Chapter 5, we discussed the use of Excel to record quantal response data. The dose can be expressed in ppm but for the analyses dose is coverted to nanogram/gram insect (ng/g) using the formula below:

Dose (ng/g) = [(Dose (ppm)*amt. applied (μL)/1,000)/wt. of insect (g)]*1,000

By using ng/g in the dose variable, a negative logarithm in the independent variable can be avoided.

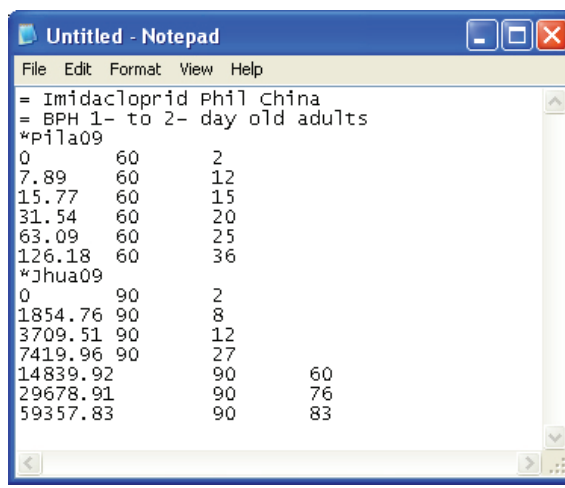
Moreover, PoloPlus© reads data from a space-delimited text file. To convert data recorded in Excel to PoloPlus© data, the following procedures can be used:

1. First, open the Excel data file and highlight the values of Dose (ng/g), Total insects, and # Dead. Either click on the file menu or right-click on the mouse to copy the data file.

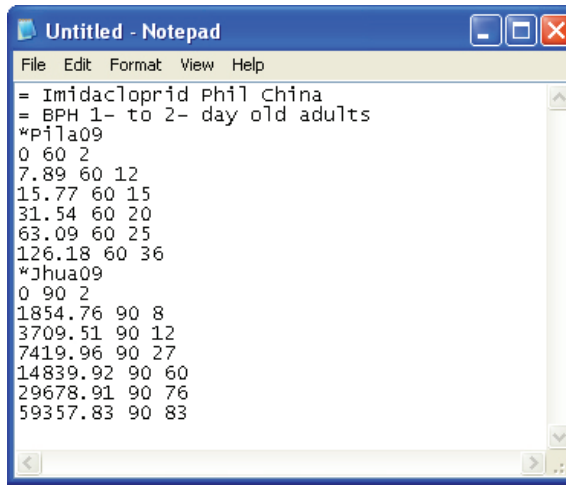


	A	B	C	D	E	F	G
1	**Location: Pila**				**Amount of Acetone droplet**		**0.24µl**
2	**Date: 16 Sept. 2009**						
3	**Chemical: Imidacloprid**				**Insect weight total (in mg)**		**45.65**
4					**no.of insects**		**20**
5					**weight per insect (in g)**		**0.00228**
6							
7	**Dose (ppm)**	**Dose (ng/g)**	**Total insects**	**# Dead**	**% Mortality**	**Corr. Mortality**	**Probit**
8	0	0	60	2			#DIV/0!
9	0.075	7.89	60	12			4.06
10	0.15	15.77	60	15			4.24
11	0.3	31.54	60	20			4.51
12	0.6	63.09	60	25			4.74
13	1.2	126.18	60	36			5.22
14							
15	**Location: Jinhua**						**0.24µl**
16	**Date: 22 Sept. 2009**						
17	**Chemical: Imidacloprid**						**77.25**
18							**30**
19							**0.00258**
20							
21	**Dose (ppm)**	**Dose (ng/g)**	**Total insects**	**# Dead**		**tality**	**Probit**
22	0	0	90	2			#DIV/0!
23	19.9	1854.76	90	8			3.51
24	39.8	3709.51	90	12			3.79
25	79.61	7419.96	90	27			4.43
26	159.22	14839.92	90	60			5.41
27	318.43	29678.91	90	76	84.44	84.09	6.00
28	636.86	59357.83	90	83	92.22	92.05	6.41

2. One option is to copy the data file in Microsoft Notepad. On the first two lines, enter the title or comment designated by an equal (=) sign. On the third line, enter an 8-character preparation line (insecticides or locations) designated by an asterisk (*). Then, paste the first data file (from step 1) on the fourth line and the next data file on the succeeding lines. The text file will appear as below.

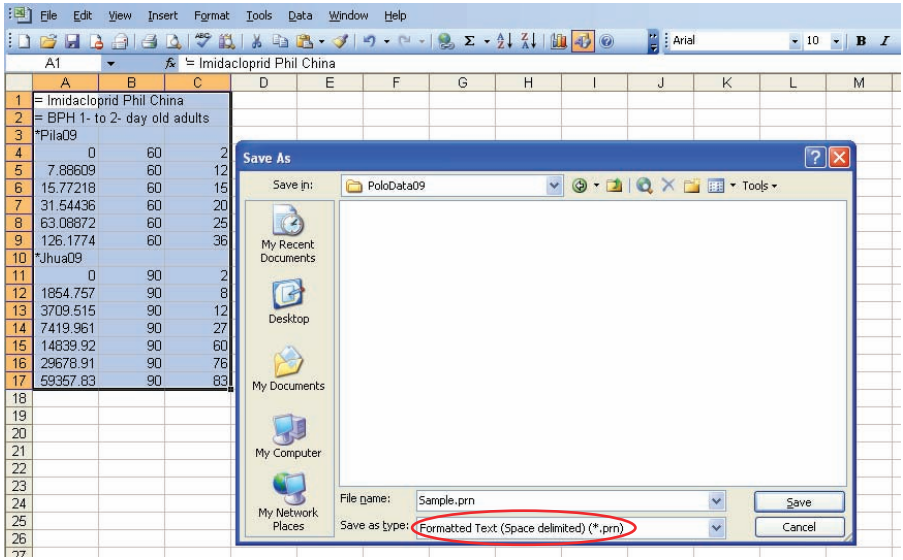


3. There is a need to further format this tab-delimited file to a space-delimited text file. To do this, highlight the space in between the Dose (ng/g) and the Total insects and then right-click on the mouse and select Copy. Next, go to the Edit menu and select Replace. In the "Find what" box, select Paste. In the "Replace with" box, press the Spacebar button once, then click Replace All.
4. The tabs between each data field now appear closer. Save the file. The data format below can be read by PoloPlus©.

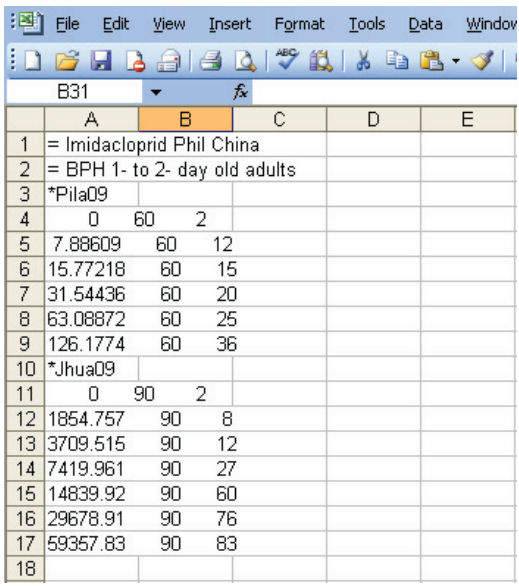


Another option is to save the data as a text file in Microsoft Excel and the steps are given below:

1. Follow step 1 and step 2 above but, instead of using a Notepad, use a new Excel worksheet to file the data.
2. The Excel data file may have formulas, so select Paste Special to copy the file (from step 1) and choose Values, then click OK.
3. After the data files have been copied, highlight the data file, click the File menu, and select Save as. Choose where to file the data in Save in, enter the File name, and, for Save as type, choose Formatted Text (Space delimited).



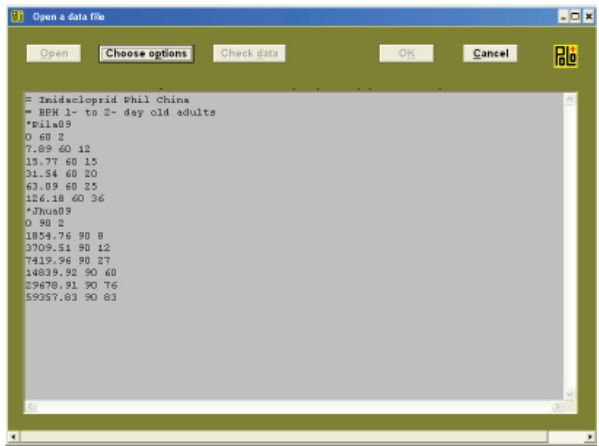
4. Save the file. Choose OK and Yes for the messages that will appear on the screen. The data can now be read by PoloPlus©.



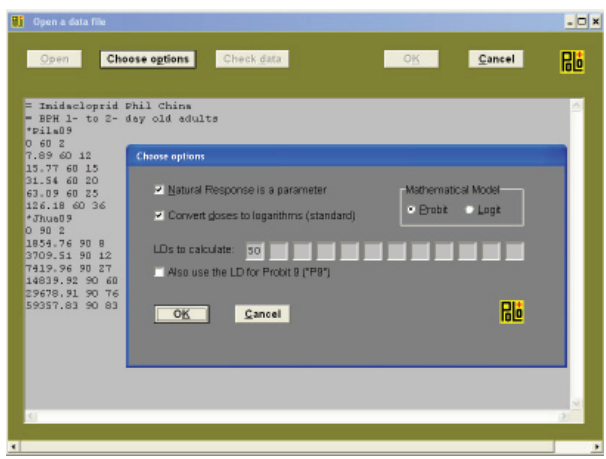
Using PoloPlus©

The quantal response data that have been saved to the specified text format can now be analyzed using the PoloPlus© program, with the following steps.

