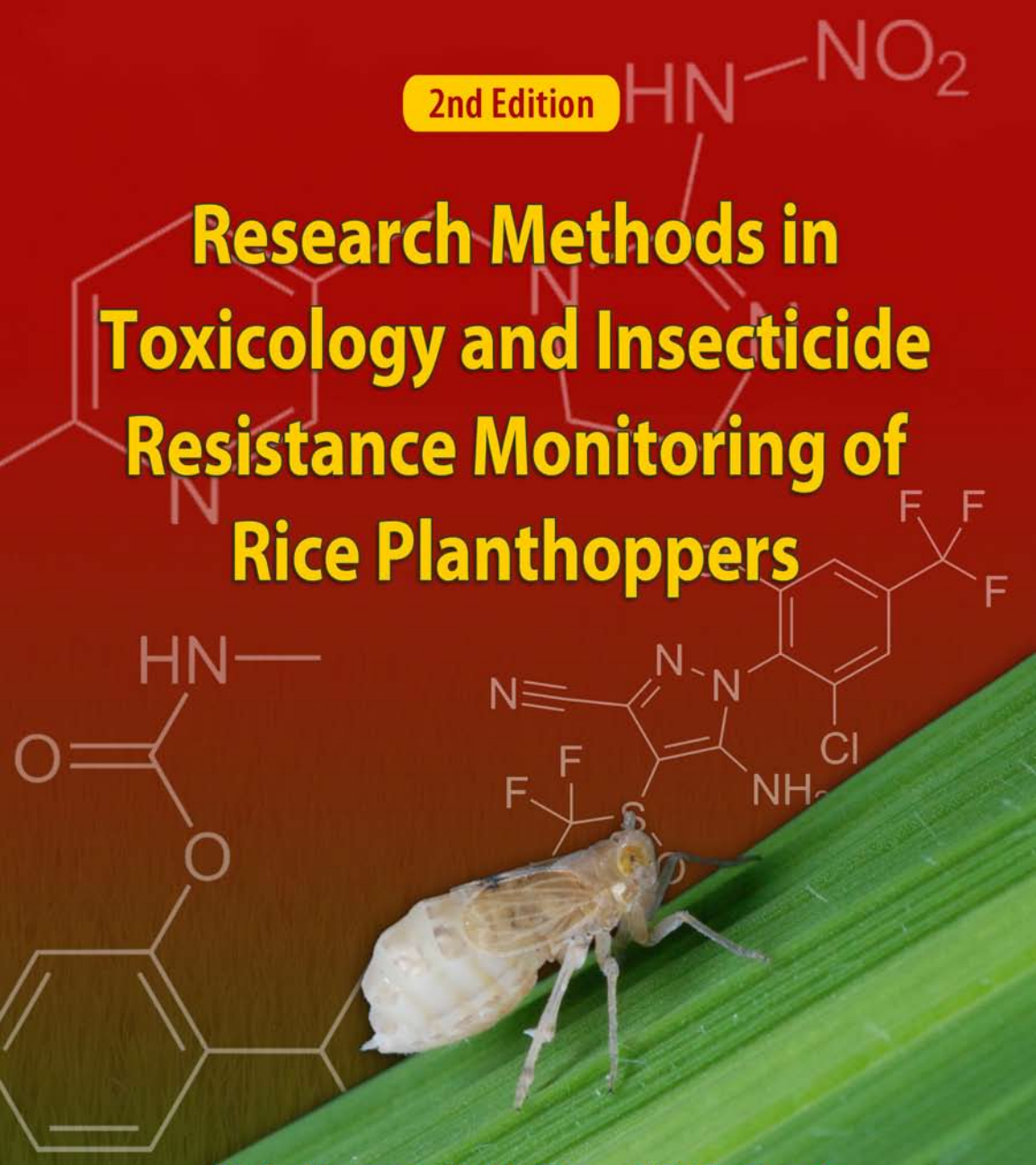
2nd Edition

Research Methods in Toxicology and Insecticide Resistance Monitoring of Rice Planthoppers



K. L. Heong, K. H. Tan, C.P.F. Garcia,
Z. Liu, and Z. Lu

IRRI
Rice Planthopper Project
ADB

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2013

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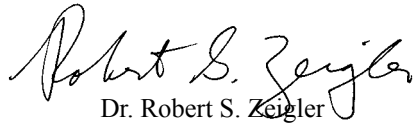
Contents

Foreword

Insect pests continue to be a major threat to the sustainability of rice production. One of the most destructive pests today is the rice planthopper group. During the Green Revolution of the 1970s and 1980s, planthoppers were a serious problem. Today, these species are once again causing serious damage across Asia. In the past 10 years, more than 20 million hectares of rice have been destroyed by planthoppers. These insects are developing resistance to many insecticides, including those from new chemistry such as the neonicotinoids, at a rapid rate. Scientists need to constantly monitor this development of resistance in order to come up with strategies for managing these pests. For cross-border comparison of data and research findings, scientists will also need to develop standardized methods and protocols. This book provides such methods, protocols for preparation of materials, and procedures to conduct statistical analyses and interpret results. Besides those, numerous examples assist the reader in practical application.

The second edition of this book builds on the first edition, incorporating more research methods with examples and new interpretation of data. The book will be a valuable resource for scientists, university professors, researchers, and students involved in insecticide toxicology and insecticide resistance research.

I would like to express our thanks to the Asian Development Bank (ADB) for providing financial support to this study as well as for the publication of this book.



Dr. Robert S. Zeigler
Director General

Preface

Insecticide resistance in rice planthoppers is developing rapidly, especially to the neonicotinoids, like imidacloprid. In China where planthoppers have acquired hundreds of folds resistance, the Ministry of Agriculture had removed this active ingredient from the rice market. Resistance reporting has been rather inconsistent differing in methods, equipment used, insect conditions, stages and statistical analyses. Since the manuscript of the first edition was completed in 2010, research partners in the ADB-IRRI Rice Planthopper Project have been conducting routine monitoring as well as evaluating repellents, studying reversion of resistance and using molecular techniques. Several workshops were held to compare results and discuss the methods, analyses and interpretation. The second edition of this book contains the modifications we have made based on the feedbacks. We have replaced authorship with Professor Z. Liu of Nanjing University joining and added 4 more chapters and references. Although the contents of Chapters 1 to 7 remain similar we have edited and updated them with new information wherever appropriate. As in the first edition, we continue to draw from the works of Busvine (1971), Finney (1977) and Robertson et al (2005) and provide step-by-step procedures for readers to design experiments and use the program for analyses.

Chapter 8 focuses on analyzing quantal response data with multiple explanatory variables and the use of PoloEncore© while Chapter 9 describes other forms of dose- response analyses with some examples. The determination of LC50s and LT50s are discussed. In Chapter 10 we describe insecticide resistance reversion and the use of the resistance stability point as a more stable baseline for comparing resistance in space and time. We also describe the use of molecular tools to detect field resistance. The molecular technique is easier to apply and can also detect resistance due to target mutation sometimes known as the second phase in resistance development. In Chapter 11 we describe the evaluation of repellent effects using methyl eugenol as an example.

This second edition of the book is now more complete and provides readers the whole range of research methods in toxicology and insecticide resistance monitoring. Although all the methods described are on rice planthoppers, the principles and methods can be generally applied to other test organisms.

Acknowledgments

In preparing the second edition many people have made significant contributes. In particular the authors would like thank the following

- The Asian Development bank (ADB) for providing the technical assistance TA7493-REG - Addressing the Pre- and Postharvest Challenges of the Rice Supply Chain (RETA 14) for us to continue the insecticide resistance monitoring network, conduct the data gathering, the training workshops, preparation and printing of the second edition.
- Ms Sylvia Villareal for all the photography in the book
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- Collaborating scientists from Zhejiang Academy of Agricultural Sciences, China, Rice Department, Thailand and the Plant Protection Department, Vietnam. In particular we would like to thank Ms Wantana Sriratanasak, Dr Ho Van Chien, and Mr Phan Van Tuong.

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 nar-

cotic. Toxicology 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])

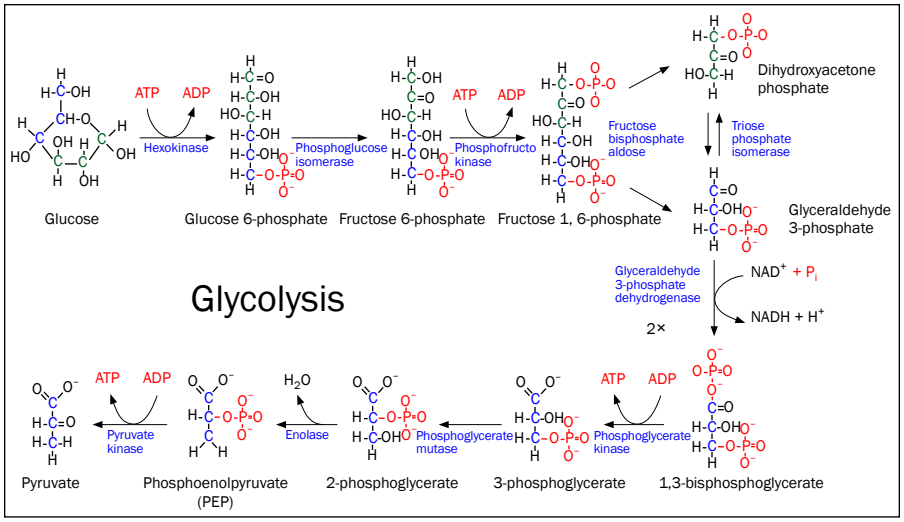


Fig. 1.1. Glycolysis pathway (it consists of 10 steps, each catalyzed by an enzyme).

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.

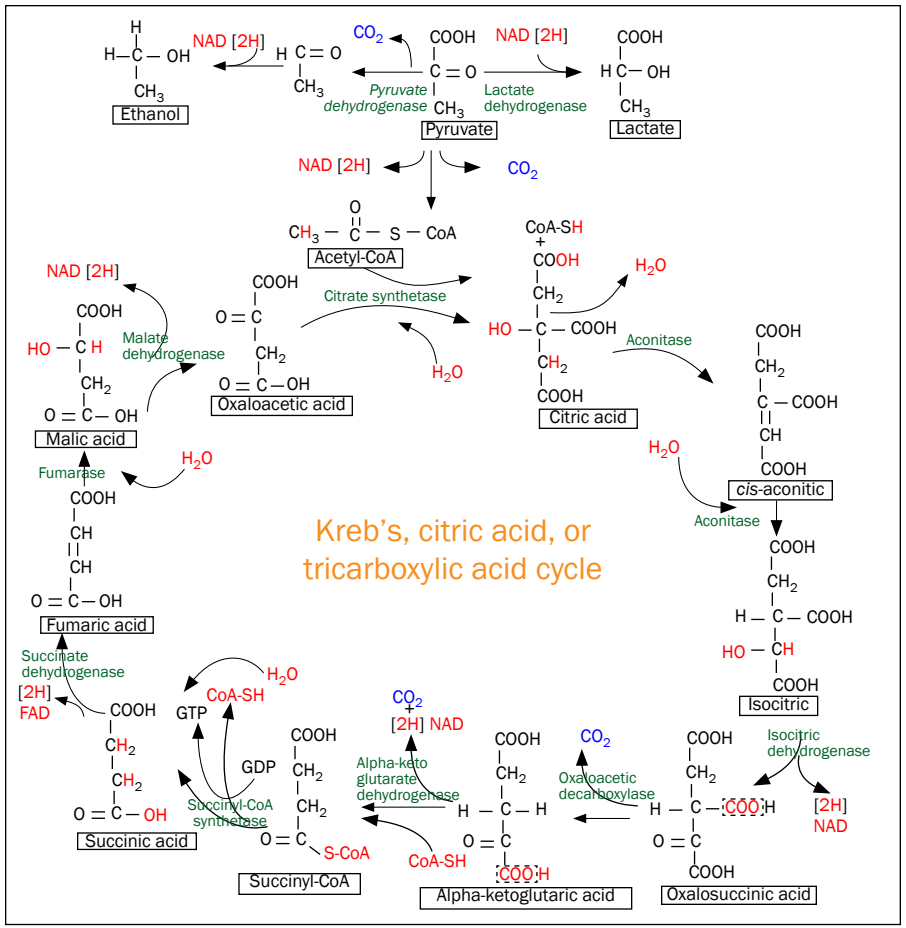


Fig. 1.2. Kreb's cycle.

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.

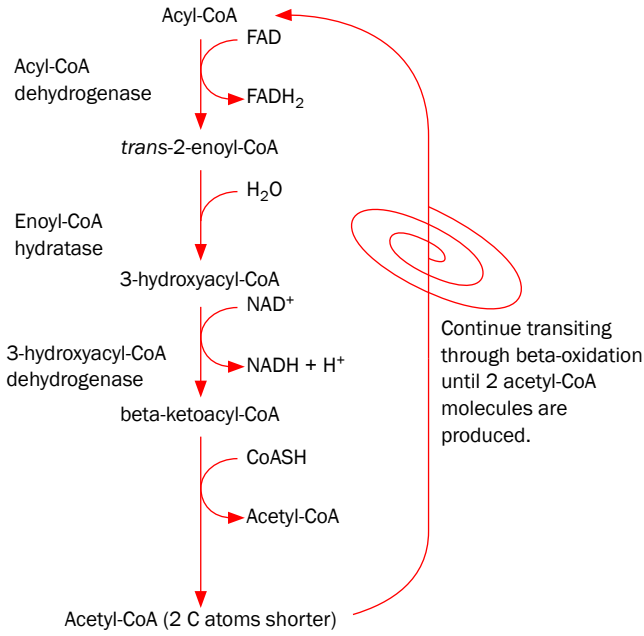


Fig. 1.3. ß-oxidation.

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

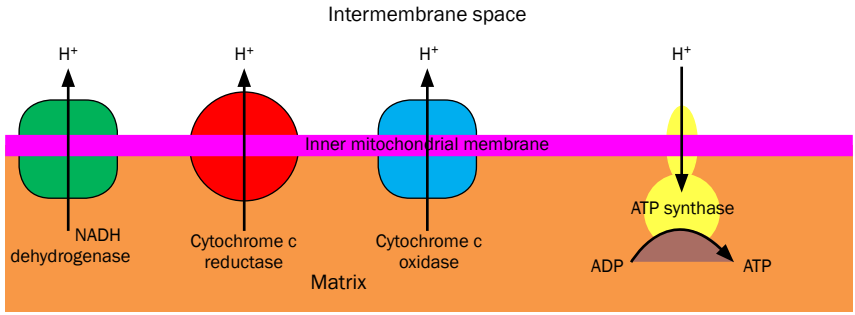


Fig. 1.4. ATP synthesis within a mitochondrion.

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:

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:

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:

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.

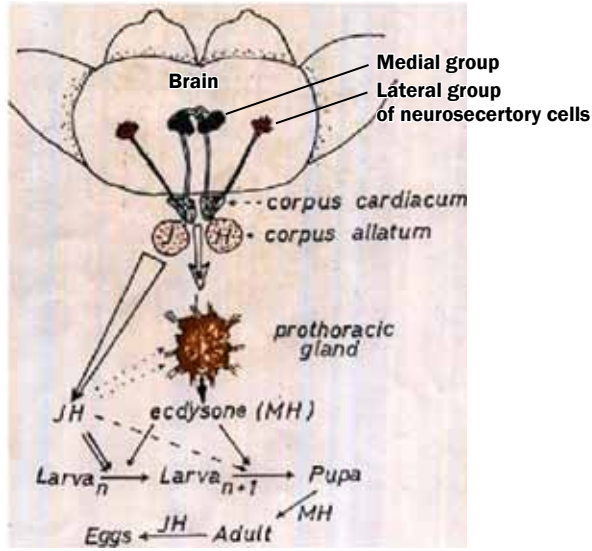


Fig. 1.5. The insect neuroendocrine system that regulates development.

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

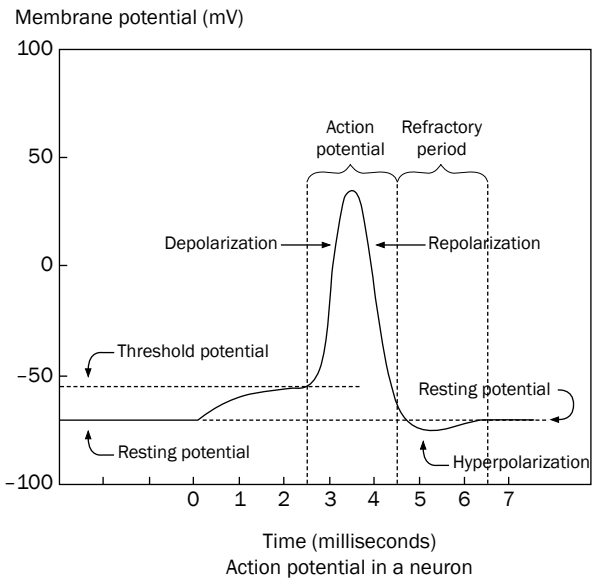


Fig. 1.6. Initiation of a nerve impulse in a neuron.

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:

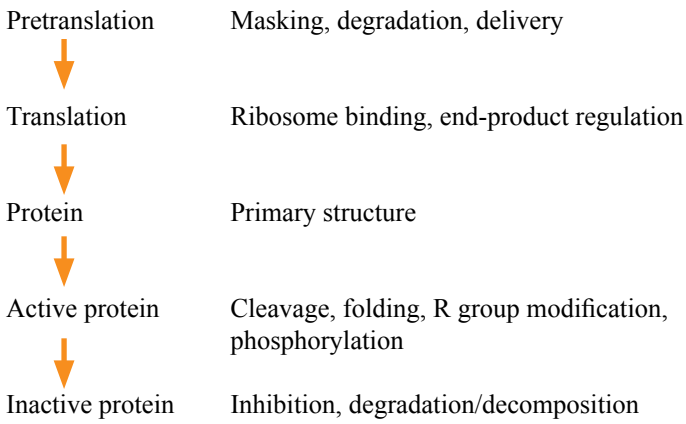
a) Nucleus

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)		Moderately toxic chemicals (50–500 mg/kg)	
Botulinum toxin	0.00001 (= 10 ng)	Paraquat	95
Dioxin	0.1	Caffeine	200
Parathion	13.0	Carbaryl	270
Strychnine	30.0	Malathion	370
Nicotine	50.0	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 non-transmission 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 from different chemical classes have the same mode of action (Matsumura et al 2008).

[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/.

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 negative 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.

Synergism should not be confused with additive effects that occur when mixing two pesticides that may provide the same response as the combined effects of each material when applied separately. Each substance in the mixture neither synergizes nor antagonizes the other. Such mixture of pesticides is used to save on labor, time, and equipment use in pest control. However, it should be emphasized that mixing of pesticides may also lead to antagonism.

Antagonism

This phenomenon occurs when a mixture of two pesticides produces less control than if each is applied separately. This is shown in several mixtures of an organophosphorous insecticide (OP and a pyrethroid against a homopteran pest, *Bemisia tabaci,* e.g., i) profenofos + any of three pyrethroids - cypermethrin, bifenthrin, and λ-cyhalothrin; ii) methyl parathion + deltamethrin; iii) triazophos + bifenthrin (Mushtag 2007). In the same study, chlorpyrifos + cypermethrin had antagonistic effects; but the OP had an additive effect with fenpropathrin against several populations of *Bemisia tabaci*.

It should be emphasized that in addition to decreasing control of a pest, antagonistic responses of a mixture may also increase phytotoxicity to plants. Furthermore, in developing countries where there are weak or no regulatory systems to manage unscrupulous practices of mixing pesticides, mixtures of pesticides—particularly insecticides with fungicides or herbicides—may be disguised for the disposal of unsold or banned insecticides.

The table below represents an example of the antagonism effect of mixing two pesticides. A 2:1 chlorpyrifos and buprofezin mixture is commonly used in the market. Based on the median lethal concentration (LC_{50}), it can be clearly seen that the toxicity of each chemical was affected by the other, thus giving the mixture less efficacy. Analysis of chemical mixtures is further explained in Chapter 7.

Table 2.1. Antagonism effect of 2:1 chlorpyrifos-buprofezin mixture.

Chemical	LC_{50} (ppm)	Fiducial limits (95%)	Slope	Heterogeneity	Tabulated Chi-Square	Computed Chi-Square
Buprofezin	1.02	0.77 to 1.29	2.151	0.099		
Chlorpyrifos	4.56	2.99 to 6.13	1.54	0.569		
Chlorpyrifos + Buprofezin mixture	2.22	1.63 to 2.82	2.27 (0.32)	0.25	11.1	1.923

Sublethal effect of insecticides: antifeeding, knockdown, and repellency

Invariably after application, all insecticides will eventually deteriorate to sublethal doses and some will also induce antifeeding, knockdown, and/or repellency in insects.

Knockdown is a state of partial paralysis or moribund that may precede death caused by an insecticide. It is common for pyrethroids to have a knockdown effect within a few seconds, in contrast to the slower action of other compounds. Knockdown effect may also be observed in insects that have picked up sufficient sublethal doses or when there is a drastic drop in environmental temperature due to the negative temperature coefficient (Wickham et al 1974). Pyrethroids, e.g., cypermethrin and permethrin, can also induce antifeeding effects as shown by *Pieris brassicae* larvae when leaves are treated at sublethal doses (Tan 1981).

Repellancy was shown by bifenthrin for up to seven days against silverleaf whitefly, *Bemisia argentifolii,* on tomato plants. The number of whitefly eggs also significantly decreased on bifenthrin-treated leaves (Liu and Stansly 1995). Irritancy responses as shown by *P. brassicae* larvae exposed to pyrethroids can also lead to repellency (Tan 1982).

New insights into the sublethal effects of insecticides—including effects on behavior, e.g., learning performance and neurophysiology in beneficial arthropods, particularly in honeybees and natural enemies of pests—have been reviewed by Desneux et al (2007). More recently, sublethal doses of neonicotinoids have been shown to disrupt honeybee learning, behavior, and cognitive functions (Palmer et al 2013).

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 randomly 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 with rice seedlings and then 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 laboratory 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 collected insects are brought back to the laboratory and reared in a greenhouse or insectary, maintained at 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, for which susceptible rice varieties without resistant genes 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 with a 10-cm diameter. Fifteen days after transplanting, 2 g of ammonium sulfate fertilizer is applied per pot. One week prior to planthopper collection, the potted plants are cleaned with tap water and the outer leaf sheaths and tillers removed in order to eliminate possible infestations of nymphs and eggs of other insects, such as leafhoppers or natural enemies such as mirid bugs and spiders. The prepared plants are covered with mylar cages and kept inside the greenhouse to avoid further infestation by other insect pests and natural enemies.

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 1000–1400 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 wall are covered with fine-mesh nylon cloth for ventilation. Insects are cultured on rice seedling mats (measuring about 22 cm x 28 cm) and 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.

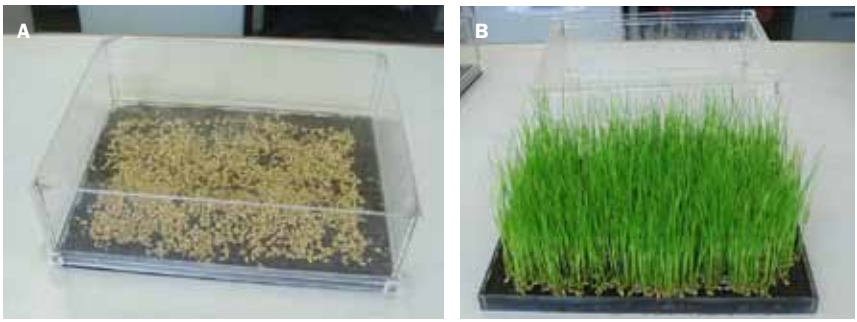


Fig. 4.5. A seedling mat (A) with germinated seeds and covered with rectangular flexi-glass; (B) a 1-week-old seedling mat.

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, size, and physiological condition of the insects affect their susceptibility to insecticides. After field collection, bioassays can be done using planthoppers from the second 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

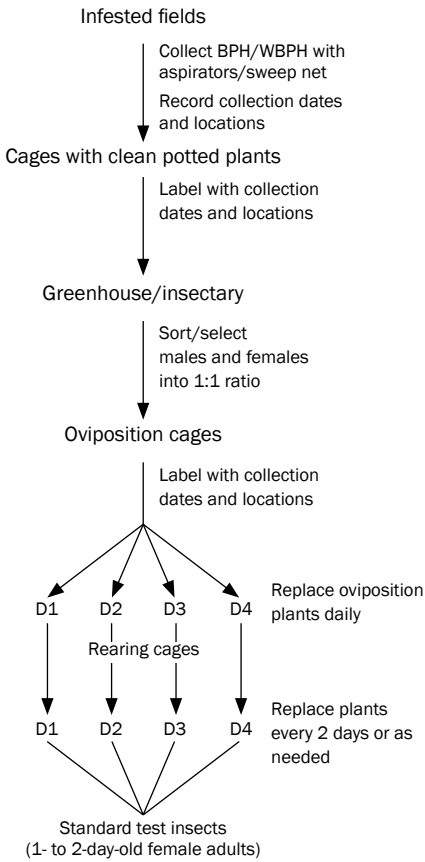


Fig. 4.6. Schematic diagram of collecting and rearing planthoppers for insecticide bioassays.

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.

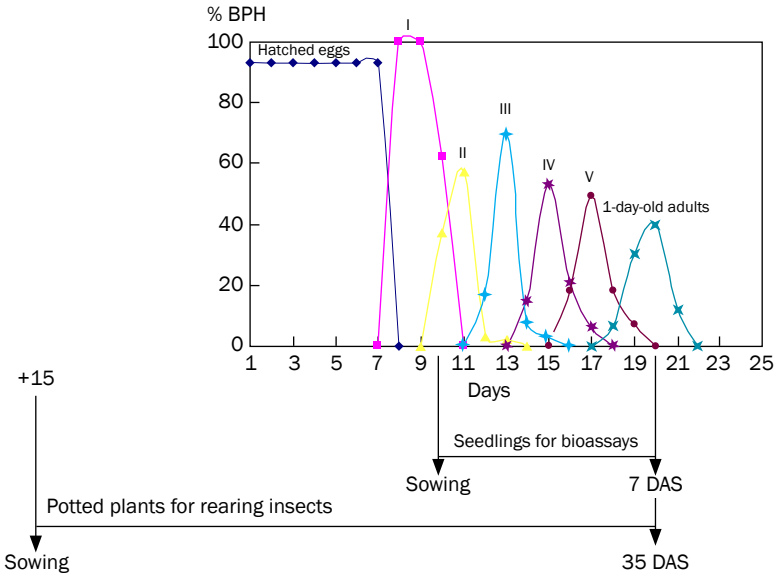


Fig. 4.7. Schematic diagram of BPH life cycle in a controlled room (26 °C) and the preparation of plant materials for the BPH cultures and bioassays.

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

A) For a laboratory without access to volumetric flasks

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.

B) For a modern laboratory with access to volumetric flasks

Pipetting may cause experimental errors (though small in some cases, the errors add up when multiple steps are involved). Therefore, to minimize pipetting errors, we recommend the use of an adjustable volume pipettor and volumetric flasks (5 or 10 mL) to prepare a stock solution (SS) and further serial dilutions. To prepare a required concentration of SS, weigh accurately a quantity (Q = ~10 mg for every 1 mL volume, i.e., ~50 and ~100 mg for 5- and 10-mL volumetric flasks, respectively) of the technical grade insecticide of known purity in a clean and dry volumetric flask using the following simple formula:

$$Y = Q / V \times 1 / CF$$

where CF is the correction factor, as in item (A) above, Q is the weight of the technical grade insecticide, V is the volume of the volumetric flask in use, and Y is the actual concentration of a.i. (in mg/mL).

Acetone is added up to the required mark using a pasteur pipette and with the correct stopper, and sealed with parafilm before the flask is shaken and labeled for storing in the freezer.

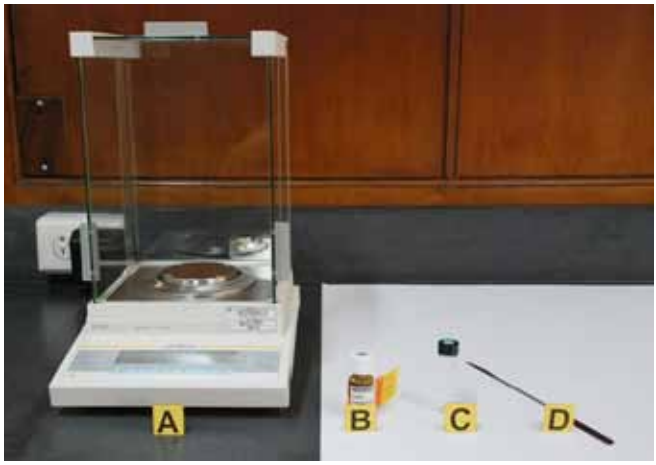


Fig. 5.1. Materials for the preparation of stock solution: (A) analytical weighing balance, (B) technical-grade insecticide, (C) 6-mL screw cap vial, (D) spatula.

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.

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.

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).

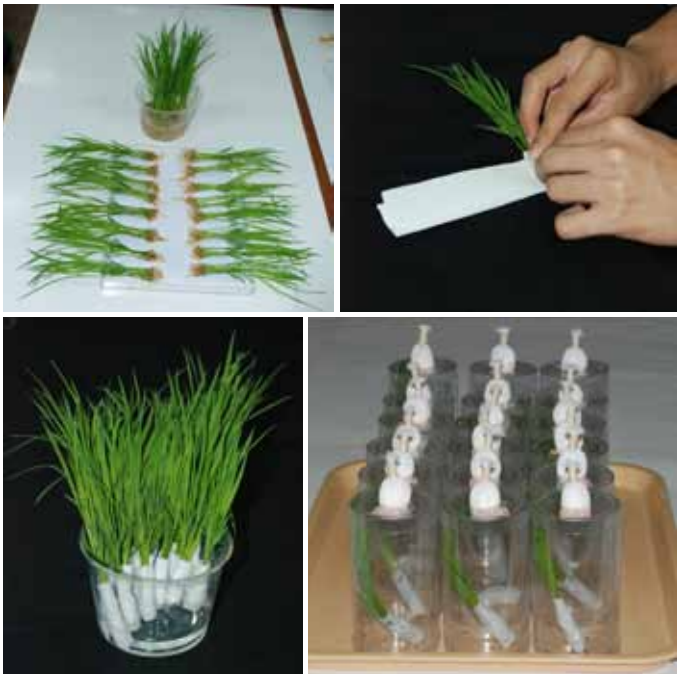


Fig. 5.3. Recovery cage preparation: (A) 7- to 10-day-old rice seedlings of any local susceptible variety, (B) wrapping seedling roots with paper towel, (C) 15 rice seedlings wrapped with paper towel and soaked in water, (D) clear tumbler cages (12.5 cm tall, 7 m in diam.) with seedlings and cotton stopper.

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.

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.

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.

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

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.

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 repeated every 24 hours for up to 5 days after treatment in some insecticide groups, such as with insect growth regulators (IGRs).

From these data, LD50 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©

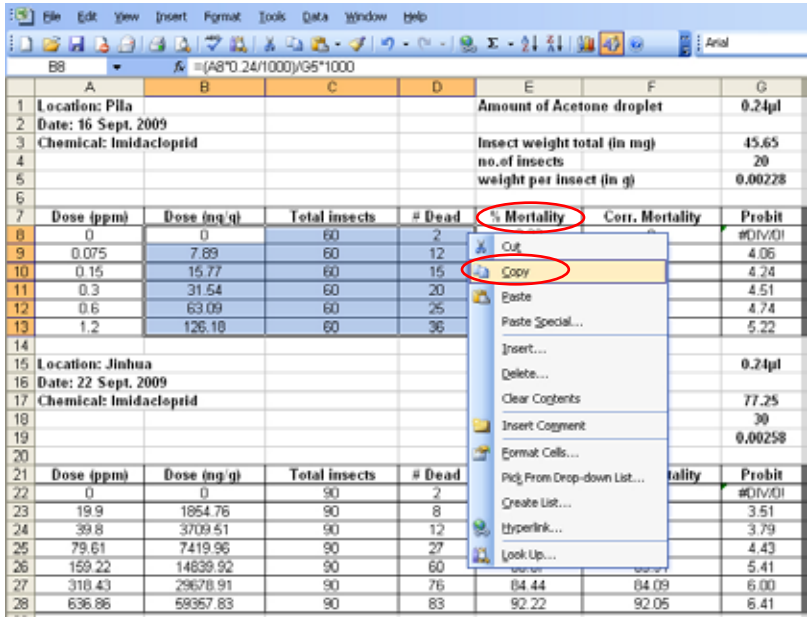
In Chapter 5, we discussed the use of Excel to record quantal response data. The dose can be expressed in ppm but for the analyses, dose is converted to nanogram/gram insect (ng/g) using the formula below:

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.

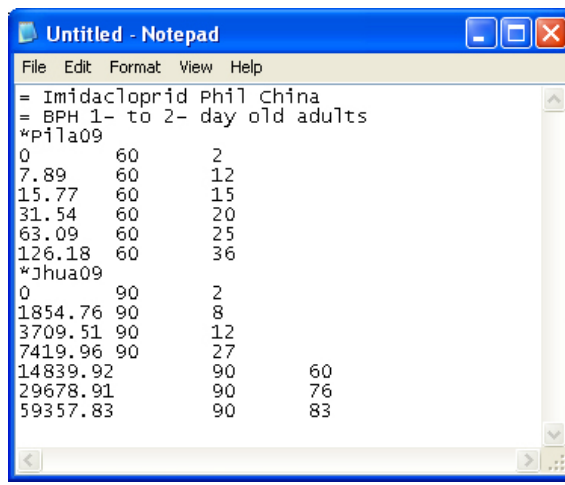
Moreover, PoloPlus© reads data from a space-delimited text file. To convert data recorded in Excel to PoloPlus© data, the following procedures can be used:

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.

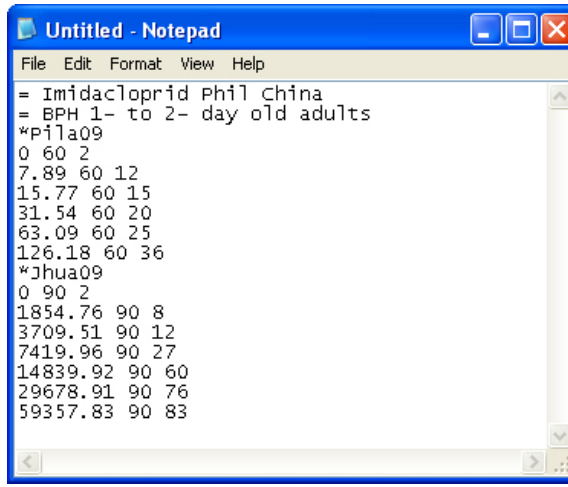


	A	B	C	D	E	F	G
1	Location: Pila				Amount of Acetone droplet		0.24pl
2	Date: 16 Sept. 2009						
3	Chemical: Imidacloprid				Insect weight total (in mg)		45.65
4					no.of insects		20
5					weight per insect (in g)		0.00228
6							
7	Dose (ppm)	Dose (ng/g)	Total insects	# Dead	% Mortality	Corr. Mortality	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						0.24pl
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			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.