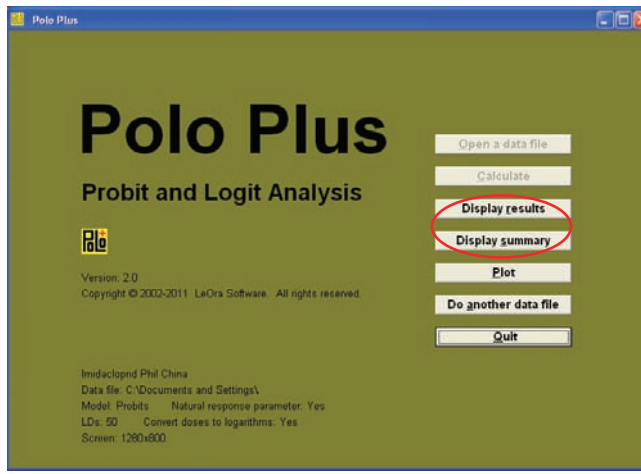
1. On the opening screen, click Open a data file.
2. Choose Open and select the data file (saved to text format) to be analyzed.
3. When the correct data file appears on the screen, click Choose options.



4. On the Choose options screen, select the Probit for the Mathematical Model and Natural Response is a parameter (unless the controls are not included). Then, enter the desired LDs (lethal doses) needed to be calculated and click OK.



5. Next, select Check data to verify whether there are no errors. If the message "No errors were found in the data file" appears, click OK. On the other hand, if the message indicates that there are errors in the data set, go to the file and follow the instructions provided in the message. Rerun PoloPlus© using the corrected data file.
6. Again click OK on the menu and the opening screen will reappear. Click Calculate and the word "Crunching" appears to indicate processing of the data.
7. After the data analyses, the Display results and Display summary options can be chosen to view the results and summary, respectively. In addition, options to Print or Save as are also available to allow storing the outputs in either Microsoft Word or PowerPoint.
8. The program also has Plot output of corresponding probit lines, but other options to plot the data using PowerPoint will be discussed later.



Interpretation of results

Detailed discussion on the interpretation of the results displayed by PoloPlus© is found in Robertson et al (2007, p 39-45). Here, we discuss the interpretation of the specific data used (Fig. 6.1).

The parameters chosen in the example are probit as the model, the natural response to be estimated, the doses to be converted to logarithms, and the LD_{50} value to be estimated (lines 10–12). The header of the data sets, Imidacloprid Phil China, is on line 16. In the two data sets (Pila09 and Jhua09), the intercepts and the slopes are to be estimated from the respective data (line 19 and line 50). Likewise, the natural response will be estimated (Pila09, line 20, and Jhua09, line 51) from the data observed in the control. However, for a data set that has no observed control mortality, the statement "not estimating natural response" will be displayed.

= Imidacloprid Phil China
= BPH 1- to 2- day old adults

PoloPlus Version 2.0
Date:
Imidacloprid Phil China
Data file: C:\Documents and Settings
Number of preparations: 2
Number of dose groups: 11
Model: Probit
Natural Response Parameter: yes
Convert doses to logarithms: yes
LDs: 50
~~~~~~

Imidacloprid Phil China

Intercepts and slopes unconstrained. Preparation is (1) Pila09
Estimating natural response

	parameter	standard error	t ratio
Pila09	-1.895	0.351	-5.401
NATURAL	0.034	0.024	1.437
SLOPE	0.961	0.207	4.650

Variance-Covariance matrix

	Pila09	NATURAL	SLOPE
Pila09	0.123170	-0.318132E-02	-0.698269E-01
NATURAL	-0.318132E-02	0.575595E-03	0.126780E-02
SLOPE	-0.698269E-01	0.126780E-02	0.426886E-01

Chi-squared goodness of fit test

prep	dose	n	r	expected	residual	probab	std resid
Pila09	7.890	60.	12.	10.80	1.204	0.180	0.405
	15.770	60.	15.	15.29	-0.291	0.255	-0.086
	31.540	60.	20.	20.86	-0.861	0.348	-0.233
	63.090	60.	25.	27.21	-2.211	0.454	-0.573
	126.180	60.	36.	33.87	2.130	0.565	0.555
NATURAL		60.	2.	2.07	-0.068	0.034	-0.048

chi-square: 0.864 degrees of freedom: 3 heterogeneity: 0.288

Effective Doses

		dose	limits	0.90	0.95	0.99
LD50	Pila09	93.958	lower	63.203	59.100	51.931
			upper	174.421	208.932	338.095

Intercepts and slopes unconstrained. Preparation is (2) Jhua09
Estimating natural response

	parameter	standard error	t ratio
Jhua09	-9.066	0.846	-10.715

Fig. 6.1. Output results.
~~~~~~

```
55   NATURAL      0.027        0.017          1.573
56   SLOPE        2.231        0.201         11.103
57
58   Variance-Covariance matrix
59                    Jhua09          NATURAL         SLOPE
60    Jhua09        0.715907       -0.764468E-02   -0.169269
61    NATURAL      -0.764468E-02    0.300705E-03    0.170227E-02
62    SLOPE        -0.169269        0.170227E-02    0.403634E-01
63
64   Chi-squared goodness of fit test
65   prep        dose          n     r     expected    residual   probab  std resid
66   Jhua09    1854.760      90.    8.      5.77        2.228    0.064    0.958
67              3709.510      90.   12.     14.25       -2.253    0.158   -0.650
68              7419.960      90.   27.     31.58       -4.579    0.351   -1.011
69             14839.920      90.   60.     54.49        5.506    0.605    1.187
70             29678.910      90.   76.     74.13        1.872    0.824    0.518
71             59357.830      90.   83.     85.02       -2.024    0.945   -0.934
72   NATURAL                  90.    2.      2.46       -0.455    0.027   -0.294
73
74   chi-square: 5.0010    degrees of freedom: 4    heterogeneity: 1.2502
75
76   Effective Doses
77                         dose     limits    0.90      0.95      0.99
78   LD50  Jhua09        11596.    lower    9291.0    8588.0    6489.9
79                                 upper    14063.    14887.    17602.
80
81   ---------------------------------------------------------------------
82
83   HYPOTHESIS OF EQUALITY (equal slopes, equal intercepts): REJECTED (P<0.05)
84     (chi-square: 216.,  degrees of freedom: 2,  tail probability: 0.000)
85
86   ---------------------------------------------------------------------
87
88   HYPOTHESIS OF PARALLELISM (equal slopes): REJECTED (P<0.05)
89     (chi-square: 22.37,  degrees of freedom: 1,  tail probability: 0.000)
90
91   ---------------------------------------------------------------------
92
93
94
95   Lethal dose ratio (LD50)
96                ratio    limits    0.95
97   Jhua09       0.008    lower     0.004
98                         upper     0.015
99
```

Fig. 6.1. Output results. (Cont.)

The values for the regression line, −1.895 (intercept), 0.034 (natural response), and 0.961 (slope), are on lines 23–25 (Pila09), with their respective columns for standard error and t-ratio. The same parameters as above are estimated for Jhua09 data sets (lines 54–56).

In case the t-ratio of any slope is <1.96, this may indicate insignificant regression, and the treatment has no effect and further analysis of the data is not necessary.

The values of the variance-covariance matrix of Pila09 (lines 27–31) are estimates of variance of intercept (0.123), variance of slope (0.442688), and covariance of intercept and slopes (0.698). These values are the basis of 95% confidence intervals for ratios and of the significant differences between preparations.

The chi-square test for goodness of fit for Pila09 (lines 33–40) and Jhua09 (lines 64–72) shows the residuals (the difference between the observed and expected values) and the standardized residuals, which could be plotted to examine the goodness of fit. Plotting of standardized residuals against the predicted values that lie within the horizontal line around zero (95% between −2 and 2) represents a good fit (Fig. 6.2); otherwise, plots could represent a lack of fit.

In line 42, Pila09 chi-square (0.864) divided by degrees of freedom (3) gives the heterogeneity (0.2880). A heterogeneity of <1.0 indicates that the Pila09 data fit the model. The parameters for Jhua09 are listed on line 74. A heterogeneity of >1.0 (Fig. 6.3) may indicate that the data do not fit the model and plots of data with heterogeneity of 2.97 (as in Chainat, Thailand) may reveal outliers that cause the lack of fit (Fig. 6.4).

For Pila09 and Jhua09 populations, the estimated LD_{50} value and its upper and lower limits (at 90%, 95%, and 99%) are listed on lines 44–47 and lines 76–79, respectively.

Another vital aspect of quantal data analysis is the testing of the hypotheses. The hypothesis of equality (lines 83–84) tests the sameness of the slopes and intercepts of the regression lines. If it is rejected, as in the example, the lines are significantly different.

On the other hand, the likelihood ratio (LR) test of parallelism (lines 88–89) compares whether the slopes of the lines are similar. In the example, the hypothesis is

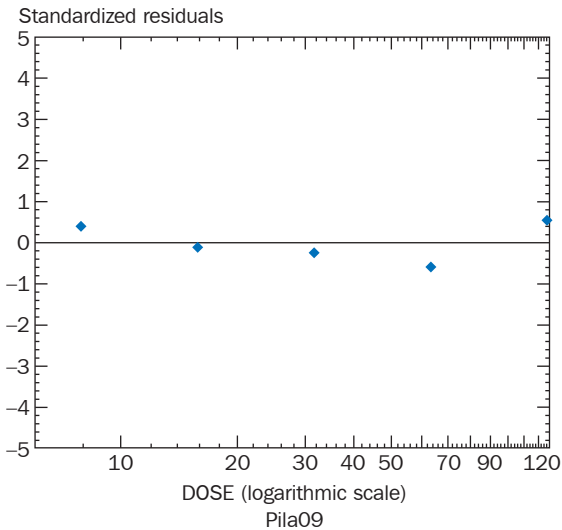


Fig. 6.2. Plot of residuals for Pila09.

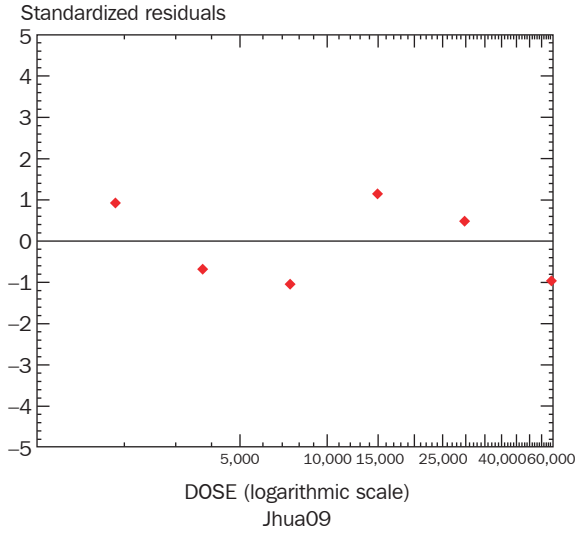


Fig. 6.3. Plot of residuals for Jhua09.