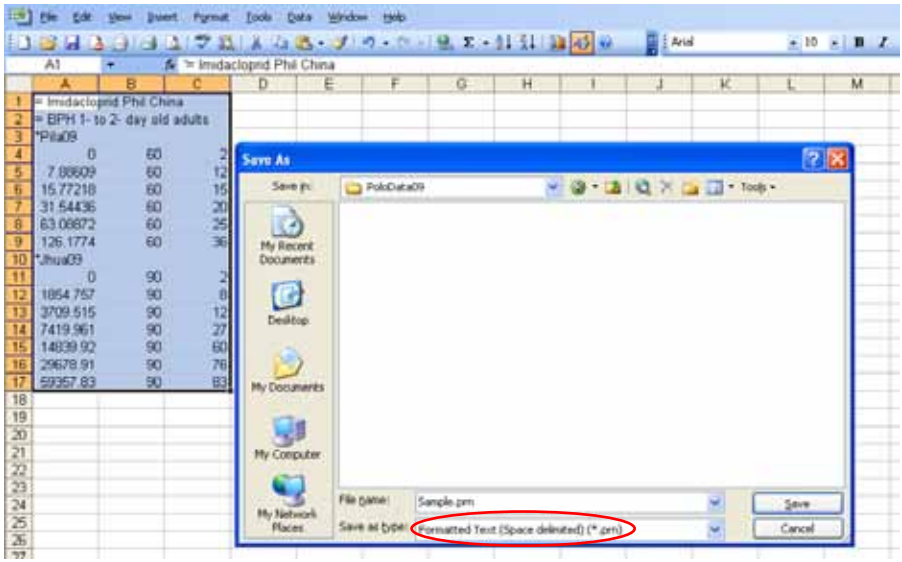


3. There is a need to further format this tab-delimited file to a space-delimited text file. To do this, highlight the space in between the Dose (ng/g) and the Total insects and then right-click on the mouse and select Copy. Next, go to the Edit menu and select Replace. In the "Find what" box, select 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©.

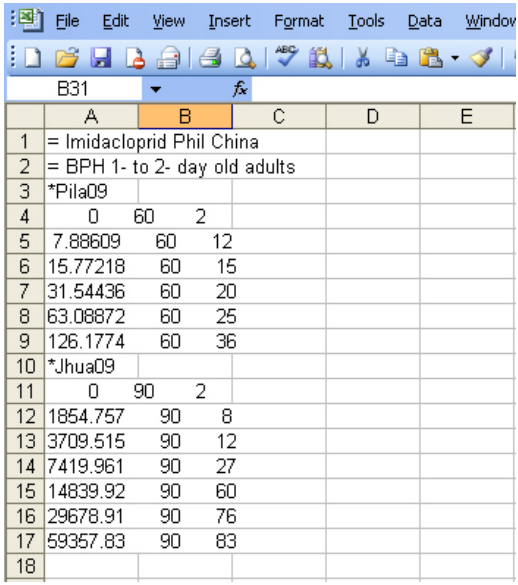


Another option is to save the data as a text file in Microsoft Excel and the steps are given below:

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©.

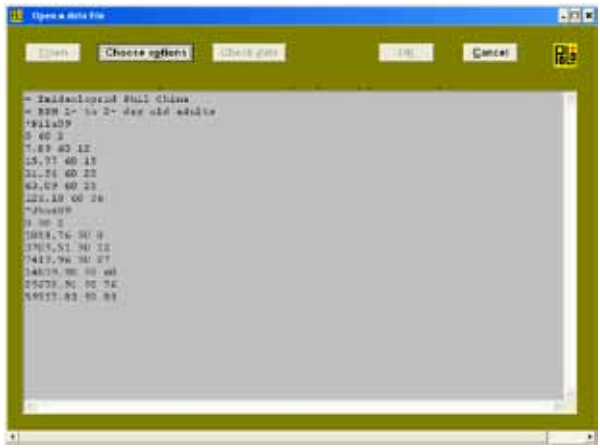


	A	B	C	D	E
1	= Imidacloprid Phil China				
2	= BPH 1- to 2- day old adults				
3	*Pila09				
4	0	60	2		
5	7.88609	60	12		
6	15.77218	60	15		
7	31.54436	60	20		
8	63.08872	60	25		
9	126.1774	60	36		
10	*Jhua09				
11	0	90	2		
12	1854.757	90	8		
13	3709.515	90	12		
14	7419.961	90	27		
15	14839.92	90	60		
16	29678.91	90	76		
17	59357.83	90	83		
18					

Using PoloPlus©

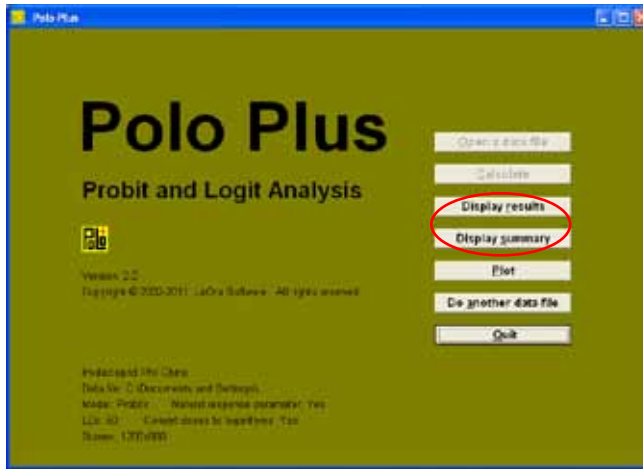
The quantal response data that have been saved to the specified text format can now be analyzed using the PoloPlus© program, with the following steps.

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

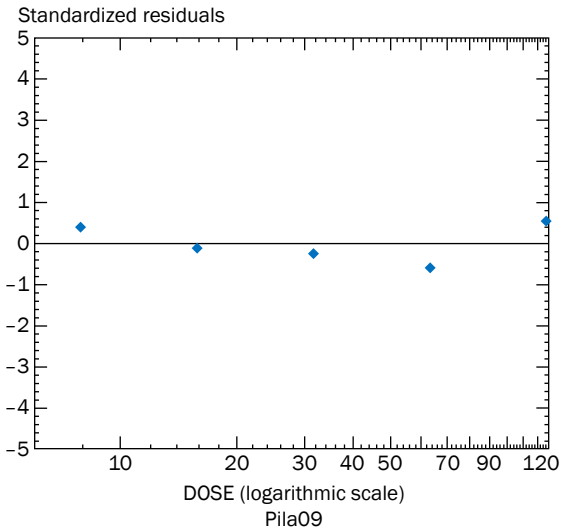


Fig. 6.2. Plot of residuals for Pila09.

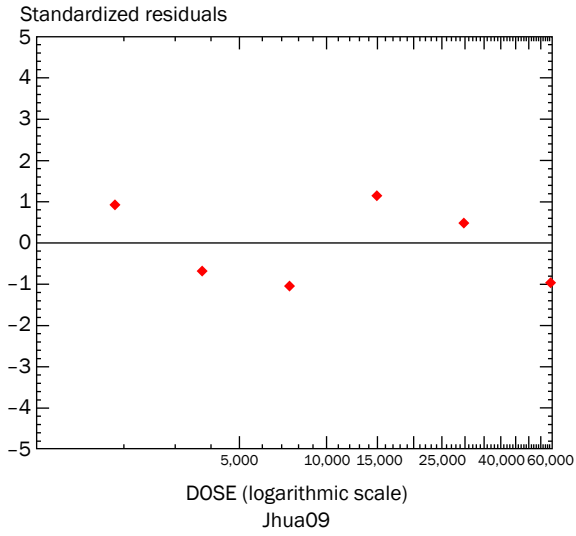


Fig. 6.3. Plot of residuals for Jhua09.

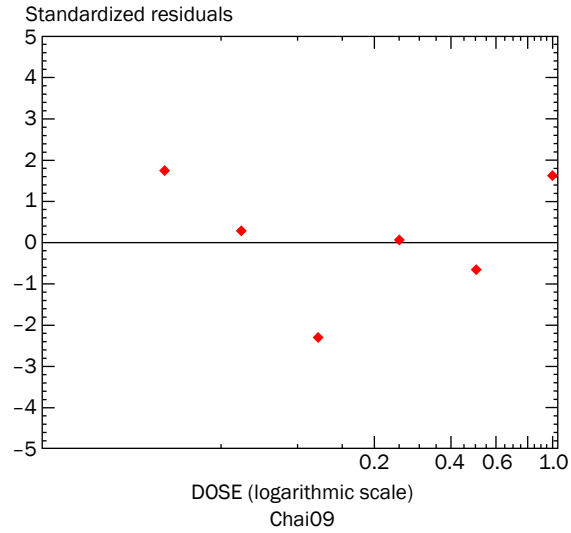


Fig. 6.4. Plot of residuals for Chai09.

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

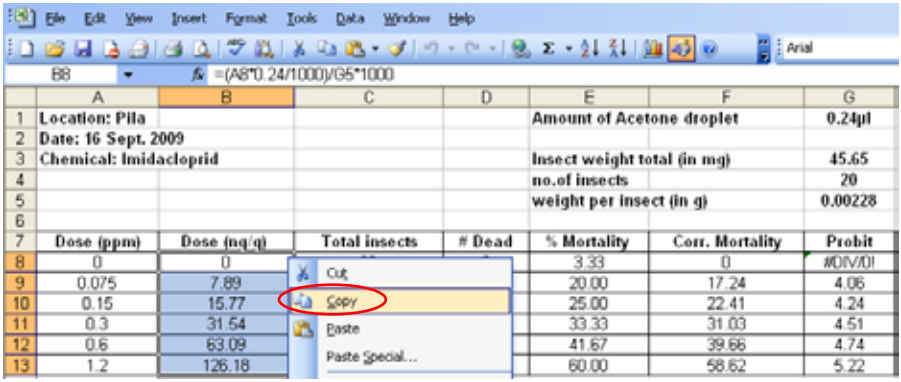
The lethal dose ratio on lines 95−98, with their upper and lower 95% confidence limits, compares the response of the second population in relation to the first population. These ratios can be used to determine the relative toxicity of the insecticide to the populations. In the example, imidacloprid is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

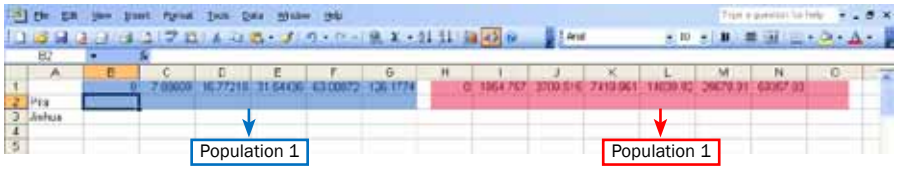
1. Using the quantal response Excel file, highlight and Copy the Dose (ng/g) values.



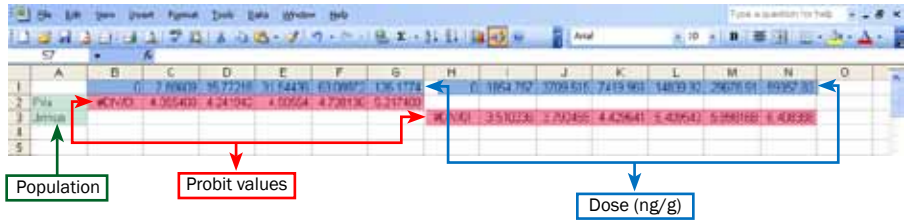
	A	B	C	D	E	F	G
1	Location: Pila				Amount of Acetone droplet		0.24µl
2	Date: 16 Sept. 2009						
3	Chemical: Imidacloprid				Insect weight total (in mg)		45.65
4					no.of insects		20
5					weight per insect (in g)		0.00228
6							
7	Dose (ppm)	Dose (ng/g)	Total insects	# Dead	% Mortality	Corr. Mortality	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.

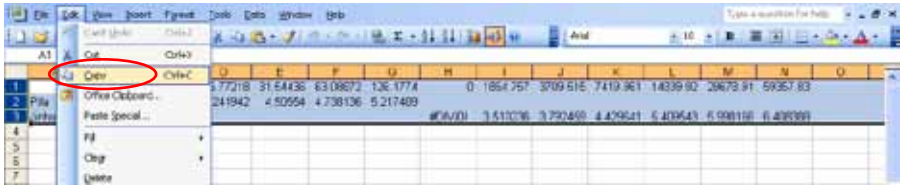
Note: Row 1 contains the Dose (ng/g), *which is the X-value.*



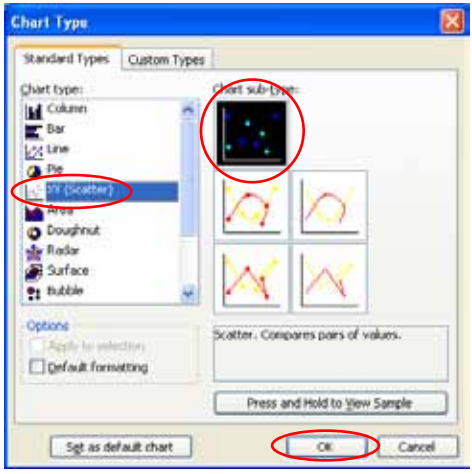
4. For the Probit values, follow step 1 to step 3 above.
   Note: The Probit values of the respective populations should vertically match with the Dose (ng/g) *values.*



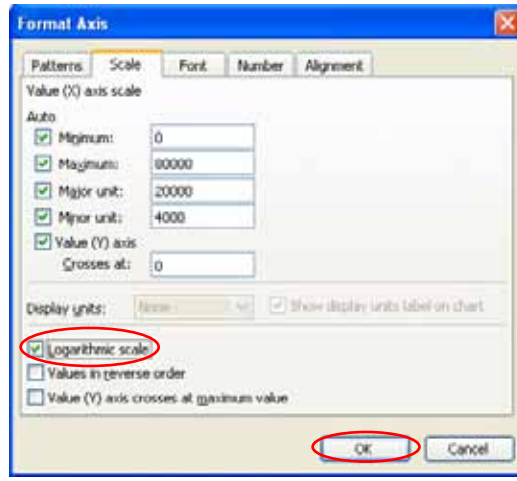
5. These are the data sets needed to plot the probit lines in PowerPoint. In the Excel file, highlight the data sets, go to the Edit menu, and click Copy.



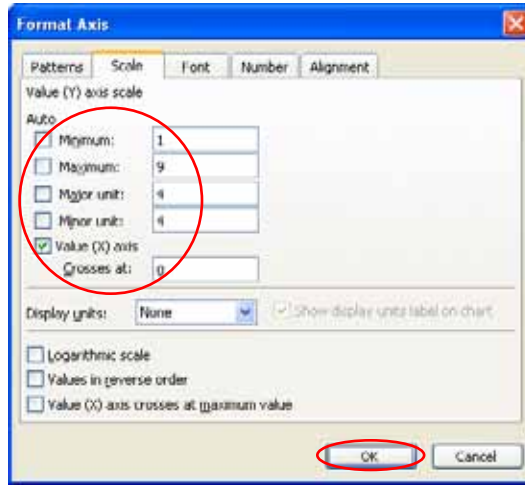
6. Then, open PowerPoint and, on the Insert menu, choose Chart and a sample bar chart will appear.
7. On the Chart menu, click Chart type. On the Standard Types option, choose XY (Scatter) chart and the first box of chart subtype, and then click OK.



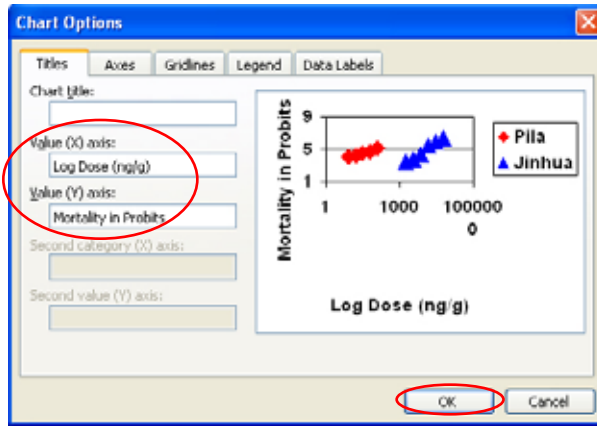
8. Then, Paste the Excel data sets on the PowerPoint data sheet.
9. Click the chart's x-axis and on the Format menu choose Selected Axis. On the Scale tab, check the Logarithmic scale and then click OK.



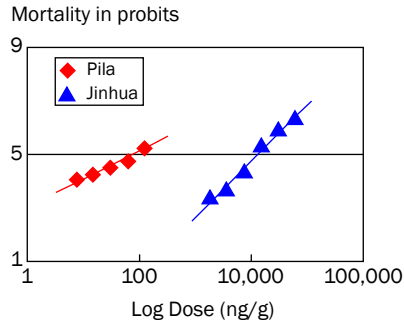
10. Next, select the chart's y-axis and click on the Format Axis. On the Scale tab, make the following adjustments: Minimum = 1, Maximum = 9, Major unit = 4, Minor unit = 4, and then click OK.



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.

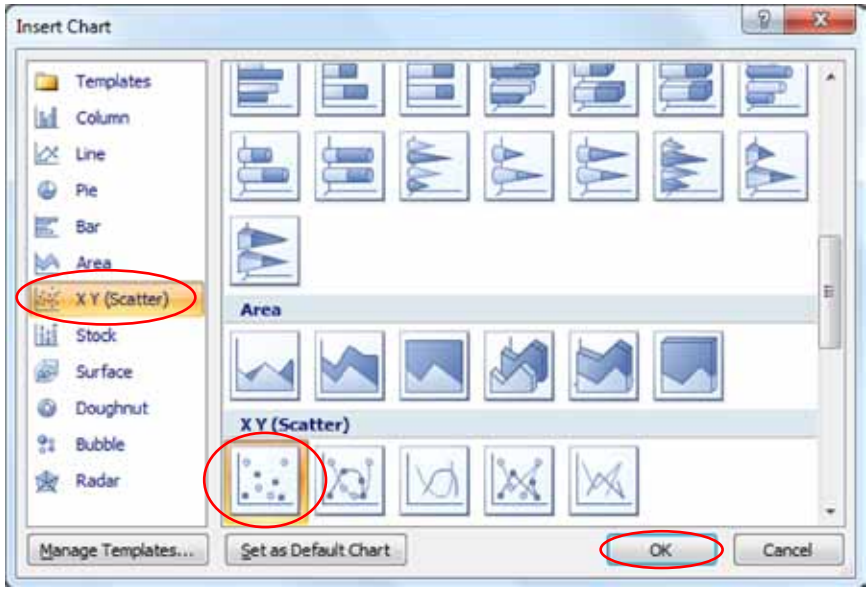


12. Lastly, choose the Line tool and a line to represent a good fit is drawn along the points of the respective data sets. The final graph is shown below.

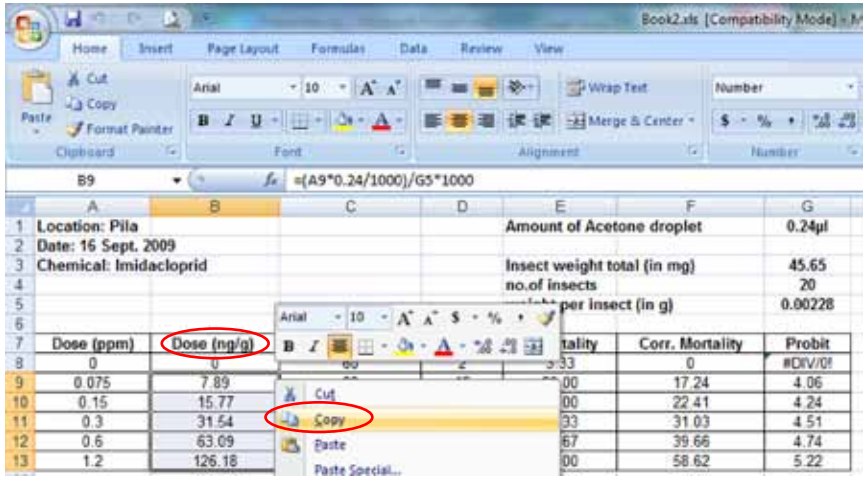


For Microsoft Office 2007 and later versions:

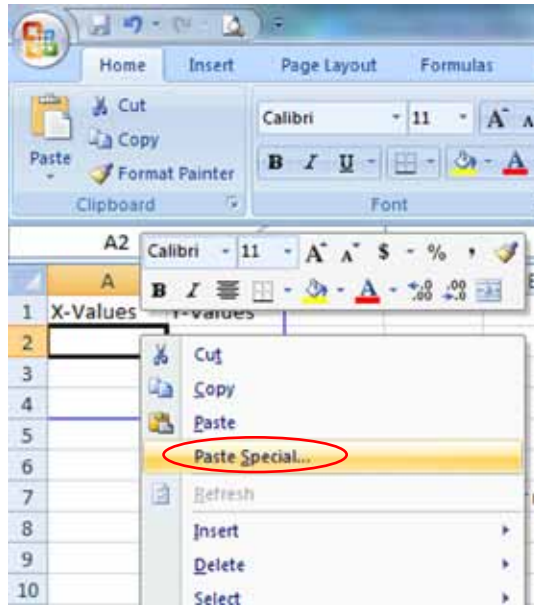
1. Open Microsoft PowerPoint 2007.
2. Insert a chart by choosing the Insert menu and clicking on the Chart icon. Several chart types will appear. Choose X Y (Scatter), click Scatter with only Markers, and then click OK. An Excel worksheet for the X and Y values of the chart will appear.



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
Insert
Page Layout
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Copy
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Paste
Clipboard
Calibri
11
Font
A2
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Paste Special...
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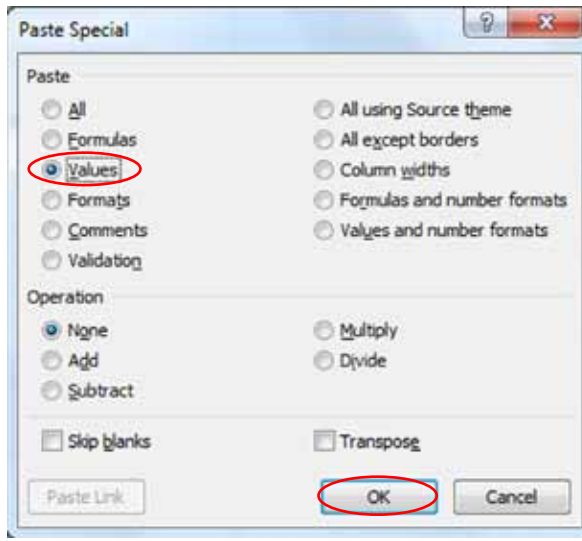
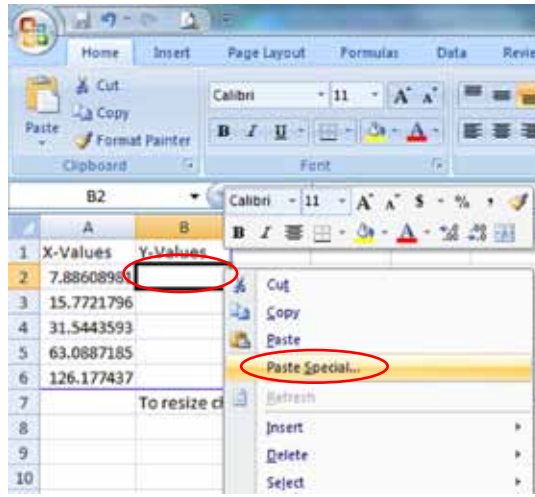
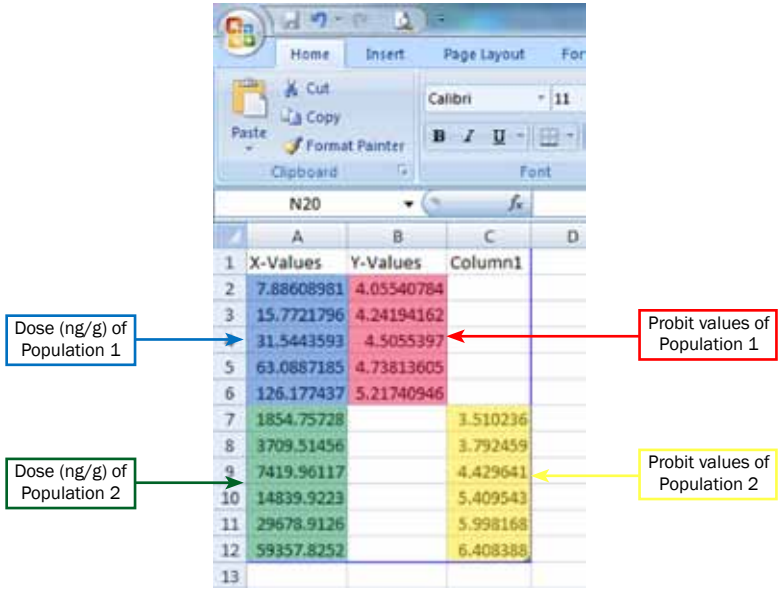

Paste Special
Paste
All
Formulas
Values
Formats
Comments
Validation
All using Source theme
All except borders
Column widths
Formulas and number formats
Values and number formats
Operation
None
Add
Subtract
Multiply
Divide
Skip blanks
Transpose
Paste Link
OK
Cancel

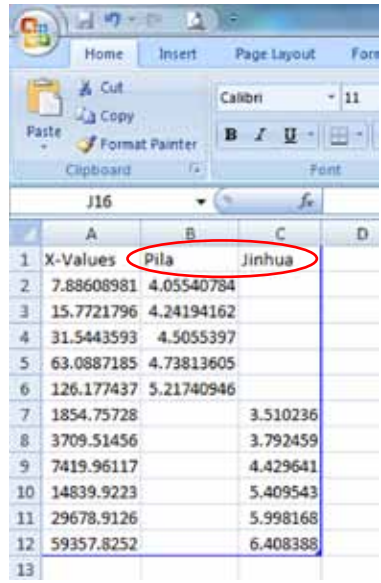
5. Highlight and Copy the Probit values, excluding the probit value for Zero ng/g. Go to the chart Excel worksheet, right-click cell B2, choose Paste Special, select Values, and click OK.



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.

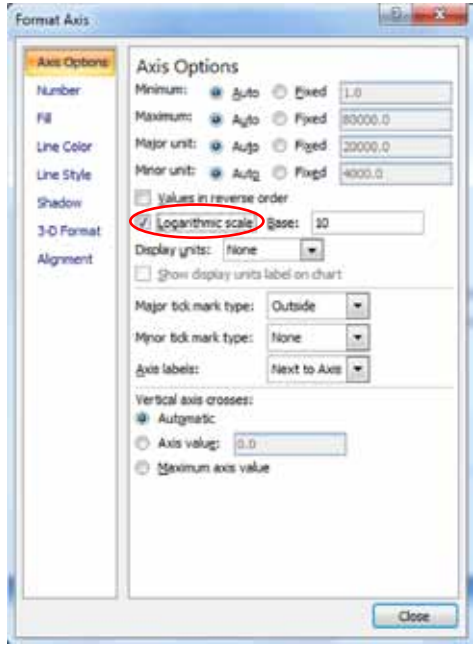


7. Label the populations by changing the first rows for each column starting from column B and so on.

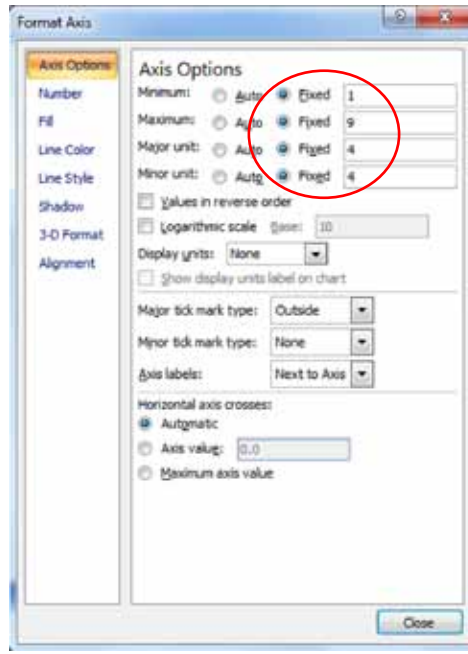


	A	B	C	D
1	X-Values	Pila	Jinhua	
2	7.88608981	4.05540784		
3	15.7721796	4.24194162		
4	31.5443593	4.5055397		
5	63.0887185	4.73813605		
6	126.177437	5.21740946		
7	1854.75728		3.510236	
8	3709.51456		3.792459	
9	7419.96117		4.429641	
10	14839.9223		5.409543	
11	29678.9126		5.998168	
12	59357.8252		6.408388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

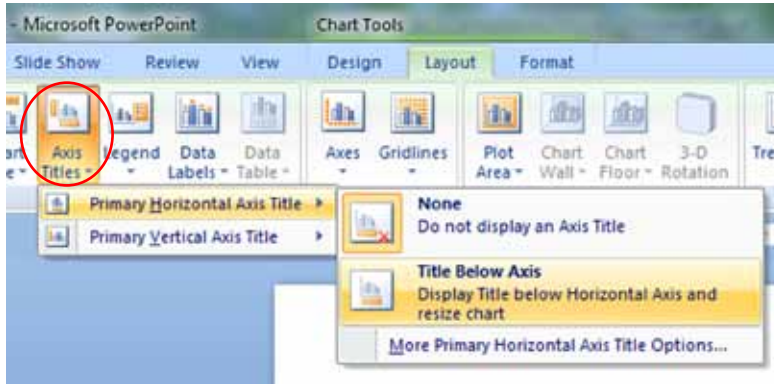
8. Go to the Microsoft PowerPoint chart. Right-click the x-axis and click Format Axis. On the Axis Options, check the Logarithmic scale option, and click Close.



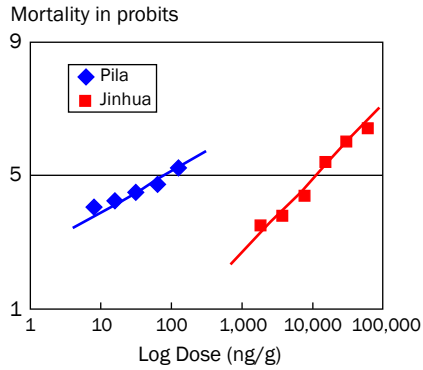
9. Right-click the y-axis, and click Format Axis. On the Axis Options, make the following adjustments: Minimum = 1, Maximum = 9, Major unit = 4, Minor unit = 4, and then click Close.



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.

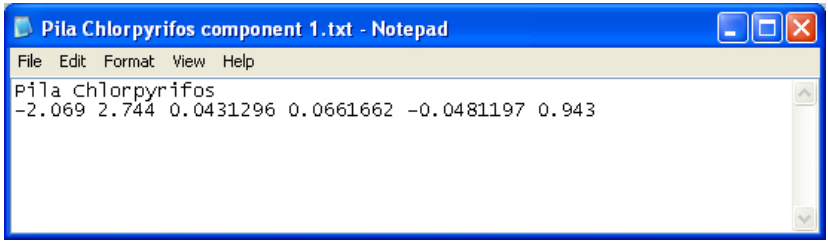
Data files

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)

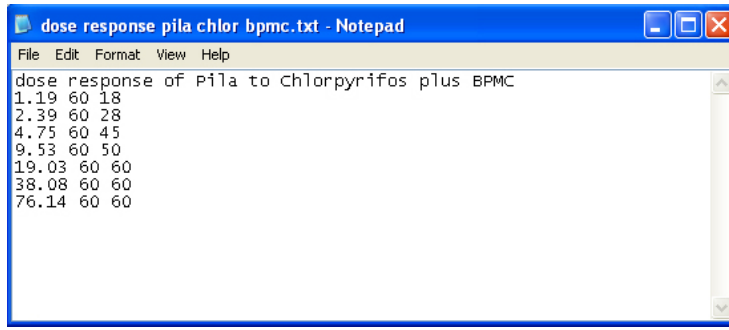
Effective Doses

	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).

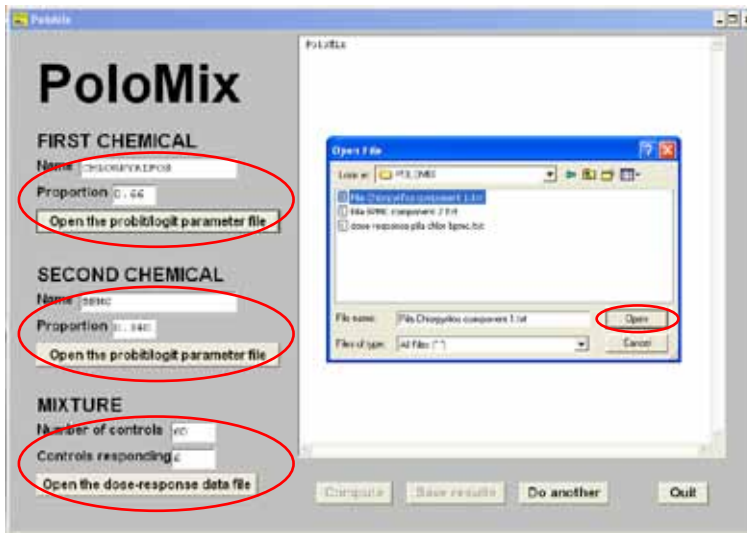
2. The same procedures as above are followed to create another file for chemical 2 (BPMC).
3. Then, a dose-response data file is created for the mixture (Chlorpyrifos plus BPMC).
   a. Likewise, the first line is for identification, followed by the dose-response data.
   b. Each of the data lines has 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.

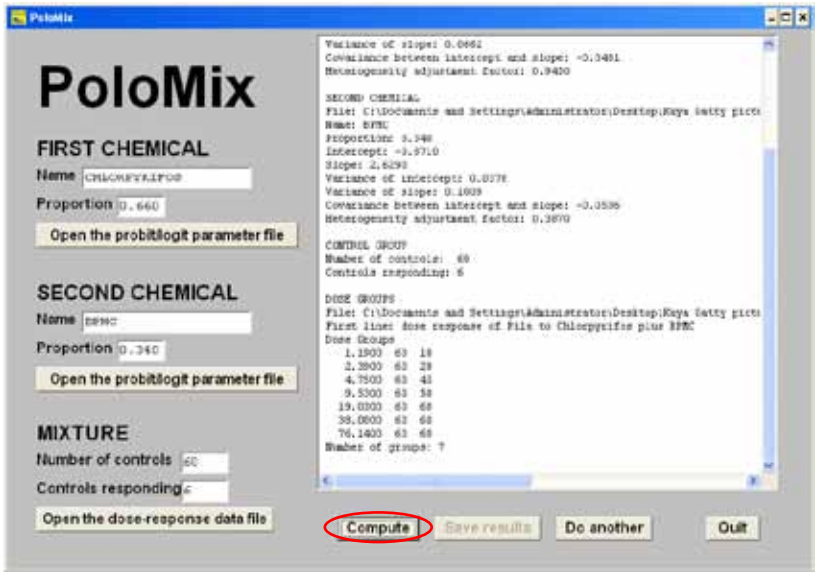


Using the PoloMix© *program*

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.