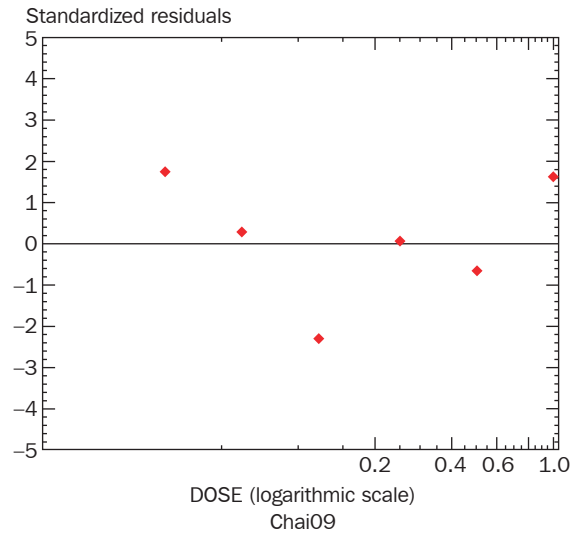


Fig. 6.4. Plot of residuals for Chai09.

rejected; thus, the slopes are not parallel. This may indicate that the relative response of the two populations is not the same.

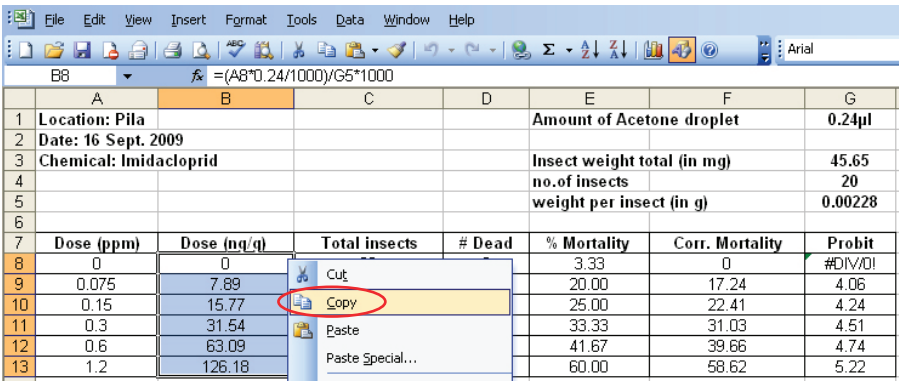
The lethal dose ratio on lines 95–98, with their upper and lower 95% confidence limits, compares the response of the second population in relation to the first population. These ratios can be used to determine the relative toxicity of the insecticide to the populations. In the example, imidacloprid is more toxic to the Pila09 population than to the Jhua09 population.

Plotting probit lines using PowerPoint

The probit lines are plots of the relationship between the doses applied and the corresponding mortality expressed in probits. One way of doing this is to use PowerPoint software and the steps for Microsoft Office 2003 and 2007 are given below.

For Microsoft Office 2003:

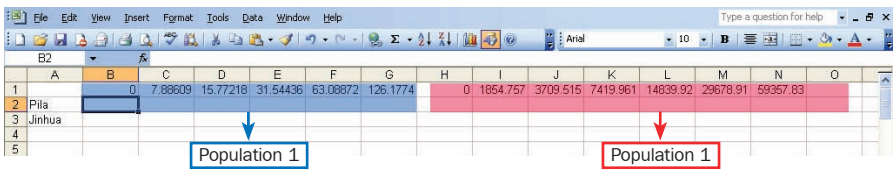
1. Using the quantal response Excel file, highlight and Copy the Dose (ng/g) values.



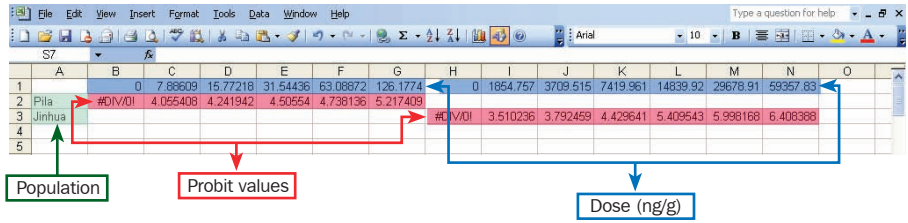
	A	B	C	D	E	F	G
1	Location: Pila				Amount of Acetone droplet		0.24µl
2	Date: 16 Sept. 2009						
3	Chemical: Imidacloprid				Insect weight total (in mg)		45.65
4					no.of insects		20
5					weight per insect (in g)		0.00228
6							
7	Dose (ppm)	Dose (ng/g)	Total insects	# Dead	% Mortality	Corr. Mortality	Probit
8	0	0			3.33	0	#DIV/0!
9	0.075	7.89			20.00	17.24	4.06
10	0.15	15.77			25.00	22.41	4.24
11	0.3	31.54			33.33	31.03	4.51
12	0.6	63.09			41.67	39.66	4.74
13	1.2	126.18			60.00	58.62	5.22

2. On a new Excel worksheet, the names of selected populations are entered consecutively on the second row of the first column. *Note: The first blank cell (A1) must be kept blank.*
3. Then, select cell B1 and go to the Edit menu. Choose Paste Special and select Values. Next, check Transpose and click OK.

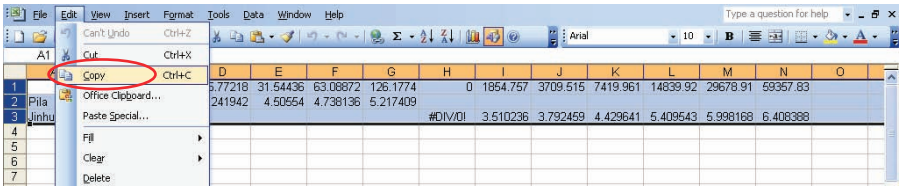
   Note: Row 1 contains the Dose (ng/g), *which is the X-value.*



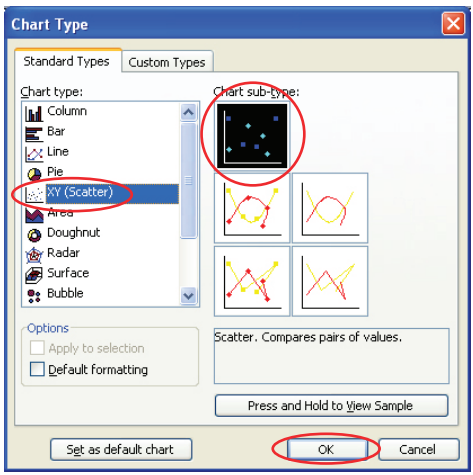
4. For the Probit values, follow step 1 to step 3 above.
   Note: The Probit values of the respective populations should vertically match with the Dose (ng/g) *values.*



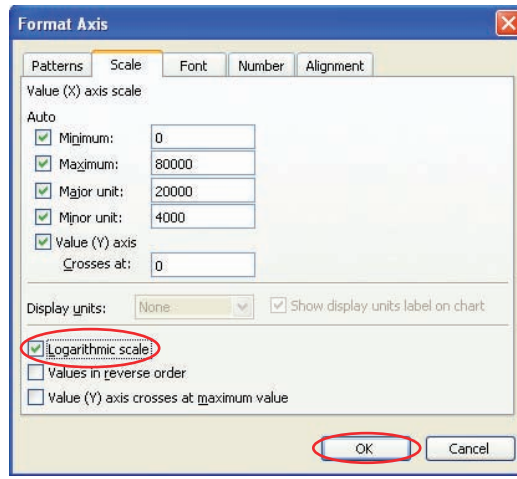
5. These are the data sets needed to plot the probit lines in PowerPoint. In the Excel file, highlight the data sets, go to the Edit menu, and click Copy.



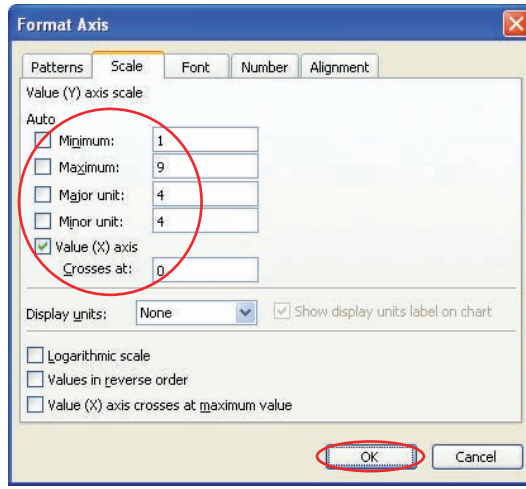
6. Then, open PowerPoint and, on the Insert menu, choose Chart and a sample bar chart will appear.
7. On the Chart menu, click Chart type. On the Standard Types option, choose XY (Scatter) chart and the first box of chart subtype, and then click OK.



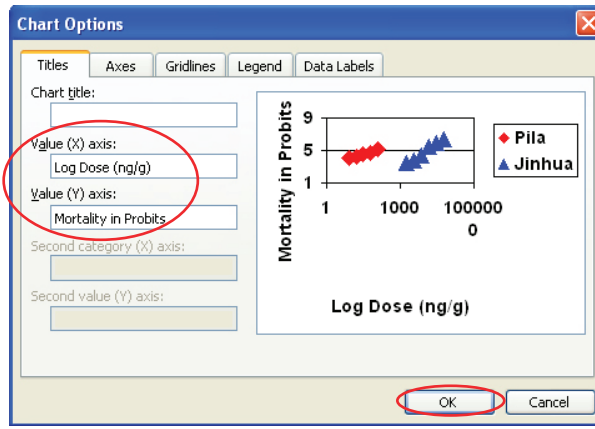
8. Then, Paste the Excel data sets on the PowerPoint data sheet.
9. Click the chart's x-axis and on the Format menu choose Selected Axis. On the Scale tab, check the Logarithmic scale and then click OK.