Antagonism

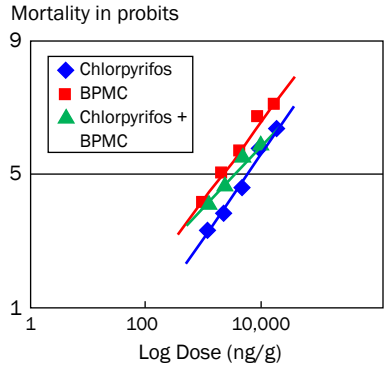


Fig. 7.3. Probit lines of two chemicals (Chlorpyrifos and BPMC) and the mixture.

Table 7.1. PoloPlus© outputs of Chlorpyrifos, BPMC, and the mixture.

Chemical	LD50 (ng/g)	Fiducial limits (95%)	Slope (+se)	Heterogeneity
Chlorpyrifos	5676.6	4890.17 to 6618.16	2.75 (0.26)	0.94
BPMC	2146.26	1683.63 to 2612.15	2.63 (0.32)	0.39
Chlorpyrifos + BPMC (2:1)	2875.11	1332.02 to 4471.33	2.25 (0.29)	1.48

Synergism

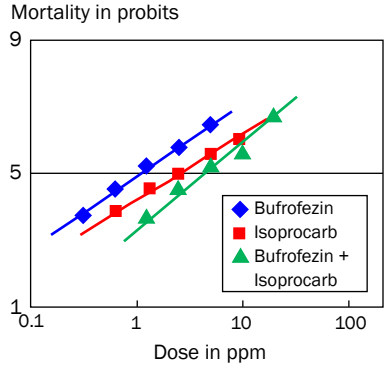


Fig. 7.4. Probit lines of a Buprofezin-Isoprocarb mixture using dipping method.

Table 7.2. PoloPlus© outputs of Buprofezin, Isoprocarb, and the mixture.

Chemical	LD50 (ppm)	Fiducial limits (95%)	Slope (+se)	Heterogeneity
Buprofezin	1.02	0.77 to 1.29	2.15 (0.28)	0.1
Isoprocarb	2.38	1.69 to 3.18	1.63 (0.24)	0.37
Buprofezin + Isoprocarb (1:4)	3.92	2.96 to 4.94	2.20 (0.28)	0.86

CHAPTER 8:

Analyzing quantal response data with multiple explanatory variables with PoloEncore©

An insect's response when exposed to an insecticide may sometimes be due to more than just the insecticide dosage. Factors can be rearing method, body weight of the insect, temperature, and methods of insecticide exposure. Discussions in the previous chapters centered on using a single explanatory variable. To incorporate the possible effects of other factors, experiments can be set up with more than one variable and the data can be analyzed using a multiple regression model or probit plane (Finney 1971).

PoloEncore© is a statistical software developed by LeOra that focuses on binary response models with multiple explanatory variables. This program offers a useful technique to test the significance of more than one variable on a specific response in a single experiment. The probit and logit line is paired with additional variables besides the dose. Examples of additional variables such as rearing condition (controlled room or greenhouse), form of diet, and type of bioassay method (dipping or topical application) are discussed.

The experiments

Two experiments were conducted to illustrate the binary response of brown planthoppers with multiple explanatory variables. In the first experiment, the variables used were (1) type of bioassay method (topical application and dipping) and (2) dose to create responses, which are mortality and physical abnormalities. The insecticide used is buprofezin, an insect growth regulator. The second experiment consists of two variables: (1) rearing method or condition (seedling box in controlled room and potted plants in the greenhouse) and (2) dose.

Bioassay methods and dosage used

Two separate and simultaneous bioassay setups were prepared. The first setup used the dipping method and the second, topical application. Collection and rearing of test insects were based on the methods described in Chapter 4.

a) Dipping method

Third instar nymphs were used in this setup. The stock solutions and procedures of the method were prepared as described in Chapter 9. The responses were recorded for 3 days on a data sheet (Table 8.1).

Table 8.1. Responses of third instar BPH nymphs to buprofezin using the dipping method.

Day 1				
	Mortality/nymphs with malformations			
Dose (ppm)	Rep 1	Rep 2	Rep 3	Total
0	1	0	0	1
0.6	1	1	0	2
1.2	3	2	2	7
2.4	3	1	3	7
4.9	2	3	2	7
9.8	5	3	3	11

Day 2				
	Mortality/nymphs with malformations			
Dose (ppm)	Rep 1	Rep 2	Rep 3	Total
0	1	0	0	1
0.6	2	3	3	8
1.2	2	5	2	9
2.4	3	4	3	10
4.9	4	4	4	12
9.8	4	4	2	10

Day 3				
	Mortality/nymphs with malformations			
Dose (ppm)	Rep 1	Rep 2	Rep 3	Total
0	2	0	0	2
0.6	4	3	3	10
1.2	5	7	5	17
2.4	5	7	5	17
4.9	7	6	6	19
9.8	6	8	9	23

b) Topical application

Nymphs in their third instar were also used in the setup. The test solutions and procedures for the topical application were prepared using the steps described in Chapter 5. The responses were also recorded for 3 days on a data sheet (Table 8.2).

Table 8.2. Responses of third instar BPH nymphs to buprofezin using the topical application method.

Day 1				
	Mortality/nymphs with malformations			
Dose (ppm)	Rep 1	Rep 2	Rep 3	Total
0	5	4	4	13
1.2	5	5	5	15
2.4	6	5	4	15
4.9	3	6	7	16
9.8	6	6	5	17
19.5	7	5	5	17

Day 2				
	Mortality/nymphs with malformations			
Dose (ppm)	Rep 1	Rep 2	Rep 3	Total
0	5	4	5	14
1.2	5	6	5	16
2.4	6	5	5	16
4.9	6	7	7	20
9.8	8	8	5	21
19.5	8	7	7	22

Day 3				
	Mortality/nymphs with malformations			
Dose (ppm)	Rep 1	Rep 2	Rep 3	Total
0	5	4	5	14
1.2	6	6	5	17
2.4	6	6	6	18
4.9	6	7	8	21
9.8	8	8	5	21
19.5	8	9	7	24

The data from Tables 8.1 and 8.2 were converted to a readable format for Polo-Encore© (steps will be described on the succeeding pages). The likelihood ratio (LR) tests showed that the hypotheses of parallelism, equality given parallelism, and equality are all not rejected. This indicated that the two variables or planes were parallel and equal. We can infer that, in testing buprofezin, the same responses could be obtained by both experimental methods.

Insect rearing methods and dose

This experiment consisted of two variables: (1) rearing methods (seedling boxes in a controlled room and in potted plants in the greenhouse) and (2) dose. Two setups were prepared: the first was for rearing of insects from a seedling box and the second for rearing insects from potted plants. Collection and rearing of insects from seedling boxes and potted plants were based on the methods described in Chapter 4. The topical application technique with fipronil as the active ingredient was used for the bioassays; the procedures for this method are found in Chapter 5.

a) Test insects from seedling boxes

One-day-old brachypterous females reared in seedling boxes inside a controlled room (27 $\pm$ 2 °C) were used in this setup. Topical application was used and mortality was recorded on a data sheet after 24 hours (Table 8.3).

Table 8.3. Responses to fipronil of 1-day-old brachypterous females reared in a seedling box.

Dose (ppm)	Total insects	# dead
0	60	2
0.3	60	9
0.6	60	21
1.2	60	32
2.4	60	45
4.9	60	55

b) Test insects from potted plants

One-day-old brachypterous females reared in potted plants inside a greenhouse were used in this experiment. The topical application results are shown in Table 8.4.

Table 8.4. Responses to fipronil of 1-day-old brachypterous females reared in potted plants inside a greenhouse.

Dose (ppm)	Total insects	# dead
0	60	1
0.3	60	11
0.6	60	21
1.2	60	34
2.4	60	48
4.9	60	57

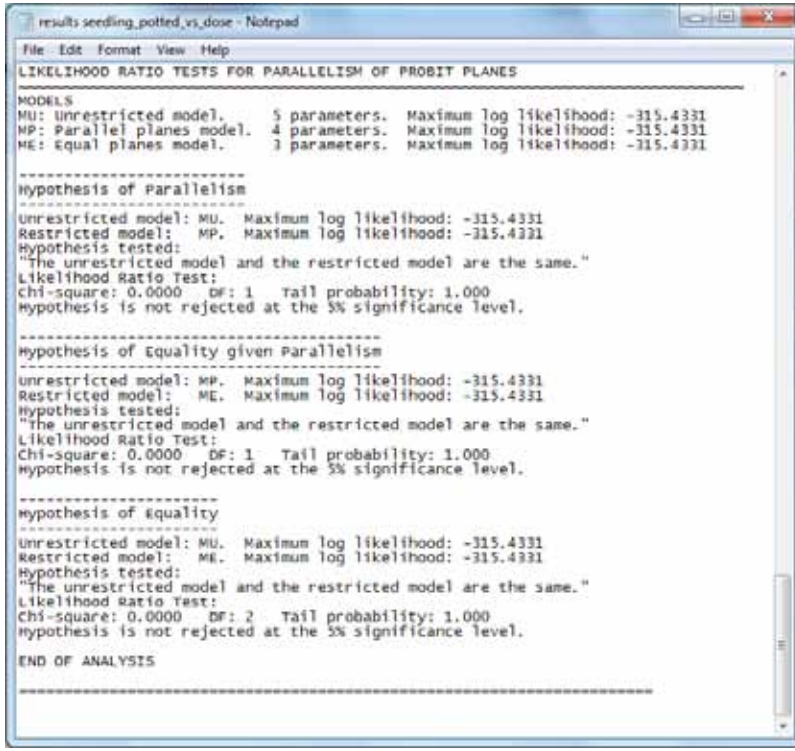


Fig. 8.1. Sample results generated by PoloEncore©.

Based on the likelihood ratio (LR) tests (Fig 8.1), the hypotheses of parallelism, equality given parallelism, and equality are all not rejected. This implies that the type of rearing method and condition has no significant effect on the responses of BPH to fipronil. The two variables or planes are parallel and equal.

Installation of PoloEncore©

PoloEncore© is compatible with a Windows 95 operating system and more recent versions. The packaged software comes in a CD together with other Polo statistical software. The steps in installing the software follow:

1. Place the Polo CD in the drive and go to My Computer.
2. Select and double-click on “SetupPoloEncore.exe.”
3. Choose “No-Questions-Asked” Installation.
4. Click on “Thanks” to end the installation.
5. Double-click on the PoloEncore icon to start the program.

Creating a data file

PoloEncore© runs only on data created with a .txt extension file. The simplest program that can be used to generate a .txt file is Microsoft Notepad (Fig. 8.2).

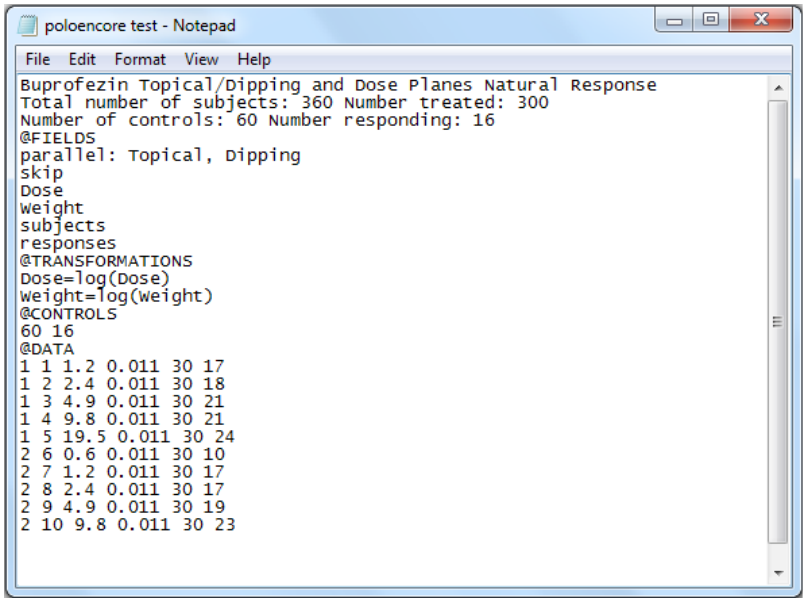


Fig. 8.2. Example of a readable PoloEncore© data file created in Microsoft Notepad.

Comment line

The first lines are the title and comments usually describing the data. The number of lines that can be used for the title and comment line is unlimited but note that the beginning of these lines should not contain the symbol @. In the example above, the title and comments are found on lines 1 to 3.

@FIELDS

This part presents the column headers of the text file. In Figure 8.2, the first header title describes the two variables paired with the dose. The word "parallel" is used to test the hypothesis of parallelism. In the sample data, number 1 represents the topical application and number 2 the dipping method. The word "skip" represents the number of lines. "Dose" and "Weight" are next, in which the doses (without the control) are

lined up together with the average individual weight of the insect. The "subjects" are the total number of insects treated. This is followed by the number of test subjects with responses in the "responses" portion. One must note that, in the @FIELDS section, the labels must(1) appear in the same order as in the @DATA section, (2) there should be no repetition among the field labels, and (3) only labels containing letters, numbers, or a combination of the two are allowed.

@TRANSFORMATIONS

This section uses different kinds of formulas similar to those used in graphing calculators and in other programming languages. Examples follow:

1. Addition, subtraction, multiplication, and division are represented by the symbols +, -, *, and /, respectively.
2. Logarithm base 10 and square root functions are written as log and sqrt.
3. The ^ symbol represents exponential forms (e.g., x^y, which means x to the power of y).

The symbol for calculations can be used by adding any name from the @FIELD section with an equal (=) sign on the right followed by the formula. In Figure 8.2, log (Dose) is calculated from the Dose the same as the Weight. Take note that writing the parameters from the @FIELD section for calculation on the @TRANSFORMATION requires case sensitivity©—meaning that upper case is different from lower case, for example, "Weight" is different from "weight."

@CONTROLS

This part requires only one line made up of two groups of numbers separated by a single space: the total number of controls and the total number of controls with responses.

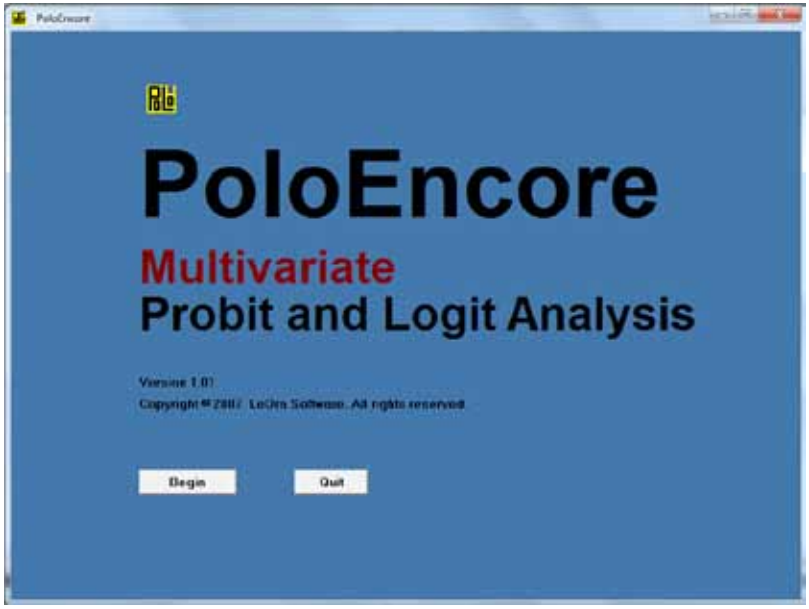
@DATA

This section represents the columns matched to the order of the labels in the @FIELDS section. Any number is allowed but scientific notations; for example, 2.345e3, are not readable. In the example illustrated in Figure 8.2, the first column represents the parallel fields (topical application and dipping method). The second column contains the number of lines. The next columns are for the doses, weight, total number of test subjects, and total number of subjects with responses.

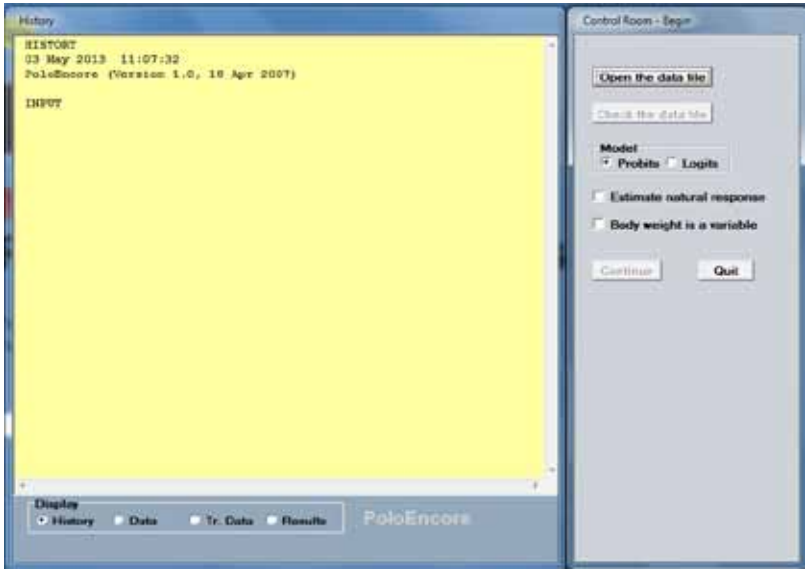
Running the data file in PoloEncore©

After creating the data file in Microsoft Notepad, open the PoloEncore© program by double-clicking on its icon.