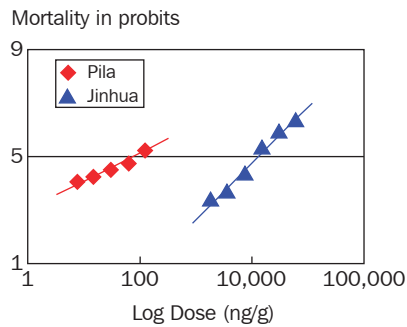
10. Next, select the chart's y-axis and click on the Format Axis. On the Scale tab, make the following adjustments: Minimum = 1, Maximum = 9, Major unit = 4, Minor unit = 4, and then click OK.



11. Go to the Chart menu and select Chart Options. On the Titles tab, type the label for Value (x) axis – Log Dose (ng/g) and for Value (y) axis – Mortality in Probits, then click OK.

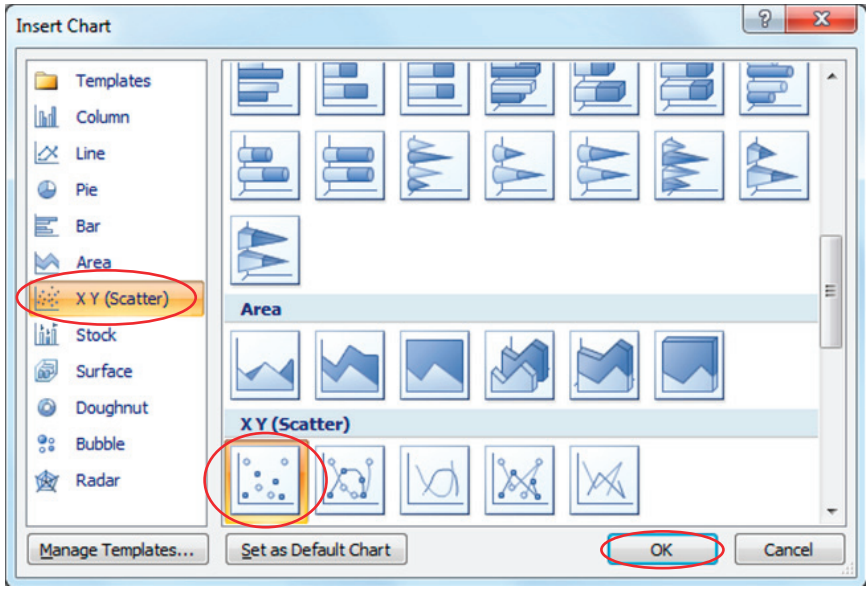


12. Lastly, choose the Line tool and a line to represent a good fit is drawn along the points of the respective data sets. The final graph is shown below.

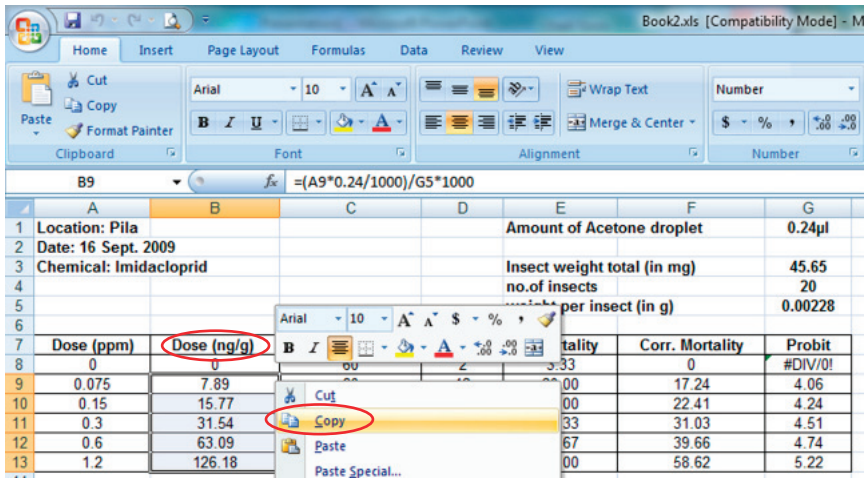


For Microsoft Office 2007:

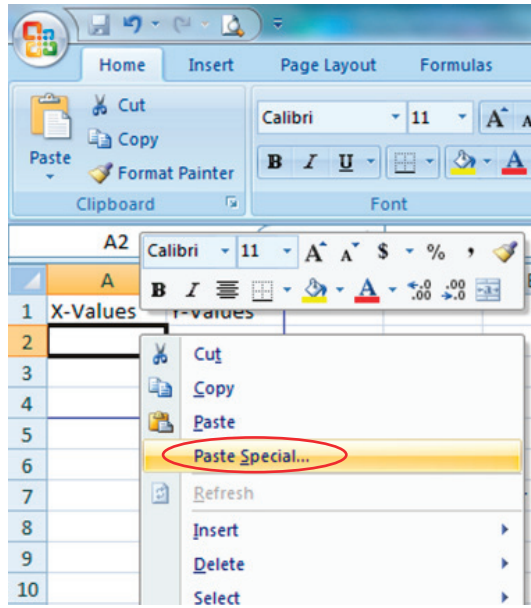
1. Open Microsoft PowerPoint 2007.
2. Insert a chart by choosing the Insert menu and clicking on the Chart icon. Several chart types will appear. Choose X Y (Scatter), click Scatter with only Markers, and then click OK. An Excel worksheet for the X and Y values of the chart will appear.



3. For the first population, open the quantal response Excel file. Highlight and Copy the Dose (ng/g) values, excluding the Zero value.



4. Go to the chart Excel worksheet, right-click cell A2, choose Paste Special, select Values, and click OK.


Home
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Page Layout
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Paste
Clipboard
Calibri
11
Font
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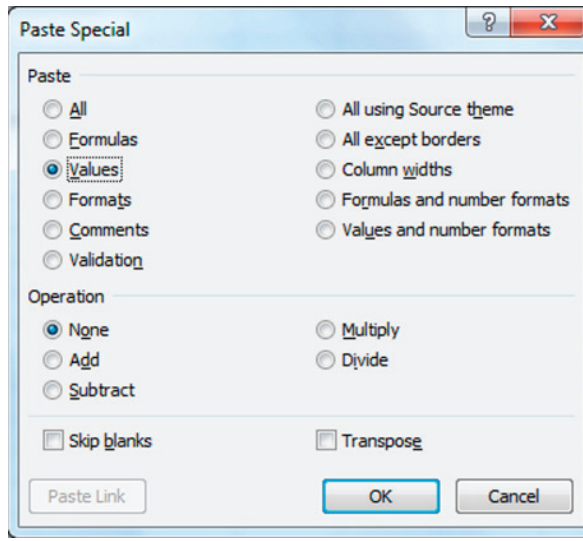
Paste Special
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All
Formulas
Values
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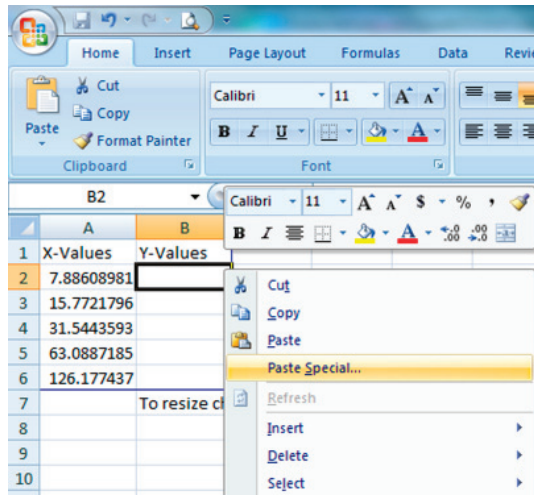
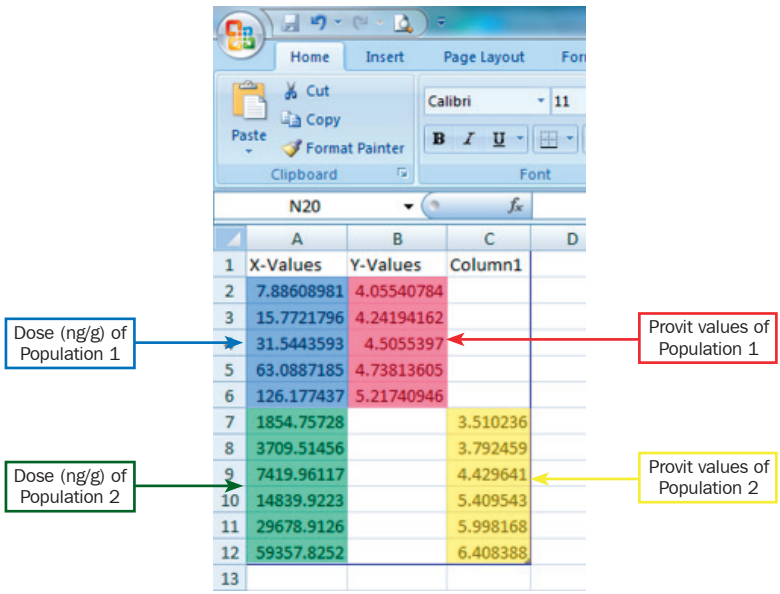
Comments
Validation
All using Source theme
All except borders
Column widths
Formulas and number formats
Values and number formats
Operation
None
Add
Subtract
Multiply
Divide
Skip blanks
Transpose
Paste Link
OK
Cancel

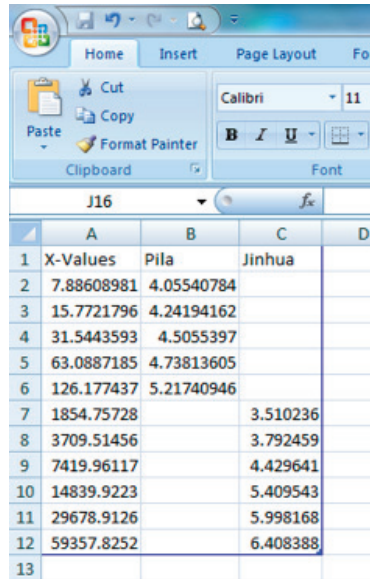
5. Highlight and Copy the Probit values, excluding the probit value for Zero ng/g. Go to the chart Excel worksheet, right-click cell B2, choose Paste Special, select Values, and click OK.



6. For the succeeding populations, the Dose (ng/g) values must be pasted immediately below the Dose (ng/g) values of the previous population. The probit values of the respective populations must be pasted on columns B and so on and should horizontally match their Dose (ng/g) values.

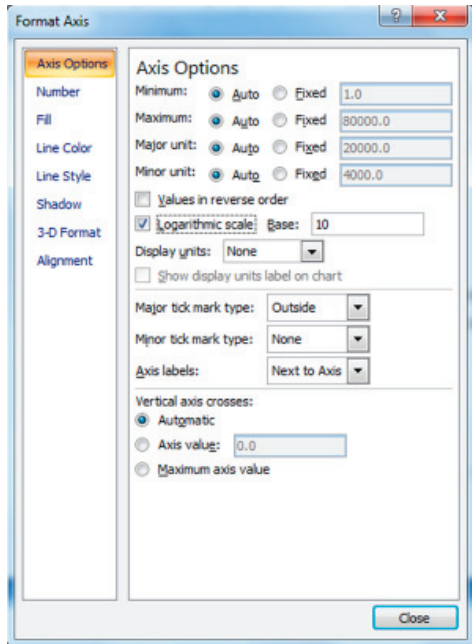


7. Label the populations by changing the first rows for each column starting from column B and so on.

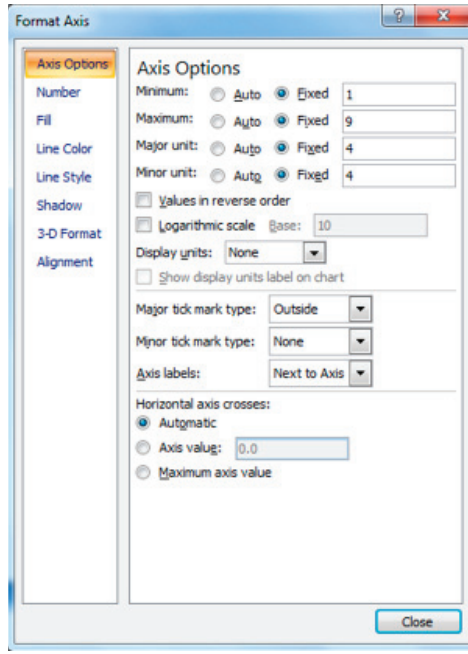


	A	B	C	D
1	X-Values	Pila	Jinhua	
2	7.88608981	4.05540784		
3	15.7721796	4.24194162		
4	31.5443593	4.5055397		
5	63.0887185	4.73813605		
6	126.177437	5.21740946		
7	1854.75728		3.510236	
8	3709.51456		3.792459	
9	7419.96117		4.429641	
10	14839.9223		5.409543	
11	29678.9126		5.998168	
12	59357.8252		6.408388	
13				

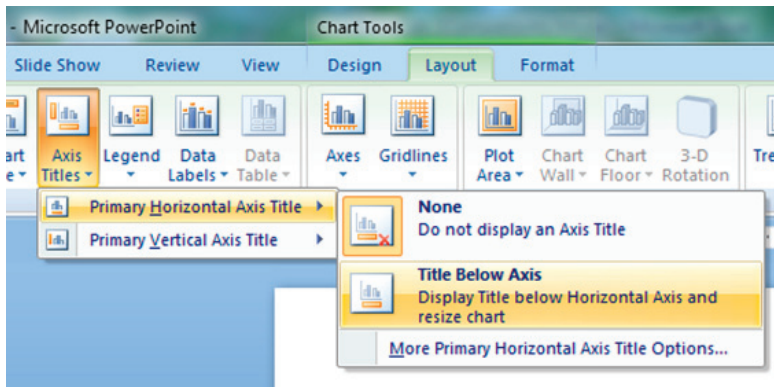
8. Go to the Microsoft PowerPoint chart. Right-click the x-axis and click Format Axis. On the Axis Options, check the Logarithmic scale option, and click Close.



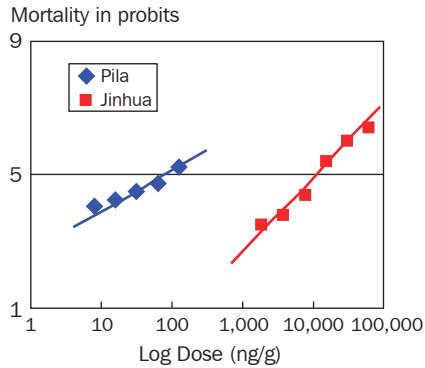
9. Right-click the y-axis, and click Format Axis. On the Axis Options, make the following adjustments: Minimum = 1, Maximum = 9, Major unit = 4, Minor unit = 4, and then click Close.



10. Put in axis titles by selecting each axis and choosing the Axis Titles option on the Layout tab.



11. Choose the Line tool, draw a line, and estimate a good fit along the points of the respective data sets. The final graph is shown below.



CHAPTER 7:

Analyzing joint action of insecticide mixtures with PoloMix©

PoloMix© is another software developed by LeOra Software that uses chi-square (x^2) statistics to test the hypothesis of independent, uncorrelated joint action in a mixture of chemicals. This hypothesis has been defined by Bliss (1935) and based on the assumption that the toxicity of one chemical in a mixture is not correlated to the toxicity of the other chemical.

Installation

The same computer requirements and installation procedures described in Chapter 6 for the PoloPlus© program can be followed except that the SetupPoloMix.exe program is chosen.

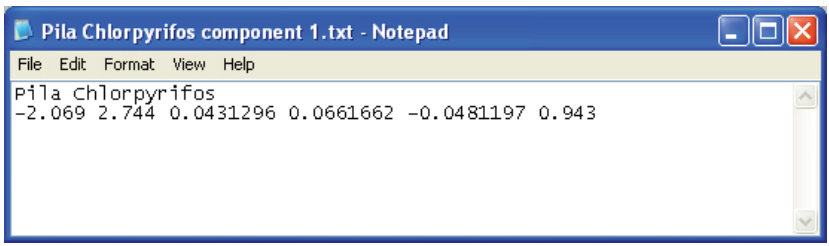
Data files

PoloMix© requires data to be encoded in any word-processing program and saved to a text format. The program uses two data files from the PoloPlus© output of chemical 1 (Fig. 7.1) and chemical 2. The third data file is the dose-response data of the mixture.

The PoloPlus© output of the Chlorpyrifos Pila (chemical 1) parameter estimates is labeled as follows: 1 = intercept, 2 = slope, 3 = variance of intercept, 4 = variance of slope, 5 = covariance of intercept and slope, and 6 = heterogeneity.

Creating data files

1. a. A Microsoft Notepad is used to create an example data file.
   b. The name of chemical 1 (Pila Chlorpyrifos) is entered on the first line for identification. On the second line, the values obtained from PoloPlus© analysis (labeled in Fig. 7.1), the estimated intercept, slope, variance of the intercept, variance of the slope, covariance of the slope and intercept, and the heterogeneity factor, are entered (left to right) consecutively.
   c. Then, on the File menu, the Save as option is selected and a file name is entered to save the data file.



= Chlorpyrifos Pila
= BPH 1- to 2- day old adults

PoloPlus Version 2.0
Date: 2010
Chlorpyrifos Pila
Data file: C:\Documents and Settings\
Number of preparations: 1
Number of dose groups: 5
Model: Probit
Natural Response Parameter: no
Convert doses to logarithms: yes
LDs: 50

~~~~~~

Chlorpyrifos Pila

	parameter	standard error	t ratio
Pila (1)	-2.069	0.208	-9.963
SLOPE (2)	2.744	0.257	10.667

Variance-Covariance matrix

	Pila	SLOPE
Pila (3)	0.431296E-01	-0.481197E-01 (4)
SLOPE (5)	-0.481197E-01	0.661662E-01

Chi-squared goodness of fit test

prep	dose	n	r	expected	residual	probab	std resid
Pila	1.180	60.	3.	1.84	1.163	0.031	0.872
	2.350	60.	8.	8.80	-0.798	0.147	-0.291
	4.720	60.	21.	24.78	-3.777	0.413	-0.990
	9.420	60.	47.	43.61	3.386	0.727	0.981
	18.850	60.	55.	55.42	-0.419	0.924	-0.204

chi-square: 2.830 degrees of freedom: 3 heterogeneity: 0.943 (6)

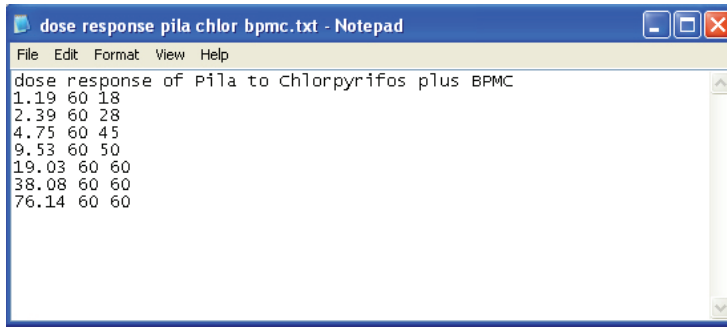
Effective Doses

	dose	limits	0.90	0.95	0.99
LD50 Pila	5.677	lower	5.010	4.890	4.659
		upper	6.451	6.619	6.971

Fig. 7.1. PoloPlus© output (chemical 1).

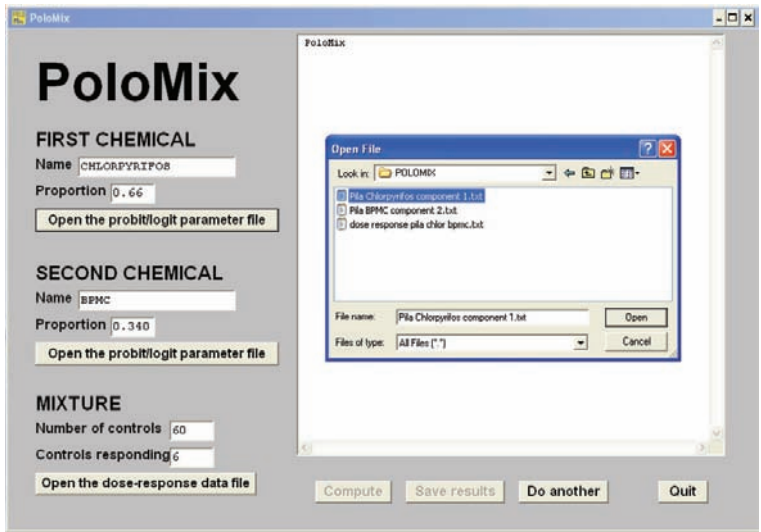
2. The same procedures as above are followed to create another file for chemical 2 (BPMC).
3. Then, a dose-response data file is created for the mixture (Chlorpyrifos plus BPMC).
   a. Likewise, the first line is for identification, followed by the dose-response data.
   b. Each of the data lines has three columns separated by a space (the dose, the number of test subjects, and the number that responded).
~~~~~~

c. Lastly, on the File menu, the Save as option is chosen and a file name is entered.