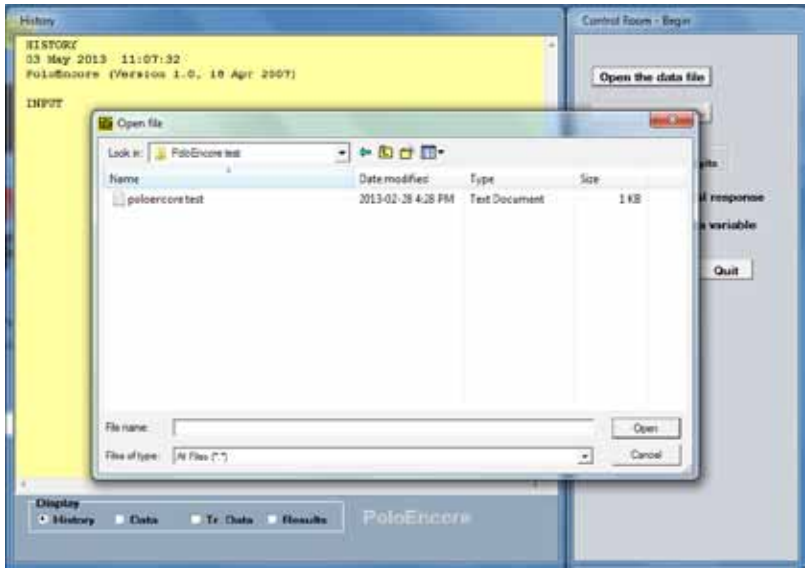
1. On the title screen, click on "Begin" to start the program.



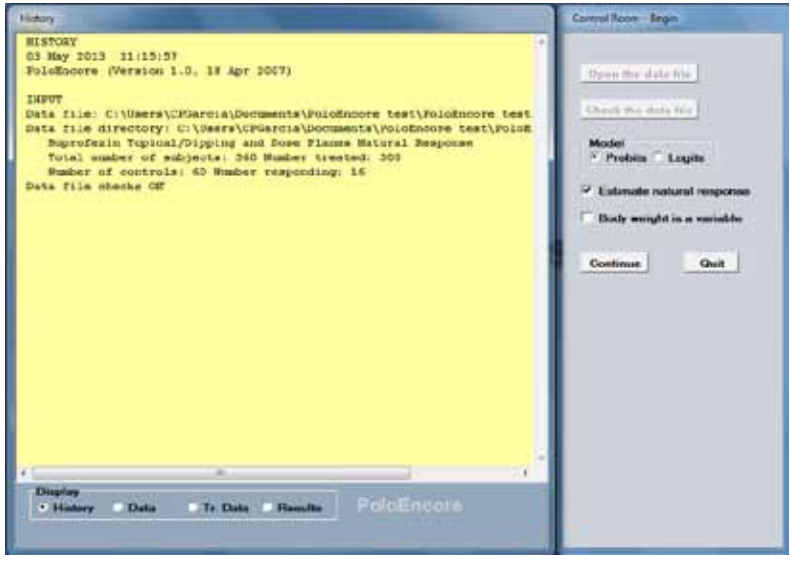
2. A two-window screen appears after "Begin" is selected: the "Control Room" on the right and "History" on the left. The "History" window identifies the program and displays the current date and time of day. The "Control Room" window provides the "Open the data file" option to access the data files for analysis. It also has a probits or logits option and boxes to select if natural response is a parameter or if body weight is a variable.



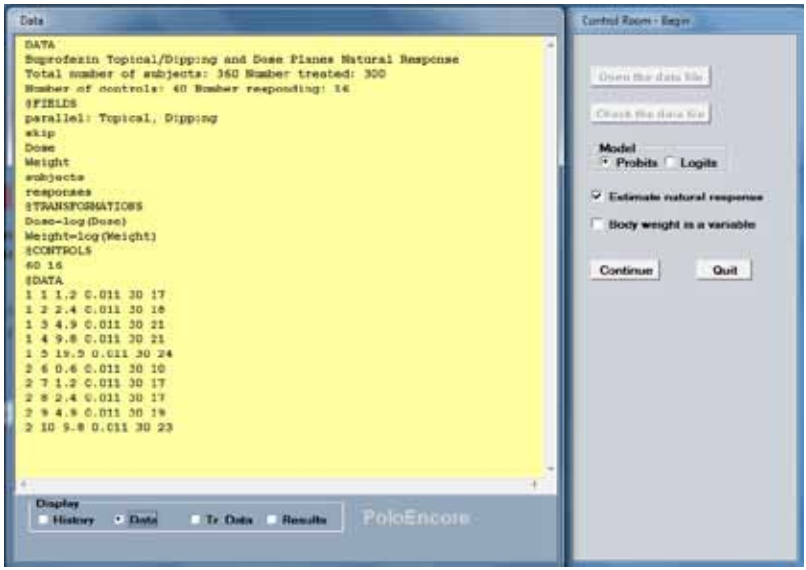
3. Begin by clicking on the “Open data file” button on the Control Room window. Locate the .txt file and click on “Open.”



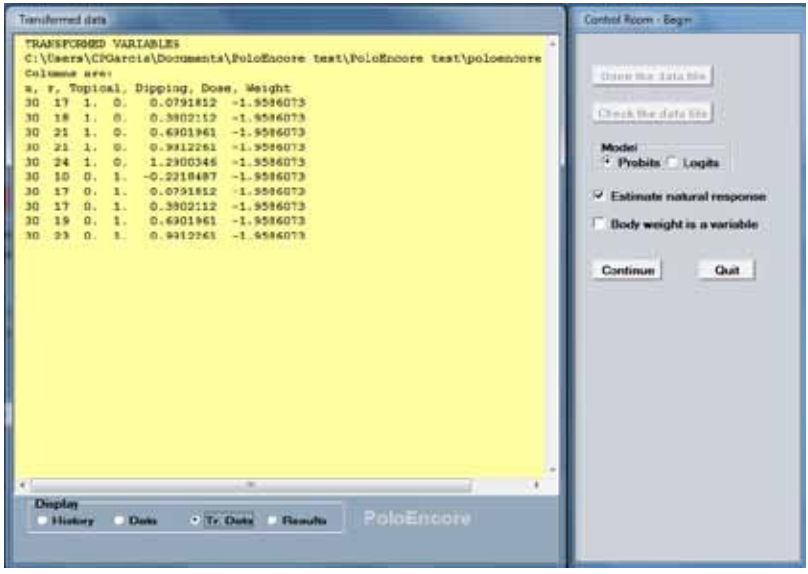
4. After uploading the file, click on “Check the data file” to check if there are significant errors in the data set. If there are any errors, a pop-up window will appear to indicate the specific error in the data file (e.g., Error in line number 16). Otherwise, the program acknowledges the data and displays “Data file checks OK” on the history window.



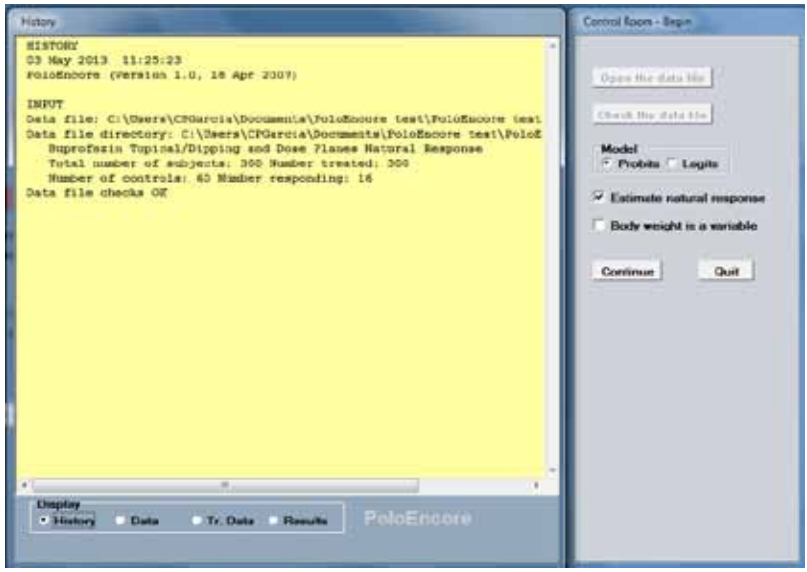
5. Before running all the data for analysis, you can view the display options found below the window named “History,” “Data,” and “Tr. Data.” “Results” can be viewed after pressing the “Continue” button.
6. “Data” show the whole data file that is uploaded for review.



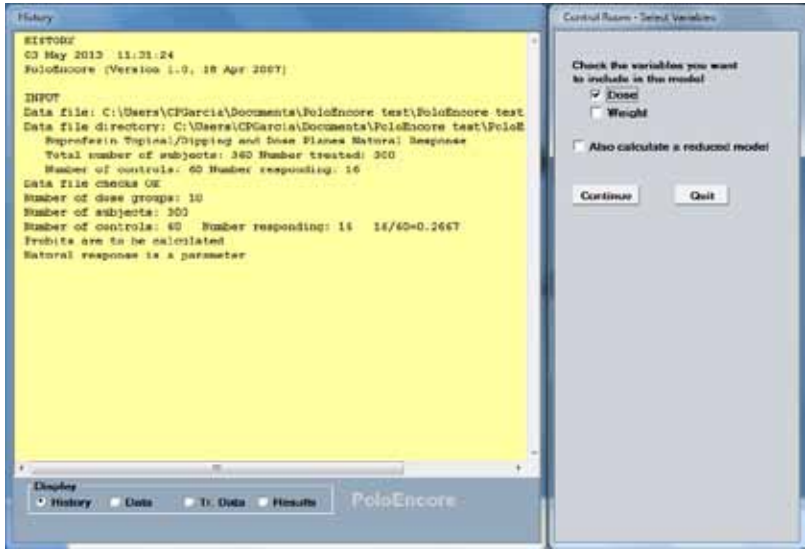
7. “Tr. Data” displays the data with the labels from @FIELDS presented on top of each @DATA column.



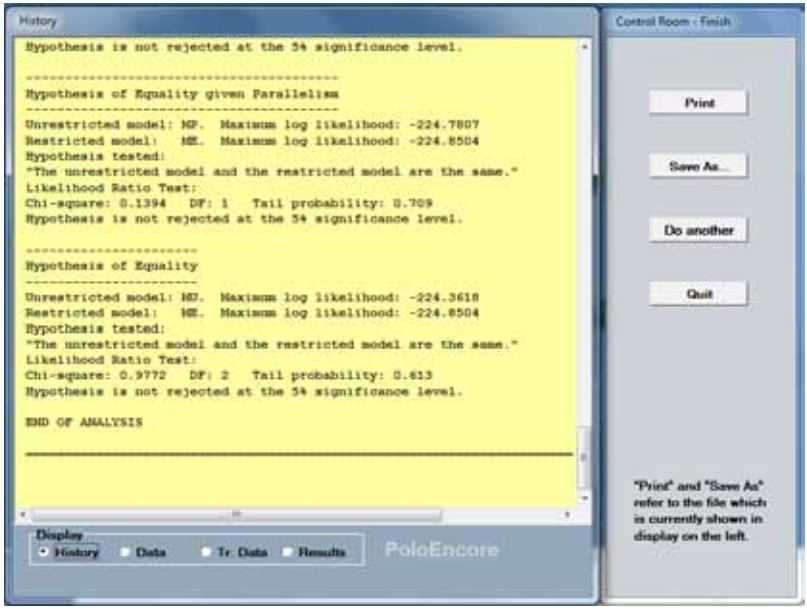
8. After reviewing the data file, click on “Continue” on the Control Room window.



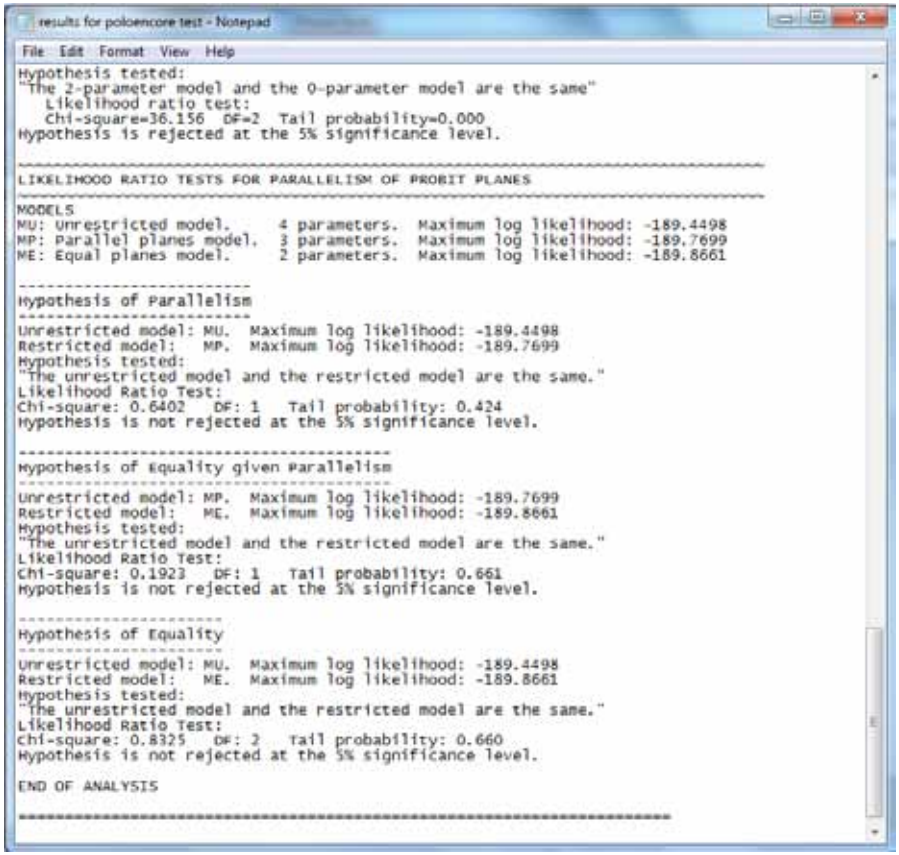
9. Next, choose the variables to be included in the results. In the example, the dose should be included in relation to the type of bioassay method (topical or dipping). In this example, Dose is chosen and Weight not chosen, since this is not included as the test variable.



10. Click on “Continue” to run the analysis. The results are displayed next. Several options in the Control Room can be chosen: (1) Print—for printing the results directly; (2) Save As—saves all the results into a .txt extension file; (3) Do another—for uploading and running another data file; and (4) Quit—to exit the whole program.



Interpretation of results



PoloEncore© generates three likelihood ratio (LR) tests of the hypotheses of parallelism, equality given parallelism, and equality. In the example above, the LR tests are all not rejected, indicating that the two variables or planes tested are parallel and equal. We can now conclude that the two treatment methods, dipping and topical application, generated the same responses.

CHAPTER 9:

Other forms of dose-response

The discussions and examples prepared in the last four chapters have focused on dose-response and estimating LD_{50}s. Although these experiments can be conducted easily in the laboratory, occasions occur when the insecticides active ingredients have low solubility or the solvent itself is affecting the test insects significantly. Some insecticides are systemic and are absorbed by the plant, distributed throughout the plant sap, and affect the insects feeding on them. Various experiments to evaluate systemic insecticides can be found in Busvine (1971). Some insecticides act on insects indirectly by interfering with the molting process, such as buprofezin. The solubility of buprofezin in acetone is low and data collected from dose-response experiments were inconsistent. Also, mortality does not occur immediately after treatment. In such cases, concentration-response experiments can be used. Here, we describe one such experiment to estimate the median lethal concentration (LC_{50}).

Estimating the median lethal concentration (LC_{50}) using the dipping method

Preparation of test insects

A known rice field is identified for planthopper collection. The initial population is reared up to its second generation using the method described in Chapter 4. The age of planthoppers is monitored and third instar nymphs are used for the experiment.

Preparation of insecticide solution

Insecticide stock solutions and dilutions are prepared using a 0.05% dilute aqueous detergent (Tween 20) solution as the solvent. A known technical grade of the insecticide (95−99% pure) is used for the test. Weighing of the chemical and the preparation of the stock solution are done using the methods described in Chapter 5. The chemical is transferred into a 2-mL standard volumetric flask and is dissolved using a small quantity of acetone. Additional acetone is added to reach the 2-mL mark. The flask containing the insecticide is again dissolved in an Erlenmeyer flask with 500mL distilled water mixed with 0.05% dilute aqueous detergent (Tween 20). This is now labeled as the stock solution. Another five Erlenmeyer flasks each containing 250mL water with 0.05% dilute aqueous detergent are prepared, the serial dilutions (1:1) are performed, and six concentrations are prepared (Fig. 9.1).

Fig. 9.1. Preparation of stock solution: (A) technical grade insecticide dissolved inside a standard volumetric flask; (B) Erlenmeyer flasks with insecticide dilutions.

Insecticide treatment

Thirty-day-old TN1 plants are used for the dipping method. The plants with secondary tillers removed and leaves cut are dipped in the respective insecticide solutions for 30 seconds and afterwards allowed to air-dry for 30 minutes on top of a mylar film under a shaded area. The treated tillers are placed in a large test tube containing 2mL rice nutrient solution (Yoshida et al 1976). Ten third instar nymphs are introduced into each tube and then covered with a mesh cloth (Fig. 9.2). Three replicates (from different cohorts of brown planthoppers) for each concentration are used. Mortalities and physical abnormalities (as determined by external deformed appearance) are observed and recorded daily from 1000 to 1100 for 5 days.

Analyzing the median lethal concentration (LC_{50}) using PoloPlus©

The concentrations (in ppm) of the insecticides and the insect responses are recorded using a word-processing program compatible with PoloPlus©. The data format and running on PoloPlus© are described in Chapter 6.