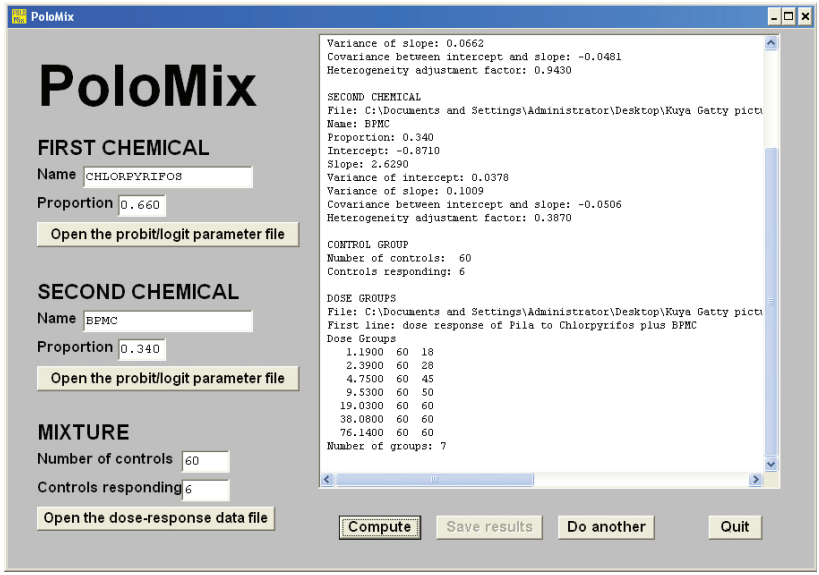


Using the PoloMix© *program*

1. On the opening screen, the names of the first and second chemicals (chlorpyrifos and BPMC) and their corresponding proportions (66 and 34) are entered, respectively. Then, the corresponding parameter files are opened when the Open the probit/logit parameter file is selected for each chemical file.
2. For the mixture, the total number of controls and the number responding are entered and then the Open the dose-response data file is selected.



3. Then, the Compute button is selected. The analysis will appear and the Save results button can be selected to save the analysis.



Program output

The parameter estimates from PoloPlus© probit analysis are listed on lines 5–11 (for chlorpyrifos) and on lines 17–23 (for BPMC) of the PoloMix© sample output (Fig. 7.2). The calculations of the expected mortality and x^2 values of each dose (lines 36–42) are listed in the last two columns of the mixture data. Lastly, the computed x^2 value and degrees of freedom are on line 44. This computed x^2 value can be compared with the tabular x^2 value (Appendix C) to determine whether there is significant departure from the null hypothesis at the corresponding probability level. When the computed x^2 value is less than the tabular x^2 value, the null hypothesis cannot be rejected. On the other hand, the hypothesis of independent joint action is rejected if the computed x^2 value is greater than the tabular x^2 value. In the example, the computed x^2 value of 23.175 (for df = 7 and $P = 0.05$) is greater than the tabular x^2 value; thus, the hypothesis of independent joint action is rejected.

Chemical 1
File: C:\Documents and Settings\Administrator\POLOMIX\Pila Chlorpyrifos component 1.txt

Name: CHLORPYRIFOS
Proportion: 0.660
Intercept: -2.0690
Slope: 2.7440
Variance of intercept: 0.0431
Variance of slope: 0.0662
Covariance between intercept and slope: -0.0481
Heterogeneity adjustment factor: 0.9430

Chemical 2
File: C:\Documents and Settings\ POLOMIX\Pila BPMC component 2.txt

Name: BPMC
Proportion: 0.340
Intercept: -0.8710
Slope: 2.6290
Variance of intercept: 0.0378
Variance of slope: 0.1009
Covariance between intercept and slope: -0.0506
Heterogeneity adjustment factor: 0.3870

Controls
Number of controls: 60
Controls responding: 6

Dose groups
File: C:\Documents and Settings\POLOMIX\dose response pila chlor bpmc.txt

Calculations

Dose	Subjects	Observed Responding	Expected Mortality	Chi-square Mortality	Contribution
1.1900	60	18	0.3000	0.1337	7.9839
2.3900	60	28	0.4667	0.2701	7.5439
4.7500	60	45	0.7500	0.5710	6.0306
9.5300	60	50	0.8333	0.8706	0.6553
19.0300	60	60	1.0000	0.9840	0.9201
38.0800	60	60	1.0000	0.9993	0.0407
76.1400	60	60	1.0000	1.0000	0.0006

Chi-square: 23.175 Degrees of freedom: 7

Fig. 7.2. PoloMix© output.

CHAPTER 8:

Presenting results

The previous chapters provide the methodology that produces the essential information needed for analyzing quantal response data with accuracy. Here we discuss how this information provided can be used for reporting the results. Each probit analysis is described by the LD_{50} or LD_{90} estimates and the related statistics. When toxicities of two populations or two preparations are compared, statistics related to the tests for equality and parallelism are also provided.

In fitting the quantal response data to the regression model, PoloPlus© generates several parameters, which can be presented in a table as shown below.

In the tests for parallelism, the χ^2 value with 1 degree of freedom was 22.37 and the null hypothesis of equal slopes had to be rejected. Similarly, in the test of equality, the χ^2 value was 216 and the null hypothesis of equal slopes and equal intercepts was rejected. From Table 8.1 by examining the confidence limits and standard values in parentheses, it is obvious that the LD_{50} and slopes differed. In this case, the two probit lines are not comparable and the relative potency value is not valid, as the lines need to be parallel for the comparison to be valid (Busvine 1971).

Insect responses to different insecticides can have valid comparisons when the slopes of the regression lines are parallel. Table 8.2 presents the results of brown planthopper populations from three countries obtained in 2009, for which the probit lines are parallel. In this case, the relative potency values are valid.

An additional way to present toxicological data visually is by using probit plots. Data from Table 8.1 are shown in Chapter 6 and Figure 8.1 shows the probit lines from Table 8.2.

Examples of how toxicity data are presented in the literature can be found in Robertson et al (2007), Ishaaya et al (2003), and Matsumura et al (2008).

Table 8.1. Toxicities and relative potencies of imidacloprid to brown planthopper from the Philippines and China.

Location	LD_{50} in μg/g insect (95% confidence limits)	Slope (SE)	Heterogeneity	Relative potency
Pila, Philippines	0.094 (0.059–0.209)	0.96 (0.21)	0.29	1.0
Jinhua, China	11.596 (8.588–14.887)	1.59 (0.18)	2.23	125[a]

[a]Since the slopes of the two regression lines are not parallel, the relative potency value is not valid.

Table 8.2. Toxicities and relative potencies of imidacloprid to brown planthopper from the Philippines, China, and Vietnam for which the probit lines are parallel.

Location	LD_{50} in μg/g insect (95% confidence limits)	Slope (SE)	Heterogeneity	Relative potency
IRRI, Philippines	0.245 (0.175–0.327)	1.68 (0.26)	0.78	1.0
Tien Giang, Vietnam	2.891 (2.225–4.196)	1.78 (0.32)	0.06	11.8
Guilin, China	6.800 (5.192–8.421)	1.59 (0.18)	0.12	27.8

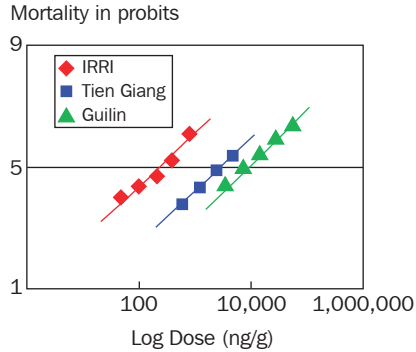


Fig. 8.1. Probit lines for imidacloprid on BPH populations in IRRI (Philippines), Tien Giang (Vietnam), and Guilin (China).

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Appendix A Table 1. Transformation of percentages to probits.