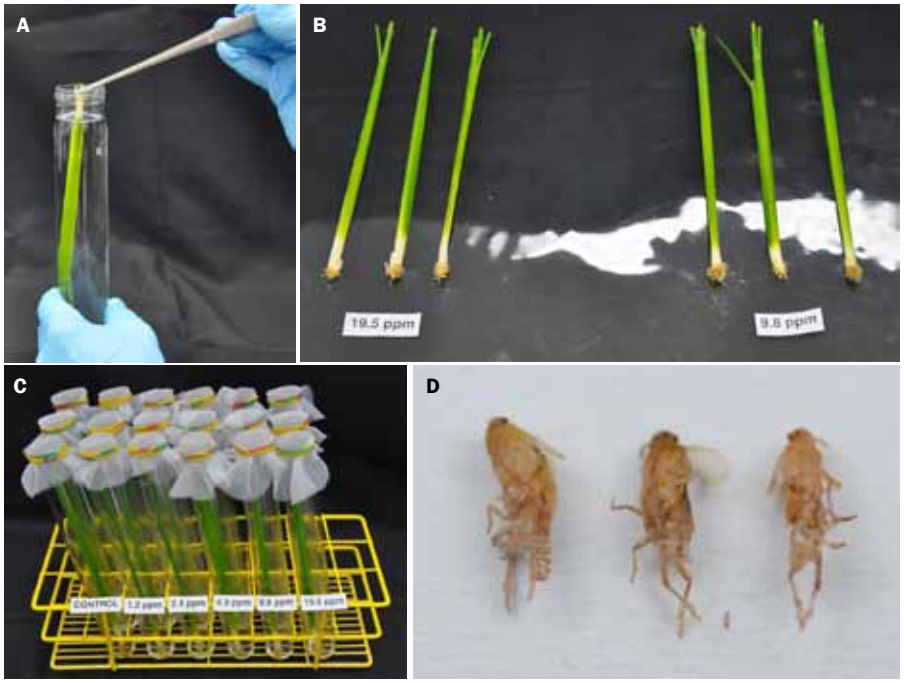


Fig. 9.2. (A) Rice plant dipped in a large test tube with insecticide solution, (B) rice plants dried on a mylar cage, (C) experimental setup, and (D) sample of physical abnormalities.

Table 9.1. LC_{50} of buprofezin on planthopper third instar nymphs.

Population	LC_{50} (in ppm)	Fiducial limits (95%)	Slope (SE)	Heterogeneity
San Pablo, Philippines	1.42	0.76 to 2.04	2.41 (0.52)	0.81

Table 9.1 presents the parameters generated by the PoloPlus© program using the response data on the fifth day after treatment. The LC_{50} of buprofezin in planthopper nymphs was found to be 1.42 ppm per insect.

Estimating the median lethal exposure time (LT_{50}) in planthoppers' tolerance of high temperature

Time-dose and insect mortality are another relationship in the study of insect responses to time exposed to stresses. Insecticides may express speed of kill and residual activity. The response is the exposure time that an insect is tolerant of physical stresses such as high temperature or submergence or high pressure. Here, we describe a time-dose-response experiment to determine the LT_{50} of planthoppers to high temperature.

Temperature response studies in the phytotron have shown that brown planthopper survival rates changed dramatically when exposed to a constant temperature of 40°C

(Heong et al 1995). A bioassay can be used to estimate the median lethal exposure time at this extreme temperature to estimate the population's tolerance of high temperature.

Preparation of test insects

Insects were collected and reared for five generations using the method described in Chapter 4. One-day-old brachypterous and macropterous females were collected from the culture and 10 of each were placed into cylindrical mylar cages with a 60-day-old rice plant trimmed to a single tiller. For each adult form, 35 replicated mylar cages were placed in an oven set at 40°C. At 5-hour intervals, five randomly selected cages were removed from the chamber and insect mortality was observed. A similar setup at room temperature was used as the control. The same experiment was performed in Los Baños, Philippines, and in Khon Kaen, Thailand, to determine the differences in planthoppers' responses to high temperature between these two locations.

The time-dose was time in hours the insects had remained in the 40°C chamber and the response was mortality. The time-dose-response data were analyzed using probit analysis done by PoloPlus© and the LT_{50}s were estimated (Table 9.2).

Table 9.2. Median lethal exposure time (LT_{50}) of the brown planthopper to 40°C (data from Heong et al 1995).

	Location	LT_{50} (h)	Fiducial limits
Brachypterous females	Los Baños	16.9	14.8–17.1
	Khon Kaen	285.9	129.4–2,038
Macropterous females	Los Baños	47.3	38.6–72.1
	Khon Kaen	140.2	86.5–372

As shown in Table 9.2, the planthopper population in Khon Kaen had higher tolerance of high temperature.

CHAPTER 10:

Monitoring insecticide resistance stability point

As new insecticides are introduced into the market and used frequently, pest populations adapt and develop resistance. Chapter 1 provides some discussion on insecticide resistance and its mechanisms. In intensive rice production systems where insecticides are routinely applied, resistance builds up rapidly and causes a decline in chemicals' efficacy. Reports of multiple-fold development of resistance and parallel resistance are found in many rice-growing countries (Matsamura et al 2008).

Resistance is a genetic change in response to selection by insecticides. More than 500 insect pest species have developed resistance to at least one insecticide in the last 40 years, and the number of resistant species is growing exponentially. Resistance management is thus essential, and, as new management tactics are deployed, such as a new chemical or a new rice variety, improved resistance management strategies should be used to prevent or slow the development of resistance. The goals of resistance management are to avoid developing resistance, to slow down the rate of resistance development, and to revert back to susceptible populations by withdrawing the sales of active ingredients.

Reversion and resistance stability point

When insecticide pressure is removed, insect populations that had acquired resistance often revert and resistance declines—this is known as resistance reversion. The resistance may not revert back to that before the insecticide was introduced. When field-collected populations are reared in an insecticide-free laboratory, there is usually a rapid decline in resistance in the first few generations and a stable level is often established after 10−15 generations—the resistance stability point. This is an estimate of how well resistance persists in the insect population when an insecticide is no longer used. The rate of reversion to this stability point varies enormously between insect populations and active ingredients.

To determine the resistance stability point of a test population for a particular insecticide, we collected field planthoppers and reared them through 30 generations. At periodic generation intervals, we took sample insects, measured LD_{50}s, and plotted them with the generations (Fig. 10.1).

Rearing and preparation of test insects

The rearing and collection methods follow the same procedure described in Chapter 4. The insects are continuously mass-reared inside a flexi-glass cage and provided with TN1 rice seedlings as feeding plants. Resistance is monitored from the second to the fifth generation and at every 10 generations using the topical application method described in Chapter 5.

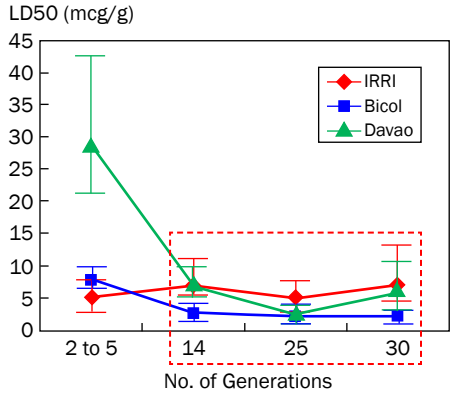


Fig. 10.1. Resistance stability points of three populations for Fenobucarb.

Estimating the LD_{50}s

The procedures for preparing stock solutions and the topical application are based on the procedures described in Chapter 5. Mortalities are recorded at 24 hours after treatment and the data are analyzed using the PoloPlus© program.

Resistance stability point

The resistance stability point of an insecticide is the LD_{50} of the pest population that will not change significantly with increasing generations in insecticide-free conditions. This point represents the resistance that the pest population had retained.

When researchers compare the resistance of insecticides, LD_{50}s of field populations after two to five generations are often used. Here, we suggest that "resistance stability point" recorded in the 14th generation after field-collected planthoppers are reared under insecticide-free conditions be used as the reference. Using the LD_{50}s determined from freshly collected insects after a few generations may provide a biased estimate of the resistance factor.

Figure 10.1 shows the decline in LD_{50}s of planthoppers from the second to fifth generation to the 30th generation. From the 14th to the 30th generation, the differences in LD_{50}s were not significant. The resistance stability point for BPMC of three populations collected in the Philippines seems to converge to about 5 mcg/g.

Table 10.1 shows the resistance stability points of different populations in three countries for fipronil, imidacloprid, and BPMC in the 14th generation.

Using the PCR method for detecting target-site insecticide resistance in brown planthopper

The main objective in monitoring insecticide resistance is to develop and implement resistance management strategies to slow down the development of insecticide

Table 10.1. Resistance stability points of different populations in China, the Philippines, and Thailand for three active ingredients in the 14th generation.

Active Ingredient	Population	Stability point (LD_{50} in ng/g)	Fiducial limits (95%)	Slope (+SE)	Heterogeneity
Fipronil	Hangzhou	166.77	77.09 to 292.48	1.06 (0.16)	0.41
	Jinhua	146.83	87.01 to 246.13	1.15 (0.16)	0.02
	IRRI	111.49	91.69 to 129.43	4.16 (0.68)	0.24
	Ang Thong	87.23	62.14 to 111.94	2.21 (0.34)	0.69
Imidacloprid	Hangzhou	34.87	16.69 to 64.62	0.79 (0.11)	0.37
	Jinhua	372.55	207.11 to 693.42	1.00 (0.15)	0.57
	IRRI	9.29	6.81 to 11.88	1.99 (0.30)	0.30
	Chainat	1,908.02	1,327.11 to 2,547.96	1.70 (0.25)	0.06
BPMC	Hangzhou	2,525.61	1,867.25 to 3,317.32	1.80 (0.22)	0.61
	Jinhua	1,423.85	1,008.34 to 1,962.85	2.14 (0.31)	0.47
	IRRI	7,061.07	4,952.81 to 11,536.45	1.62 (0.39)	0.95
	Nakhon Ratchasima	1,586.08	1,100.75 to 2,108.96	1.84 (0.31)	0.64

resistance. Effective resistance management depends on early detection using rapid, inexpensive, and simple methods. In this respect, bioassay methods are well established and commonly used. However, these tests require facilities for insect rearing and conducting bioassays that often take considerable time in field collections and maintaining insect cultures. In addition, the tests are often unable to differentiate the mechanisms of resistance in a resistant population. With recent advances in molecular techniques, a method that can potentially be used is the application of polymerase chain reaction (PCR). The advantages of PCR methods include the ease in specimen handling, less time in conducting the tests, and the ability to detect target-site resistance. However, the disadvantage of PCR is that it requires highly sophisticated laboratory equipment and suitable metabolic markers.

We describe in this chapter the PCR methods used to detect target-site resistance of brown planthopper (BPH) to imidacloprid and fipronil. Insect nicotinic acetylcholine receptors (nAChRs) are the target sites of neonicotinoid insecticides, such as imidacloprid. A target mutation, Y151S, in the BPH nAChR α1 subunit contributing to imidacloprid resistance had been identified (Liu et al 2005). In contrast, the mode of action of fipronil involves the disruption of chloride ion flow by interacting at the GABA (gamma-aminobutyric acid)-gated chloride ionophore of the central nervous system. A point mutation, A302S in GABA receptor subunit RDL, is responsible for fipronil resistance (Liu et al 2013). These metabolic markers can be used to indicate the presence of the specific target-site mutation.

1. Materials and methods

1.1 Insects used

For imidacloprid target-site resistance detection. The susceptible strain (S) of BPH obtained from the Institute of Plant Protection of the Jiangsu Academy of Agricultural Sciences had been reared continuously in insecticide-free laboratory conditions. The resistant strain (R) was collected from a field of hybrid rice in Jiangpu (Jiangsu, China) in August 2000 and reared continuously under imidacloprid selection in a laboratory for 35 generations and the resistance factor had reached 250-fold.

The strain with intermediate resistance (M) was collected from a field of hybrid rice in Jiangpu, and reared under imidacloprid selection in the laboratory for 25 generations and had a resistance ratio of about 75-fold.

Field populations were collected in Jiangpu from July to September in 2000, 2001, and 2002.

For fipronil resistance detection. The susceptible strain of BPH was from the same source in Jiangsu while the nt strain (BPH-FR) was collected from a field of hybrid rice in Hangzhou (Zhejiang, China) in August 2007 and continuously reared under fipronil-selected conditions in the laboratory and had a resistance ratio of about110-fold.

BPH-SX-13 and BPH-SX-25 populations are the populations under fipronil selection conditions for 13 and 25 generations, respectively.

The BPH-FR/S-f2 population is the first-generation offspring of hybridization between strain BPH-FR and strain S.

1.2 Designing gene-specific primers

For imidacloprid resistance detection. The gene-specific primers of imidacloprid and fipronil were designed according to the protocols (Liu and Han 2006a, Liu et al 2013) and optimized principles of Bi-PASA (Liu and Han 2006a, Liu et al 2013), based on the DNA sequences containing point mutation (Table 10.2).

Table 10.2. Gene-specific primers for Bi-PASA detection of imidacloprid and fipronil resistance.

Primer type	Primer name	Primer sequence
	Imidacloprid	
Outer primer	P	5'-ACA CGT CCC CAG TGA GCA-3'
	Q	5'-GTC GGT GGA ATG ATC TCT GC-3'
Inner primer	A	5'-GCC GTT TGG ATC CTG TAC ATC-3'
	B	5'-GCG CAT GAT TGC CGT CGT-3'
	Fipronil	
Outer primer	P	5'- GGC TGA TCG TCA TCA TAT CGT GG -3'
	Q	5'- GCA ACG ACG CGA ACA CCA TGA CG -3'
Inner primer	A	5'- TGC GAC ACC GGC ACG AGT GT -3'
	B	5'- CGG TGG TGA CGC CGA GTG C -3'

1.3 Extraction of genome DNA of a single planthopper

For genome DNA extraction, the following steps were used:

1. Preparation of buffer A: 1%（g/mL）SDS, 50 mmol/L Tris-HCl, 25 mmol/L NaCl, 25 mmol/L EDTA; all ingredients were dissolved in ultra-pure water.
2. Preparation of buffer B: 3 mol/L KAc (kalium acetate) dissolved in ultra-pure water; then, its pH was adjusted to 7.2 with NaOH (sodium hydrate).
3. A single brown planthopper was squashed with a sterilized toothpick in liquid nitrogen in a 1-mL microcentrifuge tube; then, 60 μL of buffer A was added, and the toothpick was rinsed with 60 μL of buffer A inside the centrifuge tube.
4. The tube was incubated at 65 ℃ for 45 min and vortexed every 15 min.
5. 120 μL of buffer B was added and mixed well by vortexing; then, the tube was incubated on ice for 1−2 hours.
6. 480 μL of prechilled ethanol was added and mixed well, and then incubated at −20 ℃ for 1−2 hours.
7. The solution was centrifuged at 10,000 ×g for 15 min, after which the supernatant was carefully removed and discarded. The pellet was carefully rinsed with 70% (mL/mL) ethanol.
8. Repeat step 8 and then dry at 37 ℃ or lyophilize (freeze-dry) the pellet, which now contains the genome DNA of BPH. Dissolve the pellet in double-distilled water and store the sample at −80 ℃ for later use.

1.4 PCR

PCRs were performed with 2.5 μL 10×PCR buffer (Promega), 1.25 U Ex-Taq DNA polymerase, 2.5 μL genome DNA of BPH, 0.2 mmol/L dNTPs, 1 mmol/L $MgCl_2$, 1 μmol/L outer and 1 μmol/L inner GSP. Then, the volume was supplemented with double-distilled water to 25 μL. Thermal cycling conditions were 94°C for 3 min followed by 20 cycles of 94 ℃ for 30 s, 67−58 ℃ for 30 s, 1 ℃ was reduced every two cycles, and 72 ℃ for 1 min. These were followed by 10 cycles of 94 ℃ for 30 s, 58 ℃ for 30 s, and 72 ℃ for 1 min. The last cycle was followed by final extension at 72 ℃ for 5min.

1.5 Electrophoresis

A 10-μL product of PCR was used. Electrophoresis was performed with 1% (g/mL) agarose gel at a constant current of 80 mA for 1.5 h.

2. Results

2.1 For imidacloprid resistance detection

The following steps are used: first check the gel under UV. If two bands of 900 bp (PQ) and 370 bp (AQ) are observed, the BPH is likely to be a resistant homozygote; if two bands of 900 bp (PQ) and 540 bp (PB) are observed, the BPH is likely to be a susceptible homozygote; if three bands of 900 bp (PQ), 540 bp (PB), and 370 bp (AQ) are observed, the BPH is likely to be a heterozygote.

The results (Fig. 10.2) show that all individuals from strain R were resistant homozygotes (1−3), individuals from strain S are susceptible homozygotes (4−6), and

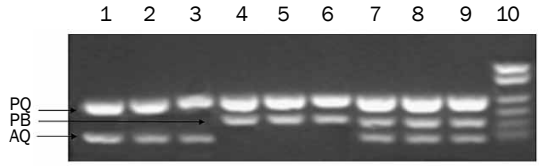


Fig. 10.2. Electrophoresis of Bi-PASA products: 1–3: resistant homozygotes (strain R); 4–6: susceptible homozygotes (strain S); 7–9: heterozygotes (strain M); 10: DNA marker.

individuals from strain M are mainly heterozygotes (7–9). The sequencing results of segments of susceptible homozygotes, resistant homozygotes, and heterozygotes are consistent with the Bi-PASA detection.

Strains S, R, and M together with field samples of BPH collected from Jiangpu, Nanjing, in July 2000, August 2001, and July 2002, respectively, were detected by the technique of Bi-PASA described above.

The results (Table 10.3) show that individuals from strain S were susceptible homozygotes and individuals from strain R were resistant homozygotes. No susceptible homozygous individuals were detected in strain M and most individuals (84.3%) were heterozygotes. Almost all of the individuals from field populations were susceptible homozygotes. Two from 107 individuals were found to be heterozygotes in the 2002 population. In general, the results showed no target resistance for imidacloprid from the field populations. However, the finding of heterozygotes suggests that there is a high tendency toward developing target resistance for imidacloprid in field populations.

2.2 For fipronil resistance detection

The following steps are used: first check the gel under UV. If two bands of 200 bp (PQ) and 150 bp (AQ) are observed, the BPH is likely to be a resistant homozygote; if two bands of 200 bp (PQ) and 80 bp (PB) are observed, the BPH is likely to be a susceptible homozygote; if three bands of 200 bp (PQ), 150 bp (PB), and 80 bp (AQ) are observed, the BPH is likely to be a heterozygote.

Table 10.3. Bi-PASA detection of resistance frequencies in lab strains and field populations.

Population		Individuals tested	Homozygote wild type	Homozygote mutant	Heterozygote
S		60	60	0	0
R		79	0	79	0
M		70	0	11	59
Field populations	2000	40	40	0	0
	2001	38	38	0	0
	2002	107	105	0	2

The results (see Fig. 10.3) show that all individuals from the BPH-FR strain were resistant homozygotes (1−5); individuals from the S strain were susceptible homozygotes (6−10); and individuals from the BPH-FR/S-F_2 population were mainly heterozygotes (11−15). The sequencing results of segments of a susceptible homozygote, resistant homozygote, and heterozygote are consistent with the Bi-PASA detection.

Randomly selected samples from populations of BPH-SX-13, BPH-SX-25, and BPH-FR/S-F_2 were evaluated with the method described above and the results are shown in Figure 10.4. After 13 generations of fipronil selection, the population did not have a high frequency of mutation, with one heterozygote from five individuals (1−5). After continuous selection for 15 generations, most individuals were heterozygotes (6−10). The offspring (BPH-FR/S-F_2) of hybridization between the resistant strain (BPH-FR) and susceptible strain (S) showed variations, including susceptible homozygotes, and both resistant homozygotes and heterozygotes.

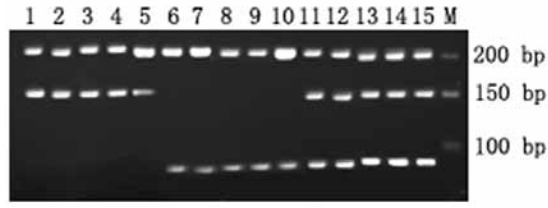


Fig. 10.3. Electrophoresis of Bi-PASA products: 1−5: resistant homozygotes (strain BPH-FR); 6−10: susceptible homozygotes (strain S); 11−15: heterozygotes (BPH-FR/S-F2); M: DNA marker.

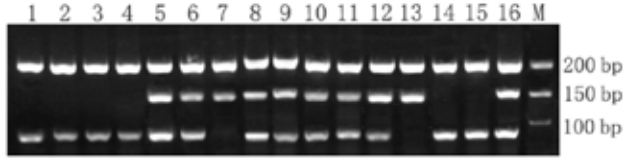


Fig. 10.4. Electrophoresis of Bi-PASA products: 1−5: BPH-SX-13; 6−10: BPH-SX-25; 11−16: BPH-FR/S-F2; M: DNA marker.

2.3 Detecting mutations in imidacloprid and fipronil resistance in field populations from some countries in Asia

Using the methods described above, the frequencies of Y151S mutation for imidacloprid resistance and A302S mutation for fipronil resistance were evaluated in 16 populations from four countries (Fig. 10.5). The Y151S mutation was found in two populations from Chainat and Ang Thong in Thailand, with one mutant from 30 tested individuals in each population. The results indicated that the mutation, Y151S, was present in Thailand but not in Vietnam, China, or the Philippines. The A302S

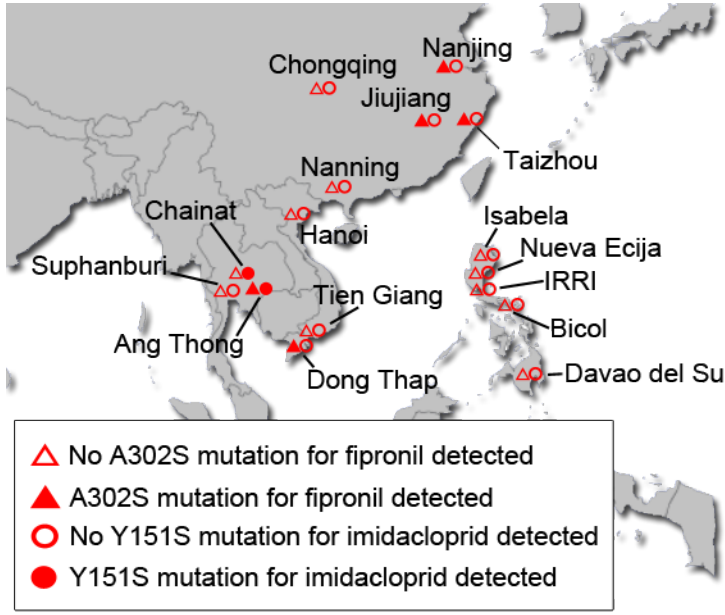


Fig. 10.5. The detection of Y151S and A302S mutations in field populations from China, Thailand, Vietnam, and the Philippines.

mutation was found in five field populations, with three (Jiujiang, Nanjing, and Taizhou) in China, one (Dong Thap) in Vietnam, and one (Ang Thong) in Thailand. Although only one mutant was found in each population, the common occurrence in different countries indicated the importance of this mutation in fipronil resistance in field populations. Neither of the two insecticide mutations was found in BPH field populations throughout the Philippines.

The detection of target-site mutations for both imidacloprid and fipronil in Central Thailand suggests that a further increase in the use of these two active ingredients can lead to rapid development of resistance in the BPH populations. In the double-S curve of insecticide resistance development (Liu et al 2006b), the detection of a target-site mutation may indicate that resistance development has entered the second stage. In China, a target mutation for imidacloprid has not been detected despite its heavy usage. Management strategies to withdraw the insecticide before resistance reaches the second phase might still be effective.

However, in the case of Thailand, the detection of target-site insensitivities for imidacloprid, a neonicotinoid, and fipronil, a phenylpyrazole, indicates that these two groups of insecticides are probably no longer useful for planthopper management.

CHAPTER 11:

Evaluating repellant effects

During the 19th century, Howlett (1912) discovered that citronella grass, *Cymbopogon nardus* (Poaceae), when used as a mosquito repellant, attracted many Bactrocera fruit flies during the daytime. Subsequently, he showed that the component responsible for the attraction was methyl eugenol (ME) (Howlett 1915). ME is found in more than 450 plant species, especially in spices, and it has many roles in nature. Besides playing an essential and important ecological role as a plant synomone in the pollination of certain wild orchid species (Tan et al 2002, 2006), it acts as an antifeedant and a repellant against certain insects, as well as an antimicrobial agent, especially against some fungi and bacteria. In a review by Tan and Nishida (2012), they found that ME is also an antifeedant to insects and this experiment evaluates such repellent effects on the brown planthopper.

Preparation of test insects

A known population was identified and 3-day-old brachypterous brown planthopper (BPH) females were used for testing. The collection and rearing procedures were conducted according to the protocol for rearing BPH described in Chapter 4.

Preparation of methyl eugenol solution

The whole procedure is conducted under a fume hood. Concentrations of ME (technical grade) needed for testing are 1.0%, 1.1%, 1.2%, 1.3%, 1.4%, 1.5%, and 0% (control). Seven 6-mL vials are prepared and marked with the desired concentrations (Fig. 11.1). Four-mL technical grade acetone was poured into each vial as solvent. Using a micropipettor, a desired volume of ME was added to each vial based on the

Fig. 11.1. Materials for preparation of ME solution: (A) disposable mask, (B) laboratory gown, (C) disposable nitrile gloves, (D) pipettor tips, (E) micropipettor, (F) parafilm strips, (G) 6-mL screw cap vials in test-tube rack, (H) technical grade acetone, (I) labeling tape, (J) labeling pen, (K) stainless-steel scissors, (L) technical grade methyl eugenol.

concentrations needed (Fig. 11.2). The caps of the vials are secured with parafilm strips to minimize evaporation and are placed into a freezer at −20 °C. The concentrations can be stored for 2 weeks.

Fig. 11.2. Preparation of ME test solution using a micropipettor in a fume hood.

Preparation of test cages and filter paper treatment

Twenty-one transparent containers are prepared and fifteen 7- to 10-day-old TN1 seedlings wrapped with moistened tissue paper (Fig. 11.3A) are placed into each container to serve as feeding plants (Fig. 11.3B).

Twenty 3−4-day-old brachypterous females are introduced into each container and the insects are allowed to feed and settle on the plants (Fig. 11.3C). Another batch of 21 test cages is prepared. Each cage is fabricated with windows and mesh cloths for ventilation (Fig. 11.4A). Twenty-one filter papers (diam. 50mm) are set up and each is treated with 0.5mL of the required ME concentration and dried at room temperature for 15−20 minutes. The treatment of the filter paper is done on a glass petridish (Fig. 11.5) and afterwards transferred inside the fabricated test cages. Three replicates are prepared for each concentration of ME.

Small cups are put inside and on top of the filter papers to prevent the absorption of water from the feeding plants (Fig. 11.4A). A sufficient amount of tap water is poured into each small cup to prevent the seedlings from drying. Afterwards, the feeding

plants with the test insects from the transparent container are carefully transferred to the fabricated test cage and placed on top of the small cup.

The experimental setup is kept in a temperature-controlled room (25 ± 2 °C) (Fig. 11.4B) and the mortality and repellency responses are observed and recorded after 5, 10, 20, and 30 min, and 3, 6, 24, and 48 h.

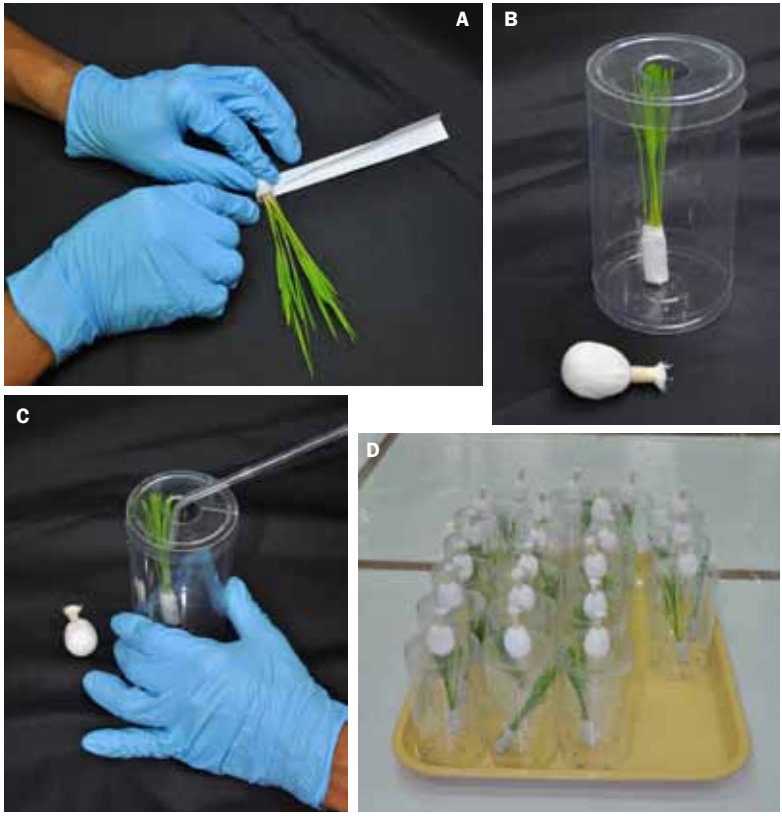


Fig. 11.3. Preparation of feeding plants and recovery cages: (A) wrapping the seedlings with tissue paper; (B) TN1 seedlings in the plastic container; (C) infestation of BPH; (D) transparent containers with TN1 seedlings infested with BPH.

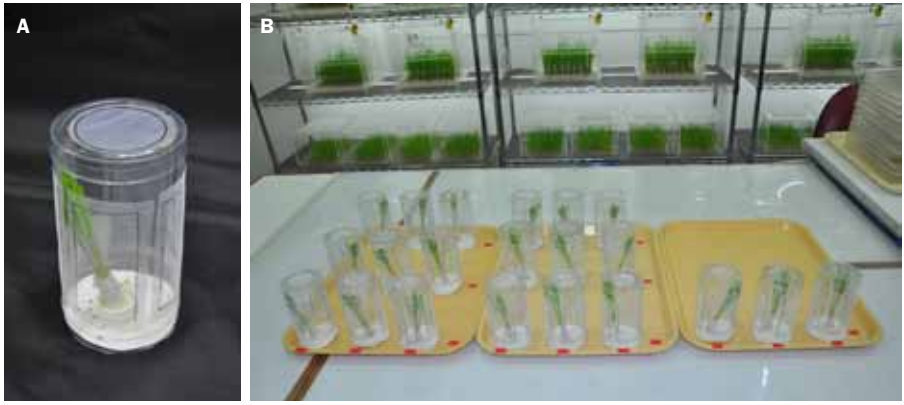


Fig. 11.4. (A) Test cage with the treated filter paper and feeding plants infested with BPH; (B) experimental setup in a temperature-controlled room.

Fig. 11.5. Filter paper treated with the desired ME concentration on a glass petri dish.

Results

Table 11.1 presents the median repellency concentration (RC_{50}) of BPH to methyl eugenol. Based on the results, repellency responses were already observed during the first 5 min and were directly proportional to the ME concentration. An RC_{50} value of 1.24% (12.4 g actual weight) was found at 20 min.

The effects of repellency and antifeeding action of ME produced significant toxicity to BPH, producing mortality after 24 hours (Table 11.2). A lethal concentration (LC_{50}) of 1.39% (13.9 g actual weight) was observed after 24 hours.

Based on these results, methyl eugenol produces repellency, antifeeding action, and mortality against brown planthoppers. The study points to a recommendation that plant species with ME can have a major role in increasing biodiversity in the rice ecosystem and ecological engineering. This insect-plant interaction method can also promote biological control, which is essential in integrated pest management.

Table 11.1. Summary of PoloPlus© outputs for repellency response.

Observation time	RC_{50} (%)	Actual wt. of RC_{50} (g)	Fiducial limits (95%)	Slope (SE)	Heterogeneity
20 min	1.24	12.4	1.07 to 1.38	5.73 (1.27)	1.05

Table 11.2. Summary of PoloPlus© outputs for mortality response.

Observation time	LC_{50} (%)	Actual wt. of LC_{50} (g)	Fiducial limits (95%)	Slope (SE)	Heterogeneity
24 h	1.39	13.9	1.29 to 1.60	7.76 (1.21)	1.46

CHAPTER 12:

Reporting 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 can be used for reporting the results.

Each probit analysis is described by the LD_{50}, LC_{50}, or LT_{50} 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 by PoloPlus©.

In fitting the quantal response data to the regression model, PoloPlus© generates several parameters, which can be presented in a table (Table 12.1).

Since the slopes of the two regression lines are not parallel, the relative potency value is not valid. The high heterogeneity of 2.23 also indicates that the responses are highly variable. This might be due to heterogeneous test insects with different ages, sexes, or sizes.

In the tests for parallelism, the $\chi 2$ 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 $\chi 2$ value was 216 and the null hypothesis of equal slopes and equal intercepts was also rejected. From Table 12.1, the confidence limits and standard values in parentheses showed 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 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 12.2 presents the results of brown planthopper populations from three countries where the probit lines are parallel. In this case, the relative potency values can be considered valid. In addition, the heterogeneity of all three probit lines was low, indicating that the responses were uniform.

An additional way to present toxicological data visually is by using probit plots. Data from Table 12.1 are shown in Chapter 6. Figure 12.1 shows the probit lines from Table 12.2.

Table 12.1. 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 12.2. 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

Further 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).

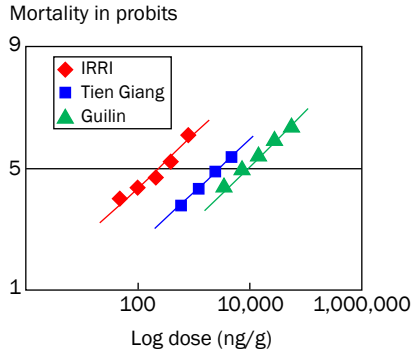


Fig. 12.1. Probit lines for imidacloprid on BPH populations at IRRI (Philippines), in Tien Giang (Vietnam), and in Guilin (China).

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Appendix A. 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	—	1.9008	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	6.6008	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*.

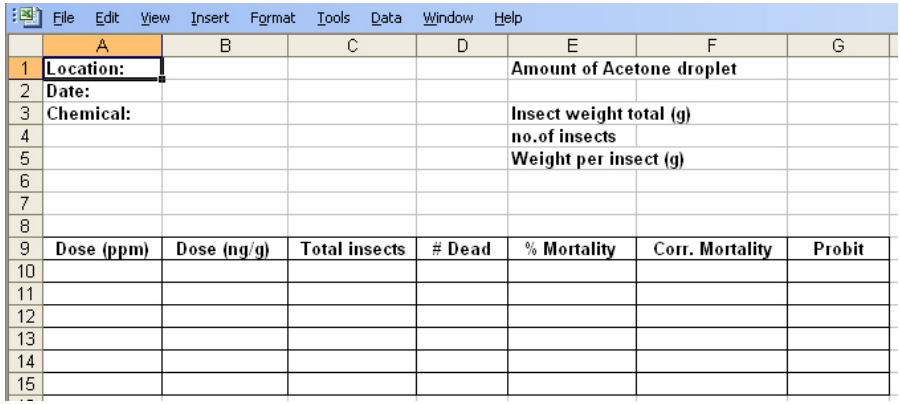
Source: Finney (1971).

Appendix 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 insects
Pc = % control mortality
Column G. Probits = computed transformed values of % mortality given in Appendix A.

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.

Appendix D. Concentration conversion table.

	Symbol	Conversion factor based on 1 g/L
Kilograms per liter	kg/L	1,000
Grams per liter	g/L	1
Milligrams per liter	mg/L	0.001
Micrograms per liter	µg/L	0.000001
Nanograms per liter	ng/L	0.000000001
Picograms per liter	pg/L	0.000000000001