%	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1	2	3	4	5
0	—	1.9098	2.1218	2.2522	2.3479	2.4242	2.4879	2.5427	2.5911	2.6344					
1	2.6737	2.7096	2.7429	2.7738	2.8027	2.8299	2.8556	2.8799	2.9031	2.9251					
2	2.9463	2.9665	2.9859	3.0046	3.0226	3.0400	3.0569	3.0732	3.0890	3.1043	For more detail see				
3	3.1192	3.1337	3.1478	3.1616	3.1750	3.1881	3.2009	3.2134	3.2256	3.2376	values for 95–100				
4	3.2493	3.2608	3.2721	3.2831	3.2940	3.3046	3.3151	3.3253	3.3354	3.3454					
5	3.3551	3.3648	3.3742	3.3836	3.3928	3.4018	3.4107	3.4195	3.4282	3.4368	9	18	27	36	45
6	3.4452	3.4536	3.4618	3.4699	3.4780	3.4859	3.4937	3.5015	3.5091	3.5167	8	16	24	32	40
7	3.5242	3.5316	3.5389	3.5462	3.5534	3.5605	3.5675	3.5745	3.5813	3.5882	7	14	21	28	36
8	3.5949	3.6016	3.6083	3.6148	3.6213	3.6278	3.6342	3.6405	3.6468	3.6531	6	13	19	26	32
9	3.6592	3.6654	3.6715	3.6775	3.6835	3.6894	3.6953	3.7012	3.7070	3.7127	6	12	18	24	30
10	3.7184	3.7241	3.7298	3.7354	3.7409	3.7464	3.7519	3.7574	3.7628	3.7681	6	11	17	22	28
11	3.7735	3.7788	3.7840	3.7893	3.7945	3.7996	3.8048	3.8099	3.8150	3.8200	5	10	16	21	26
12	3.8250	3.8300	3.8350	3.8399	3.8448	3.8497	3.8545	3.8593	3.8641	3.8689	5	10	15	20	24
13	3.8736	3.8783	3.8830	3.8877	3.8923	3.8969	3.9015	3.9061	3.9107	3.9152	5	9	14	18	23
14	3.9197	3.9242	3.9286	3.9331	3.9375	3.9419	3.9463	3.9506	3.9550	3.9593	4	9	13	18	22
15	3.9636	3.9678	3.9721	3.9763	3.9806	3.9848	3.9890	3.9931	3.9973	4.0014	4	8	13	17	21
16	4.0055	4.0096	4.0137	4.0178	4.0218	4.0259	4.0299	4.0339	4.0379	4.0419	4	8	12	16	20
17	4.0458	4.0498	4.0537	4.0576	4.0615	4.0654	4.0693	4.0731	4.0770	4.0808	4	8	12	16	19
18	4.0846	4.0884	4.0922	4.0960	4.0998	4.1035	4.1073	4.1110	4.1147	4.1184	4	8	11	15	19
19	4.1221	4.1258	4.1295	4.1331	4.1367	4.1404	4.1440	4.1476	4.1512	4.1548	4	7	11	15	18
20	4.1584	4.1619	4.1655	4.1690	4.1726	4.1761	4.1796	4.1831	4.1866	4.1901	4	7	11	14	18
21	4.1936	4.1970	4.2005	4.2039	4.2074	4.2108	4.2142	4.2176	4.2210	4.2244	3	7	10	14	17
22	4.2278	4.2312	4.2345	4.2379	4.2412	4.2446	4.2479	4.2512	4.2546	4.2579	3	7	10	13	17
23	4.2612	4.2644	4.2677	4.2710	4.2743	4.2775	4.2808	4.2840	4.2872	4.2905	3	7	10	13	16
24	4.2937	4.2969	4.3001	4.3033	4.3065	4.3097	4.3129	4.3160	4.3192	4.3224	3	6	10	13	16
25	4.3255	4.3287	4.3318	4.3349	4.3380	4.3412	4.3443	4.3474	4.3505	4.3536	3	6	9	12	16
26	4.3567	4.3597	4.3628	4.3659	4.3689	4.3720	4.3750	4.3781	4.3811	4.3842	3	6	9	12	15
27	4.3872	4.3902	4.3932	4.3962	4.3992	4.4022	4.4052	4.4082	4.4112	4.4142	3	6	9	12	15
28	4.4172	4.4201	4.4231	4.4260	4.4290	4.4319	4.4349	4.4378	4.4408	4.4437	3	6	9	12	15
29	4.4466	4.4495	4.4524	4.4554	4.4583	4.4612	4.4641	4.4670	4.4698	4.4727	3	6	9	12	14
30	4.4756	4.4785	4.4813	4.4842	4.4871	4.4899	4.4928	4.4956	4.4985	4.5013	3	6	9	11	14
31	4.5041	4.5070	4.5098	4.5126	4.5155	4.5183	4.5211	4.5239	4.5267	4.5295	3	6	8	11	14
32	4.5323	4.5351	4.5379	4.5407	4.5435	4.5462	4.5490	4.5518	4.5546	4.5573	3	6	8	11	14
33	4.5601	4.5628	4.5656	4.5684	4.5711	4.5739	4.5766	4.5793	4.5821	4.5848	3	5	8	11	14
34	4.5875	4.5903	4.5930	4.5957	4.5984	4.6011	4.6039	4.6066	4.6093	4.6120	3	5	8	11	14
35	4.6147	4.6174	4.6201	4.6228	4.6255	4.6281	4.6308	4.6335	4.6362	4.6389	3	5	8	11	13
36	4.6415	4.6442	4.6469	4.6495	4.6522	4.6549	4.6575	4.6602	4.6628	4.6655	3	5	8	11	13
37	4.6681	4.6708	4.6734	4.6761	4.6787	4.6814	4.6840	4.6866	4.6893	4.6919	3	5	8	11	13
38	4.6945	4.6971	4.6998	4.7024	4.7050	4.7076	4.7102	4.7129	4.7155	4.7181	3	5	8	10	13
39	4.7207	4.7233	4.7259	4.7285	4.7311	4.7337	4.7363	4.7389	4.7415	4.7441	3	5	8	10	13
40	4.7467	4.7492	4.7518	4.7544	4.7570	4.7596	4.7622	4.7647	4.7673	4.7699	3	5	8	10	13
41	4.7725	4.7750	4.7776	4.7802	4.7827	4.7853	4.7879	4.7904	4.7930	4.7955	3	5	8	10	13
42	4.7981	4.8007	4.8032	4.8058	4.8083	4.8109	4.8134	4.8160	4.8185	4.8211	3	5	8	10	13
43	4.8236	4.8262	4.8287	4.8313	4.8338	4.8363	4.8389	4.8414	4.8440	4.8465	3	5	8	10	13
44	4.8490	4.8516	4.8541	4.8566	4.8592	4.8617	4.8642	4.8668	4.8693	4.8718	3	5	8	10	13
45	4.8743	4.8769	4.8794	4.8819	4.8844	4.8870	4.8895	4.8920	4.8945	4.8970	3	5	8	10	13
46	4.8996	4.9021	4.9046	4.9071	4.9096	4.9122	4.9147	4.9172	4.9197	4.9222	3	5	8	10	13
47	4.9247	4.9272	4.9298	4.9323	4.9348	4.9373	4.9398	4.9423	4.9448	4.9473	3	5	8	10	13
48	4.9498	4.9524	4.9549	4.9574	4.9599	4.9624	4.9649	4.9674	4.9699	4.9724	3	5	8	10	13
49	4.9749	4.9774	4.9799	4.9825	4.9850	4.9875	4.9900	4.9925	4.9950	4.9975	3	5	8	10	13
50	5.0000	5.0025	5.0050	5.0075	5.0100	5.0125	5.0150	5.0175	5.0201	5.0226	3	5	8	10	13
51	5.0251	5.0276	5.0301	5.0326	5.0351	5.0376	5.0401	5.0426	5.0451	5.0476	3	5	8	10	13
52	5.0502	5.0527	5.0552	5.0577	5.0602	5.0627	5.0652	5.0677	5.0702	5.0728	3	5	8	10	13
53	5.0753	5.0778	5.0803	5.0828	5.0853	5.0878	5.0904	5.0929	5.0954	5.0979	3	5	8	10	13
54	5.1004	5.1030	5.1055	5.1080	5.1105	5.1130	5.1156	5.1181	5.1206	5.1231	3	5	8	10	13
55	5.1257	5.1282	5.1307	5.1332	5.1358	5.1383	5.1408	5.1434	5.1459	5.1484	3	5	8	10	13
56	5.1510	5.1535	5.1560	5.1586	5.1611	5.1637	5.1662	5.1687	5.1713	5.1738	3	5	8	10	13
57	5.1764	5.1789	5.1815	5.1840	5.1866	5.1891	5.1917	5.1942	5.1968	5.1993	3	5	8	10	13
58	5.2019	5.2045	5.2070	5.2096	5.2121	5.2147	5.2173	5.2198	5.2224	5.2250	3	5	8	10	13
59	5.2275	5.2301	5.2327	5.2353	5.2378	5.2404	5.2430	5.2456	5.2482	5.2508	3	5	8	10	13

Cont.

Cont.

%	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1	2	3	4	5
60	5.2533	5.2559	5.2585	5.2611	5.2637	5.2663	5.2689	5.2715	5.2741	5.2767	3	5	8	10	13
61	5.2793	5.2819	5.2845	5.2871	5.2898	5.2924	5.2950	5.2976	5.3002	5.3029	3	5	8	10	13
62	5.3055	5.3081	5.3107	5.3134	5.3160	5.3186	5.3213	5.3239	5.3266	5.3292	3	5	8	11	13
63	5.3319	5.3345	5.3372	5.3398	5.3425	5.3451	5.3478	5.3505	5.3531	5.3558	3	5	8	11	13
64	5.3585	5.3611	5.3638	5.3665	5.3692	5.3719	5.3745	5.3772	5.3799	5.3826	3	5	8	11	13
65	5.3853	5.3880	5.3907	5.3934	5.3961	5.3989	5.4016	4.4043	5.4070	5.4097	3	5	8	11	14
66	5.4125	5.4152	5.4179	5.4207	5.4234	5.4261	5.4289	5.4316	5.4344	5.4372	3	5	8	11	14
67	5.4399	5.4427	5.4454	5.4482	5.4510	5.4538	5.4565	5.4593	5.4621	5.4649	3	6	8	11	14
68	5.4677	5.4705	5.4733	5.4761	5.4789	5.4817	5.4845	5.4874	5.4902	5.4930	3	6	8	11	14
69	5.4959	5.4987	5.5015	5.5044	5.5072	5.5101	5.5129	5.5158	5.5187	5.5215	3	6	9	11	14
70	5.5244	5.5273	5.5302	5.5330	5.5359	5.5388	5.5417	5.5446	5.5476	5.5505	3	6	9	12	14
71	5.5534	5.5563	5.5592	5.5622	5.5651	5.5681	5.5710	5.5740	5.5769	5.5799	3	6	9	12	15
72	5.5828	5.5858	5.5888	5.5918	5.5948	5.5978	5.6008	5.6038	5.6068	5.6098	3	6	9	12	15
73	5.6128	5.6158	5.6189	5.6219	5.6250	5.6280	5.6311	5.6341	5.6372	5.6403	3	6	9	12	15
74	5.6433	5.6464	5.6495	5.6526	5.6557	5.6588	5.6620	5.6651	5.6682	5.6713	3	6	9	12	16
75	5.6745	5.6776	5.6808	5.6840	5.6871	5.6903	5.6935	5.6967	5.6999	5.7031	3	6	10	13	16
76	5.7063	5.7095	5.7128	5.7160	5.7192	5.7225	5.7257	5.7290	5.7323	5.7356	3	7	10	13	16
77	5.7388	5.7421	5.7454	5.7488	5.7521	5.7554	5.7588	5.7621	5.7655	5.7688	3	7	10	13	17
78	5.7722	5.7756	5.7790	5.7824	5.7858	5.7892	5.7926	5.7961	5.7995	5.8030	3	7	10	14	17
79	5.8064	5.8099	5.8134	5.8169	5.8204	5.8239	5.8274	5.8310	5.8345	5.8381	4	7	11	14	18
80	5.8416	5.8452	5.8488	5.8524	5.8560	5.8596	5.8633	5.8669	5.8705	5.8742	4	7	11	14	18
81	5.8779	5.8816	5.8853	5.8890	5.8927	5.8965	5.9002	5.9040	5.9078	5.9116	4	7	11	15	19
82	5.9154	5.9192	5.9230	5.9269	5.9307	5.9346	5.9385	5.9424	5.9463	5.9502	4	8	12	15	19
83	5.9542	5.9581	5.9621	5.9661	5.9701	5.9741	5.9782	5.9822	5.9863	5.9904	4	8	12	16	20
84	5.9945	5.9986	6.0027	6.0069	6.0110	6.0152	6.0194	6.0237	6.0279	6.0322	4	8	13	17	21
85	6.0364	6.0407	6.0450	6.0494	6.0537	6.0581	6.0625	6.0669	6.0714	6.0758	4	9	13	18	22
86	6.0803	6.0848	6.0893	6.0939	6.0985	6.1031	6.1077	6.1123	6.1170	6.1217	5	9	14	18	23
87	6.1264	6.1311	6.1359	6.1407	6.1455	6.1503	6.1552	6.1601	6.1650	6.1700	5	10	15	19	24
88	6.1750	6.1800	6.1850	6.1901	6.1952	6.2004	6.2055	6.2107	6.2160	6.2212	5	10	15	21	26
89	6.2265	6.2319	6.2372	6.2426	6.2481	6.2536	6.2591	6.2646	6.2702	6.2759	5	11	16	22	27
90	6.2816	6.2873	6.2930	6.2988	6.3047	6.3106	6.3165	6.3225	6.3285	6.3346	6	12	18	24	29
91	6.3408	6.3469	6.3532	6.3595	6.3658	6.3722	6.3787	6.3852	6.3917	6.3984	6	13	19	26	32
92	6.4051	6.4118	6.4187	6.4255	6.4325	6.4395	6.4466	6.4538	6.4611	6.4684	7	14	21	28	35
93	6.4758	6.4833	6.4909	6.4985	6.5063	6.5141	6.5220	6.5301	6.5382	6.5464	8	16	24	31	39
94	6.5548	6.5632	6.5718	6.5805	6.5893	6.5982	6.6072	6.6164	6.6258	6.6352	9	18	27	36	45
95	6.6449	6.6546	6.6646	6.6747	6.6849	6.6954	6.7060	6.7169	6.7279	6.7392					
	97	100	101	102	105	106	109	110	113	115					
96	6.7507	6.7624	6.7744	6.7866	6.7991	6.8119	6.8250	6.8384	6.8522	6.8663					
	117	120	122	125	128	131	134	138	141	145					
97	6.8808	6.8957	6.9110	6.9268	6.9431	6.9600	6.9774	6.9954	7.0141	7.0335					
	149	153	158	163	169	174	180	187	194	202					
98.0	7.0537	7.0558	7.0579	7.0600	7.0621	7.0642	7.0663	7.0684	7.0706	7.0727	2	4	6	8	11
98.1	7.0749	7.0770	7.0792	7.0814	7.0836	7.0858	7.0880	7.0902	7.0924	7.0947	2	4	7	9	11
98.2	7.0969	7.0992	7.1015	7.1038	7.1061	7.1084	7.1107	7.1130	7.1154	7.1177	2	5	7	9	12
98.3	7.1201	7.1224	7.1248	7.1272	7.1297	7.1321	7.1345	7.1370	7.1394	7.1419	2	5	7	10	12
98.4	7.1444	7.1469	7.1494	7.1520	7.1545	7.1571	7.1596	7.1622	7.1648	7.1675	3	5	8	10	13
98.5	7.1701	7.1727	7.1754	7.1781	7.1808	7.1835	7.1862	7.1890	7.1917	7.1945	3	5	8	11	14
98.6	7.1973	7.2001	7.2029	7.2058	7.2086	7.2115	7.2144	7.2173	7.2203	7.2232	3	6	9	12	14
98.7	7.2262	7.2292	7.2322	7.2353	7.2383	7.2414	7.2445	7.2476	7.2508	7.2539	3	6	9	12	15
98.8	7.2571	7.2603	7.2636	7.2668	7.2701	7.2734	7.2768	7.2801	7.2835	7.2869	3	7	10	13	17
98.9	7.2904	7.2938	7.2973	7.3009	7.3044	7.3080	7.3116	7.3152	7.3189	7.3226	4	7	11	14	18
99.0	7.3263	7.3301	7.3339	7.3378	7.3416	7.3455	7.3495	7.3535	7.3575	7.3615	4	8	12	16	20
99.1	7.3656	7.3698	7.3739	7.3781	7.3824	7.3867	7.3911	7.3954	7.3999	7.4044	4	9	13	17	22
99.2	7.4089	7.4135	7.4181	7.4228	7.4276	7.4324	7.4372	7.4422	7.4471	7.4522	5	10	14	19	24
99.3	7.4573	7.4624	7.4677	7.4730	7.4783	7.4838	7.4893	7.4949	7.5006	7.5063	5	11	16	22	27
99.4	7.5121	7.5181	7.5241	7.5302	7.5364	7.5427	7.5491	7.5556	7.5622	7.5690	6	13	19	25	32
99.5	7.5758	7.5828	7.5899	7.5972	7.6045	7.6121	7.6197	7.6276	7.6356	7.6437					
99.6	7.6521	7.6606	7.6693	7.6783	7.6874	7.6968	7.7065	7.7164	7.7266	7.7370					
99.7	7.7478	7.7589	7.7703	7.7822	7.7944	7.8070	7.8202	7.8338	7.8480	7.8627					
99.8	7.8782	7.8943	7.9112	7.9290	7.9478	7.9677	7.9889	8.0115	8.0357	8.0618					
99.9	8.0902	8.1214	8.1559	8.1947	8.2389	8.2905	8.3528	8.4316	8.5401	8.7190					

I am indebted to Professor R. A. Fisher and Dr F. Yates, and also to Messrs Oliver and Boyd, Ltd. of Edinburgh, for permission to reprint Table I from Table IX of their book *Statistical Tables for Biological, Agricultural and Medical Research*.

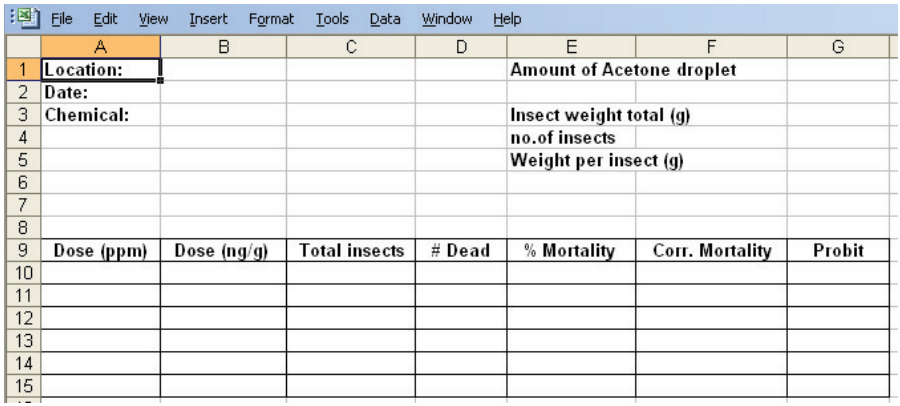
Source: Finney (1971).

Appendix Table B1. Raw data recording sheet.

File Edit View Insert Format Tools Data Window Help

	A	B	C	D	E	F
1	**Topical Application Data Sheet**					
2	Date:					
3	Chemical:					
4	Location:					
5	Temperature:					
6	RH:					
7	Insect:					
8	Age:					
9	Sex:					
10	Weight:					
11		Mortality				
12	Dose (ppm)	Rep 1	Rep 2	Rep 3		Total
13						
14						
15						
16						
17						
18						
19						
20						
21						
22						
23						
24						
25						
26						
27						
28						
29						
30						
31						
32						
33						
34						
35						
36						
37						

Appendix B2. Excel worksheet for quantal response data.



File Edit View Insert Format Tools Data Window Help

	A	B	C	D	E	F	G
1	Location:				Amount of Acetone droplet		
2	Date:						
3	Chemical:				Insect weight total (g)		
4					no.of insects		
5					Weight per insect (g)		
6							
7							
8							
9	Dose (ppm)	Dose (ng/g)	Total insects	# Dead	% Mortality	Corr. Mortality	Probit
10							
11							
12							
13							
14							
15							

Column A. Dose is the concentration of the insecticide in ppm.
Column B. Dose (ng/g) = [(Dose (ppm)*amt. applied (µL)/1,000)/wt. of insect (g)]*1,000.
Column C. Total number of insects treated in all replications.
Column D. Total number of dead insects observed.
Column E. % Mortality = total number of dead insects/100.
Column F. Corr. mortality (Abbotts) = (Po – Pc)/(100 – Pc)*100
where Po = observed mortality in treated
Pc = % control mortality
Column G. Probits = computed transformed values of % mortality given in Appendix 1.

Appendix C Table 1. The distribution of χ^2.[a]

Degrees of freedom (df)	Probability			
	0.1	0.05	0.01	0.001
1	2.7	3.8	6.6	10.8
2	4.6	6.0	9.2	13.8
3	6.3	7.8	11.3	16.3
4	7.8	9.5	13.3	18.5
5	9.2	11.1	15.1	20.5
6	10.6	12.6	16.8	22.5
7	12.0	14.1	18.5	24.3
8	13.4	15.5	20.1	26.1
9	14.7	16.9	21.7	27.9
10	16.0	18.3	23.2	29.6
11	17.3	19.7	24.7	31.3
12	18.5	21.0	26.2	32.9
13	19.8	22.4	27.7	34.5
14	21.1	23.7	29.1	36.1
15	22.3	25.0	30.6	37.7
16	23.5	26.3	32.0	39.3
17	24.8	27.6	33.4	40.8
18	26.0	28.9	34.8	42.3
19	27.2	30.1	36.2	43.8
20	28.4	31.4	37.6	45.3
21	29.6	32.7	38.9	46.8
22	30.8	33.9	40.3	48.3
23	32.0	35.2	41.6	49.7
24	33.2	36.4	43.0	51.2
25	34.4	37.7	44.3	52.6
26	35.6	38.9	45.6	54.1
27	36.7	40.1	47.0	55.5
28	37.9	41.3	48.3	56.9
29	39.1	42.6	49.6	58.3
30	40.3	43.8	50.9	59.7

[a]The values of χ^2 distribution are computed using www.fourmilab.ch/rpkp/experiments/analysis/chiCalc.html.